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Preliminary communication

Synthesis and biological activity of the phosphate and sulfate esters of naloxone and naltrexone

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Summary — Prodrugs of the two opiate antagonists naloxone and naltrexone, in particular the 3-monophosphate, 3-triphosphate, 3-monosulfate and 3,14-disulfate esters, have been synthesized and evaluated for: i) their ability to bind opioid receptors *in vitro*; and ii) their stability in both space and time upon entrapment into *ex vivo* human red blood cells (RBC). We find that, unlike the other esters, the mono- and triphosphate esters of naloxone and naltrexone retain high (in the nmol range) affinities especially for μ- and κ-opioid receptors. Owing to their hydrophilic nature, the two esters could possibly help in certain types of pharmacological experiments. Moreover, upon entrapment into human RBC, the triphosphate esters of naloxone and naltrexone display considerable *ex vivo* intracellular stability.

phosphate and sulfate esters of naloxone and naltrexone / opiate antagonists / μ- δ- and κ-opioid receptors / equilibrium binding / encapsulation / red blood cells / metabolic stability

Introduction

Naloxone and naltrexone are two potent antagonists of narcotic analgesics that have been shown to prevent the development of opiate dependence in several animal models [1–3]. The major limitation on the use of these drugs is their short-term effect and lack of incentive for the patient to keep taking the medication [4] and consequently the need of a long-term medical follow-up. Therefore, we considered that the use of carrier erythrocytes as a potential slow release system could block narcotic effects for a long period of time and have a major potential advantage over current oral preparations (for naltrexone) that require dosing daily or 3 times a week [5]. Moreover, the encapsulation of opioid antagonists such as naloxone and naltrexone into red blood cells (RBC) may be a useful administration mode for psychologically fragile subjects because it would prevent long-term repetition of detoxicant drugs. Along these lines, a general method for the encapsulation of substances of biological and/or therapeutic interest into RBC has recently been developed and is presently being applied to a variety of compounds [6–8].

Although large-scale entrapment of drugs into resealed RBC is readily achieved under continuous-flow dialysis [6], the procedure is not directly applicable to naloxone or naltrexone because their hydrophobic nature (they cross the blood–brain barrier) causes them to strongly interact with the erythrocyte's plasma membrane, thus precluding efficient internalization. From a similar approach showing the importance of the hydrophobic nature of naltrexone [9], less hydrophobic derivatives (prodrugs) of naloxone and naltrexone are required which should, among other characteristics, ultimately exhibit: i) high levels of encapsulation and stability within both *ex vivo* and *in vivo* RBC, *ie* following the loading and re-transfusion steps; and ii) the ability to be transformed back into their parent [10, 11]; pharmacologically active and bioavailable drugs should have a natural 'death' ($t_{1/2} \approx 120$ d in humans) in *in vivo* RBC.

In this report, we describe the synthesis of some prodrugs of naloxone and naltrexone, in particular the phosphate and sulfate esters with 3- and/or 14-OH functions. We also report on: i) the affinities of these prodrugs for μ-, δ- and κ-opioid receptor sites *in vitro*; and ii) their stability following their internalization in human RBC.

Chemistry

Phosphates

Initial attempts to synthesize morphine phosphate esters included a phosphorylation procedure with 2-cyanoethylphosphate as the phosphorylating agent [12]. However, this method resulted in poor yields and was not readily adaptable to large-scale preparations. In this paper, we describe an improved and efficient procedure to prepare phosphorylate naloxone **1** and naltrexone **2**.

Monomethylphosphate monosodium salt
Phosphorylation of naloxone or naltrexone with dimethylchlorophosphate [13] as the phosphorylating agent yielded the 3-dimethylmonophosphate ester. Removal of the methyl group from the ester was readily achieved upon the action of sodium iodide [14], which selectively generates the monosodium salt of the 3-methylphosphate.

Monophosphate disodium salt
The disodium salt of the monophosphate was synthesized by *Method A* or *B*.

Method A is based on a phosphite chemistry approach using 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite. This reagent was originally developed by Köster *et al* [15] for the synthesis of DNA and has recently been used to synthesize α-L-fucopyranosyl phosphate [16] and branched ribonucleotides [17]. Hence, naloxone (or naltrexone) was treated with 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite in dichloromethane to give the phosphoramidite derivative. Treatment of the latter with tetrazole and 2-cyanoethanol yielded the phosphite triester. Oxidation of the phosphite with *tert*-butyl hydroperoxide [18] gave the phosphate triester. Finally, β-elimination of the two 2-cyanoethyl groups was achieved by sustained ammonolysis at 50°C for 3 h.

In *Method B* the 3-phosphate group was efficiently introduced by treatment of naloxone (or naltrexone) with NaH [19, 20] in THF at rt or lithium diisopropylamide [21] in THF at –78°C, followed by direct phosphorylation with tetrabenzyl pyrophosphate (TBPP) [22] as a phosphorylating agent. The labile phosphate triester was obtained in excellent yield. The benzyl esters were rapidly cleaved by trimethylsilylbromide (Me_3SiBr) [20] at 0°C in the case of the naloxone-phosphate, and by catalytic hydrogenolysis in the case of the naltrexone-phosphate.

Triphosphate
Among the many procedures described for the preparation of nucleoside triphosphates from nucleoside monophosphates, we selected that using the carbonyldiimidazole method [23–25].

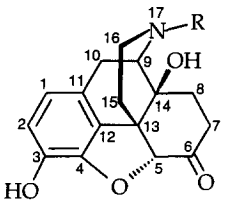
The monophosphate was first converted into its tri-*N*-butylammonium salt, activated with 1,1'-carbonyldiimidazole (CDI) and then treated with the tri-*N*-butylammonium salt of pyrophosphate to yield the expected triphosphate upon purification.

