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REVIEW

Insights into Peptide and Protein Function: A Convergent Approach[1†]

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Abstract: From viruses to multicellular organisms, life is inseparable from the genetic instructions aimed at regulating its maintenance, division, multiplication, differentiation and death (apoptosis). Over the past 15 years, structural studies have begun to resolve the complex reactions involved in these fundamental processes in biology. The three-dimensional representations of the complexes formed with peptides and/or proteins have allowed interpretation of the biochemical data and formulation of novel hypotheses about the control and execution of these processes. Moreover, they have opened the way to rational approaches for designing compounds able to interfere with these crucial events in normal or pathological conditions. Various results obtained in our laboratory in these fields are briefly summarized in this review.

Keywords: analgesia; apoptosis; CCK_8; HIV-1 proteins; metallopeptidase-inhibitors; peptidomimetics; rational drug design

There is a large number of data suggesting that experience with adverse life events contributes to promote behavioural impairments in humans. Regulatory peptides, particularly neuropeptides in the central nervous system, seems to play a critical role in modulating hyperexpression of classical neurotransmitters induced by various stressors, thus acting as physiological regulators of behavioural adaptations. In addition, as illustrated with various neuropeptides, such as enkephalins, cholecystokinin or substance P, stimulation or blockade of peptidergic pathways could induce specific pharmacological and therapeutical responses, independently of neurotransmitters, as recently illustrated with inhibitors of neuropeptide-metabolizing enzymes and SP antagonists as new antidepressants. Moreover, inhibition of peptidases involved in enkephalins inactivation or in regulation of vascular tone and fluid homeostasis represent promising approaches in analgesia, depression and in treatment of severe cardiovascular diseases, respectively (reviews in [1–3]). This will be discussed in the first

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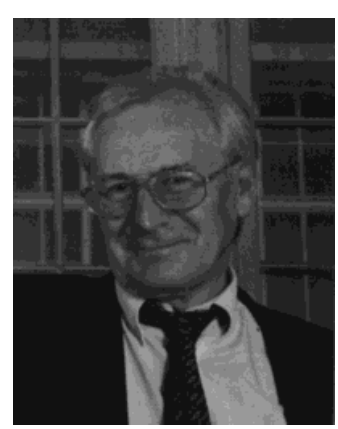

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part of this paper. The second part will focus on protein–protein interactions.

IS CONFORMATIONAL ANALYSIS OF NEUROPEPTIDES IN SOLUTION USEFUL FOR DESIGNING POTENT AND SELECTIVE RECEPTOR LIGANDS?

In the 1970–1976 period, numerous neuropeptides have been discovered in mammalian central nervous system (CNS), and were also shown very often to be present in the gastro-intestinal tract. Thus in 1975, the two endogenous morphine-like peptides, Met- and Leu-enkephalins were isolated and characterized in the brain [4], and in the same year, cholecystokinin was found to be present in the brain [5], and soon after shown to correspond to the tyrosine sulphated octapeptide CCK_8: Asp-Tyr(SO_3H)Met-Gly-Trp-Met-Asp-Phe-NH_2. These peptides were demonstrated to interact with at least two different receptors, which were cloned and sequenced about 10 years later (review in [6]). There are now more and more biochemical and pharmacological results suggesting that these receptors, belonging to the class of G-protein coupled receptors with seven transmembrane domains, could exist under different conformations in slow equilibrium [7]. This process is somewhat similar to the allosteric mechanism earlier proposed for the cholinergic receptor [8], and could account for numerous features such as: (i) existence of various affinity states for a single receptor triggering different pharmacological responses, likely related to differences in G-protein coupling; (ii) non-identical rates of receptor internalization; (iii) or putative differences in dimerization of receptors. These mechanisms should extend the multiplicity of responses conveyed by neuronal networks.

The interaction between a receptor and a ligand (agonist or antagonist) is the result of a thermodynamical-dependent process which, respectively, takes into account: the energies of the solvated peptide, and the free protein and that of their complex. Obviously, one parameter in the recognition process is the adaptation of the ligand during the binding process, which is more or less dependent on its conformational properties in solution. This is the reason for which short peptides, assumed to be very flexible, such as the enkephalins or cholecystokinin, were studied by NMR spectroscopy, and then by crystallography, and found somewhat unexpectedly, to exist preferentially under folded forms [9–11]. As the receptor binding of these peptides is mainly achieved (as illustrated by crystallographic analysis of enzyme-inhibitor complexes) by interactions of their constituting amino acid side-chains with those of the protein target, it was

Table 1 Binding Affinities of Various Peptides for Rat Brain Opioid μ and δ Receptors or for Guinea Pig Brain CCK-A and CCK-B Receptors

		K_i (nM) μR	δR
Tyr-Gly-Gly-Phe-Met	Met-Enk	11	4.2
Tyr-D.Ser-Gly-Phe-Leu-Thr	DSLET	31	4.8
Tyr-D.Thr-Gly-Phe-Leu-D.Thr	DTLET	25	1.3
Tyr-(D.Ser-OtBu)-Gly-Phe-Leu-Thr	DSTBULET	374	2.81
Tyr-D.Ser-(OtBu)-Gly-Phe-Leu-Thr(OtBu)	BUBU	480	1.69
Tyr-D.Pen-Gly-Phe-D.Pen	DPDPE	980	8.8
	Morphine	4.2	49
		K_D (nM) CCK_BR	CCK_AR
Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2	(CCK_8)	0.30	0.91
Asp-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2	(BDNL)	0.23	0.93
Boc γ-D.Asp-Tyr(SO_3H)-Nle-D.Lys-Trp-Nle-Asp-Phe-NH_2	(BC 197)	5.10	910
Boc-D.Asp-Tyr(SO_3H)-Nle-D.Lys-Trp-Nle-Asp-Phe-NH_2	(BC 254)	0.49	970
Boc γ-D.Asp-Tyr(SO_3H)-Nle-D.Lys-Trp-Nle-Asp-Phe-NH_2	(open BC 254)	301	> 3000
Boc-Tyr (SO_3H)-*gNle-mGly*-Trp-(Me)Nle-Asp-Phe-NH_2	(BC 264)	0.15	75
Boc-Phe(pCH_2SO_3H)-*gNle-mGly*-Trp-(Me)Nle-Asp-Phe-NH_2		0.46	1100

