tually observing signals from the bound ligand and not from the enzyme, impurities in the enzyme sample can be tolerated.

The precision of the structure that was determined using this approach was quite high with the average rmsd of all heavy atoms from a calculated average structure of 0.45 ± 0.17 Å for the 73 final structures. Furthermore, the NMR-derived structure of bound ascomycin was found to be virtually identical to the recently determined X-ray crystal structure of FK-506 bound to FKBP.[7] Superposition of the common heavy atoms of the two structures, Figure 6, gives an rmsd of 0.56 Å, a value which is within the standard deviation of the rmsd for the 73 final NMR structures. However, the conformation of ascomycin when bound to FKBP was found to be quite different from the conformation of the free ligand either in solution or in the solid state. These results are similar to those obtained for cyclosporin A (CsA) in which the conformation of CsA when bound to cyclophilin was found to be very different from the conformation of free CsA.[23-25] Thus, the three-dimensional structure of the uncomplexed molecule may not be a suitable template for designing new molecules and may be inappropriate for rationalizing structure–activity relationships.

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"Mixed Inhibitor–Prodrug" as a New Approach toward Systemically Active Inhibitors of Enkephalin-Degrading Enzymes

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In order to evaluate the possible advantages of potentiating the effects of the endogenous enkephalins, to obtain analgesia without the serious drawbacks of morphine, it was essential to design systemically active compounds which inhibit the two metabolizing enzymes, aminopeptidase N (APN) and neutral endopeptidase 24.11 (NEP). A new concept combining the idea of "prodrug" and "mixed inhibitor" was therefore developed. Given the high efficiency of β-mercaptoalkylamines as APN inhibitors and of *N*-(mercaptoacyl) amino acids as NEP inhibitors, compounds associating these molecules through disulfide or thioester bonds, which are known to increase lipophilicity and to favor passage across the blood–brain barrier, have been synthesized. An HPLC study indicated that the disulfide bridge was resistant to serum enzymes but was cleaved by brain membrane homogenates, suggesting that the active inhibitors were released in the central nervous system. The validity of the approach was verified by the efficient antinociceptive responses obtained in the hot plate test in mice after iv administration of disulfide-containing inhibitors (ED_{50}s of from 4 to 26 mg/kg on the jump latency time). The analgesic potencies of the "mixed inhibitor–prodrug" RB 101 [$H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOCH_2Ph$] after iv administration were three times greater than those of a similar combined dose of its two constitutive moieties. The separation of the two diastereoisomers constituting RB 101 showed that the analgesia has a stereochemical dependence, the (*S,S,S*)-isomer being more active than the (*S,R,S*)-isomer. Furthermore, in the tail flick test in the rat, RB 101 gave 38% analgesia at a dose of 80 mg/kg. Due to its high efficiency and its longer pharmacological effect, RB 101 was selected for a complete study of its analgesic properties.

Introduction

The antinociceptive and behavioral responses to various physical or mental stresses evoked by interaction of the endogenous opioid peptides enkephalins with opioid receptors have been shown to be potentiated by protecting the peptides from enzymatic inactivation (review in ref 1). The enkephalins are rapidly hydrolyzed in vivo by two well-defined enzymes, neutral endopeptidase, NEP (EC 3.4.24.11), and aminopeptidase N, APN (EC 3.4.11.2). Inhibition of only one of these enzymes, NEP, by thiorphan[2] or SCH 32615,[3] or APN, by bestatin,[4] does not increase the endogenous enkephalin concentration to a level sufficient to induce strong analgesic responses, even after icv administration. To overcome this problem, we have developed the concept of mixed inhibitors, i.e. molecules able to inhibit both enzymes. Among these compounds, hydroxamate-containing inhibitors, such as kelatorphan[5] and especially RB38A,[6] were shown to produce, after icv administration, naloxone-reversible antinociceptive re-

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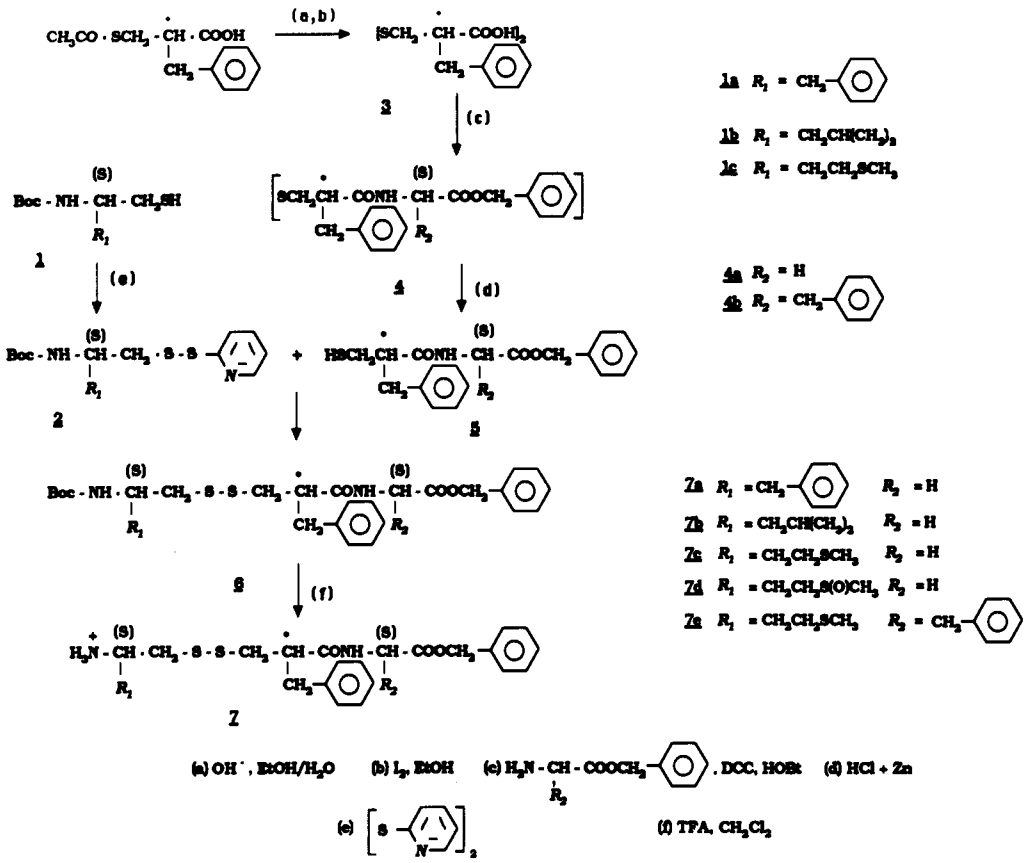

Figure 1. Scheme for the synthesis of "mixed inhibitor–prodrugs" of series A.

sponses significantly higher than those obtained with a mixture of bestatin and thiorphan, in all the pharmacological tests used to screen morphine-like compounds.[7]

Moreover, only limited tolerance effects were observed after chronic icv administration of RB 38A.[8] However, owing to their high hydrophilicity, the mixed hydroxamate inhibitors were unable to enter the brain and were therefore inactive after iv administration. Because of this, no critical evaluation could be made of the possible advantages of potentiating the effects of the endogenous enkephalins by compounds which might induce analgesia without the serious drawbacks of morphine. However, hydrophilic molecules can be transformed into lipophilic prodrugs capable of crossing the blood–brain barrier, with the subsequent liberation of the active form of the inhibitor. Thus, thiorphan was transformed into a more hydrophobic compound, acetorphan [$CH_3COSCH_2CH(CH_2Ph)CONHCH_2COOCH_2Ph$], by the introduction of mercapto- and carboxyl-protecting groups, which are removed in brain tissue by esterases. Likewise we have recently observed that, when associated with acetorphan, various systemically administered β-amino thiols are very efficient at reducing pain stimuli, when they are administered as disulfide forms $[SCH_2CH(R)CHNH_3^+]_2$.[9] These results suggest that the disulfide bridge, which endowes the molecules with sufficient hydrophobicity to cross the blood–brain barrier, is cleaved by a biologically-dependent process, releasing the active compound.[9]

