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Selective Lymphocyte Activation and Inhibition of *In Vitro* Tumor Cell Growth by Novel Morphinans

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Abstract: Opioids can suppress immune functions and increase susceptibility to developing cancer and infectious diseases. Recently, novel opioid compounds have been synthesized that lack immunosuppressive effects. We evaluated the effects of morphinans with substituted pyrimidine (methyl, phenyl, hydroxy, and amino groups) and pyrazole groups on *in vitro* rat thymic lymphocyte and splenic macrophage functions, and tumor cell growth. We observed that morphinans with methyl, phenyl, hydroxy, amino, and pyrazole groups at concentrations from 10^{-10}M to 10^{-5}M plus Con A (2.5 μg/ml) significantly ($P < 0.01$) induced 2- to 2.9-, 2.3- to 6.4-, 2.4- to 3.4-, 2.6- to 3.4-, and 2.6- to 3.2-fold increases respectively in thymic lymphoproliferation compared with Con A alone; this effect was reversed by naloxone. Macrophage nitric oxide production was not altered by morphinans. In addition, we observed that all tested morphinans were associated with significant ($P < 0.01$) *in vitro* tumor cell growth inhibition of J774A (18-41%), L929 (12-36%), L5178 (9-15%) cell lines in a dose-dependent manner, at doses ranging from 10^{-11}M to 10^{-5}M. Morphinans may be applied in clinical situations where immunosuppression is undesirable.

Key words: Rodent, spleen, thymus, monocytes/macrophages, T lymphocytes, nitric oxide, tumor immunity.

INTRODUCTION

Opioids represent a major source of relief for most chronic moderate to severe nonmalignant pain[1]. However, they can alter immune function and consequently increase susceptibility to developing cancer and infectious diseases[2-4]. Novel opioid compounds have been synthesized that have analgesic capacity, but lack immunosuppressive effects or even potentiate immune function[5-9]. In this respect, Nowak *et al.* (1998) reported that the non-peptide agonist SNC80 did not alter NK cell, lymphocyte, and macrophage functions following intracerebroventricular administration[7]. However, intravenous administration of SNC80 was associated with *ex vivo* immunopotentiation[5], and significant *in vitro* stimulation of lymphoproliferation (up to 174% stimulation) of resident and concanavalin A (Con A)-treated lymphocytes[9]. Furthermore, SNC80 was recently shown to increase survival of tumor-bearing mice and significantly reduce tumor size[10]. In addition, novel non-peptide naltrindole and naltrexone derivatives, and the tetrahydroquinoline CGPM-9 have shown immunoenhancing properties *in vitro*[6,8,9]. Non-peptide opioid agonists have proven to overcome major disadvantages of peptidic opioids (rapid degradation and their low potential to cross the blood brain barrier), because they are not only highly selective and potent, but are also proteolytically stable, thus increasing their clinical applications[11]. In addition, their usefulness as non-immunosuppressive analgesics has been demonstrated[12].

The μ-opioid receptor selective morphinans, such as levallorphan, cyclorphan and butorphanol, are oxymorphon derivatives first introduced by Grewe in 1946. They are similar in structure to the morphine analogues, but lack the E ring found in the naturally occurring opioids, as well as the 6-OH and the 7,8-double bond. Although μ agonists have been associated with immunosuppression[3,13-16] several novel non-peptide opioid compounds have shown immunopotentiating effects[5,6,8,9-11].

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The present study was undertaken to evaluate the structure and function relationship of the non-peptidic opioid morphinans on *in vitro* lymphocyte and macrophage functions, and *in vitro* tumor cell growth. We found that depending upon their structure, morphinans differentially enhanced lymphoproliferation without altering macrophage functions, and inhibited *in vitro* cell growth of various human and murine tumor cell lines.

MATERIALS AND METHODS

Reagents and culture media: Penicillin-streptomycin solution, L-glutamine, RPMI 1640, and AIM-V media were obtained from Life Technologies (Grand Island, NY). Morphinans with substituted pyrimidino (methyl, phenyl, hydroxy, and amino groups) and pyrazole groups (Fig. 1) were synthesized by William J. Dunn III from the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, Chicago, Illinois. Concanavalin A (Con A), LPS from *Escherichia coli* serotype 0128:B12, DMSO, HCl, MTT, red blood cell lysing buffer, sodium dodecyl sulfate (SDS), dimethylformamide (DMF), and fetal bovine serum were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals: Fischer 344N male rats (200-220g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). They were housed in a pathogen free environment, 2-4 animals per cage, fed an *ad libitum* diet of laboratory food pellets and water and were maintained on a schedule of 12 hours light (0600-1800) and 12 hours dark (1800-0600) at 21 ± 3 °C. Animals were euthanized by asphyxiation in 100% CO_2 chamber.