Sulfates

The syntheses of naloxone 3,14-disulfate and naloxone 3-monosulfate have been described by Linder *et al* [26]. By slight modification of the published procedure (reaction times, reagent concentrations), we easily obtained excellent yields. This sulfating procedure was also applied to naltrexone, an analog of naloxone whose sulfate derivatives were previously unknown.

Disulfate
In order to obtain the naloxone and naltrexone disulfates, the 3- and 14-hydroxyl groups were directly sulfated in the presence of excess sulfuric acid using dicyclohexylcarbodiimide (DCC) as the coupling agent.

Monosulfate
Synthesis of 3-monosulfate began with the preparation of the 3,14-diacetate derivative which required heating under reflux for 1 h with excess acetic anhydride. The 3,14-diacetate was then hydrolyzed using 4% aqueous sulfuric acid to remove the 3-acetate group selectively. Finally the 14-acetate was converted into the 3-sulfate derivative by sulfatation followed by alkaline hydrolysis of the 14-acetate group.



1: R = $CH_2CH{=}CH_2$
2: R = CH_2-C-C_3H_5

Scheme 1.

NMR spectral assignments

For analysis and further studies, the phosphate and the sulfate ammonium salts of naloxone and naltrexone (table I) were converted into the sodium salts by ion-exchange chromatography.

The structures of prodrugs of naloxone and naltrexone were determined from ^{1}H-NMR spectra: i) in deuterated dimethylsulfoxide for the sulfate esters and monophosphate esters; ii) in deuterated oxide for the triphosphate esters; and iii) in deuterated chloroform for the acetate and dibenzylphosphate esters. A chemical downfield shift for the doublet corresponding to proton H_2 was observed for the derivatives bearing a sulfate or phosphate group in the 3-position (see table II). A downfield shift was also observed for the H_9 doublet, regardless of the nature of the ester group at position 14. This shift could be considered as the result of the conformation of the ester group at the position 14, and the interaction with the nitrogen in the piperidine ring. These important chemical shifts permit the detection and differentiation of the substitutions of the hydroxyl groups at positions 3 and 14.

Spectral data of **1** and **1f–j** were in accordance with the values obtained by Linder *et al* [26].

Pharmacological evaluation and discussion

Equilibrium binding studies

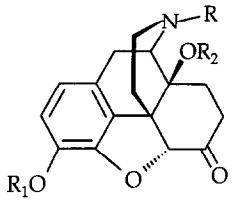
The phosphate and sulfate esters of naloxone and naltrexone were tested for their ability to compete with equilibrium binding of the nonselective opiate antagonist [^{3}H]diprenorphine in a crude membrane fraction of rabbit cerebellum, which contains mostly μ-opioid sites [27], NG 108–15 hybrid cells, which express exclusively δ-opioid sites [28], or guinea-pig cerebellum, which contains essentially κ-opioid sites [29]. The results are shown in table III.

Naloxone **1** and naltrexone **2** were found to display 3–4-fold and 20–40-fold the apparent affinity for μ- than they did for κ- and δ-sites, respectively. Yet naltrexone had a slightly higher affinity for the 3 types of opioid binding sites than naloxone.

In comparison, the 3-sulfate (**1j** and **2j**) and, more so, the 3,14-disulfate (**1f** and **2f**) derivatives of the 2 opiate antagonists showed considerably decreased apparent affinity not only for μ- but also for δ- and κ-opioid sites. This loss of affinity appeared to be mostly due to sulfatation of the 3-OH group.

The monomethylphosphate esters of naloxone **1b** and naltrexone **2b** also exhibited substantially decreased affinity toward the 3 types of opioid binding sites. In contrast, the 3-mono- (**1d** and **2d**) and 3-triphosphate (**1e** and **2e**) esters of the two opiate antagonists retained excellent apparent affinity especially for μ- and κ-receptor sites. In comparison with their parent drugs, **1e** and **2e** showed only a moderately (3–6-fold) decreased ability to bind μ-, δ- and κ-opioid sites. Interestingly, monophosphorylation of the 3-OH groups of naloxone **1d** and naltrexone **2d** led to opposite effects: decreased affinity of **2d** and increased affinity of **1d** for μ- and κ-opioid binding sites.

Table I. Derivatives of compounds **1** and **2**.



Compound	R	R_1	R_2
1	$CH_2CH{=}CH_2$	H	H
1a	$CH_2CH{=}CH_2$	$P(O)(OCH_3)_2$	H
1b	$CH_2CH{=}CH_2$	$P(O)(OCH_3)O^-$	H
1c	$CH_2CH{=}CH_2$	$P(O)(OBn)_2$	H
1d	$CH_2CH{=}CH_2$	$P(O)O_2^=$	H
1e	$CH_2CH{=}CH_2$	TP[a]	H
1f	$CH_2CH{=}CH_2$	SO_3^-	SO_3^-
1g	$CH_2CH{=}CH_2$	Ac	Ac
1h	$CH_2CH{=}CH_2$	H	Ac
1i	$CH_2CH{=}CH_2$	SO_3^-	Ac
1j	$CH_2CH{=}CH_2$	SO_3^-	H
2	CH_2-c-C_3H_5	H	H
2a	CH_2-c-C_3H_5	$P(O)(OCH_3)_2$	H
2b	CH_2-c-C_3H_5	$P(O)(OCH_3)O^-$	H
2c	CH_2-c-C_3H_5	$P(O)(OBn)_2$	H
2d	CH_2-c-C_3H_5	$P(O)O_2^=$	H
2e	CH_2-c-C_3H_5	TP	H
2f	CH_2-c-C_3H_5	SO_3^-	SO_3^-
2g	CH_2-c-C_3H_5	Ac	Ac
2h	CH_2-c-C_3H_5	H	Ac
2i	CH_2-c-C_3H_5	SO_3^-	Ac
2j	CH_2-c-C_3H_5	SO_3^-	H

[a]TP = triphosphate.