Taken together, these observations led us to develop a new concept, combining the idea of "prodrug" and "mixed inhibitor". The objective was to chemically associate two inhibitors by the intermediate of an ester, thioester, or disulfide bond, in order to increase the global hydrophobicity of each molecule and consequently to facilitate their passage across the blood–brain barrier. After entering the brain, the associated molecules would be physiologically separated by an enzymatic process, simultaneously releasing the two active entities. This approach has the advantage of allowing two inhibitors with different activities to be associated and, therefore, their respective efficiencies to be modulated. Moreover, this overcomes the problem of using two compounds with different pharmacokinetics and bioavailibilities.

Using thiol inhibitors of APN and NEP, three types of "mixed inhibitor–prodrugs" were designed. In the first series (A) a disulfide bridge was formed between the free mercapto groups of both inhibitors, leading to compounds containing a free amino group and a hydrophobic benzyl ester. In the second series (B), a thioester linkage was

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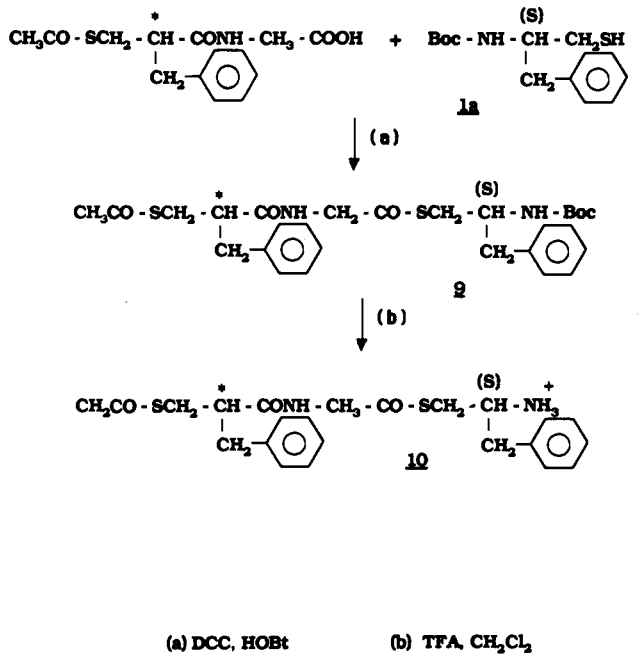

Figure 2. Scheme for the synthesis of the "mixed inhibitor–prodrug" of series B.

formed between the free carboxylate of the NEP inhibitor, whose mercapto group is acetylated, and the thiol group of the β-amino thiol. In the last series (C), the two mercaptans were linked together under thioester forms using a succinyl spacer. The release of the active inhibitors from the prodrugs belonging to the A series was studied by HPLC after incubation with serum and brain tissue. These mixed inhibitor–prodrugs are the first compounds to induce strong antinociceptive responses after systemic administration by protecting the endogenous enkephalins from enzymatic inactivation.

Results

1. **Synthesis.** The first series of mixed inhibitors (A) of general formula **7** (Figure 1) was obtained by coupling *N*-(mercaptoacyl)amino esters **5**, corresponding to the NEP inhibitor moiety, with activated Boc-amino disulfides **2**, prepared by condensation of 2,2′-dithiodipyridine[10] with the Boc-amino thiols **1**. The fully protected inhibitor **6** obtained was transformed to **7** by trifluoroacetic acid treatment. The intermediate *N*-(mercaptoacyl)amino esters **5** were synthesized by coupling 3,3′-dithiobis(2-benzylpropanoic acid) (**3**) with various amino esters by the classical DCC/HOBt method, followed by reduction of the disulfide bond by Zn and hydrochloric acid.

For the second series of inhibitors (B) (Figure 2) a thioester linkage between the Boc-amino thiol **1a** and acetylthiorphan **8**, was achieved using the DCC/HOBt method. Deprotection of the amino function of **9** by trifluoroacetic acid led to compound **10**.

In the last series (C) (Figure 3), the succinyl spacer was firstly introduced on the free thiol group of thiorphan benzyl ester **5a**, leading to compound **11**, which after deprotection of the *tert*-butyl ester and condensation of the Boc-phenylalaninethiol **1a** by the DCC/HOBt method led to the desired inhibitor **13** after deprotection of the amino function.

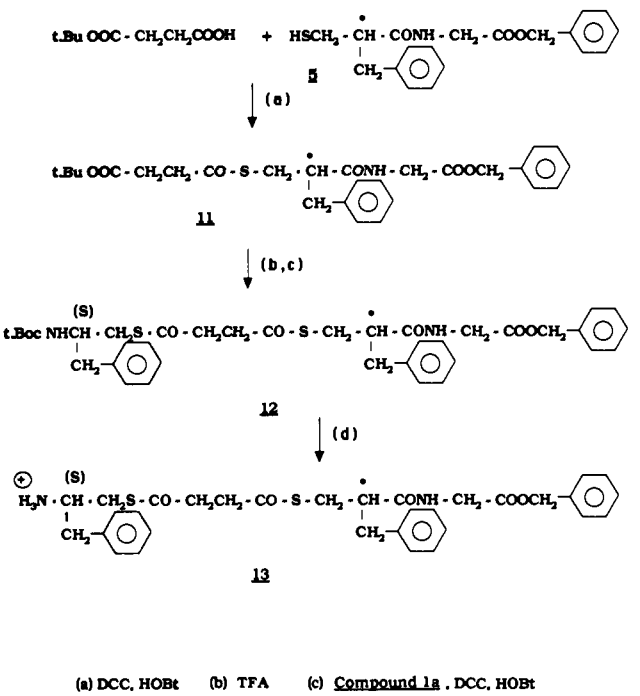

Figure 3. Scheme for the synthesis of the "mixed inhibitor–prodrug" of series C.

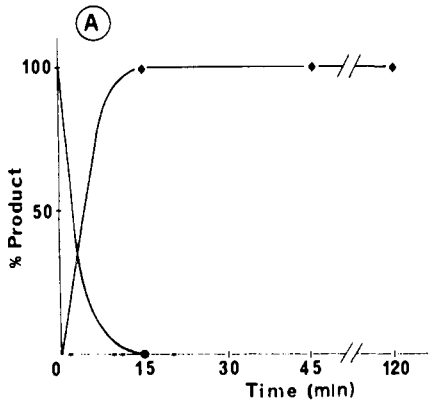

Figure 4. HPLC study of the metabolism of the "mixed inhibitor–prodrug" **7e** ($C = 10^{-4}$ M) in rat serum (3 mg of protein/mL): (●) compound **7e** = $H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOCH_2Ph$, (♦) compound **14** = $H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$.

In all these compounds, the APN inhibitor moieties were derived from α-amino acids of L configuration. Conversely, the NEP blocking counterparts were introduced as a mixture of enantiomers in compounds **7a–d**, **10**, and **13** or as a mixture of diastereoisomers in compound **7e**. In order to evaluate the influence of the stereochemistry of the NEP inhibitors on their in vivo activity, the (*S*)- and (*R*)-isomers of the 2-(mercaptomethyl)-3-phenylpropanoic acid were resolved as previously described[11] and incorporated separately in compound **7e**. The pure stereoisomers obtained were designated **7e**(*S*) [(*S*,*S*,*S*)-isomer] and **7e**(*R*) [(*S*,*R*,-*S*)-isomer]. Their enantiomeric purity was verified by both HPLC and ^1H NMR spectroscopy.

