R. C. Tsang, P. M. Steiner, *Pediatr. Res.* **11**, 957 (1977).
10. T. J. C. Boulton, I. H. Craig, G. Hill, *Acta Paediatr. Scand.* **68**, 363 (1979).
11. D. W. Bilheimer, N. J. Stone, S. M. Grundy, *J. Clin. Invest.* **64**, 524 (1979).
12. Cord plasma levels of cholesterol in low-density, high-density, and very low density lipoprotein were determined according to the techniques of the National Institutes of Health, Manual of Laboratory Operations Lipid Research Clinics Program [*Lipid and Lipoprotein Analysis*, U.S. Department of Health, Education, and Welfare, Publ. No. 75-628 (1974)]. The levels of DS in umbilical cord plasma were determined by radioimmunoassay [J. E. Buster and G. E. Abraham, *Anal. Lett.* **5**, 543 (1972)].
13. L. Nachtigall, M. Bassett, U. Hogsander, M. Levitz, *Am. J. Obstet. Gynecol.* **101**, 638 (1968).
14. L. Miyakawa, I. Ikeda, A. Nakayama, M. Makyama, *J. Endocrinol.* **69**, 291 (1976).
15. K. Benirschke, *Obstet. Gynecol.* **8**, 412 (1956).
16. G. E. Andersen and B. Friis-Hansen, *Acta Paediatr. Scand.* **66**, 355 (1977); *Pediatrics* **62**, 8 (1978).
17. ———, *Acta Paediatr. Scand.* **65**, 369 (1976); H. A. Cress, R. M. Shaher, R. Laffin, K. Karpowicz, *Pediatr. Res.* **11**, 19 (1977).
18. We thank J. Sonenthal and J. Morrison for technical assistance and M. Arnold for editorial assistance. We also thank J. Buster for the generous gift of the DS antiserum. This research was supported, in part, by NIH grants 1-RO1-HD13234, AG-00306, and HD-11149.

18 December 1979; revised 8 February 1980

Opiate Analgesia: Evidence for Mediation by a Subpopulation of Opiate Receptors

Abstract. *Naloxazone, a hydrazone derivative of the opiate antagonist naloxone, has a high affinity for opiate receptor binding sites. Naloxazone injections reduce opiate receptor binding to extensively washed mouse brain membranes for more than 24 hours, suggesting that the effect is irreversible. High-affinity binding sites are abolished by this treatment, whereas low-affinity sites are unaffected. Naloxazone treatment blocks the analgesic effects of morphine for at least 24 hours but does not prevent death from high doses of morphine. Thus analgesic but nonlethal opiate effects may be mediated by the high-affinity subpopulation of opiate receptors.*

Various pharmacological (*1*) and biochemical (*2*) studies support the existence of multiple opiate receptors. Distinct high- and low-affinity opiate receptor binding sites have been reported (*2*). Sodium selectively inhibits opiate agonist binding by abolishing high-affinity binding, with minimal effects on low-affinity sites; manganese ions enhance agonist binding by increasing the binding capacity of high-affinity opiate receptor sites. Low concentrations of protein-modifying reagents, such as *N*-ethylmaleimide and iodoacetamide, also lower agonist binding by destroying predominantly high-affinity agonist binding sites (*3*, *4*).

Irreversibly acting drugs can often be used as probes for characterizing the functional correlates of receptors. The opiate antagonist chlornaltrexamine and the agonist chloroxymorphamine appear to act irreversibly (*5*). Naloxazone is a monosubstituted C-6 hydrazone derivative of naloxone (Fig. 1) (*6*). In the present study, we show that naloxazone blocks high-affinity binding of ^{3}H-labeled opiates selectively and irreversibly in vivo. Since naloxazone markedly attenuates morphine analgesia with little effect on the lethality of narcotic overdose, it may prove possible to identify highly analgesic opiates, acting at high-affinity sites, that have a lesser potential for lethal effects and are mediated via low-affinity receptors.