Table II. ^{1}H-NMR chemical shifts (δ ppm/TMS)[a] of the phosphate, sulfate, acetate and dibenzylphosphate esters.

Compound	H position H_1	H_2	H_5	H_9
Determined in $CDCl_3$				
1	6.53	6.59	4.80	(-)[b]
1a	6.75	7.35	4.65	(-)
1c	6.61	7.06	4.65	(-)
1g	6.65	6.80	4.65	4.40[c]
1h	6.55	6.70	4.70	4.40
2	6.51	6.57	4.76	(-)
2a	6.75	7.36	4.70	(-)
2c	6.63	7.05	4.67	(-)
2g	6.60	6.75	4.65	4.45
2h	6.65	6.70	4.70	4.40
Determined in DMSO-d_6				
1	6.55	6.71	4.67	(-)
1b	6.75	7.40	4.75	(-)
1d	6.57	7.25	4.81	(-)
1f	6.75	7.35	5.00	4.55
1i	6.80	7.40	5.00	4.50
1j	6.70	7.30	4.90	(-)
2	6.54	6.71	4.68	(-)
2b	6.80	7.40	4.75	(-)
2d	6.65	7.25	4.80	(-)
2f	6.80	7.35	5.00	4.60
2i	6.80	7.40	5.00	4.55
2j	6.75	7.35	5.00	(-)
Determined in (D_2O + CF_3COOH)				
1[d,e]	6.76	6.80	5.01	3.90
1e	6.77	7.26	4.93	3.83
2[d]	6.79	6.84	5.03	4.13
2e	6.78	7.25	4.95	4.06

[a]Determined at 300 MHz; [b]the exact chemical shift of proton H_9 could not be determined because of the superposition of its signal with those of methylene protons; [c]the H_9 appeared as a downfield doublet; [d]as hydrochloride salt; [e]H_{18} protons of compound **1** appeared as a downfield shift at 4.03–4.11 ppm.

Stability of the mono- and triphosphate esters of naloxone and naltrexone in ex vivo human RBC

A prerequisite for prodrugs of naloxone and/or naltrexone to be suited to long-term delivery by RBC is that they remain stable, in both space and time upon entrapment in RBC. *Ex vivo* stability experiments were therefore carried out, in particular with the phosphate esters of the two opiate antagonists. The prodrugs were encapsulated in human RBC and assayed for with HPLC upon incubation of the loaded RBC with autologous plasma (35% final haematocrit) at 4°C and 37°C. Figure 1 shows that at 4°C, there was little if any decrease in the RBC content of the 4 prodrugs tested: the phosphates 3-mono- and 3-triphosphates of naloxone and naltrexone. In contrast, there was a rapid decrease in the intracellular levels of the two monophosphate esters of the two drugs at 37°C. Interestingly, at this physiological temperature, the triphosphate esters of the two opiate antagonists remained nearly totally entrapped for as long as 48 h. Not shown here was the observation that at 37°C most of the monophosphate esters of naloxone and naltrexone were recovered in plasma as 'free' naloxone and naltrexone.

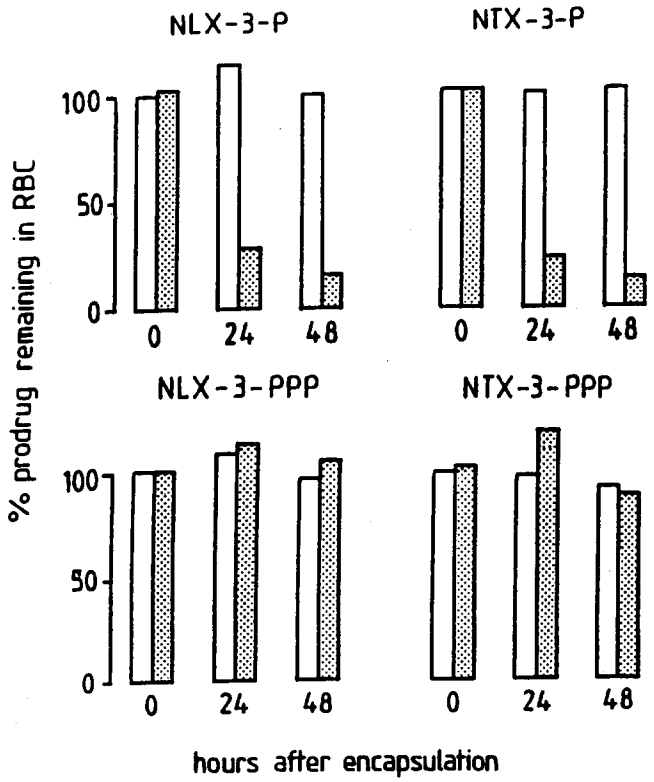


Fig 1. Compared stabilities of mono- and triphosphate esters of naloxone and naltrexone following entrapment in human RBC. The esters (prodrugs) were encapsulated in RBC and assayed for by HPLC upon incubation of the loaded RBC with autologous plasma (35% final haematocrit) at 4°C (white rectangles) and 37°C (shaded rectangles). The results are expressed as percentages of the RBC content of prodrug at the start. NLX: naloxone; NTX: naltrexone; P: phosphate group.

Table III. Opioid receptor binding activities.