2. **HPLC Studies of the Biologically Dependent Activation of the Mixed Inhibitor–Prodrug 7e in Rat**

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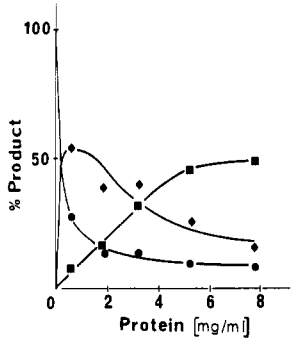

Figure 5. HPLC study of the biological activation of the "mixed inhibitor–prodrug" **7e** ($C = 10^{-4}$ M) by a rat brain membrane preparation at different concentrations of proteins: (●) compound **7e** = $H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOCH_2Ph$, (♦) compound **14** = $H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$, (■) compound **15** = $HSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$.

Serum and Rat Brain Membranes. The prodrug inhibitor **7e**, incubated at a final concentration of 10^{-5} M with rat serum (3 mg of protein/mL) at 37 °C, was completely transformed into a single product after 15 min (Figure 4). A comparison with synthetic standards showed that the compound formed, **14** [$H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$], had lost the benzyl ester group with no alteration of the disulfide bond. The same result was obtained with a longer incubation time (3 h) or using a higher serum concentration (6 mg/mL).

Incubation of compound **7e** with a homogenate of brain membranes gave a different set of peaks. At a protein concentration of 2.7 mg/mL, **7e** disappeared progressively with formation of the disulfide acid **14** arising from hydrolysis of the benzyl ester. However, in contrast to incubations with serum, a second product appeared more slowly. This product, **15**, essentially formed to the detriment of **14**, corresponded to the thiorphan analog $HSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$, indicating disulfide bond cleavage. After 45 min, a plateau was obtained with about 60% of **14** and 25% of **15** formed (not shown). At the same time a signal corresponding to the aminopeptidase inhibitor appeared, but its low sensitivity to HPLC detection methods precluded a quantitative estimation of its levels.

The same experiment, performed with a higher concentration of membrane proteins (Figure 5) and with an incubation time of 45 min, showed that the release of the NEP inhibitor **15** was dependent on protein concentration (around 50% formed for 8 mg/protein). Furthermore, the cleavage of the disulfide bond of **7e** was prevented by heating the membrane homogenate at 80 °C for 15 min before incubation.

3. Analgesic Properties. In a first series of experiments, the various inhibitors were tested on the hot-plate test in mice 15 min after iv administration.

The percentage of analgesia obtained with compounds **10** (series B) and **13** (series C), which contain thioester linkages, are reported in Table I. For compound **10**, significant antinociceptive responses were obtained with an ED_{50} of around 8 mg/kg (1.4×10^{-5} mol) in the jump test. However, as there were some signs of toxicity at doses starting at 20 mg/kg, it was not studied in more detail. Compound **13** was much less efficient, giving only 21% analgesia at 20 mg/kg.

The dose–response curves obtained with the five inhibitors containing a disulfide linkage (compounds **7a–e**) are shown in Figure 6. All the compounds gave highly efficient antinociceptive responses on the jump latency. The cutoff time (240 s) was reached with doses from 20 (compound **7c**) to 60 mg/kg (compound **7a**). The statistical analysis of the curves showed that they can be considered as parallel, allowing the relative efficiencies of the inhibitors to be compared using their ED_{50}s. The order of increasing efficacity was **7a** (26 mg/kg, 5×10^{-5} mol) < **7d** (13 mg/kg, 2.5×10^{-5} mol) < **7e** (9 mg/kg, 1.5×10^{-5} mol) < **7b** (5 mg/kg, 1×10^{-5} mol) < **7c** (4 mg/kg, 0.8×10^{-5} mol). The two separate stereoisomers **7e**(*S*) and **7e**(*R*) were tested in parallel and a comparison was made for each compound with the mixture of isomers **7e** using a single dose of 20 mg/kg. Under these conditions, the percentages of analgesia measured were 60 ± 8% for **7e**, 43.6 ± 7.6% for **7e**(*R*), and 68.5 ± 7% for **7e**(*S*), respectively. For all the compounds tested, the antinociceptive responses were prevented by a prior administration of naloxone (0.1 mg/kg sc) (data not shown).

Table I. Antinociceptive Responses Induced after Intravenous Administration of Compounds **10** and **13** in the Hot-Plate Test (55 ± 1 °C) in Mice (Jump Latency Time)

compd	dose (mg/kg)	jump latency time (s)	% analgesia
10	0	56.7 ± 4.4	
	1	74.1 ± 7.7	9.0 ± 4.0
	5	80.4 ± 8.6	13 ± 5
	10	142.8 ± 18.3	63 ± 13
13	0	48.7 ± 3.0	
	5	46.5 ± 7.0	NS
	10	71.7 ± 7.3	12 ± 3.8
	20	90.1 ± 18.4	21.6 ± 9.6

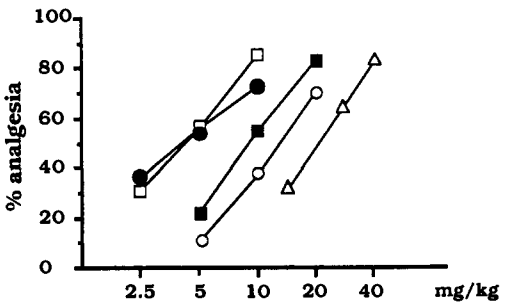

Figure 6. Analgesic dose–response curves observed 15 min after iv administration of the "mixed inhibitor–prodrugs" in the hot-plate test in mice: (□) compound **7c** = $H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH_2COOCH_2Ph$, (●) compound **7b** = $H_2NCH(CH_2CH(CH_3)_2)CH_2SSCH_2CH(CH_2Ph)CONHCH_2COOCH_2Ph$, (■) compound **7e** = $H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOCH_2Ph$, (○) compound **7d** = $H_2NCH(CH_2CH_2S(O)CH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH_2COOCH_2Ph$, (△) compound **7a** = $H_2NCH(CH_2Ph)CH_2SSCH_2CH(CH_2Ph)CONHCH_2COOCH_2Ph$.

Furthermore, in order to verify the validity of our approach, we compared the antinociceptive properties induced by compound **7e** and its two constitutive moieties, ($[SCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOCH_2Ph]_2$) **4b** and ($[H_3N^+CH(CH_2CH_2SCH_3)CH_2S]_2$) **16** (9) combined at the same dose after iv administration (Table II). The mixture of these compounds led to an antinociceptive response which was about 3 times lower than that obtained with the "mixed prodrug" inhibitor **7e**.