Naloxazone was synthesized by adding naloxone to a tenfold excess of hydrazine in ethanol. After 2 hours, sodium borate solution was added and the product extracted into chloroform. After recrystallization from chloroform and petroleum ether, thin white crystals of free base with a melting point of 163°C were obtained; mass spectroscopy showed a single parent-ion with a mass-to-charge ratio (m/e) of 341 (M^+) corresponding to the molecular weight of 341; elemental analysis of C (66.61); H (6.90); O (14.36); N (12.13) compared favorably to the theoretical value for $C_{19}H_{23}O_3N_3$ (C, 66.84; H, 6.79; O, 14.06; N, 12.31). Nuclear magnetic resonance showed a downshift of 0.17 part per million for the hydrogen at C-5. Infrared spectroscopy showed disappearance of the ketone absorbance at 1700 cm^{-1} of naloxone, and a positive test with trinitrobenzylsulfonic acid indicated the presence of free $^-NH_2$. Thin-layer chromatography of naloxazone yielded a single spot with an R_F of 0.74 on silica gel (chloroform, methanol, and concentrated ammonium hydroxide at 132:12:0.9) and an R_F of 0.44 on alumina (chloroform and methanol at 9:1). The R_F of naloxone was 0.86 in the silica gel system and 0.74 in the alumina system.

Table 1. Morphine analgesia and death in naloxone- and naloxazone-treated mice. Mice treated with naloxone or naloxazone were given intraperitoneal injections of morphine sulfate 16 to 20 hours later; survivors were tested for analgesia with the tail-flick assay. Each animal received only one injection of morphine. A mouse was considered insensible to pain if the tail-flick latency was twice as long after morphine administration as before. Control latencies ranged from 3 to 5 seconds.

Morphine dose (mg/kg)	Preliminary treatment			
	Naloxone (200 mg/kg)		Naloxazone (200 mg/kg)	
	Analgesia	Death	Analgesia	Death
2.5	1/10			
5	4/10			
10	14/19		0/10	
25			1/10	
30			0/8	
60			3/9	
90			7/9	
100			8/10	
400		3/8		3/7
500		13/16		10/16

Solutions of naloxone or naloxazone in 0.9 percent NaCl were made with glacial acetic acid (final pH, 7.0) and injected subcutaneously into the back of the neck. Binding assays were performed as previously described, using extensive washing techniques that have been demonstrated to remove reversible ligands (*4*). The effects of naloxazone on morphine analgesia were examined by treating mice with naloxone, naloxazone, or saline, giving them various doses of morphine sulfate, and testing them with the tail-flick assay (*7*); morphine lethality was determined through intraperitoneal injection of high doses of morphine sulfate in groups of mice treated in the same manner.

Opiate receptor binding assays in vitro show that naloxazone competes with both the narcotic agonist [^{3}H]dihydromorphine and the antagonist [^{3}H]naloxone. The inhibition constant of naloxazone for both the [^{3}H]naloxone and [^{3}H]dihydromorphine binding sites is 2 to 4 nM (four replications with 10 to 20 percent variation) and is unaffected by either sodium or manganese ions. In the experiments, single concentrations of the ^{3}H-labeled ligands were used, with no separation of high- and low-affinity sites. Since sodium ions decrease agonist inhibition of [^{3}H]naloxone binding and manganese ions increase it (*3*), the absence of any effect by these ions supports naloxazone's antagonistic character.

One day after the subcutaneous injection of naloxazone (200 mg/kg), [^{3}H]-naloxone binding in whole brain membranes was reduced by 40 percent. Binding remained reduced despite several washings, resuspension, and incubation at 37°C to remove any drug only

0036-8075/80/0502-0514$00.50/0

loosely attached to the membranes (*4*). We attribute this reduction in binding to complete abolition of the high-affinity sites; the effects on low-affinity binding sites were negligible (Fig. 2). Naloxazone induced the same selective reduction of high-affinity binding of [^{3}H]-dihydromorphine and tritiated D-2-Ala-Met-enkephalin. By contrast, binding in naloxone-treated mice was the same as in saline-injected controls.