Compound	$K_{I\mu}$	*nM* $K_{I\delta}$	$K_{I\kappa}$
1	1.3 ± 0.2 (2)	22 ± 0.7 (2)	4.5 ± 0.8 (2)
1b	260 ± 28 (2)	996 ± 427 (3)	615 ± 93 (3)
1d	0.9 ± 0.08 (2)	96 ± 22 (3)	1.2 ± 0.3 (3)
1e	5.2 ± 0.5 (3)	43 ± 12 (3)	27 ± 3.8 (3)
1f	5200 ± 900 (3)	> 3745 (3)	> 25 000 (3)
1j	1070 ± 98 (4)	2170 ± 593 (3)	7100 ± 1175 (4)
2	0.3 ± 0.05 (3)	13 ± 4.7 (4)	1.2 ± 0.2 (2)
2b	33 ± 2.3 (5)	206 ± 59 (4)	68 ± 8.8 (4)
2d	5.6 ± 0.5 (5)	21 ± 7.5 (3)	7.5 ± 0.8 (4)
2e	1.3 ± 0.1 (3)	21 ± 2.6 (3)	3.8 ± 0.4 (3)
2f	1960 ± 325 (3)	> 3745 (3)	9700 ± 1375 (4)
2j	385 ± 27.5 (3)	397 ± 192 (3)	550 ± 67.5 (3)

Parameters characterizing the inhibition of equilibrium binding of [^{3}H]diprenorphine (0.3 nM) by phosphate and sulfate esters of naloxone and naltrexone in a crude membrane fraction from rabbit cerebellum (μ-opioid sites), NG 108–15 hybrid cells (δ-opioid sites) and from guinea-pig cerebellum (κ-opioid sites); IC_{50} (concentration of unlabelled ligand causing 50% inhibition) values were measured from a modified Hill linear regression analysis transformed by individual inhibition curves whose number is indicated in parentheses; K_I values were calculated from $K_I = IC_{50}\ (1 + L/K_D)^{-1}$, L being the concentration of free [^{3}H]diprenorphine and K_D (0.1 nM in cerebellar tissues [30], 0.6 nM in NG cell membranes [28]), the equilibrium dissociation constant of the [^{3}H]diprenorphine–receptor complex; all values are expressed as means ± SD.

Discussion

The synthesis of phosphate and sulfate esters of naloxone and naltrexone has been detailed in this report. These compounds might constitute prodrugs to be used in a novel sustained release delivery procedure (see *Introduction*) whereby they must be biotransformed back into naloxone or naltrexone. In themselves, they are not expected to cross the blood–brain barrier and reach central opioid receptors upon peripheral administration. However, since these compounds are new (except for **1** and **1f–j**), it was of interest to evaluate their ability to bind with opioid receptors *in vitro*. An interesting issue of this study was that the 3-mono- and triphosphate derivatives of naloxone and naltrexone and, in particular, naloxone 3-phosphate and naltrexone 3-triphosphate, retain high affinity for μ- and κ-opioid receptors. In addition to being less hydrophobic than naloxone and naltrexone, these compounds could be obtained in the ^{32}P-labelled form, and may therefore represent useful probes in biochemical studies on μ- and κ-opioid receptors *in vitro*. They might also be useful in local microinjection experiments in the brain where diffusion of opiate antagonists away from their sites of application is a complicating factor in the interpretation of data.

The main objective of the present study was to design prodrugs of naloxone and naltrexone, which would be suited to sustained long-term delivery by RBC, *ie* i) exhibiting metabolic and intracellular stability once entrapped in RBC; and ii) susceptible to hydrolysis back to the parent drugs once released by RBC *in vivo*. Our data indicate that, unlike their monophosphate esters, the triphosphate esters of naloxone and naltrexone fulfill the first criterion: they do show substantial *ex vivo* intracellular and metabolic stability at physiological temperature (37°C) upon entrapment into human RBC. These triphosphate esters therefore appear to be good candidates as a form for the long–term administration of opiate antagonists.

Experimental protocols

Chemistry

IR spectra were obtained with a Perkin–Elmer 279 infrared spectrophotometer. ^{1}H-NMR spectra were recorded at ambient temperature with a Bruker Model AM–300 spectrophotometer with Me_4Si (TMS) as internal standard. Thin-layer chromatography (TLC) was performed on Merck silica-gel plates. Visualization was accomplished with Dragendorff's solution [31]. HPLC analysis was performed on a Spectra–Physics SP8800 liquid chromatograph, using a Supelco C_{18} column (5 μM; 4.6 mm x 150 or 250 mm). Compounds were detected at 280 nm with the use of a Spectra–Physics 100 variable-wavelength detector. Elution was carried out at a flow rate of 1 ml/min using various percentages of MeOH in aqueous 0.1 M KH_2PO_4. Naloxone hydrochloride and naltrexone hydrochloride were obtained from Francopia–Sanofi and Sigma. The naloxone and naltrexone hydrochlorides were used for the sulfatation procedure, but liberated free bases were used for the syntheses of the phosphates and the acetate derivatives. Microanalyses for the elements indicated were within 0.4% of theoretical values.

Naloxone 3-dimethylphosphate ***1a***
$(CH_3O)_2P(O)Cl$ (216 mg, 1.5 mmol) in benzene (4 ml) was added at –10°C to naloxone (327 mg, 1 mmol) in triethylamine (0.28 ml, 2 mmol). The mixture was then allowed to warm to rt and stirred for 48 h after which the solvent was removed *in vacuo*. The residue was taken up in CH_2Cl_2, washed with aqueous 5% bicarbonate then with water, dried over $MgSO_4$ and the solvent removed *in vacuo* to yield the crude final product. The latter was purified by liquid chromatography on silica gel (eluent: MeOH/CH_2Cl_2, 3:97) to give the pure product (83%). IR (KBr): ν 1240 (P=O), 1720 cm^{-1} (C=O). ^{1}H-NMR ($CDCl_3$): δ 3.90 (d, 2 x OCH_3, J = 11.7 Hz); 4.65 (s, H_5); 5.10–5.30 (m, gem vinylic H); 5.60–5.90 (m, vinylic H); 6.75 (d, H_1, J = 8 Hz); 7.35 (d, H_2, J = 8 Hz). Anal $C_{21}H_{26}NO_7P$ (C, H, N, P).