In a second series of experiments, the antinociceptive properties of compounds **7b** and **7e** were compared by the tail flick test in rats. As shown in Figure 7, iv administration of the two compounds gave significant responses, which were reversed by pretreatment with naloxone (0.1 mg/kg sc). A shift in the dose–response curve was ob-

Table II. Comparison of the Antinociceptive Responses Induced by Compound 7e and Its Two Constitutive Inhibitors, 16 and 4b, under Their Disulfide Forms on the Hot-Plate Test (55 ± 1 °C) in Mice (Jump Latency Time)

compd			dose (mol/mice)	jump latency time (s)	% analgesia
control				71.6 ± 8.9	
16	$(H_3\dot{N}CHCH_2S]_2$ $CH_2CH_2SCH_3$	(10 mg/kg)	0.75×10^{-6}		
+				126.0 ± 11.0	32.2 ± 6.6
4b	$[SCH_2CHCONH—CHCOOCH_2-C_6H_5]_2$ $CH_2-C_6H_5$ $CH_2-C_6H_5$	(10 mg/kg)	0.46×10^{-6}		
7e		(20 mg/kg)	0.57×10^{-6}	235.5 ± 10.5	92.1 ± 8.5

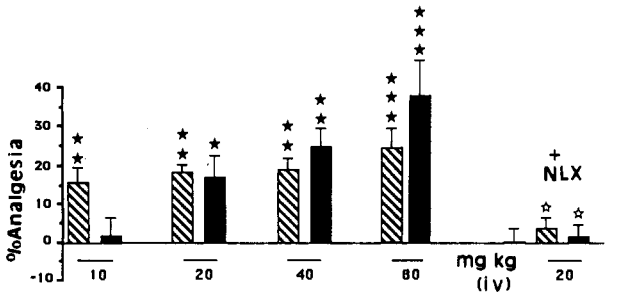

Figure 7. Analgesic dose-response curves observed after iv administration of two "mixed inhibitor-prodrugs" in the tail-flick test in the rat: (striped) compound 7b, (solid) compound 7e (RB 101), (+ NLX) effect of naloxone (0.1 mg/kg, s.c.) administered 10 min before iv injection (20 mg/kg) of the inhibitors.

served for 7e as compared to 7b: at 10 mg/kg, compound 7b gave 15% analgesia, while at the same dose compound 7e was inactive. However at higher doses, compound 7e was significantly more efficient, giving 38% analgesia at 80 mg/kg vs 25% for 7b.

Discussion

In order to obtain mixed inhibitors of enkephalin-degrading enzymes that are active after systemic administration, three series of compounds, connecting highly efficient inhibitors of NEP and APN, have been designed. Thus, thiorphan and its more lipophilic analog $HSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$ (15) (described in the Experimental Section) which inactivate the enzyme in the nanomolar range, and which can be easily used in the construction of a prodrug, were selected as NEP inhibitors. For APN inhibition, we used some recently described efficient inhibitors ($IC_{50} \sim 10^{-8}$ M) which also contain a thiol group as the zinc-coordinating moiety.[9]

The two thioester bonds of compound 10 are probably cleaved in the brain with subsequent release of the active inhibitors, as it acted as an efficient analgesic after iv administration. However, the side effects observed at doses higher than 20 mg/kg precluded a complete study of this molecule. These effects may be due to the intrinsic toxicity of phenylalaninethiol or might reflect the rapid formation of a toxic metabolite.

The low analgesic activity measured after iv injection of compound 13 indicated that this type of prodrug was not well-adapted. It is possible that the protection was too labile, leading the inhibitors to be very rapidly released in the blood. Alternatively the penetration or deprotection rates in the brain could be so slow that only small amounts of the active inhibitors were formed.

For compounds 7a–e, highly significant antinociceptive responses were obtained on the jump latency time in the hot-plate test, with ED_{50} values in the 4–26 mg/kg range (8–50 μmol/kg), indicating that both the disulfide bridge and the benzyl ester were useful for an efficient prodrug construction. The differences observed between these compounds reflected both the variation in the potencies of their constitutive inhibitors in vitro and their own pharmacokinetic properties, but all of them led to an almost maximum (cutoff time) analgesic effect. In the same test, morphine, iv administered, has an ED_{50} of 1.6 mg/kg (4 μmol/kg), which is only 2 times smaller than the ED_{50} of compound 7c. The importance of the pharmacokinetic parameters was also shown when the activity of 20 mg/kg of compound 7e (0.57×10^{-6} mol/mice) was compared to the activity of a mixture of its constitutive inhibitors in their disulfide forms, 0.75×10^{-6} mol/mice for 16 and 0.46×10^{-6} mol/mice for 4b (Table II). The 32% analgesia measured with the mixture of NEP and APN inhibitors was three times lower than that obtained with the mixed inhibitor prodrug 7e. Among other factors, this could indicate a large shift in the peak of activity of one inhibitor with regard to the other, and confirms the necessity of using a single compound to reduce the problem of bioavailability.

The antinociceptive responses obtained after iv administration of compounds 7a–e indicated clearly their ability to enter the brain. It was therefore interesting to characterize the metabolism of these prodrugs by the various enzymes present in the blood or in the brain. In the serum a single chemical transformation corresponding to the hydrolysis of the benzyl ester was observed (Figure 4). A similar result has been previously obtained by incubating the prodrug acetorphan ($CH_3COSCH_2CH(CH_2Ph)CONHCH_2COOCH_2Ph$) with serum, which led to the rapid formation of the monoprotected form, $CH_3COSCH_2CH(CH_2Ph)CONHCH_2COOH$. With brain membrane homogenates, the hydrolysis of the benzyl ester was followed by the cleavage of the disulfide bond. From the kinetics of this latter reaction, it would seem to be due to a biologically dependent process. This was shown by both the plateau observed in the formation of compound 15, reflecting the saturation of the biological process, and the protein concentration dependence of this formation (Figure 5). It was also confirmed by the absence of this bioactivation after prior treatment of the brain homogenates at 80 °C to denature the proteins. These in vitro data suggest that the mixed inhibitor–prodrugs could cross the blood–brain barrier, mainly as their disulfide forms, and that the active compounds are released in the various regions of the central nervous system implicated in pain control more homogenously than after icv administration.

An interesting point, which should be underlined, was the influence of the stereochemistry of the 2-(mercaptomethyl)-3-phenylpropanoyl moiety contained in thiorphan or in the thiorphan analog $HSCH_2CH^*(CH_2Ph)CONHCH(CH_2Ph)COOCH_2Ph$ (5b) on the analgesic activity of the corresponding inhibitors. We have previously

demonstrated[12] that the (S)- and (R)-enantiomers of thiorphan, and of some of their analogs, are equipotent as NEP inhibitors. These data, obtained in vitro, have been confirmed by several laboratories.[13,14] However, it has been reported that in in vivo experiments, (R)-thiorphan, which corresponds to a nonnatural amino acid, is more efficient than the (S)-isomer,[13] while the two enantiomers of acetorphan were equiactive in vivo.[14] The stereoselective synthesis of the pure stereoisomers of compounds **7e**, designated **7e**(S) and **7e**(R), in reference to the absolute configuration of the asymmetric carbon of the 2-(mercaptomethyl)-3-phenylpropanoyl moiety, allowed the antinociceptive properties of each derivative to be measured. As shown here, the isomer **7e**(S) was significantly more active than the **7e**(R) isomer. These data are in agreement with those obtained in our laboratory with the two isomers of thiorphan, which displayed significantly different antinociceptive responses after icv injection since 100 μg of (S)-thiorphan gave 48% analgesia and (R)-thiorphan 20% analgesia in the hot-plate test in mice (cutoff time 240 s) (unpublished results). Taking into account the specificity of enzyme recognition in vivo, these stereochemical dependences are more logical that those previously proposed. For compound **7e**, the difference in the activity of the two stereoisomers was less important than with thiorphan, but differences in the ability to enter the brain and in the catabolism of the inhibitor prodrugs[15] could explain this result. The simultaneous inhibition of NEP and APN led to a series of systematically active compounds (A), which gave strong antinociceptive responses, resulting very likely from protection of extracellularly released endogenous enkephalins, since in all the antinociceptive tests used, the analgesic responses were prevented by prior administration of naloxone. This shows that, following their protection from metabolizing enzymes, the endogenous opioid peptides interact with centrally located opioid receptors to reduce the nociceptive stimuli. This interpretation has been clearly confirmed by in vivo binding experiments in mice showing that intracerebroventricular administration of the mixed inhibitor kelatorphan[16] or iv injection of RB 101[17] induced a large decrease in the binding to brain membranes of the μ agonist [^{3}H]DAMGO or the nonselective antagonist [^{3}H]diprenorphine, respectively.

