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RAPID COMMUNICATION

EXAMINATION OF THE POSSIBLE MEDIATION OF ANTINEOPLASTIC EFFECTS OF OPIATES THROUGH THE INHIBITION OF TYROSINE-SPECIFIC PROTEIN KINASES

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It has been known for a number of years that opiates and compounds of related structure can influence the course of neoplastic processes. Opioid agonists and antagonists have been shown to modulate the growth of a variety of tumors *in vivo* and *in vitro* in a stereospecific manner [1,2]. In addition, compounds possessing opioid structures such as several (+)-morphinans [3,4] and an unusual polyoxygenated morphinan [5] have also been shown to have antitumor properties. The opioids exert many of their analgesic actions through interaction with specific receptors which have as their endogenous ligands a family of tyrosine-containing peptides, the enkephalins and the endorphins [6]. It is not surprising, then, that enkephalins and enkephalin-like peptides also have antimetastatic and growth regulatory properties [7,8]. While opioid peptides and receptors are expressed in a number of human tumors, the precise role that they play in tumor growth is not known.

Opioid peptides universally contain a tyrosine residue, and classical structure-activity studies on morphine and related compounds have shown the importance of a hydroxyl-bearing aromatic ring and a basic nitrogen for potent analgesic activity [9]. Such features reflect the ability of these non-peptide compounds to mimic specific tyrosine residues of the endogenous opioid peptides (Fig. 1). The carbocyclic opioids underscore the importance of non-peptide molecules which mimic peptide ligands at their receptors in physiological systems. Such mimicking has provided the basis for potent and specific non-peptide inhibitors in other systems. For example, the extremely potent benzodiazepine-based inhibitors of cholecystokinin (CCK) and gastrin exert their activity by mimicking portions of the CCK/gastrin peptides and provide examples of the potential value of developing non-peptide ligands for peptide receptors [10].

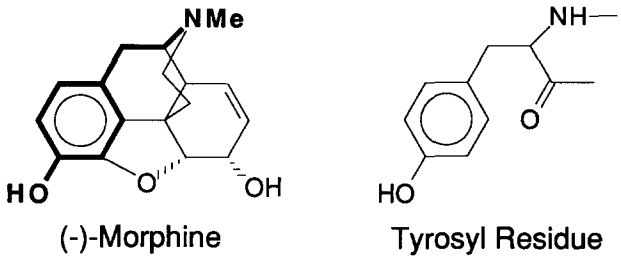

Fig. 1. Structural features of (-)-morphine which mimic a tyrosyl residue.

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Tyrosine-specific protein kinases (TPK) are an important class of enzymes which play central roles in growth and differentiation, and they have been implicated in a number of neoplastic processes [11]. Signal transduction by these enzymes occurs through the binding of tyrosine-containing proteins which are subsequently phosphorylated at the phenolic 4-hydroxyl of specific tyrosine residues. Compounds which inhibit the function of TPKs by competing with substrate binding to the enzyme have been shown to be antiproliferative [12], and TPK inhibitors provide potential approaches toward the development of antineoplastic drugs. It is interesting to speculate whether at least some of the antiproliferative effects observed with certain opioids can be attributed to inhibition *in situ* of TPKs. Such activity could occur through competition with endogenous substrates by mimicking tyrosine-containing peptides. To investigate this, a study was undertaken to examine the ability of a wide range of structurally diverse opioids to inhibit epidermal growth factor receptor (EGFR) tyrosine kinase.