Naltrexone 3-dimethylphosphate ***2a***
This compound was prepared by the procedure described for compound **1a** with a final yield of 85%. IR (KBr): ν 1240 (P=O), 1720 cm^{-1} (C=O). ^{1}H-NMR ($CDCl_3$): δ 4.00 (d, 2 x OCH_3, J = 11.8 Hz); 4.70 (s, H_5); 6.75 (d, H_1, J = 7.9 Hz); 7.36 (d, H_2, J = 7.9 Hz). Anal $C_{22}H_{28}NO_7P$ (C, H, N, P).

Naloxone 3-monomethylphosphate monosodium salt ***1b***
Naloxone 3-dimethylphosphate (196 mg, 0.45 mmol) was dissolved in a mixture CH_2Cl_2/acetone (1:1, 5 ml). The solution was supplemented with NaI (335 mg, 2.23 mmol) and the resulting mixture was allowed to stand for 24 h at rt. A copious white precipitate formed which was filtered, washed with acetone and allowed to dry *in vacuo* over P_2O_5 (95%). IR (KBr): ν 1240 (P=O), 1720 cm^{-1} (C=O). ^{1}H-NMR (DMSO–d_6): δ 3.80 (d, OCH_3, J = 11.8 Hz); 4.75 (s, H_5); 5.15–5.30 (m, gem vinylic H); 5.80–5.95 (m, vinylic H); 6.75 (d, H_1, J = 8 Hz); 7.40 (d, H_2, J = 8 Hz). Anal $C_{20}H_{23}NO_7PNa$ (C, H, N, P).

Naltrexone 3-monomethylphosphate monosodium salt ***2b***
The demethylation procedure was the same as above. The yield was 96%. IR (KBr): ν 1240 (P=O), 1720 cm^{-1} (C=O). ^{1}H-NMR (DMSO–d_6): δ 3.90 (d, OCH_3, J = 11.8 Hz); 4.75 (s, H_5); 6.80 (d, H_1, J = 8 Hz); 7.40 (d, H_2, J = 8 Hz). Anal $C_{21}H_{25}NO_7PNa$ (C, H, N, P).

Naloxone 3-phosphate disodium salt ***1d***

Method A: direct phosphorylation of naloxone. Naloxone (180 mg, 0.55 mmol) was treated with diisopropylamino(2-cyanoethyl)chlorophosphine (0.16 ml, 0.72 mmol) and diisopropylethylamine (0.19 ml, 1.07 mmol) in dichloromethane (5 ml) to yield the 3-monophosphoramidite in ≈ 99% yield. Reaction of the phosphoramidite with tetrazole (46 mg, 0.65 mmol) and 2-cyanoethanol (0.04 ml, 0.55 mmol) yielded the 3-monophosphite triester. Oxidation with anhydrous *tert*-butyl hydroperoxide (0.2 ml, 1.68 mmol) afforded quantitatively the 3-monophosphate triester. Finally, complete ammonolysis by heating to 50°C with NH_4OH for 3 h removed the 2-cyanoethyl groups to give the phosphate ester as ammonium salt which was further converted into sodium salt by elution from a Dowex 50 x 8 (sodium form) ion-exchange column (55%). IR (KBr): ν 1240 (P=O), 1720 cm^{-1} (C=O). ^{1}H-NMR (DMSO–d_6): δ 4.81 (s, H_5); 5.22–5.37 (m, gem vinylic H); 5.88–6.02 (m, vinylic H); 6.57 (d, H_1, J = 8 Hz); 7.25 (d, H_2, J = 8 Hz). Anal $C_{19}H_{20}NO_7PNa_2$ (C, H, N, P).

Method B: synthesis and debenzylation of naloxone-3-dibenzylphosphate ***1c***. There are two procedures for the synthesis of naloxone 3-dibenzylphosphate **1c**:

In the first, to a solution of lithium diisopropylamide (0.16 mmol) at –78°C was added a solution of naloxone (50 mg, 0.15 mmol) in dry THF (5 ml). After 15 min, TBPP (97 mg, 0.18 mmol) was added and the resulting solution was kept at –78°C under an argon atmosphere for 1 h. The reaction was warmed to 0°C over 1.5 h, quenched with 3 ml of saturated aqueous $NaHCO_3$ and partitioned between CH_2Cl_2 and H_2O. The organic layer was separated, dried over $MgSO_4$ and evaporated. The crude product was chromatographed on silica gel with MeOH/ CH_2Cl_2 (3:97) as the eluent to give 75 mg of pure **1c** (84%) as a gum. IR (film): ν 1245 (P=O), 1725 cm^{-1} (C=O). ^{1}H-NMR ($CDCl_3$): δ 4.65 (s, H_5); 5.15–5.35 (m, $PhCH_2O$ and gem vinylic H); 5.73–5.87 (m, vinylic H); 6.61 (d, H_1, J = 8 Hz); 7.06 (d, H_2, J = 8 Hz); 7.27–7.44 (m, aromatic H). Anal $C_{33}H_{34}NO_7P$ (C, H, N, P).

In the second procedure, sodium hydride (60% dispersion in oil; 32 mg, 1.33 mmol) was added in 1 portion to a stirred solution of naloxone (435 mg, 1.33 mmol) in dry THF (20 ml) under an argon atmosphere at rt. After 10 min, TBPP (1045 mg, 1.94 mmol) in dry THF (5 ml) was added and the mixture was allowed to stand for 18 h. The reaction was quenched with 10 ml of saturated aqueous $NaHCO_3$, and partitioned between H_2O and CH_2Cl_2. The organic layer was dried and evaporated. Chromatography on silica gel with MeOH/ CH_2Cl_2 (3:97) as the eluent gave compound **1c** in high yield (90%).