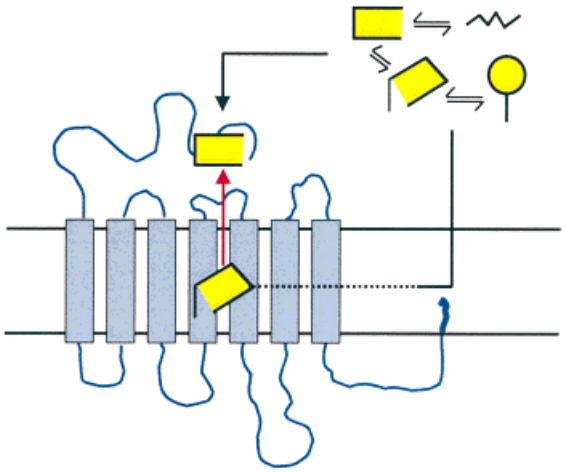


Plate 1 Different models of ligand-G-protein coupled receptor recognition: (i) by initial adsorption into the phospholipid bilayers of the cell membrane followed by diffusion inside the receptor; (ii) by transconformation of the ligand during the binding process or preselection of the final bound structure (see discussion in the text about these models).

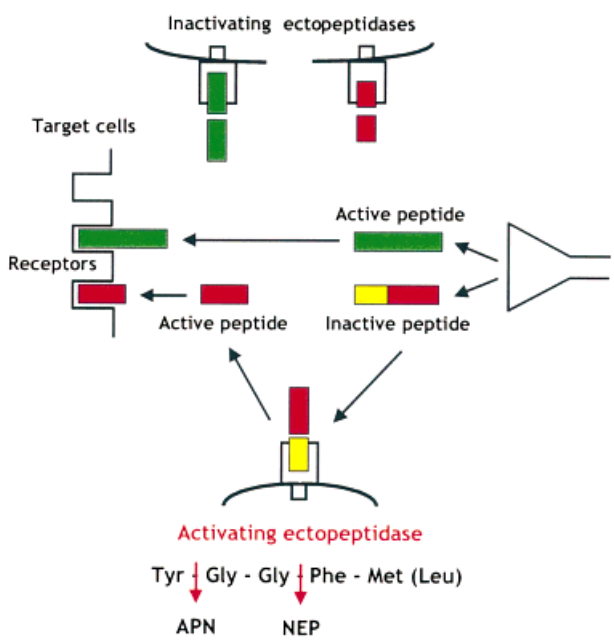


Plate 2 Schematic representation of a peptidergic pathway in which the peptide messenger could be released under inactive form processed by activating enzyme(s) to give the active ligand. After binding to their specific receptor(s), the active peptides are mainly inactivated by metabolizing enzyme(s). Very often several enzymes are involved in the inactivation of peptides as shown with enkephalins (APN: aminopeptidase *N*; NEP: neutral endopeptidase).

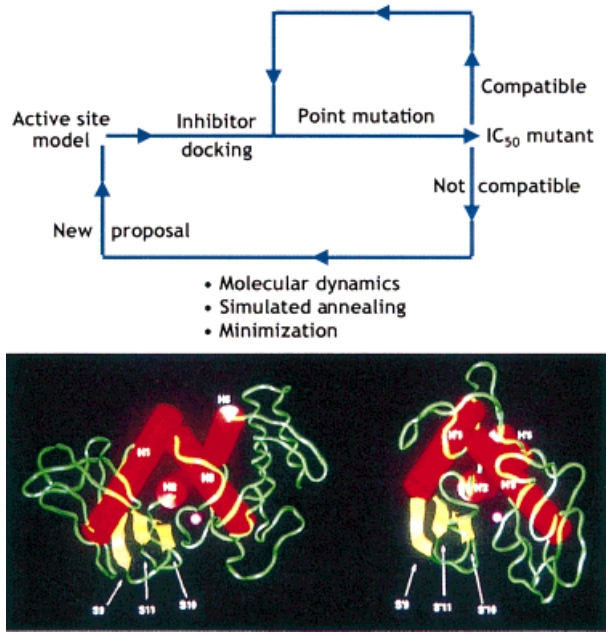


Plate 3 Schematic representation of the strategy used to propose a model of the active site of NEP. Top: Site-directed mutagenesis were performed in parallel in TLN (315 amino acids), the structure of which was known at the atomic level [31] and NEP (749 amino acids). In addition, sequence comparison and two-dimensional structure prediction were used. This has resulted in a model [38] roughly close to the structure recently determined by X-ray cristallography [39]. Bottom: Similarities in the spatial arrangment of the active sites of TLN (left) and of the NEP model (right).

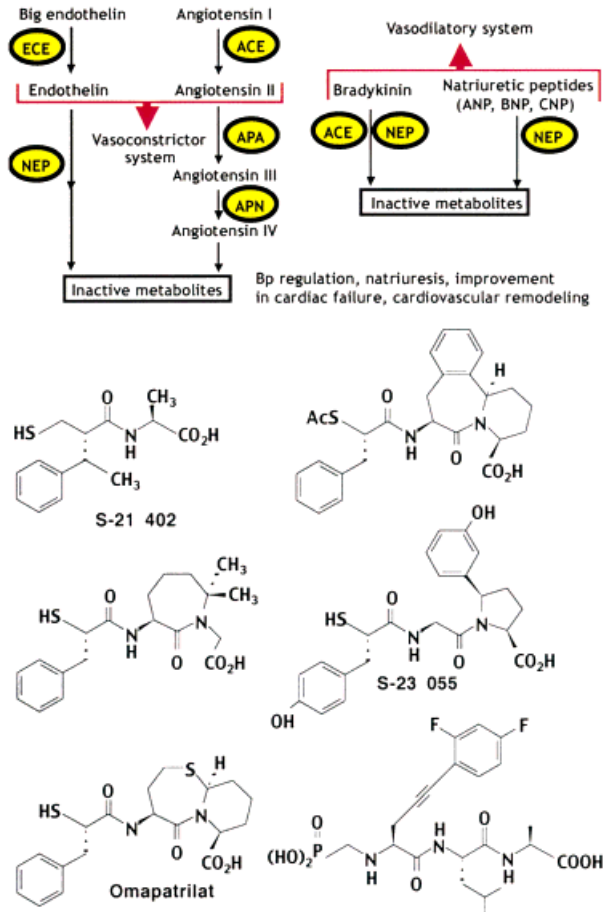


Plate 4 Top: Schematic representation of the metabolism of peptides involved in the two counteracting systems regulating blood pressure and fluid homeostasis. Bottom: Some described dual inhibitors of NEP/ACE, most of them being in clinical trials [2].