Drug preparation: Morphinans were prepared and diluted to appropriate concentrations in AIM-V medium.

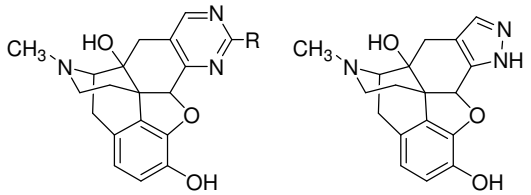
Cell preparation and culture: Single-cell suspensions were prepared by disrupting the spleen and thymus in RPMI 1640 medium. Cell suspensions were then washed three times in this medium, suspended and adjusted at appropriate densities with AIM-V medium, which has been observed to support cell culture[17].

T cell proliferation assay: T cell proliferation was determined by [^{3}H]-thymidine uptake as previously reported[18]. Immediately after rat death, single-cell thymus suspensions were prepared as described above, and adjusted to 1 x 10^7 cells/ml. Cell suspensions (100 µl) were added to round-bottomed 96-well plates (Becton Dickinson) containing triplicate cultures (100 µl) of AIM-V medium (unstimulated control) or the mitogen Con A at submaximal and maximal concentrations of 0.625, 1.25, and 2.5 µg/ml in the presence or absence of morphinans. After incubation for 44 h at 37°C with 5% CO_2, [^{3}H]-methylthymidine (6.7 Ci/mmol, ICN Pharmaceuticals Inc., Costa Mesa, CA) was added (1 µCi/10 µl/well), and cultures were incubated for additional 4 h. Cell cultures were then harvested with a semiautomatic cell harvester (Tomtec, Orange, CT), and cell-incorporated radioactivity was determined by liquid scintillation spectrophotometry using a Microbeta Plus liquid scintillation counter (model 1450, Wallac Oy, Turku, Finland) with a counting efficiency for tritium of 35%. Proliferative responses of thymic lymphocyte to maximal and submaximal concentrations of Con A were used for data analysis.

Nitrite determination: Accumulation of nitrite in the supernatants of macrophage cultures was used as an indicator of nitric oxide production by resident or activated cells[19]. One hundred-microliter splenic cell suspensions, prepared as explained above, were adjusted to 1 x 10^7 cells/ml in AIM-V medium and plated in flat-bottomed 96-well plates (Becton Dickinson) for 2 h at 37°C. Non-adherent cells were then removed, 50 µl of red cell lysing buffer (Sigma) was added to all wells, followed by the addition of 50 µl of AIM-V medium. After removal of the supernatant, adherent cells (about 10% of the input cells) were then incubated overnight in AIM-V medium in the presence or absence of morphinans. After incubation, macrophage monolayers were washed once in this culture medium. The final cell monolayers consisted of > 95% macrophages as judged by Giemsa's stain procedures. Morphinan-treated and untreated splenic macrophages were incubated in the presence or absence of LPS (25 ng/ml) in triplicate, in a total volume of 200 µl AIM-V medium per well for 3 days. After incubation, supernatants were obtained and nitrite levels were determined with the Griess reagent[20], using $NaNO_2$ as standard. Optical densities at 540 nm were then determined in a microplate reader (Molecular Devices Corporation, Palo Alto, CA).

Antitumor activity: The effects of morphinans on in vitro growth of human and murine tumor cell lines of different lineage were determined. Lymphoid (L5178Y-R, JURKAT and H-9), myeloid (U937 and J774A-1) and fibroblastoid (L929) tumor cells were adjusted to 5 x 10^4 cell/ml, and were incubated in the presence or absence of morphinans for 44 h. At the end of the incubation period, MTT (final concentration of 0.5 mg/ml) was added to all wells, and cell cultures were incubated for additional 4 h. After this, 100 µl of cell lysing buffer (20% SDS in 50% each DMF and water) was added to all wells, and cultures were incubated for 16 h. Optical densities were then read at 540 nm, and percentages of cell growth inhibition induced by morphinans compared to untreated control were calculated.