The effects of naloxazone were dose-dependent, with decreases in binding of 20 to 30 percent apparent at 75 mg/kg. At 200 mg/kg, virtually all high-affinity binding was blocked after 24 hours; this dose is commonly used in experiments. Experiments with [^{3}H]naloxazone indicated that its entry into the brain is only 0.1 percent of the administered dose, whereas the entry of identically administered [^{3}H]naloxone is twice as great. Although 200 mg/kg is close to the lethal dose for naloxone, there is negligible mortality in animals receiving as much as 300 mg of naloxazone per kilogram. After naloxazone administration (200 mg/kg), mice evince a moderate reduction in locomotor activity.

Complete abolition of high-affinity binding of [^{3}H]naloxone was apparent 4, 12, and 24 hours after administration of naloxazone. After 48 hours, high-affinity binding of [^{3}H]naloxone was reduced only 50 percent, whereas after 76 hours binding returned to normal levels. By contrast, naloxone (200 mg/kg) failed to inhibit [^{3}H]naloxone binding after 4, 12, or 24 hours. This time course resembles the prolonged reduction in opiate receptor binding after mice are treated with the irreversibly acting agents chlornaltrexamine and chloroxymorphamine (*5*).

To ensure specificity, naloxazone's effects on muscarinic acetylcholine and alpha- and beta-noradrenergic and benzodiazepine binding were studied with [^{3}H]quinuclidinyl benzilate, [^{3}H]WB-4101, [^{3}H]dihydroalprenolol, and [^{3}H]-flunitrazepam used as ligands (*8*). Preliminary treatment with naloxazone produced no decrease in binding of any of these ^{3}H-labeled ligands despite a 33 percent inhibition of [^{3}H]naloxone binding in the same tissue samples.

To determine the pharmacological relevance of naloxazone's long-lasting blockade of high-affinity opiate receptors, we used a tail-flick assay to measure the antinociceptive effect of morphine on mice 16 to 20 hours after injection of naloxone or naloxazone (200 mg/kg) (Table 1). The median effective dose (ED_{50}) for morphine analgesia in the naloxazone-treated mice was 70.3 ± 4.0 mg/kg, whereas it was only 6.1 ± 0.1 mg/kg in naloxone-treated control animals [determined with a least-squares fit to a log-probit plot; the lines are parallel at the 95 percent level of confidence as determined by the method of Litchfield and Wilcoxon (*9*)]. Thus morphine's analgesic potency was reduced more than 11-fold by naloxazone treatment. As high-affinity binding returned in the naloxazone-treated mice, sensitivity to morphine analgesia also increased. After 48 hours the ED_{50} for morphine analgesia decreased to 27.9 ± 3.6 mg/kg, and after 72 hours (when binding had increased to control levels) the ED_{50} was 7.6 ± 0.05 mg/kg. In naloxone-treated controls, the ED_{50} was 6.4 ± 2.5 and 5.2 ± 1.4 mg/kg after 48 and 72 hours, respectively.

Unlike its effects on analgesia, naloxazone does not induce long-lasting blockade of morphine's lethal effects. Sixteen hours after injection of naloxazone (200 mg/kg), the median lethal dose of morphine sulfate was 435 mg/kg, about the same as for the naloxone-treated controls (Table 1). However, if the pretreated animals were given a dose of naloxone (50 mg/kg) 15 minutes before the lethal dose of morphine, the mice were fully protected from the lethal effects. Thus the lethal effects of morphine in these mice appeared to be opiate-specific since they were reversed with naloxone.

In summary, naloxazone elicits a long-lasting selective reduction in the binding capacity of high-affinity opiate receptor sites. Since the reduction persists despite extensive washing, it appears likely that the drug binds irreversibly to the receptors by means of covalent linkages. Chlornaltrexamine and chloroxymorphamine, which possess reactive alkylating groups, reduce opiate receptor binding with a similar time course; it is unclear whether they affect high- and low-affinity sites differentially. The 3 days required for restoration of binding after administration of these three drugs might reflect the time required for regeneration of new opiate receptors, thus providing an index to the turnover rate of the receptors.