tempting to use the results of conformational analysis for designing potent and selective ligands. This strategy was initially used for the development of linear peptides such as DSLET, DTLET, DSTBULET or BUBU and cyclic peptides such as DPDPE or DPLPE, which were the first more or less selective opioid delta agonists, all deriving from the native enkephalins [12,13]. The same strategy was followed in synthesizing the cyclic derivatives of CCK_8, BC 197 and BC 254 [14], which remain, with BC 264, the most potent and selective agonists for the CCK-B (or CCK_2) receptor (Table 1).

In spite of our increasing knowledge of the three-dimensional structure of the G-protein coupled receptor [15], the computerized models of receptor-ligand complexes are unable to confirm or to refute the hypotheses used during the early development of selective opioid or CCK ligands. Nevertheless, the plasticity of the seven transmembrane helices shows, for example, that the same hydrophobic hole, surrounded by the same constituting amino acids, is present in both opioid (mu, delta) receptors, and within the opioid-related neurotensin receptor [16]. Therefore, this somewhat large binding site could fit enkephalins and derivatives under folded or cyclic forms. This is one of the reasons for which a conformational analysis is now currently done for any newly discovered peptide.

On the other hand, the binding of a ligand to its receptor could be even more complicated if the initial step corresponds to the adsorption of the peptide into the membrane, followed by its diffusion towards the receptor [17] (Plate 1). However, this proposed model of receptor-recognition suffers from various inconsistencies. First, the release of a neuropeptide is ensured by fusion of large secretory vesicules with the external membrane of the cell, and many studies have shown the difficulty for peptides in entering or exiting the cells by crossing the phospholipid bilayer. Moreover, it is difficult to understand why a peptide interacting with the external loops of the receptor would have to diffuse from the lipid bilayers inside the interior of the 7 TM helices of the receptor, and then merge outside, in order to find its external binding site. Nevertheless, this model is interesting and cannot be completely rejected, particularly for highly hydrophobic ligands, such as endogenous agonists of the cannabinoid receptors.

In conclusion, at present, caution must be taken to propose the rules of the receptor-recognition process, rational design of ligands or improvement of their affinities from modelled, bound receptors owing to the few crystal data for G-protein-coupled receptors. Of course this does not mean that, in the future, the expected *in silico* design of ligands based on reconstituted targets issued from human genome analysis is impossible, but it could require more time than is often claimed.

The Presence of Counteracting Neuropeptide Systems: Opponent Processes are Needed for Homeostasis Equilibrium

Although far from perfect in terms of bioavailability (except BC 264), the derived highly potent and selective agonists for delta opioid and CCK_2 receptors (Table 1) allowed the most important responses associated with stimulation of one type of receptor to be characterized. Thus, the major role of mu opioid receptor in control of almost all types of nociceptive stimuli was evidenced by the linear correlation observed between the residual affinities for mu sites of delta agonists and their analgesic potencies [18]. This was recently confirmed by using mice with genetically-induced invalidation of the mu receptor [19]. Furthermore, the critical role of the mu receptor in respiratory function and the relationships with the CCK system have been demonstrated. At the intestinal level, the role of delta receptors in control of fluid secretion was dissociated from the role of mu binding sites in smooth muscle contraction. This has resulted in the introduction of the enkephalin degrading enzyme inhibitor, thiorphan, on the market as a new antidiarrheal agent, as will be discussed further.

Nevertheless, the most interesting results obtained by using systemically active delta probes, such as DSTBULET and BUBU or CCK_2 agonists like BC 264 and BC 197, were: (i) the demonstration of the highly significant antidepressant properties resulting from delta receptor activation [20], a result recently confirmed using mice with disruption of this receptor [21]; (ii) the improvement in mnesic processes initiated by stimulation of CCK_2 receptor by BC 264, and the anxiogenic properties in rodents of BC 197 leading to the proposal of the occurrence of two affinity binding states for the CCK_2 receptor [22]; (iii) BC 264-induced release of dopamine in the *N. Accumbens* and striatum measured by microdialysis in freely moving rats [23], suggesting interesting possible applications in DA-deficient diseases (Parkinsons', Schizophrenia . . .); (iv) the occurrence of a physiological antagonism between the opioid and CCK systems, exemplified by the potentiation of analgesic or antidepressant effects of opioid

agonists or endogenous enkephalins protected from their metabolizing enzymes, by selective CCK_2 antagonists (reviewed in [24]).

Several of these results indicate clearly that new drugs could be designed aimed at re-establishing imbalances between neuropeptide-dependent equilibria, which control important adaptational processes very likely impaired in severe depression, general anxiety etc. The recent clinical development as a new promising antidepressant of an antagonist for the substance P NK1 receptor [25] should be followed by that of other drugs targeting peptidergic systems.

INTERRUPTION OF THE PEPTIDERGIC TRANSMISSION

As will be discussed further in more detail, one of the peculiarities of peptidergic transmission is that, in contrast to classical neurotransmitter, the message conveyed by the peptide messenger is mainly, but not exclusively, abolished by action of ectopeptidases anchored in the external membrane of the cell (Plate 2) (reviews in [1,3]).

This mechanism was obviously rapidly taken into account to synthesize, for instance, the first peptidase-resistant enkephalins, and in our case, to design BC 264 (Table 1), a compound completely insensitive to CCK_8 metabolizing enzymes. In addition, this peptide is endowed with favourable pharmacokinetic properties, demonstrated by the presence of the intact tritiated counterpart of BC 264 ([^{3}H]pBC 264) in the brain after systemic administration. A great number of experiments were done with this peptide, which was shown to improve vigilance in monkeys without apparent anxiogenic properties (review in [26]). This is in contrast with BC 197, which was shown to induce anxiety in rodents and behavioural responses [24,26], mimicking the panic attacks evoked in humans by administration of CCK_4 (review in [27]). Shorter peptides derived from CCK_4 and incorporating the structural particularity of BC 264, i.e. the *gNle-mGly* motif have been designed [28], and seem to have similar properties than the longer peptide, although it remains to be verified if the very good affinity (~ 0.2 nM) of BC 264 for the CCK_2 receptor of various species, including humans, is preserved in the smaller sequences.