A solution of naloxone 3-dibenzylphosphate **1c** (76 mg, 0.13 mmol) in 3 ml CH_2Cl_2 at 0°C was treated with a solution of 0.078 ml (0.59 mmol) Me_3SiBr and 0.062 ml (1.48 mmol) pyridine in 2 ml CH_2Cl_2 at 0°C for 2.5 h H_2O (3 ml) was added and the aqueous layer was separated, cooled to 0°C and made alkaline with 1.3 ml (1.29 mmol) 1 N NaOH. After standing for 4.5 h, this solution was applied directly to a column of Duolite AP 143/1096 and eluted with a linear gradient of NH_4HCO_3 (0.0–0.5 M). The appropriate fractions were collected, the solvent removed *in vacuo* and the residue co-evaporated with H_2O several times to give the product as the ammonium salt. Cation exchange (Dowex 50 x 8, Na^+ form) afforded the sodium phosphate salt **1d** (68%) as described in *Method A*.

Naltrexone 3-phosphate disodium salt ***2d***

Method A. In this method the phosphorylation procedure was identical to that described for the naloxone 3-phosphate **1d**. The yield of the final product was 55%. IR (KBr): ν 1250 (P=O), 1730 cm^{-1} (C=O). ^{1}H-NMR (DMSO–d_6): δ 4.80 (s, H_5); 6.65 (d, H_1, J = 7.9 Hz); 7.25 (d, H_2, J = 7.9 Hz). Anal $C_{20}H_{22}NO_7PNa_2$ (C, H, N, P).

Method B. Naltrexone 3-dibenzylphosphate **2c** was prepared by the same procedure as that described for compound **1c**. The yields were 84% (LDA procedure) and 95% (NaH procedure). IR (film): ν 1250 (P=O), 1730 cm^{-1} (C=O). ^{1}H-NMR ($CDCl_3$): δ 4.67 (s, H_5); 5.22 (d, $PhCH_2O$, J = 8.2 Hz); 6.63 (d, H_1, J = 8 Hz); 7.05 (d, H_2, J = 8 Hz); 7.28–7.45 (m, aromatic H). Anal $C_{34}H_{36}NO_7P$ (C, H, N, P).

A solution of naltrexone 3-dibenzylphosphate **2c** (96 mg, 0.16 mmol) in MeOH (8 ml) was shaken with palladium on carbon (10%, 40 mg) under hydrogen atmosphere. When the reaction was complete, the catalyst was removed by filtration. Evaporation of the solvent under reduced pressure resulted in the free acid which was then converted to the sodium salt **2d** by passing through a column of Dowex 50 x 8 (Na^+ form) (84%) as described in *Method A*.

Naloxone 3-triphosphate sodium salt ***1e***. *Preparation of standard solution of tri-N-butylammonium pyrophosphate*
A suspension of anhydrous pyrophosphoric acid (1.78 g, 10 mmol) in 6 ml acetonitrile was cooled in an ice bath and

treated with tri-*N*-butylamine (1.85 g, 10 mmol). The reaction mixture was stirred until the solid dissolved and then allowed to warm to rt. Addition of acetonitrile to give a final volume of 10 ml afforded a 1.0 M standard solution of tri-*N*-butylammonium pyrophosphate.

Tri-N-butylammonium salt of naloxone 3-monophosphate
The free acid of naloxone 3-monophosphate **1d** (250 mg, 0.5 mmol) was suspended in a mixture of 5 ml MeOH and 5 ml EtOH and tri-*N*-butylamine (93 mg, 0.5 mmol) was added. The reaction mixture was heated until the solid had dissolved. The solvent was removed *in vacuo*. After several co-evaporations with dioxane, the dried tri-*N*-butylammonium salt was obtained in quantitative yield.

Preparation of naloxone 3-triphosphate
The naloxone 3-monophosphate tri-*N*-butylammonium salt (0.5 mmol) was taken up in 20 ml of acetonitrile and CDI (325 mg, 2 mmol) was added. The reaction mixture was stirred at rt under an argon atmosphere for 1 d. The solution was treated with dry MeOH (0.06 ml, 1.5 mmol) to remove excess CDI. This gave the monophosphate activated as the imidazolate. After 30 min, an aliquot of the standard pyrophosphate solution (described above) (2 ml, 2 mmol) was added to the CH_3CN solution of the activated monophosphate. After 45 h, the solution was evaporated to dryness *in vacuo*. The residue was chromatographed on a Duolite AP 143/1096, elution with a linear gradient of NH_4HCO_3 (0.0–0.8 M). The appropriate fractions were lyophilized and the residue was co-evaporated several times with H_2O to give the ammonium salt. This was passed through a column of Dowex 50 x 8 (sodium form) resin to give **1e** as the sodium salt (68%). IR (KBr): ν 1245 (P=O), 1725 cm^{-1} (C=O). ^{1}H-NMR (D_2O/CF_3COOH): δ 3.83 (d, H_9, J = 5.8 Hz); 3.90–4.00 (m, two H_{18}); 4.93 (s, H_5); 5.43–5.55 (m, gem vinylic H); 5.68–5.87 (m, vinylic H); 6.77 (d, H_1, J = 8 Hz); 7.26 (d, H_2, J = 8 Hz). Anal $C_{19}H_{20}NO_{13}P_3Na_4$ (C, H, N, P).

*Naltrexone 3-triphosphate sodium salt **2e***
This compound was prepared by the procedure described for compound **1e**. Compound **2e** was obtained in 70% yield. IR (KBr): ν 1240 (P=O), 1720 cm^{-1} (C=O). ^{1}H-NMR (D_2O/CF_3COOH): δ 4.06 (d, H_9, J = 5.9 Hz); 4.95 (s, H_5); 6.78 (d, H_1, J = 8 Hz); 7.25 (d, H_2, J = 8 Hz). Anal $C_{20}H_{22}NO_{13}P_3Na_4$ (C, H, N, P).