The long-lasting blockade of analgesia by naloxazone suggests that high-affinity receptor sites mediate opiate analgesia. The failure of naloxazone to protect for

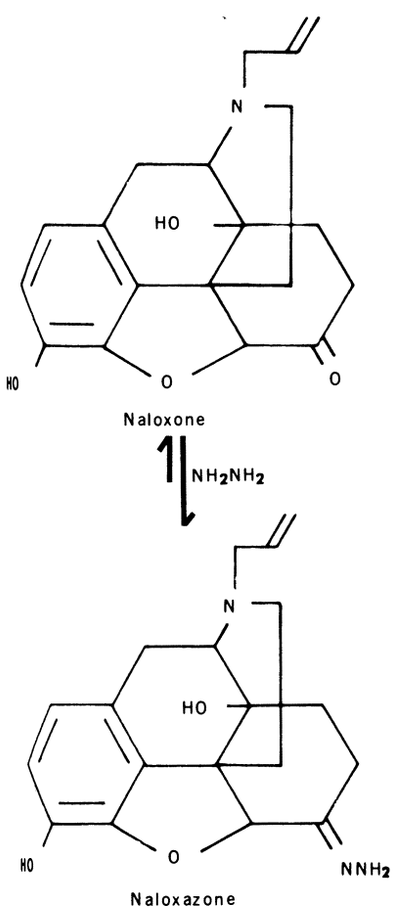

Fig. 1. Synthesis of naloxazone.

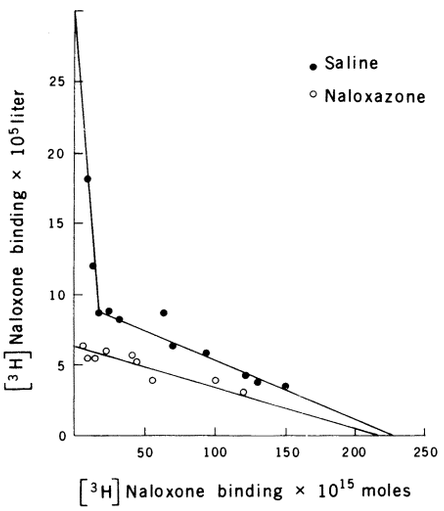

Fig. 2. Scatchard analysis of [^{3}H]naloxone binding in naloxazone-treated mice. Mice were injected subcutaneously with naloxazone (200 mg/kg), saline, or naloxone (200 mg/kg) and killed 24 hours later. The brains were homogenized and washed to remove any reversibly bound drug. Brain tissue (20 mg, wet weight) was then assayed with [^{3}H]naloxone (88 pmole to 5.4 nmole). Saline controls have about 24 fmole of high-affinity binding sites with an affinity constant of 0.84 n*M* and 205 fmole of low-affinity binding sites with an affinity constant of 2.4 n*M*. The naloxazone-treated mice showed no high-affinity binding and had 218 fmole of low-affinity binding sites with an affinity constant of 3.4 n*M*. All values represent stereospecific binding only. The experiment was repeated three times, with the affinity constant and maximum number of binding sites for high- and low-affinity sites varying by less than 20 percent.

long periods against morphine lethality suggests that the high-affinity sites do not mediate the lethal effects, which presumably include respiratory depression and interference with cardiovascular function (*10*). The ability of naloxazone to differentiate between analgesia and opiate mortality suggests that different subpopulations of receptors mediate various pharmacological effects. The distinct high- and low-affinity receptors described here do not appear to correspond to mu, kappa, and sigma receptors identified on pharmacological grounds (*1*) or to enkephalin or morphine binding sites (*2*). Conceivably, opiates with affinities for certain subpopulations of receptors might be selected to elicit analgesia without respiratory depression.