In order to further improve resistance to hydrolysis of CCK_8 and derivatives, such as BC 264 (Table 1), without a large change in their affinities, we have developed two non-hydrolysable derivatives of sulphated and phosphorylated tyrosine by replacement of the ester group by a $-CH_2-SO_3H$ or $-CH_2-PO_3H_2$ groups [29]. The latter compound, para-phosphomethyl phenylalanine (PmpF) is currently used in the replacement of the phosphorylated tyrosine, for example, in inhibitors of tyrosine kinase, the new amino acid being resistant to phosphatases.

Selective, Dual or Plurifunctional Inhibitors of Zinc Metallopeptidases as Therapeutical Agents

Generation and/or interruption of messages conveyed by neuropeptides was shown to be essentially, although not exclusively, achieved by one or several membrane-bound enzymes, giving rise to the concept of dual versus selective inhibitors (review in [30]). Most of these enzymes are zinc metallopeptidases, leading to a structure-based design of inhibitors [1]. The physiological role of neuropeptides and their relationships with other peptide systems can be investigated with peptidase inhibitors and selective antagonists, and results compared with those obtained using mice deleted for genes encoding the various components of a peptidergic system. In addition to providing valuable insights into the roles of peptides and their metabolizing enzymes, some inhibitors are in clinic as antihypertensives and antidiarrhoeal agents, and there is a potential future for other inhibitors as new analgesics or antidepressants, and in the treatment of severe hypertension and heart failure.

The bacterial peptidase thermolysin (TLN), is the representative of this group of zinc metalloectopeptidases. TLN has been crystallized and co-crystallized with different groups of inhibitors, all able to interact in a monodentate or bidentate manner to the zinc atom in the catalytic site. A mechanism of action was proposed for this class of enzymes from structural studies on TLN [31], and recently revisited to take into account the occurrence of a network of hydrogen bonds revealed by site-directed mutagenesis [32].

Neutral endopeptidase, NEP, one of the two peptidases inactivating enkephalins, was the first physiological zinc peptidase of this family to be cloned and sequenced (review in [1]). Comparison with TLN showed the occurrence of two consensus sequences, HexxH and ExxxD [33], which contain the catalytic glutamate in the first sequence and the third zinc ligand, Glu, in the second one (Figure 1). Soon after NEP, several other metallopeptidases such as ACE, ECE, APN, APA were cloned. More

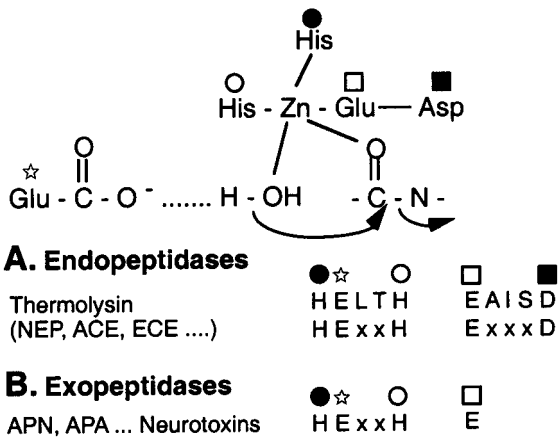


Figure 1 The cloning and sequencing of zinc metalloproteases have shown the occurrence of consensus sequences bearing amino acids critically involved in the catalytic process. The figure shows a simplified model of peptide bond hydrolysis by zinc metallopeptidases.

recently, peptidases belonging to the group of Metzincins (stromelysin for example) were also cloned and sequenced. The mechanisms of peptide bond hydrolysis of all these enzymes is similar, and ensured by polarization of a water molecule by the catalytic glutamate.

Thiorphan, HS-CH_2-CH($CH_2\Phi$)*CONH*-CH_2-COOH ($K_I = 2$ nM) was the first synthetic inhibitor of NEP [34]. It was designed, based on the observation by Ondetti and Cushman, that introduction of a zinc chelating group on short peptide or pseudopeptide recognizing the S'_1-S'_2 subsites of ACE, leads to potent and selective inhibitor such as captopril [35].

One of the problems encountered with thiorphan was its recognition by ACE ($K_I \sim 140$ nM). For this reason, and using data from the litterature on the ACE pharmacophore, we designed retrothiorphan HS-CH_2-CH($CH_2\Phi$)-*NHCO*-CH_2-COOH ($K_I = 4$ nM on NEP and > 10000 on ACE) based on the hypothesis that retroinversion of the amide bond could provide the same hydrogen bonding network than thiorphan in the active site of NEP, but not in that of ACE [36]. However, thiorphan and its prodrugs, such as acetorphan, were unable to produce significant antinociceptive responses owing to the partial protection of the enkephalins which are also inactivated by aminopeptidase *N* (APN) (Plate 2). In order to completely inhibit degradation of these peptides, we proposed the concept of dual (or mixed) NEP/APN inhibitors (review in 30) and we extended this concept in 1990 [37] to dual inhibition of NEP, shown to inactivate the atrial natriuretic factor, ANP and ACE generating the hypertensive peptide angiotensin II. All these enzymes are zinc metallopeptidases, facilitating the design of dual or even plurifunctional inhibitors, blocking, for instance, the action of NEP, ACE and ECE (endothelin converting enzyme). Although the sequence homology between TLN (316 a.a.) and NEP (749 a.a.) are low, secondary structure prediction associated with side-directed mutagenesis made in parallel in TLN and NEP (Plate 3) has resulted, with the aid of calculations, in a model of NEP active site [38] which was found relatively close to that found in the recently determined structure of NEP [39]. This is encouraging for the modelization of ECE or other large zinc metallopeptidases.