On the other hand, inhibition of brain-located enzymes could increase the amounts of other extracellularly released centrally active peptides such as substance P, neurotensin (NT), or CCK, which behave, in vitro, as NEP substrates. However, in vivo experiments have shown that, contrasting with their ability to strongly enhance extracellular enkephalin levels, NEP inhibitors have minor effects on the cerebral levels of SP[18] or CCK,[19] and NT seems to be mainly inactivated by a specific peptidase, EC 3.4.24.16.[20] This does not exclude that a part of the analgesic effects induced by the mixed inhibitors could be due to an indirect modulation by the protected enkephalins of the release of peptides involved in pain control such as SP in the spinal cord[21] and/or CCK in particular brain regions.[22,23]

NEP and APN were selected as targets for several reasons. In in vivo experiments, a minor cleavage of the enkephalins at the Gly^2-Gly^3 bond by dipeptidylaminopeptidase activity (DAP) has been observed.[24,25] However intracerebroventricular injection of Tyr-Phe-NHOH, a potent and selective DAP inhibitor, did not produce any antinociceptive effect or potentiate the analgesia induced by the association of thiorphan and bestatin.[26] This argues against a major role of DAP in enkephalin catabolism. In addition to NEP, several membrane-bound aminopeptidases have been shown capable of releasing the N-terminal tyrosine from enkephalins under in vitro conditions. Among these enzymes, APN seems to play a major role in enkephalin inactivation. In rat brain, APN is located on both cerebral microvessels and neuronal membranes.[27,28,29] Moreover, as compared to bestatin and

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thiorphan, the more efficient protecting effect of the mixed inhibitor kelatorphan toward circulating endogenous enkephalins[30] and its significantly higher antinociceptive potency support a preferential involvement of APN in in vivo enkephalin degradation. Kelatorphan has an inhibitory potency for APN which is more than 100 fold higher than for the total membrane-bound aminopeptidases.[31]

In conclusion, a new concept in the design of prodrugs able to efficiently pass the blood-brain barrier before in situ bioactivation has been developed. In addition to simplifying the problems associated with differences in bioavailabilities of a mixture of two compounds, this type of prodrug has the advantage of associating selective inhibitors of two well-defined enzymes whose potencies against their own peptidase can be selected.

Among the various mixed inhibitor-prodrugs described in this paper, compound **7e** (RB 101) which had the longest pharmacological effect, was selected for a complete study of its analgesic properties.[32] It was shown to be active in all the classical tests used for the screening of opiates and, more interestingly, to be devoid of tolerance and dependence side effects after chronic administration in rodents (Noble et al., submitted). This confirms our hypothesis that potentiation of the physiological mechanisms of pain control could give analgesic responses devoid of the severe drawbacks induced by morphine.[1]

Experimental Section

HPLC Studies of Prodrug-Inhibitor 7e Bioactivation. The in vitro formation of the active components of the prodrug-inhibitor **7e** was monitored by HPLC. Compound **7e** (10 μM final concentration) was incubated for 30 min at 37 °C in the presence of rat brain membranes (2.7 and 8 mg of protein/mL) or rat serum (3 and 6 mg of protein/mL) in 450 μL of 50 mM Tris-HCl buffer (pH 7.4). The metabolic process was stopped by addition of 50 μL of 4 M $HClO_4$, and the tubes were kept at 0 °C for 10 min. Acetonitrile (200 μL) was then added to extract products adsorbed to proteins, and the mixture was vigorously agitated and centrifuged for 5 min at 100000*g*. Controls were carried out under the same conditions in the absence of protein or with protein inactivated by prior addition of 4 M $HClO_4$.

Degradation products were separated by HPLC on a Nucleosil 5 μm C8 column (4.6 × 250 mm). The mobile phase consisted of (A) 0.05% trifluoroacetic acid, and (B) acetonitrile/H_2O 7/3 with 0.05% trifluoroacetic acid. The cleavage products formed during the incubations were detected at 214 nm, identified, and quantified by comparison with synthetic markers (solubilized in Tris-HCl buffer/EtOH 90/10). Using a linear gradient rising from 50% to 100% B in 30 min at a flow rate of 1 mL/min, the elution times were 22.7 min for compound **7e**, 15.2 min for $HSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$, 11.6 and 13.6 min for $H_2NCH(CH_2CH_2SCH_3)CH_2SSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$ [(*S,R,S*)- and (*S,S,S*)-isomers], and 27.4 min for $HSCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOCH_2Ph$. In isocratic conditions (14% of B), the elution time of aminopeptidase inhibitor moiety $H_2NCH(CH_2CH_2SCH_3)CH_2SH$ was 7.5 min.

Hot-Plate Test. The test was based on that described by Eddy and Leimbach.[33] A glass cylinder (16-cm height, 16-cm diameter) was used to keep the mouse on the heated surface of the plate, which was kept at a temperature of 55 ± 0.5 °C using a thermoregulated water circulating pump. The latency period until the mouse jumped was registered using a stopwatch. Mice that did not jump within 240 s were removed from the hot-plate and a latency time of 240 s was recorded (cutoff time). Dose-response curves were established by expressing the data as a percentage of analgesia using the following equation: % analgesia = (test latency − control latency)/(cutoff time − control latency) × 100. Statistical analysis was carried out by ANOVA (analysis of variance), followed by Dunnett's *t* test. The ED_{50} was defined as the dose of inhibitor required to elicit 50% analgesia. ED_{50} values and their 95% confidence limits were calculated by log-probit analysis according to the method of Litchfield and Wilcoxon.[34] The antinociceptive responses were prevented by sc injection of naloxone (0.1 mg/kg) 10 min prior to iv administration of the studied compounds.

Tail-Flick Test. The antinociceptive responses were determined by measuring the time required to respond to a painful radiating thermal stimulus, according to the method of D'Amour and Smith.[35] The rat was restrained so that the radiant heat source was focused onto the base of the tail. An automatized tail-flick analgesymeter (Apelex) was used. The cutoff time was set at 15 s. For each rat, three determinations were carried out prior to drug injection (control latency). The tail-flick-latency responses were expressed as a percentage of analgesia calculated by %analgesia = (test latency−control latency)/(cutoff time − control latency) × 100. The intensity of the thermal stimulus was adjusted to obtain a control latency between 4 and 6 s. Results were analyzed by Student's paired *t* test. The antinociceptive responses were prevented by sc injection of naloxone (0.1 mg/kg) 10 min prior to iv administration of the studied compounds.

Chemistry. Amino acids were from Bachem (Bubendorf, Switzerland). Reagents were from Aldrich Chemie (Steinheim, Germany). The solvents were from SDS (Peypin, France).