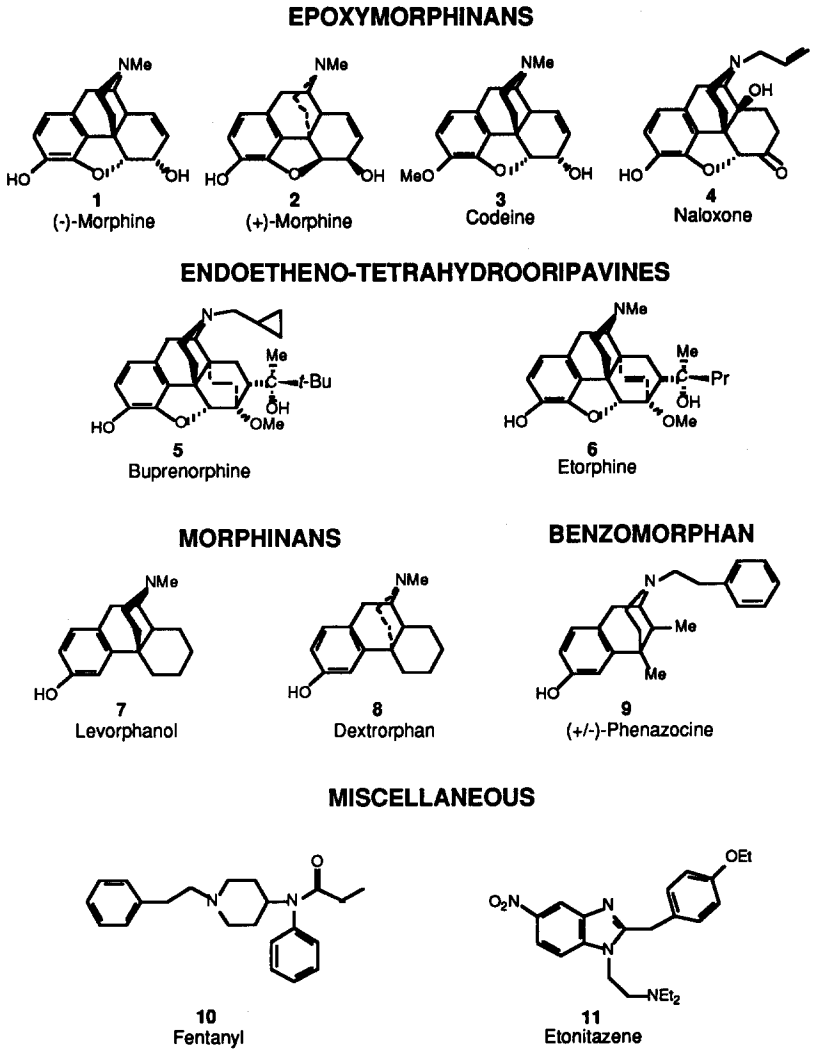

Fig. 2. Structures of opioids tested for inhibition of EGFR autophosphorylation.

MATERIALS AND METHODS

The synthesis of (+)-morphine hydrobromide (**2**) has been described previously [13]. A-431 cells were grown in Dulbecco's Modified Eagle Medium (D-MEM, Gibco). Cells were lysed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) NP40, 2 mM ethylenediaminetetraacetic acid, 100 μM orthovanadate, and 10 mg/mL each of aprotinin and leupeptin. Insoluble cellular debris was removed by centrifugation and 20 μg of cell lysate was immunoprecipitated on ice with 5 μL (0.1 μg/μL) of monoclonal anti-human EGF receptor IgG (Upstate Biotechnology, Inc.). Immune complexes were collected with formalin-fixed *Staphylococcus aureus* cells precoated with affinity-purified rabbit anti-mouse immunoglobulin. Following three washes with lysis buffer, one wash with lysis buffer containing 1 M NaCl, one wash with lysis buffer without NaCl and one wash with kinase buffer (with $MnCl_2$), immune complex kinase assays were performed by adding 20 mM morpholinopropane sulfonic acid, pH 7.5, 5 mM $MnCl_2$, 10 μM ATP, 20 μCi [γ-^{32}P]ATP (3000 Ci/mmol, New England Nuclear), and inhibitors at various micromolar concentrations. The typical concentrations of inhibitors were: 0, 0.1, 0.5, 1, 5, 10, 25, 50, 100, 250, 500, and 1000 μM. Samples were analyzed on 8% sodium dodecyl sulfate-polyacrylamide gels, and radioactive bands were detected by autoradiography. The percentage of autophosphorylated EGFR was plotted against the concentration of the inhibitors.

RESULTS AND DISCUSSION

The structures of compounds tested are shown in Fig. 2. Representative examples of the major classes of opioid nuclei included epoxymorphinans [(-)-morphine (**1**), (+)-morphine (**2**), codeine (**3**) and naloxone (**4**)]; endoetheno-tetrahydrooripavines [buprenorphine (**5**) and etorphine (**6**)]; morphinans [levorphanol (**7**) and dextrorphan (**8**)]; a benzomorphan [(+/-)-phenazocine (**9**)] as well as the anilidopiperidine, fentanyl (**10**) and the benzimidazole, etonitazene (**11**). With the exception of (+/-)-phenazocine (**9**), none of these compounds significantly inhibited the autophosphorylation of EGFR up to concentrations of 1000 μM. A plot of the inhibition versus concentration for (-)-morphine, which is typical of the inactive opiates, is shown in Fig. 3. (+/-)-Phenazocine itself exhibited marked inhibition (IC_{50} = 12 μM) in the range of the potent tyrosine kinase inhibitor erbstatin (**12**) [14] which was run as a control in these assays (IC_{50} = 5 μM; Fig. 3).