Dual Inhibition of NEP and APN as a Means to Fill the Gap Between NAIDS and Opioid Analgesics

A large number of selective NEP inhibitors were synthesized (review in [1]), and Figure 2 shows several new structures able to recognize, with a high affinity, this enzyme, which was developed in our laboratory. Thus, we have extended the series of hydroxamate- or *N*-hydroxy formyl-containing inhibitors developed by Nishino and Powers [40] by incorporating a methylene group between the bidentate zinc chelating group and the moiety expected to interact with the S'_1 subsite. This has largely improved the potency of the NEP inhibitors, which interestingly, were shown unable to recognize

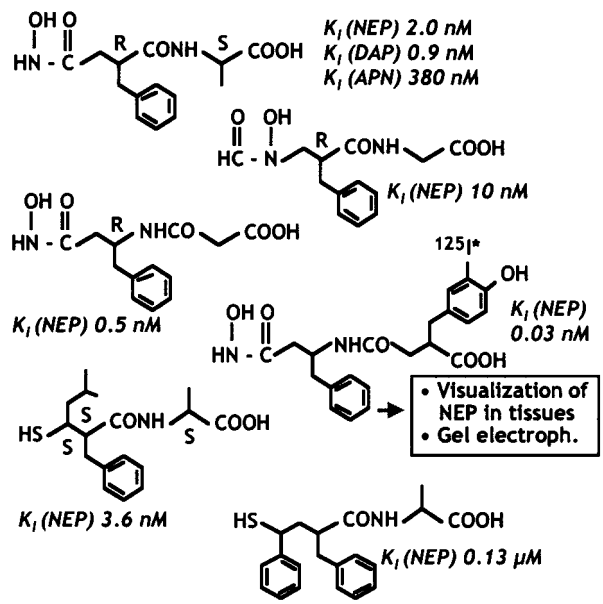


Figure 2 Some designed inhibitors with new modes of zinc metallopeptidase recognition.

the ACE active site. These kinds of hydroxamate inhibitors are now currently used to block matrix metallopeptidases. Inhibitors containing thiol of carboxyl zinc coordinating groups were useful in investigating the critical S_1-S'_2 enzyme subsites, with the aim of selectively recognizing NEP or ACE. Among the hydroxamate inhibitor, RB 104 and its radioiodinated form contain a retro-inversion of the amide bond (Figure 2). They are the most potent and selective NEP inhibitors designed so far, and are commonly used to directly visualize the peptidase in tissues, for instance, after gel electrophoresis, thus demonstrating the presence of NEP in the vascular endothelium [41]. RB 104 is also used to evaluate by competition the ability of a given inhibitor to fit the active site of NEP in various tissues following its administration by different routes. Another hydroxamate was used to perform the first visualization by autoradiography of a metallopeptidase in brain and various peripheral tissues after *in vitro* or *in vivo* use [42].

Dual inhibitors of NEP and APN were found early in the series of hydroxamates with kelatorphan HONH-CO-CH_2-CH($CH_2\Phi$)-CONH-CH(CH_3)-COOH (review in [1]), which gives strong analgesic responses although its bioavailability was too low for a possible clinical development. This was not the case of RB 101, a compound made of a highly potent APN inhibitor linked by a disulphide bond to a potent NEP inhibitor derived from thiorphan. The disulphide bond of this dual inhibitor prodrug, H_2N-CH($CH_2CH_2SCH_3$)-CH_2-S-S-CH_2-CH($CH_2\Phi$)-CONH-CH($CH_2\Phi$)-CONH-$CH_2\Phi$, is cleaved in a biologically dependent manner to release the two thiol inhibitors, each inhibiting their own peptidase (review in [1]).

RB 101 was shown to be the first compound active in all animal models where morphine is active, including severe pain, inflammatory and neurogenic pain (Figure 3). Moreover, the compound is devoid of the side effects of morphine, because, in contrast to the alcaloid which activates ubiquitously all opioid receptors, RB 101 protects the enkephalins only in tissues where there is a tonic or phasic release of the endogenous morphine-like peptides (reviews in [1,3]). Very recently, true dual NEP/APN inhibitors fulfilling criteria of interaction with the S_1 subsite of APN (presence of an amino group interacting with a conserved glutamate in S_1 of the enzyme) and of strong binding to the zinc atom in both enzymes were synthesized [43] (Table 2).

MAIN TYPES OF PAIN AND RELATED ANIMAL MODELS : EFFECTS OF VARIOUS COMPOUNDS

	Animal models	Opiates	Antalgics	Dual inhibitors
Severe	Tail flick Hot plate Electric stimulation	++	0	+
Inflammatory	Formalin Arthritic rats Carrageenin	++	±	++
Neuropathic	Sciatic nerve constriction Diabetic rats	±	0	+
Visceral	Writhing	++	+	++

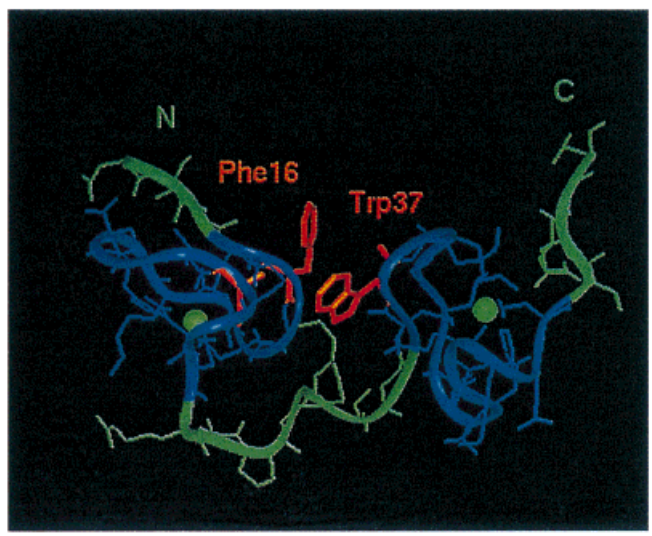
Figure 3 Different animal models used to demonstrate the efficiency of dual NEP/APN inhibitors [1] that are, at present, the only compounds filling the gap between NSAIDs and opiate analgesics.