The purity of the compounds synthesized was checked by thin-layer chromatography on silica gel plates (60F 254, Merck) [using the following solvent system = (v/v): (A) cyclohexane/EtOAc/acetic acid 8/2/0.5, (B) cyclohexane/EtOAc/acetic acid 7/3/0.5, (C) cyclohexane/EtOAc/acetic acid 9/1/0.5, (D) CH_2Cl_2/MeOH 9/1, (E) $CHCl_3$/MeOH/acetic acid 9/1/0.5, (F) CH_2Cl_2/MeOH 9/0.5, (G) CH_2Cl_2/MeOH 7/3] and by HPLC using a reverse-phase Nucleosil C_8 column (SFCC) with CH_3CN/TFA 0.07% buffer (pH = 4.0) as an eluent. The eluted peaks were monitored at 210 nm. The structure of all the compounds synthesized was confirmed by ^1H NMR spectroscopy (Brüker WH, 270 MHz) in DMSO-d_6 solutions (5×10^{-3} M) and by mass spectroscopy (Nermag R10C, DIC NH_3 mode). Satisfactory analyses were obtained (C, H, N) for all compounds. Mass spectra and analysis were carried out on the deprotonated amine forms of the inhibitors. Melting points of the crystallized compounds were determined on an Electrothermal apparatus and are reported uncorrected. The following abbreviations are used: THF, tetrahydrofuran, TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; and HOBt, 1-hydroxybenzotriazole.

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General Procedure for the Synthesis of the *N*-Boc-β-mercaptoalkylamines, Compounds 1. N-Boc-β-(acetylthio)-alkylamine, synthesized as previously described,[9] was dissolved in degassed EtOH. A solution of degassed 1 M NaOH (1.5 equiv) was added at 0 °C under an inert atmosphere. After 1 h at 0 °C and 2 h at room temperature, the mixture was acidified with 3 M HCl, and the solvents were evaporated in vacuo. The residue was taken off in degassed water and extracted with $CHCl_3$. The organic layer was washed with H_2O, dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude compounds were used for the following step of the synthesis.

Compound **1a** ($R_1 = CH_2Ph$): oily product (97%); R_f (A) 0.51; ^1H NMR δ 1.25, ($(CH_3)_3C$) 2.18 (S*H*), 2.72 (CH_2(SH)), 2.61 and 2.77 ($CH_2\beta$), 3.56 ($CH\alpha$), 6.76 (N*H*), 7.16 (Ph). Compound **1b** ($R_1 = CH_2CH(CH_3)_2$): oily product (95%); R_f (B) 0.61; ^1H NMR δ 0.81 ($(CH_3)_2$), 1.25 ($CH_2\beta$), 1.38 ($(CH_3)_3C$), 1.52 ($CH\gamma$), 2.08 (*H*S), 2.40 (CH_2(SH)), 3.41 ($CH\alpha$), 6.62 (N*H*). Compound **1c** ($R_1 = CH_2CH_2SCH_3$): oily product (97%); R_f (B) 0.51; ^1H NMR δ 1.30 ($(CH_3)_3C$), 1.53 and 1.71 ($CH_2\beta$), 1.95 (SCH_3), 2.12 (S*H*), 2.36 ($CH_2\gamma$), 2.45 (CH_2(SH)), 3.41 ($CH\alpha$), 6.13 (N*H*).

General Procedure for the Synthesis of the *N*-Boc-1-alkyl-2-(2-pyridyldithio)ethylamine, Compounds 2. To a solution of 2,2'-dithiodipyridine (40 mmol) in EtOH (20 mL) were added, under an inert atmosphere, CH_3COOH (1 mL) and the Boc-amino thiol (10 mmol) in EtOH (10 mL). The mixture was stirred overnight at room temperature. After evaporation of the solvent, the residue was chromatographed on a silica gel column using cyclohexane/EtOAc/acetic acid 9/1/0.5 as eluent.

Compound **2a** ($R_1 = CH_2Ph$): oily product (51%); R_f (C) 0.51. Compound **2b** ($R_1 = CH_2CH(CH_3)_2$): oily product (72%); R_f (A) 0.42. Compound **2c** ($R_1 = CH_2CH_2SCH_3$): oily product (75%); R_f (C) 0.43.

Synthesis of 3,3'-Dithiobis(2-benzylpropanoic acid), Compound 3. To a solution of 20 g (84 mM) of 3-(acetylthio)-2-benzylpropanoic acid in 250 mL of $EtOH/H_2O$ were added, at 0 °C, 340 mL (4 equiv) of 1 M NaOH. After 30 min at 0 °C and 1 h at room temperature, a solution of I_2 in EtOH (0.3 M) was added until a persistant yellow color was observed. The solvents were evaporated; the residue was dissolved in water, acidified to pH 2, and extracted with EtOAc. The organic layer was washed, dried over Na_2SO_4, filtered, and evaporated in vacuo to yield a white solid (70%): mp 120 °C; R_f (D) 0.18.

Synthesis of *N*-[2-(Mercaptomethyl)-3-phenylpropanoyl]-L-α-amino Acid Benzyl Esters Compounds 5. To a solution of compound **3** (1 equiv) in dry THF were added successively, at 0 °C, a solution of the benzyl ester of the amino acid (2 equiv) and triethylamine (2 equiv) in $CHCl_3$, a solution of 1-hydroxybenzotriazole (2 equiv) in dry THF, and a solution of dicyclohexylcarbodiimide (2.2 equiv) in $CHCl_3$. The mixture was stirred overnight at room temperature. The dicyclohexylurea was filtered, and the solvents were evaporated in vacuo. The residue was taken off in EtOAc, washed with water, a 10% citric acid solution, water, a 10% $NaHCO_3$ solution, water, and a saturated NaCl solution, and dried over Na_2SO_4. After filtration and evaporation of the solvents, compounds **4** were isolated. Compound **4a** (R_2 = H): oily product (87%); R_f (D) 0.91. Compound **4e** ($R_2 = CH_2Ph$): white solid; mp 118 °C (84%)l R_f (A) = 0.40. Compounds **4** were dissolved in degassed MeOH, and 1 M HCl (15 equiv) and Zn (7 equiv) were added. The mixture was stirred for 2 h at room temperature and MeOH was evaporated in vacuo. The aqueous layer was extracted by degassed $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude products were used for the following step.

Compound **5a** (R_2 = H): oily product (97%); R_f (E) 0.76; ^1H NMR δ 2.14 S*H*, 2.31 and 2.56 (CH_2S), 2.63 and 2.78 ($CHCH_2Ph$), 3.80 [CH_2(Gly)], 5.03 OCH_2, 7.13 and 7.27 (Ph), 8.43 (N*H*).

Compound **5e** ($R_2 = CH_2Ph$): white solid (98%); R_f (B) 0.42; ^1H NMR δ 2.01 (SH), centered on 2.56 (CH_2CHCH_2), 2.66 and 2.90 [$CH_2\beta$ (Phe)], 4.46 [$CH\alpha$ (Phe)], 5.02 (OCH_2), 7.20 (Phe), 8.45 (N*H*).

Synthesis of *N*-[2-[[[2-Alkyl-2-(Boc-amino)ethyl]dithio]methyl]-3-phenylpropanoyl]-L-α-amino Acid Benzyl Esters, Compounds 6. To a solution of compound **2** (1 mmol) in EtOH (5 mL) was added, under an inert atmosphere, a solution of the *N*-[2-(mercaptomethyl)-1-oxo-3-phenylpropyl]amino ester **5** (1 mmol) in EtOH (4 mL). After stirring overnight at room temperature, the mixture was evaporated in vacuo and the residue chromatographed on a silica gel column using cyclohexane/EtOAc/acetic acid as eluent.