GAVRIL W. PASTERNAK
Cotzias Laboratory of Neuro-Oncology, Memorial Sloan-Kettering Cancer Center, and *Departments of Neurology* and *Pharmacology, Cornell University Medical College, New York 10021*
STEPHEN R. CHILDERS
Department of Pharmacology, University of Florida College Medical School, Gainesville 32610
SOLOMON H. SNYDER
Departments of Pharmacology and Experimental Therapeutics and *Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

References and Notes

1. W. R. Martin, C. G. Eades, J. A. Thompson, R. E. Huppler, P. E. Gilbert, *J. Pharmacol. Exp. Ther.* **197**, 517 (1976).
2. G. W. Pasternak and S. H. Snyder, *Nature (London)* **253**, 563 (1975); *Mol. Pharmacol.* **10**, 183 (1974); J. A. H. Lord, A. A. Waterfield, J. Hughes, H. W. Kosterlitz, *Nature (London)* **267**, 495 (1977); K.-J. Chang and P. Cuatrecasas, *J. Biol. Chem.* **254**, 2616 (1979); K.-J. Chang, B. R. Cooper, E. Hazum, P. Cuatrecasas, *Mol. Pharmacol.* **16**, 91 (1979).
3. G. W. Pasternak, A. M. Snowman, S. H. Snyder, *Mol. Pharmacol.* **11**, 735 (1975); H. A. Wilson, G. W. Pasternak, S. H. Snyder, *Nature (London)* **253**, 448 (1975).
4. G. W. Pasternak, H. A. Wilson, S. H. Snyder, *Mol. Pharmacol.* **11**, 478 (1975). Preparation of the brain membranes included standard washes and incubation at 37°C to facilitate dissociation of reversibly bound endogenous and exogenous ligands. (This procedure effectively removes all endogenous enkephalins and endorphins as well as high doses of narcotics.) All points were assayed in triplicate at 25°C in the presence or absence of 1 μM levallorphan and are reported here as the difference between the two sets (stereospecific binding). Triplicates routinely varied by less than 8 percent, and all experiments were repeated at least three times.
5. P. S. Portoghese, D. L. Larson, J. B. Liang, A. E. Takemori, T. Caruso, *J. Med. Chem.* **21**, 598 (1978); T. P. Caruso, A. E. Takemori, D. L. Larson, P. S. Portoghese, *Science* **204**, 316 (1979).
6. S. H. Snyder, S. R. Childers, G. W. Pasternak, in *Advances in Pharmacology and Therapeutics*, J. Jacob, Ed. (Pergamon, New York, 1978), vol. 1, pp. 39-46; G. W. Pasternak and E. Hahn, *J. Med. Chem.*, in press.
7. F. E. D'Amour and D. L. Smith, *J. Pharmacol. Exp. Ther.* **72**, 74 (1941). After determining the baseline latency, we gave the animals morphine sulfate intraperitoneally and again determined the tail-flick latency.
8. H. I. Yamamura, S. J. Enna, M. J. Kuhar, Eds., *Neurotransmitter Receptor Binding* (Raven, New York, 1978).
9. J. T. Litchfield and F. Wilcoxon, *J. Pharmacol. Exp. Ther.* **96**, 99 (1949).
10. J. Jaffe and W. R. Martin, in *The Pharmacological Basis of Therapeutics*, L. S. Goodman and A. Gilman, Eds. (Macmillan, New York, 1975).
11. Supported in part by a Pharmaceutical Manufacturers Association Foundation research starter grant and E. I. Lilly research grant to G.W.P., National Cancer Institute core grant CA-08748 to the Sloan-Kettering Institute, and Johns Hopkins Drug Abuse Research Center grant DA-00266. G.W.P. is a recipient of NINCDS teacher-investigator award 1K07NS415-01. We thank Adele Snowman, Marla Bowman, and Mary Buatti for their excellent technical assistance; Ercelia A. Kersane for secretarial help; and Drs. Jason Umans, Elliott Hahn, and Charles Inturrisi for their aid.