These aminophosphinic derivatives, which can be considered as transition state inhibitors, give a potent analgesic response in all animal models of pain, but with a long duration of action (2–3 h) as compared with RB 101. Moreover structure-activity studies has allowed the synthesis of the first highly potent and selective APN inhibitor (Table 2) used to visualize the enzyme in brain by autoradiography. In this aminophosphinic compound, Phe in the P'_2 position was replaced by a radioiodinated analogue of Tyr.

In the case of APA, the first selective and potent inhibitors were found in the series of thiols. The use of APA inhibitors has shown that in brain, A_{III} generated by the action of APA on A_{II} issued from the renin-angiotensin cascade, could be the physiological effector regulating vasopressin release and the central control of blood pressure [44].

Dual ACE and NEP Inhibitors and Triple ACE/NEP/ECE Inhibitors as Therapeutic Agents in Cardiovascular Diseases

ACE takes part in the renin-angiotensin system, catalysing the release of the vasoconstrictive peptide angiotensin-II. Selective inhibitors of ACE, such as captopril and enalaprilat, which are used in the treatment of essential hypertension, remain the most effective agents in hypertension with high renin activity, but they have no effect on hypertension resulting from sodium retention. E-24.11 inhibitors, on the other hand, by increasing the circulating levels of atrial natriuretic peptide, promote its renal effects of natriuresis and diuresis. In addition, inhibitors of either enzyme increase the circulating levels of the hypotensive peptide bradykinin. This led to the idea that dual inhibition



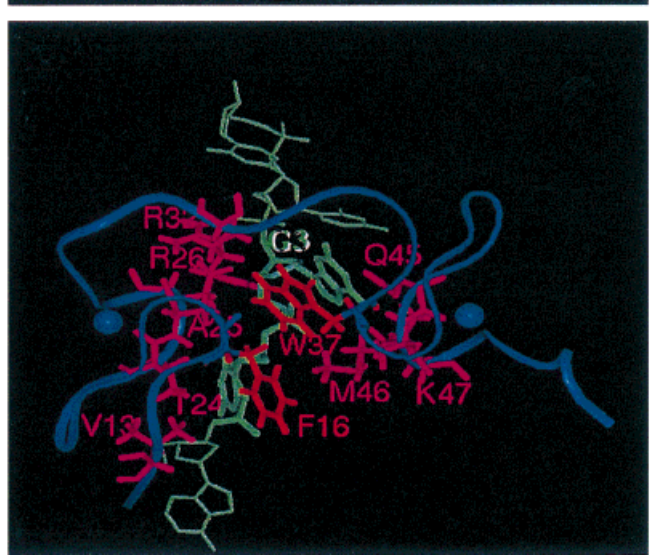


Plate 5 Top: Sequence of the nucleocapsid protein, NCp7 showing the highly conserved two successive zinc fingers. The single replacement of one amino acid in position 16, 23, 28, 31 or 37 led to structural changes in NCp7 associated with a complete loss of virus infectivity in the mutated virus illustrating the critical relationships between the structure and functions of NCp7. Middle: NMR structure of the central part of NCp7 showing the proximity of the two zinc fingers. Bottom: Structure of the complex between (12-53)NCp7 and d(ACGCC) showing the lack of important change in the three-dimensional structure of NCp7. Thus, the proximity between both zinc fingers is reinforced. Moreover, the stacking interaction between Trp^{37} and the guanine is critical for the interaction, and more generally, for the recognition of nucleic acids [49].

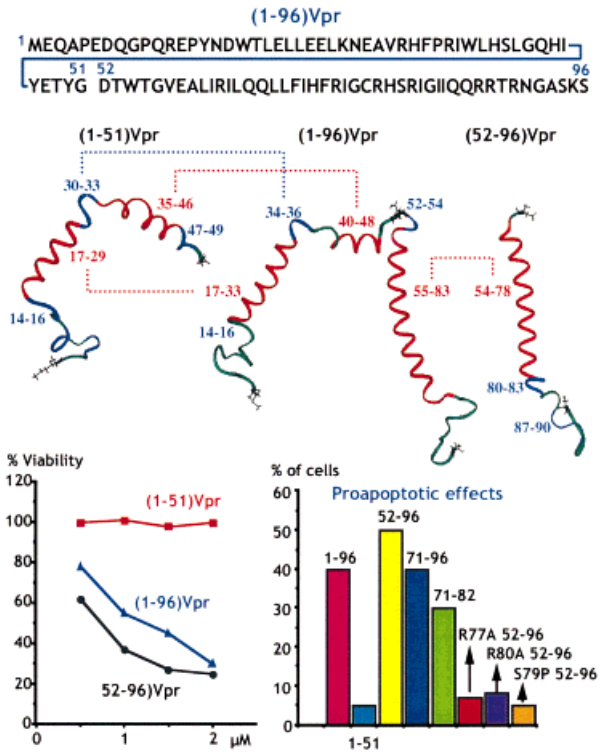


Plate 6 Top: Amino acids sequence of the HIV-1 protein Vpr obtained by SPS, and representation of the NMR structure of the protein and its *N*- and *C*-terminal domains [53]. Bottom: Vpr and even more (52-96)Vpr produce cell apoptosis by interaction with a protein of the mitochondrial pore and subsequent release of proapoptotic compounds. The short helical domain 71-82 is sufficient to induce the apoptosis [54].