Compound **6a** ($R_1 = CH_2Ph$, R_2 = H): chromatography with solvent B; oily product (61%); R_f (B) 0.54. Compound **6b** ($R_1 = CH_2CH(CH_3)_2$, R_2 = H): chromatography with solvent C; oily product (73%) R_f (A) 0.51. Compound **6c** ($R_1 = CH_2CH_2SCH_3$, R_2 = H): chromatography with solvent C; oily product (64%); R_f (A) 0.54. Compound **6e** ($R_1 = CH_2CH_2SCH_3$, $R_2 = CH_2Ph$); chromatography in solvent B; white solid; mp 112 °C (65%); R_f (B) 0.42.

Synthesis of Compound 6d ($R_1 = CH_2$)$CH_2S(O)CH_3$, R_2 = H). To a solution of compound **6c** (2.2 g) in EtOH (200 mL) was added at 0 °C a solution of 0.2 M $NaIO_4$ (1.2 equiv) and the mixture was stirred overnight at 0 °C. After evaporation, the residue was dissolved in EtOAc and the organic layer was washed with H_2O and a NaCl-saturated solution and dried over Na_2SO_4. After filtration and evaporation, the crude oily product was purified by chromatography on silica gel using CH_2Cl_2/MeOH = 10/0.5 as eluent: 1.6 g (70%); R_f (F) 0.23.

Synthesis of *N*-[2-[[(2-Alkyl-2-aminoethyl)dithio]methyl]-3-phenylpropanoyl]-L-α-amino Acid Benzyl Esters, Compounds 7. Compounds **6** were dissolved in CH_2Cl_2 (1.5 mL/mmol), and TFA (~20 equiv) was added at 0 °C. After stirring for 1 h at 0 °C and 2 h at room temperature, the mixture was evaporated in vacuo. The oily residues were purified by chromatography on silica gel column.

Compound **7a** ($R_1 = CH_2Ph$, R_2 = H) chromatographed in CH_2Cl_2/MeOH 20/1: oily product (85%); HPLC t_R (CH_3CN/TFA 0.07% 50/50) 12 and 12.1 min; MS m/z = 509 (M + 1); ^1H NMR δ 2.66 and 2.81 ($CH_2CHCH_2 + CH_2Ph + CH_2S$), 3.52 [$(CH(NH_3^+)$], 3.80 [$CH_2$ (Gly)], 5.04 (CH_2O), 7.09 and 7.20 (Ph), 7.88 (NH_3^+), 8.44 (N*H*). Anal. ($C_{28}H_{32}N_2O_2S_2$) C, H, N.

Compound **7b** ($R_1 = CH_2CH(CH_3)_2$, R_2 = H): chromatographed in CH_2Cl_2/MeOH 20/1; oily product (73%); HPLC t_R (CH_3CN/TFA 0.07% 50/50) 8.49 min; MS m/z = 475 (M + 1); ^1H NMR δ 0.82 [$CH_3\delta$ (Leu)], 1.38 [$CH_2\beta$ (Leu)], 1.65 [$CH\gamma$ (Leu)], centered on 2.76 ($CH_2S + CH_2CHCH_2$), 3.28 [$CH\alpha$ (Leu)], 3.80 [CH_2 (Gly)], 5.07 (CH_2O), 7.16 and 7.30 (Ph), 7.85 (NH_3^+), 8.55 (N*H*). Anal. ($C_{25}H_{34}N_2O_3S_2$) C, H, N.

Compound **7c** ($R_1 = CH_2CH_2SCH_3$, R_2 = H): chromatography in CH_2Cl_2/MeOH 20/1; white product (73%); HPLC t_R (CH_3CN/TFA 0.07% 60/40) 11.4 min; MS m/z = 493 (M + 1); ^1H NMR δ 1.37 and 1.60 [$CH_2\beta$ (Met)], 2.00 [CH_3S (Met)], 2.49 ($CH_2\gamma$ (Met)], 2.56 and 2.63 [CH_2CHCH_2 (Ph)], 2.86 [CH_2S (Met)], 3.28 [$CH\alpha$ (Met)], 3.83 [CH_2 (Gly)], 5.06 (OCH_2), 7.15 and 7.30 (Ph), 8.51 (N*H*). Anal. ($C_{24}H_{32}N_2O_3S_3$) C, H, N.

Compound **7d** ($R_1 = CH_2CH_2S(O)CH_3$, R_2 = H): chromatography in CH_2Cl_2/MeOH 8.5/1.5; white solid (78%); R_f (D) 0.21; HPLC t_R (CH_3CN/TFA 0.07% 50/50) 8.6 s. MS m/z = 509 (M + 1); ^1H NMR δ 1.56 and 1.80 [$CH_2\beta$ (Met)], 2.43 ($S(O)CH_3$), 2.62 and 2.86 [$CH_2CHCH_2 + CH_2S + CH_2\gamma$ (Met)], 3.25 [$CH\alpha$ (Met)], 3.78 [CH_2 (Gly)], 5.01 (CH_2O), 7.15 and 7.29 (Ph), 8.53 (N*H*). Anal. ($C_{24}H_{32}N_2O_3S_3$) C, H, N.

Compound **7e** ($R_1 = CH_2CH_2SCH_3$, $R_2 = CH_2Ph$): chromatography in CH_2Cl_2/MeOH/acetic acid 9/0.25/0.25; white solid; mp 96 °C (72%); R_f (E) 0.72 and 0.60; HPLC t_R (CH_3CN/TFA 0.7% 50/50) 11.8 and 12.6 min; MS m/z = 583 (M + 1); ^1H NMR δ 1.70 and 1.78 [$CH_2\beta$ (Met)], 1.96 (SCH_3), centered on 2.53 and 2.79 [$CH_2CHCH_2 + CH_2\beta$ (Phe) + $CH_2S + CH_2\gamma$ (Met)], 3.25 [$CH\alpha$ (Met)], 4.48 [$CH\alpha$ (Phe)], 5.05 (CH_2O), 7.15 (Ph), 7.80 (NH_3^+), 8.53 (N*H*). Anal. ($C_{31}H_{38}N_2O_3S_3$) C, H, N.

The separate synthesis of the two stereoisomers of **7e**, the (*S*,*S*,*S*)-isomer (**7e***S*) and (*S*,*R*,*S*)-isomer (**7e***R*), was performed using the same chemical steps, but with 3-(benzoylthio)-2-benzylpropanoic acid in place of 3-(acetylthio)-2-benzylpropanoic acid. The (*R*)- and (*S*)-isomers of 3-(benzylthio)-2-benzylpropanoic acid were easily separated as described by Bindra (11) using the chiral amine (+)- or (−)-α-methylbenzylamine: (*S*)-3-(Benzoylthio)-2-benzylpropanoic acid, mp 70 °C, $[\alpha]^{20}$ = −41.7°; (*R*)-3-(benzoylthio)-2-benzylpropanoic acid, mp 70 °C, $[\alpha]^{20}$ +43.4°.

Isomer **7e**(*S*): HPLC t_R (CH_3CN/TFA 0.07% 50/50) 11.8 min. Isomer **7e**(*R*): HPLC t_R (CH_3CN/TFA 0.07% 50/50) 12.6 min.