Statistical analysis: The results were expressed as mean ± SEM of the response of 4 separate rat tissues to each treatment (morphinan concentrations vs Con A concentrations) with 3 replicate determinations per treatment or experimental group (morphinan, Con A, morphinan ± Con A, and LPS) from a representative experiment. All experiments were repeated at least three times with similar results. Level of significance was assessed by Student's t test and analysis of variance (ANOVA).



1a, R = CH_3
1b, R = C_6H_5
1c, R = OH
1d, R = NH_2

2.

Fig. 1: Morphinan structures.

RESULTS

Effect of morphinans on *in vitro* thymic lymphocyte proliferation: Morphinans with methyl, phenyl, hydroxy, amino, and pyrazole groups at concentrations from 10^{-10}M to 10^{-5}M plus Con A (2.5 μg/ml) significantly (**P** < 0.01) induced 2- to 2.9-, 2.3- to 6.4-, 2.4- to 3.4-, 2.6- to 3.4-, and 2.6- to 3.2-fold increases respectively in lymphoproliferation compared with Con A alone (Fig. 2). In addition, only the morphinan with the phenyl group (at 10^{-6}M and 10^{-5}M) potentiated Con A-induced lymphocyte proliferation at Con A concentrations of 0.625 and 1.25 μg/ml (data not shown).

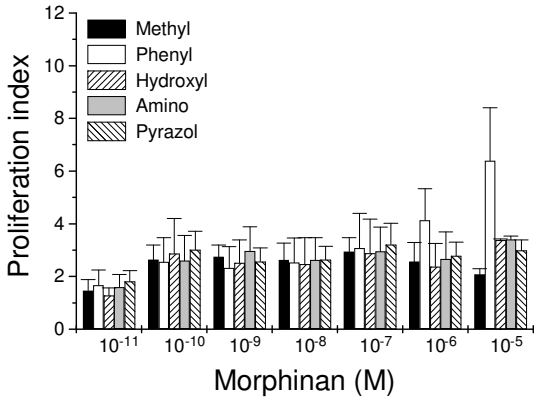


Fig. 2: Induction of lymphoproliferation by morphinans. Thymic cells were adjusted to 1 x 10^7 cells/ml, and incubated with Con A (2.5 μg/ml) in the presence or absence of morphinans for 48 h at 37°C in 5% CO_2. [^{3}H]thymidine (1 μCi/well) was added 4 h prior to the end of the incubation period. Cell cultures were then harvested, and cell-incorporated radioactivity was determined as explained in the text. Proliferation index = cpm of morphinan plus Con A-treated cells/cpm of Con A-treated cells. Data represent mean ± SEM of triplicates from a representative experiment. Thymic lymphocyte proliferation to Con A alone was 10440 ± 1310 cpm.

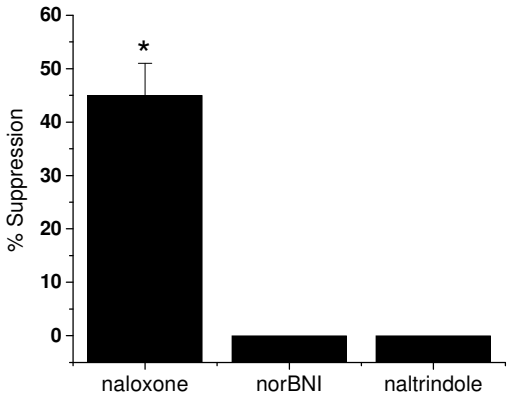


Fig. 3: Effect of opioid antagonists on morphinan-induced lymphoproliferation. Thymic cells were adjusted to 1 x 10^7 cells/ml, and incubated with Con A (2.5 μg/ml) in the presence or absence of the morphinan with phenyl substituent at 10^{-5}M, with or without the antagonist at 10^{-5}M, for 48 h at 37°C in 5% CO_2. [^{3}H]thymidine (1 μCi/well) was added 4 h prior to the end of the incubation period. Cell cultures were then harvested, and cell-incorporated radioactivity was determined as explained in the text. % Suppression represents the reduction of morphinan plus Con A-induced lymphoproliferation by the opioid antagonist. Data represent mean ± SEM of triplicates from a representative experiment. *$\mathbf{P}$ < 0.01 compared with the effect of the morphinan alone. Thymic lymphocyte proliferation to Con A alone was 10440 ± 1310 cpm.