5 December 1979

Inhibition of Cell Motility by Interferon

Abstract. *Interferon derived from human leukocytes, human fibroblasts, and mouse fibroblasts was found to inhibit the motility of cultured cells. It inhibits the tumor-induced motility of capillary endothelial cells as well as the spontaneous migration of other cell types. The ability of a given preparation of interferon to inhibit the motility of a given cell type is proportional to its antiviral activity in that particular cell type. Antiserum to human leukocyte interferon neutralizes both the motility-inhibitory activity and the antiviral activity of this preparation.*

Although interferon originally captured interest as an antiviral agent, it has become increasingly clear that this molecule can regulate other biological activities. Interferon inhibits cell multiplication in vivo and in vitro, modifies reactions of the immune system in vivo, and is currently being tested as an antitumor agent in animals and humans (*1*). Whereas much is known about the mechanism of interferon's antiviral activity (*2*), the mechanism by which it exerts antitumor activity is unclear.

Recently, in an effort to study the way in which new capillaries are induced to migrate toward a growing tumor, we developed a quantitative in vitro assay to study the motility of cultured cells. Analysis of cell motility is performed by measuring the dimensions of the phagokinetic tracks (*3*) left by cells as they move across gold-coated cover slips. It has been found that tumor cell extracts dramatically stimulate the phagokinetic movement of capillary endothelial cells (*4*). We report here that interferon

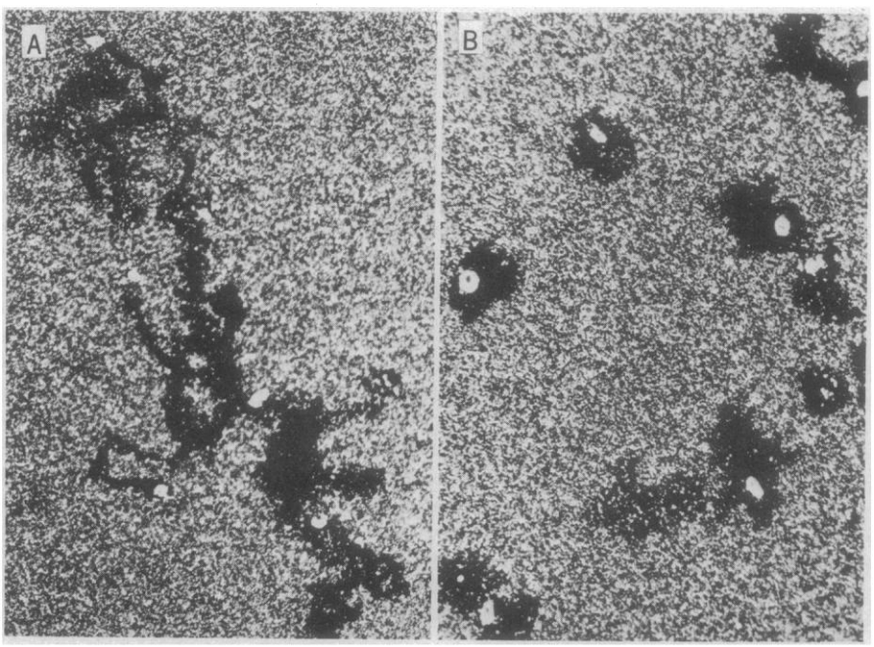

Fig. 1. Inhibition of tumor-stimulated bovine capillary endothelial cell migration by human leukocyte interferon. Bovine capillary endothelial cells (3000) were seeded onto gold-coated glass cover slips (22 mm square) prepared according to the method of Albrecht-Buehler (*3*). The cultures were incubated at 37°C in sarcoma-conditioned Dulbecco's modified Eagle medium containing 10 percent calf serum (A) or in the same medium plus 64 antiviral units of human leukocyte interferon (1.5×10^6 units per milligram of protein) (B) (*6*). After an additional 36 hours, the medium was removed and replaced with 2.5 ml of 10 percent phosphate-buffered Formalin (Fisher Scientific) to terminate the experiment. The phagokinetic tracks were observed under incident light with a Nikon MS inverted microscope and photographed with a Wild Heerbrugg MKA4 photoautomat camera (magnification, × 150).

0036-8075/80/0502-0516$00.50/0