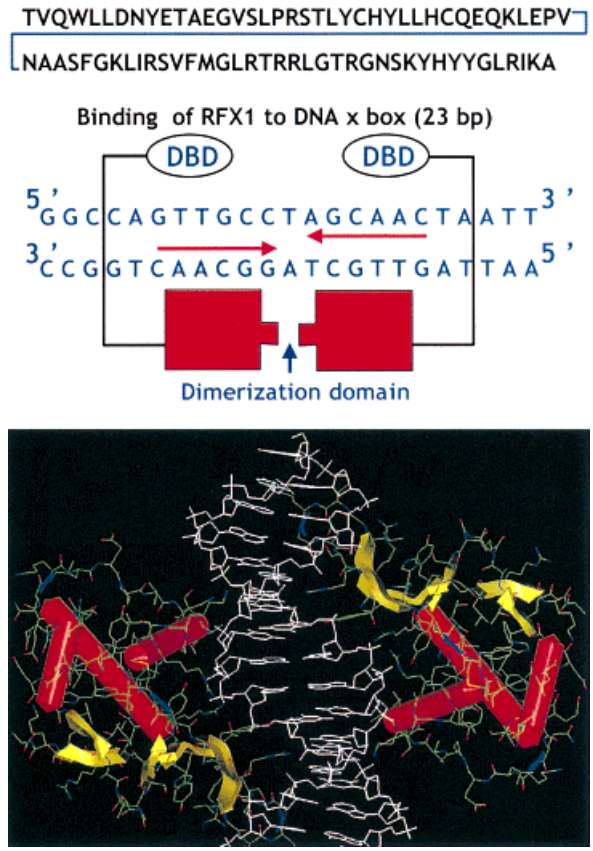
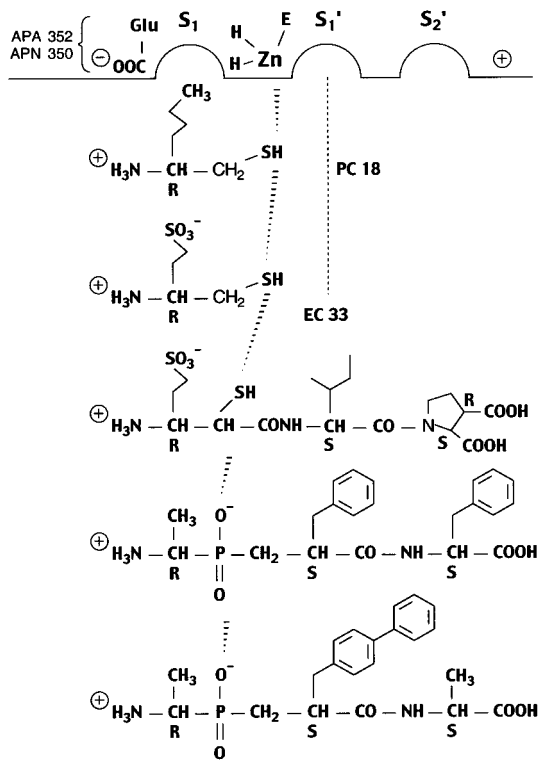


Plate 7 Top: Amino acids sequence of the DNA binding domain obtained by SPS of the RFX factor which interact with the palindromic arrangment of its DNA box [56] to induce the expression of gene encoding the MHC class II protein. Genetic defects in FRX could cause the base syndrome-characterized by a severe immunodeficiency [55]. Bottom: Crystal structure of the complex between DBA and the DNA-X box illustrating a new mode of protein-DNA recognition [57].

Table 2 Selective or Dual Inhibitors of Various Zinc Metallopeptides



	Inhibitory Potencies (nM)			
	APN	APA	NEP	ACE
PC 18	**8 ± 1.2**	> 100	> 1000	> 100
EC 33	~ 25,000	**120 ± 18**	> 1000	> 1000
	> 1000	**0.8 ± 0.07**	219 ± 10	259 ± 8
	0.6 ± 0.05	130	146 ± 2	> 1000
	2.9 ± 0.3	> 1000	**1.2 ± 0.2**	120 ± 8

of ACE and E-24.11 could have beneficial effects in the treatment of hypertension and heart failure [37].

In addition, the highly potent vasoconstrictor peptide endothelin is generated by the metallopeptidase ECE, offering the possibility of designing triple inhibitors (Plate 4).

Some dual ACE/NEP inhibitors are shown in Plate 4. Several of them are in clinical trials, with omapatrilat being in phase III (review in [2]). Pharmacological results are very promising, showing a slight improvement of the anti-hypertensive action as compared with selective ACE inhibitor with a more long-lasting action and interesting protective properties of the endothelium, decrease in cardiac overload and reduction in ischaemic injury after cardiac failure (review in [45]). One of the problems encountered with these inhibitors could be the possible side effects induced by the too efficient protection of bradykinin.

Very recently, we have shown that the metallopeptidase activity of tetanus neurotoxin occurs by an allosteric-like mechanism, still not reported for this kind of enzyme [46]. Moreover, we have designed the first highly potent and selective inhibitors ($K_I \sim$ nM) for clostridial neurotoxins such as botulinum type B (BoNT/B).

INHIBITION OF PROTEIN–PROTEIN INTERACTIONS: THE EXPANDING CHALLENGE

Most of the clinically used drugs interact with proteins (receptors or enzymes) in inhibiting the recognition by these targets of their physiological effectors, which are generally of small size. The role of these drugs is, essentially, to regulate defects in extracellular informations. However, the functioning of the biological machinery is also critically dependent on protein–protein recognition, as illustrated, for example, with cytosolic traffic of organelles, secretory processes or various signalling pathways. Owing to the size of the partners, inhibition or potentiation of such protein–protein interactions were considered for a long time as very difficult. However, structural studies have shown that, most of the time, only limited domains of the interacting proteins participate in the recognition process. These fragments obtained by molecular biology approaches or by solid phase synthesis allow us to perform: (i) structural studies of the protein–protein interaction; (ii) the rational development of inhibitors of this interaction; (iii) development of high throughput screening assays. This is

illustrated, for instance, by our recent design of compounds inhibiting, with nanomolar affinities, the binding of the adaptator protein, Grb2, critically involved in Ras signalling pathway, with the exchange factor Sos or the tyrosine kinase EGF receptor (review in [47]).

Inhibition of Nucleocapsid (NCp7)-Reverse Transcriptase (RT) Complex as a New Approach for Designing HIV-1 Antiviral Agents

Other examples of the inhibition of protein–protein interactions studied in our laboratory were in the field of virology. Combined inhibition of HIV-1 reverse transcriptase (RT) and protease (Pr) has significantly improved the treatment of AIDS. However, the emergence of resistance to these polytherapies remains a critical problem. This is owing to the well-known high plasticity of enzymatic proteins, in which a large number of mutations were shown to be capable of reducing the affinity of a given inhibitor without changing the catalytic efficiency of the mutant. This points out the need for new antiviral agents interfering with highly conserved non-enzymatic viral proteins. An attractive target is the nucleocapsid protein NCp7 (Plate 5), which contains two highly conserved zinc fingers. NMR studies have shown that the folded zinc arrays are in spatial proximity (Plate 5) [48], and that mutations inducing modifications in the general conformation of the protein, such as replacement of His^{23} by Cys, Pro^{31} by Leu or Trp^{37} by a non-aromatic residue led to a complete loss of virus infectivity of the mutated viruses (review in [49]). One possible explanation for this drastic impairment of infectivity could be that changes in NCp7 structure hinders one essential NCp7-dependent step of virus life-cycle, such as reverse transcription and provirus synthesis (review in [50]). Indeed: (i) *in vitro* HIV-RT shows unusual low processivity, suggesting that additional factors are required for efficient viral DNA synthesis *in vivo*; (ii) NCp7 activates annealing of $tRNA^{Lys,3}$ at the primer binding site level and promotes 5′-3′ viral DNA strand transfer; (iii) NCp7 reduces non-specific reverse transcription; (iv) NCp7 enhances the efficiency and processivity of RT; (v) NCp7 re-establishes strand transfer efficiency and RNAse H activity of a defective RT mutant.