Morphinans alone did not stimulate lymphoproliferation. The effect of the most active of these compounds, the morphinan with a phenyl substituent, at 10^{-5}M (a concentration that induced the highest lymphoproliferation stimulus (Fig. 2)) plus Con A (2.5 μg/ml), was antagonized by naloxone (45% inhibition of morphinan plus Con A-induced lymphoproliferation at 10^{-5}M), but not by nor-binaltorphimine and naltrindole (Fig. 3).

Effect of morphinans on *in vitro* production of nitric oxide by splenic macrophages: Mophinans were observed not to alter nitric oxide production by splenic macrophages as shown in Fig. 4.

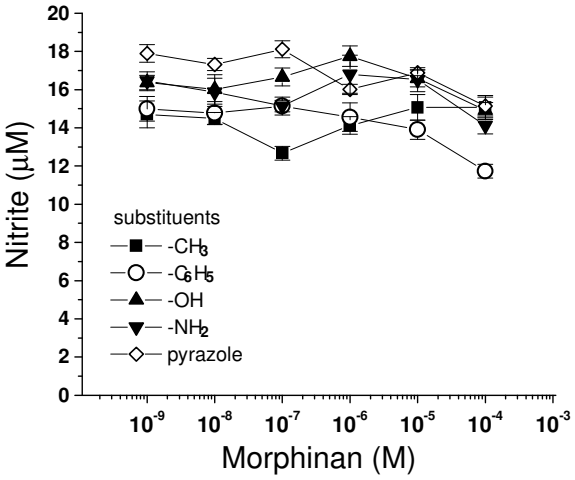


Fig. 4: Effect of morphinans on nitric oxide production by macrophages. One hundred-microliter splenic cell suspensions, were adjusted to 1 x 10^7 cells/ml in AIM-V medium and incubated for 2 h at 37°C. Non-adherent cells were then removed, and adherent cells (about 10% of the input cells or 1 x 10^6 cells/ml) were then incubated overnight in AIM-V medium in the presence or absence of morphinans. After incubation, macrophage monolayers were washed once in this culture medium. Morphinan-treated and untreated splenic macrophages were incubated in the presence or absence of LPS (25 ng/ml) in triplicate in AIM-V medium per well for 3 days. After incubation, supernatants were obtained and nitrite levels were determined with the Griess reagent. Optical densities at 540 nm were then determined in a microplate reader. Data represent mean ± SEM of triplicates from a representative experiment. LPS-treated cells alone released 15.25 ± 1.8 μM nitrite, whereas nitrite release by untreated macrophages was negligible.

Antitumor activity of morphinans: All tested morphinans (only data from phenyl-substituted morphinan is shown) were associated with significant ($\mathbf{P} < 0.01$) *in vitro* tumor cell growth inhibition of J774A (18-41%), L929 (12-36%), L5178 (9-15%) cell lines in a dose-dependent manner, at doses ranging from 10^{-11}M to 10^{-5}M (Fig. 5). However, morphinans marginally inhibited U937, H9 and Jurkat tumor cell growth (1%-10%).

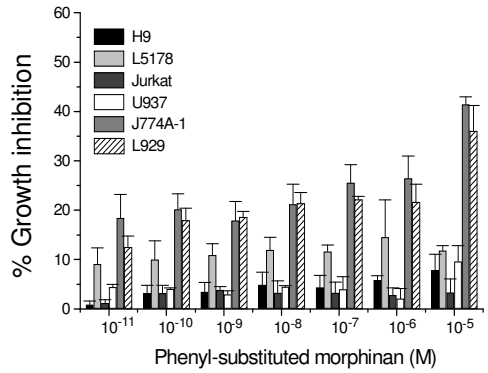


Fig. 5: Effect of morphinans on *in vitro* tumor cell growth. H9, L5178Y-R, JURKAT, U937, J774A-1, and L929 tumor cells were adjusted to 5 x 10^4 cell/ml, and were incubated in the presence or absence of morphinan with phenyl substituent for 44h. At the end of the incubation period, MTT (final concentration of 0.5 mg/ml) was added to all wells, and cell cultures were incubated for additional 4h. After this, 100 μl of cell lysing buffer were added to all wells, and cultures were incubated for 16 h. Optical densities were then read at 540 nm, and percentages of cell growth inhibition induced by the morphinan compared to untreated control were calculated.