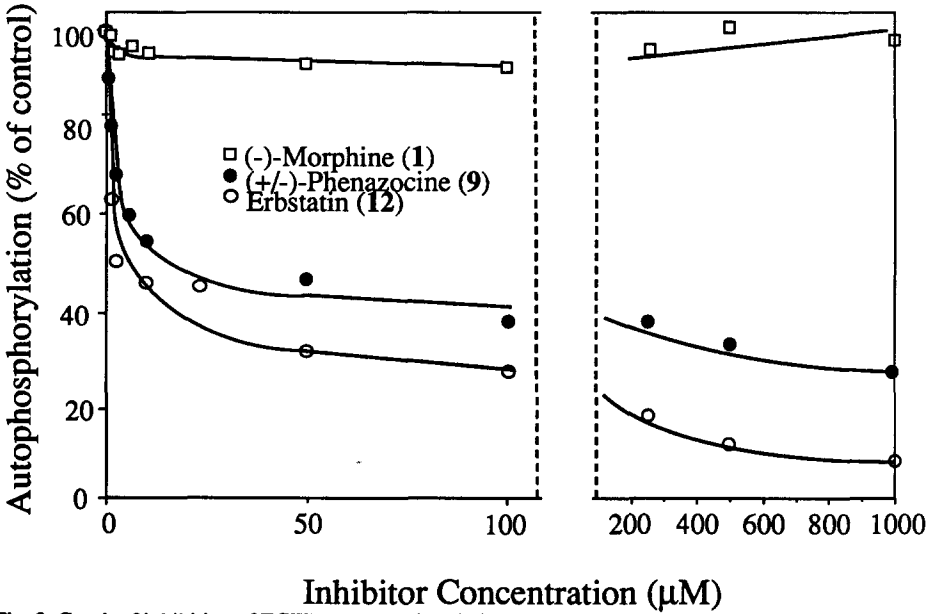

Fig. 3. Graph of inhibition of EGFR autophosphorylation versus concentration of inhibitor. Typical control values were approximately 800 cpm. Conditions are as described in the Materials and Methods.

At present few inhibitors of EGFR kinase exhibit IC_{50} values below the micromolar range [15-17] and the IC_{50} of 12 μM for (+/-)-phenazocine places it as one of the more potent inhibitors of EGFR kinase. However, at a drug concentration of approximately 200 μM, the inhibition observed with (+/-)-phenazocine reached a plateau of 65% and remained unchanged at that level even at concentrations up to 1000 μM. In contrast, erbstatin achieved inhibitory levels of approximately 90% above 500 μM (Fig. 3). The reasons for

this difference in maximum inhibition are unclear; however the inability of (+/-)-phenazocine to exceed 65% inhibition may point toward a mode of interaction with the EGFR which differs from that of erbstatin.

The apparent lack of kinase inhibition by the opiates and other related drugs in this test series indicates that, in general, such drugs do not appear to act as TPK inhibitors, and the observed antineoplastic activity of some members of the opiate family may be attributable to causes other then TPK inhibition. The anomalous ability of (+/-)-phenazocine to inhibit EGFR autophosphorylation is difficult to explain on structural grounds. As a member of the benzomorphan class of opiates [9], (+/-)-phenazocine retains partial features of both the epoxymorphinans (of which morphine, codeine and naloxone are members) and the morphinans (of which levorphanol and dextrorphan are members), all of which were not active in the test. One structural feature present in (+/-)-phenazocine which these other molecules lack is the phenylethyl substitution on nitrogen. The type of nitrogen substitution is extremely important for conferring pharmacological profiles of opiates [9] and, by analogy, the phenyl ring of phenazocine may provide a key feature for enzyme recognition by the EGFR. This is supported by the fact that several other potent inhibitors of EGFR kinase function also contain two phenyl rings [15-17]. However, since fentanyl and etonitazene also contain multiple aromatic rings but do not inhibit EGFR kinase function, additional structural features are also required for kinase inhibition.

Structurally, (+/-)-phenazocine potentially represents an important lead in the design of a new class of TPK inhibitors in that it departs from the vinylbenzene nucleus common to a large number of currently known TPK inhibitors. Further work remains to be undertaken to more fully explore the potential utility of phenazocine and related compounds both as TPK inhibitors and antineoplastic agents.

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