Synthesis of *N*-[2-[(Acetylthio)methyl]-3-phenylpropanoyl]glycine *S*-(2-amino-3-phenylpropyl) Thioester, Compound 10. To a solution of *N*-[3-(acetylthio)-2-benzyl-1-

oxopropyl]glycine (8) (0.27 g) in THF were added, at 0 °C and under N_2, a solution of *N*-Boc-phenylalaninethiol (**1a**) (0.25 g) in THF, a solution of HOBt (0.14 g) in THF, and a solution of DCC (0.23 g) in $CHCl_3$. After 1 h at 0 °C, the mixture was stirred overnight at room temperature and the reaction was determined as described for compounds **5**. The crude, oily product **9** was purified by chromatography in cyclohexane/EtOAc/acetic acid 7.5/2.5/0.5: oily compound (61%); R_f (B) = 0.45. Compound **9** (0.10 g) was dissolved in 0.3 mL of CH_2Cl_2 and 0.3 mL of TFA was added at 0 °C. After 1 h at 0 °C and 3 h at room temperature, the mixture was evaporated in vacuo and the oily residue extensively washed with Et_2O/petroleum ether 50/50: oil (98%); R_f (B) 0.32; HPLC t_R (CH_3CN/TFA) 0.07% 50/50) 12 min; MS m/z = 445 (M + 1); ^1H NMR δ 2.22 (CH_3CO), centered on 2.85 [CH_2CHCH_2 (Ph) + CH_2S and CH_2 (Ph) of phenylalaninethiol moiety], 3.46 [$CH\alpha$ (Phe-thiol)], 3.93 [CH_2 (Gly)], 7.16 (Ph), 7.93 (NH_3^+), 8.70 (N*H*). Anal. ($C_{23}H_{28}N_2O_3S_2$) C, H, N.

Synthesis of *N*-[2-[[[3-[[(2-Amino-3-phenylpropyl)thio]carbonyl]propanoyl]thio]methyl]-3-phenylpropanoyl]glycine Benzyl Ester, Compound 13. To a solution of benzyl *N*-[2-(mercaptomethyl)-3-phenylpropanoyl]glycinate (0.33 g) in THF were added successively at 0 °C and under N_2, a solution of *tert*-butyl hydrogen succinate (0.17 g) in THF, a solution of HOBt (0.15 g) in THF, and a solution of DCC (0.25 g) in $CHCl_3$. After 1 h at 0 °C, the mixture was stirred overnight at room temperature, and the reaction was treated as described for compounds **5** [68%; R_f (A) 0.32]. Compound **11** (0.24 g) was dissolved in 1 mL of CH_2Cl_2, and 1 mL of TFA was added at 0 °C. After 1 h at 0 °C and 2 h at room temperature, the mixture was evaporated in vacuo and the residue extensively washed with Et_2O/petroleum ether. An oily product was obtained [84%; R_f (G) 0.79], which was used without further purification for the following step. To a solution of the preceeding compound (0.11 g) in THF were added successively a solution of Boc-phenylalaninethiol (0.07 g) in THF, a solution of HOBt (0.04 g) in THF, and a solution of DCC (0.07 g) in $CHCl_3$, at 0 °C and under N_2. After 1 h at 0 °C and overnight at room temperature, the reaction was terminated as described for compounds **5**. Compound **12** was obtained as a white solid (0.14 g, 80%) after chromatography on silica gel, using cyclohexane/EtOAc/acetic acid 8/2/0.5 as eluent [R_f (B) 0.47]. Compound **12** (0.06 g) was dissolved in 0.15 mL of CH_2Cl_2, and 0.15 mL of TFA was added at 0 °C. After 30 min at 0 °C and 3 h at room temperature, the mixture was evaporated in vacuo. The residue was extensively washed with Et_2O/petroleum ether 50/50: oily product (60%); purified by chromatography in CH_2Cl_2/MeOH 20/1; R_f (F) 0.64; HPLC t_R (CH_3CN/TFA 0.07% 50/50) 12.6 min; MS, m/z = 593 (M + 1); ^1H NMR δ 2.45 [CH_2 (succinate)], 2.58 and 2.83 [broad massifs containing CH_2S, CH_2 (Ph) of phenylalaninethiol moiety, and CH_2CHCH_2 (Ph) of the benzyl propanoyl moiety], 3.10 [$CH\alpha$ (Phe-thiol)], 3.81 ppm, [CH_2 (Gly)], 5.04 (CH_2O), 7.11 and 7.28 (Ph), 7.90 (NH_3^+), 8.47 (N*H*). Anal. ($C_{32}H_{36}N_2O_5S_2$) C, H, N.

Registry No. **1a**, 141437-85-6; **1b**, 112157-38-7; **1c**, 141437-94-7; **2a**, 141437-86-7; **2b**, 141437-95-8; **2c**, 141437-96-9; **3**, 141437-87-8; **4a**, 141437-88-9; **4e**, 141437-97-0; **5a**, 81110-69-2; **5e**, 135949-97-2; **6a**, 135949-89-2; **6b**, 135949-87-0; **6c**, 135949-90-5; **6d**, 141437-99-2; **6e**, 141437-98-1; **7a**, 135949-57-4; **7b**, 135949-54-1; **7c**, 135949-58-5; **7d**, 135949-61-0; **7e**, 135949-60-9; **7eS**, 141507-09-7; **7eR**, 141507-10-0; **8**, 76932-19-9; **9**, 141437-89-0; **10**, 141437-90-3; **11**, 141437-91-4; **12**, 141437-92-5; **13**, 141437-93-6; APN, 9054-63-1; NEP, 82707-54-8; 2,2'-dithiopyridine, 2127-03-9; 3-(acetylthio)-2-benzylpropanoic acid, 91702-98-6; *N*-[2-(mercaptomethyl)-3-phenylpropanoyl]glycinate, 15026-17-2; *tert*-butyl hydrogen succinate, 15026-17-2; (*tert*-butoxycarbonyl)phenylalaninethiol, 141437-85-6.

New Triazine Derivatives as Potent Modulators of Multidrug Resistance

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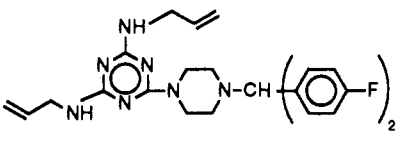
A series of 70 triazine derivatives have been synthesized and tested for their capacity to modulate multidrug resistance (MDR) in DC-3F/AD and KB-A1 tumor cells in vitro, in comparison with verapamil (VRP), a calcium channel antagonist currently used in therapy as an antihypertensive drug, which also shows MDR modulating activity. Among the 12 selected compounds, **16** (S9788) showed high MDR reversing properties in vitro (300- and 6-fold VRP at 5 μM in DC-3F/AD and KB-A1 cells, respectively) and induced a strong accumulation of adriamycin. The relationship between the increase of ADR accumulation and the fold reversal induced by these compounds and their lack of effects on the sensitive DC-3F cells suggest that they act mainly by inhibiting the P-glycoprotein (Pgp) catalyzed efflux of cytotoxic agents, as already described for a majority of MDR modulators. In vivo, in association with the antitumor drug vincristine (0.25 mg/kg), **16** (100 mg/kg) increased the T/C by 39% in mice bearing the resistant tumor cell line P388/VCR. According to these interesting properties, **16** was selected for a clinical development because it was more bioavailable than **34**, even though it was less active.

Multidrug resistance (MDR) is now recognized as a major cause of failure of cancer chemotherapy. Tumor cells having the MDR phenotype are characterized by an increased expression of an energy-dependent drug-efflux pump called P-glycoprotein (Pgp), which lowers the intracellular concentration of cytotoxic agents.[1] One current approach to circumvent this type of resistance is to inhibit this multidrug transporter by noncytotoxic compounds, thus restoring sensitivity to classical cytotoxic anticancer drugs. A wide variety of compounds have now been shown to reverse MDR in vitro, including calcium channel antagonists (verapamil, VRP), calmodulin antagonists (trifluoperazine), antihypertensive agents (reserpine), steroids (progesterone), antiparasitic agents (chloroquine), and immunosupressants (cyclosporins).[2] Among these mod-

Chart I



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