*Naloxone 3,14-disulfate sodium salt **1f***
Naloxone hydrochloride (113 mg, 0.28 mmol) and DCC (645 mg, 3.13 mmol) were dissolved in DMF (4 ml) and cooled to 0°C. A chilled 0.5-ml solution of H_2SO_4 (0.2 ml, 3.47 mmol) in DMF was then added and the resulting mixture was stirred for 1 h at 0°C. The reaction mixture was then basified (pH 9) with dilute NH_4OH, filtered (to remove dicyclohexylurea) and the filtrate was evaporated under reduced pressure. The residue was dissolved in DMF (1 ml) and filtered to remove inorganic salts. AcOEt (5 ml) was added to the filtrate to give a white precipitate of 3,14-disulfate ammonium salt. This ammonium salt was then converted into sodium salt using a Dowex 50 x 8 column (Na^+ form) (85%). The description of **1f** was in accordance with the values obtained by Linder *et al* [26].

*Naltrexone 3,14-disulfate sodium salt **2f***
The sulfatation procedure was identical to that described above. The yield of product was 86%. IR (KBr): ν 1250 (OSO_3), 1720 cm^{-1} (C=O). ^{1}H-NMR (DMSO–d_6): δ 4.60 (d, H_9, J = 6.50 Hz); 5.00 (s, H_5); 6.80 (d, H_1, J = 8.2 Hz); 7.35 (d, H_2, J = 8.2 Hz). Anal $C_{20}H_{21}NO_{10}S_2Na_2$ (C, H, N, S).

*Naloxone 3,14-diacetate **1g***
A solution of naloxone (150 mg, 0.46 mmol) in acetic anhydride (4 ml) was refluxed for 1 h. The reaction mixture was evaporated. The residue was dissolved in CH_2Cl_2 and extracted several times with 5% NaOH. The organic layer was collected, washed with water, dried and evaporated under reduced pressure. The residue was chromatographed on silica gel with $MeOH/CH_2Cl_2$ (1:9) as the eluent to give compound **1g** (91%). The description of **1g** was in accordance with the values obtained by Linder *et al* [26].

*Naltrexone 3,14-diacetate **2g***
This compound was prepared by the procedure described for compound **1g**. Compound **2g** was obtained in 83% yield. IR (KBr): ν 1720 (C=O, 14-acetate), 1760 cm^{-1} (C=O, 3-acetate). ^{1}H-NMR ($CDCl_3$): δ 2.15 (s, 14-acetate methyl H); 2.30 (s, 3-acetate methyl H); 4.45 (d, H_9, J = 6.60 Hz); 4.65 (s, H_5); 6.60–6.75 (2d, H_1, H_2, J = 8 Hz). Anal $C_{24}H_{27}NO_6$ (C, H, N).

*Naloxone 14-acetate **1h***
Naloxone 3,14-diacetate **1g** (153 mg, 0.33 mmol) was hydrolyzed with 4% aqueous H_2SO_4 (12 ml) for 24 h at rt. The reaction mixture was then adjusted to pH 8 with dilute NH_4OH and extracted with CH_2Cl_2. The solvents were evaporated under reduced pressure, and the residue was chromatographed on silica gel, with $MeOH/CH_2Cl_2$ (1:9) as the eluent to yield compound **1h** (77%). The description of **1h** was in accordance with the values obtained by Linder *et al* [26].

*Naltrexone 14-acetate **2h***
This compound was prepared by the procedure described for compound **1h**. The yield of final product was 79%. IR (KBr): ν 1730 (C=O, 14-acetate), 3400 cm^{-1} (O-H). ^{1}H-NMR ($CDCl_3$): δ 2.20 (s, 14-acetate methyl H); 4.40 (d, H_9, J = 6.6 Hz); 4.70 (s, H_5); 6.65–6.70 (2d, H_1, H_2, J = 8 Hz). Anal $C_{22}H_{25}NO_5$ (C, H, N).

*Naloxone 3-sulfate 14-acetate ammonium salt **1i***
Naloxone 14-acetate **1h** (96 mg, 0.26 mmol) and DCC (619 mg, 3 mmol) were dissolved in DMF (3 ml) and cooled to 0°C. A chilled 1-ml solution of H_2SO_4 (0.029 ml, 0.52 mmol) in DMF was then added and the mixture was allowed to stand for 1 h at 0°C with stirring. After this time the solution was taken to pH 9 with dilute NH_4OH and filtered (to remove dicyclohexylurea). The filtrate was evaporated under reduced pressure and the residue was redissolved in DMF (1 ml) and filtered to remove inorganic salts. AcOEt (5 ml) was added to the filtrate to give a white precipitate of the 3-sulfate 14-acetate ammonium salt (63%). The description of **1i** was in accordance with the values obtained by Linder *et al* [26].

*Naltrexone 3-sulfate 14-acetate ammonium salt **2i***
This compound was prepared by the procedure described for compound **1i**. The yield of compound **2i** was 50%. IR (KBr): ν 1250 (OSO_3), 1730 cm^{-1} (C=O, 14-acetate). ^{1}H-NMR (DMSO–d_6): δ 2.30 (s, 14-acetate methyl H); 4.55 (d, H_9, J = 6.60 Hz); 5.00 (s, H_5); 6.80 (d, H_1, J = 8 Hz); 7.40 (d, H_2, J = 8 Hz). Anal $C_{22}H_{28}N_2O_8S$ (C, H, N, S).

*Naloxone 3-sulfate sodium salt **1j***
Naloxone 3-sulfate 14-acetate ammonium salt **1i** (47 mg, 0.10 mmol) was dissolved in 20 ml dilute NH_4OH (pH 9),

allowed to stir for 2 h at rt. After evaporation, the residue thus obtained was dissolved in DMF (1 ml), and AcOEt (5 ml) and was then added to give a white precipitate of the 3-sulfate ammonium salt, which was converted into the sodium salt by ion-exchange chromatography (Dowex 50 x 8, Na^+ form) (65%). The description of **1j** was in accordance with the values obtained by Linder *et al* [26].