This suggested the existence of a direct interaction between RT and NCp7 which was demonstrated by using various techniques (Western blots, plasmon resonance spectroscopy, chemical cross-linking etc.). The 1/1 RT/NCp7 complex has an affinity of 0.7 μM, and NCp7 interacts with both subunits [51]. *In vivo*, this interaction could stabilize the ternary complex formed between RT, NCp7 and nucleic acids, enhancing RT-dependent processes resulting in provirus synthesis. This hypothesis is supported by the structure of the complexes formed between NCp7 and nucleic acids such as d(ACGCC) (Plate 5) or the stem-loop SL3 (review in [49]), in which the same type of structure, not very different from that of free NCp7, was observed. Based on these structural data, a cyclic hexapeptide, RB 2121, was designed to mimic the most important determinants involved in complexation of RT with NCp7. RB 2121 displays antiviral activity by inhibiting the RT-dependent production of proviral DNA [52]. Results of the structure activity of a series of cyclic peptides support the mechanism of action of RB 2121. Combinatorial chemistry and development of a high throughput screening assay are in progress to isolate a compound with favourable pharmacokinetic properties, in this new class of antiviral agents.

The HIV-1 Protein, Vpr has Strong Apoptotic Properties

The small protein (96 a.a.) Vpr appears essential for viral replication and pathogenesis *in vivo*. Moreover, Vpr seems to play several roles during the HIV-1 life cycle, the most important being: (i) participation in transfer of the provirus to the nucleus of the host cell; (ii) arrest of the cell-cycle in G2 phase. Vpr and its *N*- and *C*-terminal fragments were prepared by SPS with incorporation of 23 amino acids enriched in ^{15}N (90%) and ^{13}C (15%), to facilitate structural analyses by NMR. These studies have shown the presence of a large proportion of helices in the fragments, as well as in (52-96)(Vpr ([53]). However, in the case of the entire protein, the location of the helices are somewhat different, particularly at the center of the molecule (Plate 6). This leads to a U-shaped form for the protein, which could explain the better activity of the (52-96) *C*-terminal domain as compared with Vpr, in a reaction such as nucleic acid recognition, complexation with NCp7 or with ANT (adenine nucleotide translocase).

Thus, we have very recently evidenced in close collaboration with G. Krömer and coll., the formation of a complex between Vpr and ANT *in vitro* and *in vivo* [54]. The affinity of (52-96)Vpr for ANT is around 7 nM for a 1/1 complex and the minimum sequence of Vpr capable to bind ANT corresponds to the 71-82 domain (Plate 6). Conversely, the minimum fragment of ANT interacting with (52-96)Vpr is

(104-116)ANT. Vpr behaves as one of the most potent apoptotic factor, and seems to act by opening the mitochondrial membrane pore, resulting in release of caspase activators and other apoptotic factors which trigger a cascade of events leading to cell death.

Insights into the Regulation of MHC Class II Genes: A Potential Route to Immunodepressive Agents

The level of MHC class II expression at the surface of lymphocytes is a key parameter in T-cell activation, and is, therefore, essential for the control of immune responses. The bare lymphocyte syndrome is a genetic disease characterized by the lack of HLA class II antigens at the surface of all cells. This defect leads to severe immunodeficiency, multiple infections and, frequently, death. MHC II genes expression are on the dependence of transcription factors, in particular, the regulatory factor X, RFX discovered by Mach and coll. [55], which interacts with a DNA X box to promote MHC II gene expression. The bare syndrome is owing to mutations in RFX. All RFX proteins are large proteins containing a highly conserved domain of about 76 amino acids, designated DNA binding domain, DBD, which interacts with a palindromic DNA box to trigger the MHC II gene expression. The RFX protein binds DNA under non-covalent dimeric form. We have synthesized large quantities ($\sim$90 mg) of the DBD and several mutants. Using these large peptides, we have determined the minimum DNA binding domain. The interaction of the DBD of human RFX (hRFX1) with the palindromic 23 double stranded DNA base pairs was cooperative, and results in a large stabilization of DNA [56]. The minimum sequence of the X box corresponding to a 16 bp was used to obtain crystal data of a 2DBD/1 oligonucleotide complex. The structure of the DNA binding domain corresponds, as expected, to that of transcription factor with the classical helix-turn-helix arrangment. However, unexpectedly, the DNA recognition mode is new and does not correspond to those previously found for DNA binding factors [57].

Details of the organization of the complex (Plate 7) will be used to try to explain the lack of DNA binding of RFX mutants, particularly for those found in the bare syndrome, and as a scaffold for designing new immunosuppressive agents [55].

CONCLUSION

Inhibition of ACE by captopril has been one of the most impressive achievements in therapeutics. It is now well-established that the physiological regulation of blood pressure by inhibiting the production of A_{II} is probably the only treatment leading to a significant increase life expectancy. In clinic, ACE inhibitors seem not to be overpassed by A_{II} antagonists. There is, therefore, no theoretical reasons for which NEP/APN inhibitors or dual and triple ACE, NEP, ECE inhibitors would not be used in therapeutics, one of the most important problems being the bioavailability of most of these compounds.

Regarding protein–protein interactions, progress in our knowledge of the human genome open the way for multiple approaches aimed at regulating deficiencies in protein-dependent biological processes. There is no doubt that, for peptide and protein specialists, fascinating perspectives lie ahead.

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