Naltrexone 3-sulfate sodium salt ***2j***
This compound was prepared by the same procedure as that described for compound **1j**. The yield of compound **2j** was 74%. IR (KBr): ν 1250 (OSO_3), 1720 cm^{-1} (C=O). ^{1}H-NMR (DMSO–d_6): δ 5.00 (s, H_5); 6.75 (d, H_1, J = 8.2 Hz); 7.35 (d, H_2, J = 8.2 Hz). Anal $C_{20}H_{22}NO_7SNa$ (C, H, N, S).

Pharmacology

Opioid receptor binding
Young New-Zealand white rabbits weighing 1.3–1.5 kg were obtained from a local farm. Tri-colored guinea pigs weighing ≈ 0.3 kg were purchased from EVIC–CEBA (Blanquefort, France). NG 108–15 hybrid cells were grown under standard conditions [30] and kindly provided by J Polastron (CNRS, Toulouse).

Preparation of the crude membrane fraction
The animals were killed by decapitation. The cerebellum was rapidly excised, freed from adhering meninges, weighed and immediately processed as follows. The nerve tissue (100 mg) was homogenized at 4°C in a final volume (V_0) of 1.2 ml in 0.32 M sucrose buffered at pH 7.4 with 1 mM Tris–HCl. Homogenization was completed in a Potter–Elvehjem tissue grinder by 20 strokes of a Teflon pestle, motor-driven at 800–1000 rev/min. The crude homogenate was centrifuged at 4°C in a Beckman rotor type 30 for 35 min at 30 000 rev/min. The supernatant was discarded and the pellet was dispersed (Polytron) in a large excess of ice-cold Tris-HCl 50 mM, pH 7.4 ('buffer') and spun again as before. The supernatant was removed and the washed membranes were homogenized (Polytron) in V_0 of 'buffer' to yield the crude membrane fraction with a protein content averaging 5 mg/ml. The crude membrane fraction from NG 108–15 cells was obtained exactly as described above.

Competition experiments were carried out at 25°C in Tris–HCl 50 mM, pH 7.4. Each incubation mixture (0.5 ml) contained 0.03 ml of crude membrane suspension (≈ 0.15 mg of protein) and 0.3 nM (1 nM with NG cell membranes) of [^{3}H]diprenorphine: i) with no other addition (total binding, in sextuplate); ii) in the presence of the desired concentration of unlabelled inhibitor (in triplicate); and iii) in the presence of 10 μM levorphanol (nonspecific binding, in sextuplate).

After a 1-h incubation, the samples were rapidly filtered at rt on glass fiber discs (Whatman GF/B) and washed with 2 5-ml portions of ice-cold buffer. Millipore model 1225 sampling manifolds were used. The filters were then dried for 15 min under an IR lamp and counted for radioactivity in 4 ml of Aquasol-2 (New England Nuclear) cocktail by a Kontron model MR 300 automatic liquid scintillation system. Tritium counting efficiency was ≈ 50%.

Stability of the mono- and triphosphate esters of naloxone and naltrexone in human RBC ex vivo
Blood was collected from informed donors in standard plastic bags containing CPD (45 mM citrate, 15 mM phosphate and 130 mM dextrose). The RBC was washed 3 times with isotonic NaCl solution by centrifugation at 1000 *g* at 4°C for 10 min.

The RBC suspension was adjusted at 70% haematocrit with a NaCl solution containing the prodrug at a final concentration of 1 mg/ml.

The dialysis step was performed on 5 ml RBC suspension, in a cylindric cellulose membrane tubing placed in a bottle filled with 250 ml dialysis buffer, rotated at 4°C for 45 min. The resealing and rejuvenation of the cells were performed by incubation for 10 min at 37°C after addition (1:10, v/v) of modified PIGPA solution (1 vol 12% NaCl and 1.4 vol PIGPA solution (100 mM pyruvate, 100 mM inosine, 100 mM glucose, 100 mM phosphate, 5 mM adenine)), and then incubation for 30 min at 37°C. Finally, lysed and resealed erythrocytes were washed 3 times at 4°C with a NaCl isotonic solution. The loaded RBC were resuspended in their autologous plasma (1:1, v/v) containing streptomycin 1 g/l and bipenicillin 1 M. UI: 2 μl 1 ml (Laboratoire Diamant, France) to prevent hemolysis and incubated for 24 or 48 h at 4 or 37°C. The RBC were then collected by centrifugation (1000 *g*, 10 min, 4°C), washed with isotonic NaCl and hemolyzed with distilled water. The haemolysate was supplemented with a known amount of nalorphine (internal standard) and precipitated with 7.5% (v/v) perchloric acid. The supernatant was alkalinized with 28% ammonia. At this point and when the triphosphate esters were to be assayed, a hydrolysis step aimed at converting these esters into naloxone or naltrexone was included (24% hydrofluoric acid, 60°C, 30 min). The (alkalinized) supernatant was extracted 3 times with chloroform/isopropanol (8:2, v/v), the organic layer was dried under a stream of nitrogen and taken up in HPLC elution buffer (KH_2PO_4/K_2HPO_4, 01 M, pH 7.6 and 8% MeOH).

HPLC was carried out using a C_{18} μBondapack (Waters) column. Elution was with 8–20% methanol gradient in phosphate buffer and detection was by UV absorption at 220 nm. The following retention times were recorded: 7.8 min (naloxone), 11 min (naltrexone), 12.5 min (nalorphine), 7.5 min (naloxone-3-monophosphate) and 8 min (naltrexone-3-monophosphate).

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