DISCUSSION

Non-peptidic opioids are highly selective, potent, and proteolytically stable[11]. Morphine represents the most widely utilized of these compounds and is clinically the opioid of choice for pain relief. However, morphine is also a well known immunosuppressive agent[13-16,21]. In previous reports, we showed that novel non-peptidic μ and δ opioids have the ability to potentiate immune function[5-8]. In the present study, we demonstrated that morphinans with substituted pyrimidine and pyrazole groups at concentrations of 10^{-10} to 10^{-5} M were associated with increased Con A-stimulated rat thymic lymphocyte proliferation with the order of potency phenyl > pyrazole, hydroxy, amino > methyl > Con A alone (control). This effect was antagonized by naloxone, but not by nor-binaltorphimine and naltrindole. These results indicate that the inclusion of a phenyl substituent at the 2' position of the pyrimidine group significantly potentiated Con A-induced thymic cell proliferation, and that this effect may be mediated through μ–opioid receptor subtype. Opioids produce their effects in leukocytes via interactions with μ, δ, and κ opioid receptors. These receptors exhibit different patterns of ligand selectivity, stereoselectivity, saturability and

nanomolar affinity for opioids[22]. We observed that the effect of the most immunoactive of the morphinan compounds at 10^{-5}M plus Con A (2.5 μg/ml), was antagonized by naloxone (45% inhibition at equimolar concentration), but not by nor-binaltorphimine and naltrindole. This indicated that the morphinan action was mostly mediated through saturable μ-opioid receptors, rather than kappa or delta opioid receptors, on the surface of the lymphocytes. Presence of opioid receptors on the surface of cells is not specific and absolute (lymphocytes do not possess 100% of opioid receptors of a particular type). In fact, they will have receptors for μ, κ, and δ opioids at different densities. The use of antagonists at higher concentrations than the ligands (5 or 10 times the agonist concentration) has been tested in our laboratory with other opioids resulting in no further increase in the antagonist effect when utilized at equimolar concentration[6,8]. These facts may indirectly suggest that morphinans bind to selective, saturable, and high affinity membrane μ-opioid receptors on leukocytes[22]. This is relevant for two major reasons: a) the μ-receptor is considered to be the major site of analgesic action, since opioids used for pain management bind to this receptor with high affinity[23], and b) current use of μ opioids such as morphine for pain control are also immunosuppressive [13-16,21].

It was also demonstrated the morphinans did not alter macrophage functions, thus indicating a selective immunopotentiating effect on lymphocytes. Differential effects of opioids on leukocyte functions are commonly observed[24, 25]. In this regard, Kowalski et al. (1995) reported that enkephalins were associated with both suppressing and enhancing effects on splenic NK cell and macrophage functions related to the treatment period[24]. In addition, Pacifici et al. (1994) observed time-dependent biphasic effects of morphine, but not methadone, on immune parameters in vivo[25]. In yet other studies, dose-dependent bimodal responses of lymphocytes and macrophages to opioids have been reported[26, 27], and Ryng et al. (1999) demonstrated that non-opioid substituted phenilamides of 5-amino-3-methylisooxazole-4-carboxylic acid have differential effects on lymphocyte and macrophage functions[28]. In addition, Hicks et al. (2001) reported that the tetrahydroquinoline CGPM-9 enhanced rat thymic lymphoproliferation, but suppressed nitric oxide and tumor necrosis factor-alpha production by peritoneal macrophages[6]. In this latter study, the μ-opioid receptor selective antagonist CTOP used at equimolar doses significantly suppressed the effect of CGPM-9 on lymphocyte and macrophage functions[6]. The mechanism (s) by which morphinans enhance lymphocyte proliferative response, but not alter macrophage functions, remains to be investigated. However, potentiating lymphocyte functions, while not affecting nitric oxide (NO) production by macrophages, may be advantageous for these opioid derivatives. NO is produced during inflammation and can be both beneficial and detrimental for the organism[29-32]. Although NO has been associated with antimicrobial and antitumor properties[29, 30], it also induces immunosuppression by affecting lymphocyte and macrophage functions through direct action on these cells[31, 32], or indirectly via the central nervous system (CNS)[33, 34]. NO is also associated with CNS pathology[35]. In contrast, stimulation of lymphoproliferation by morphinan derivatives may be utilized in clinical situations where lymphocyte blood pool is significantly reduced, as in the cases of AIDS and aging[36]. Therefore, morphinan derivatives may potentiate lymphoproliferative responses, without altering macrophage functions and thus avoiding potential pathological states.

Additionally, we showed that morphinans have the ability to significantly and selectively inhibit *in vitro* cell growth of murine tumor cells, and marginally inhibited growth of human tumor cells. However, the mechanism by which morphinans inhibit tumor cell growth remains to be elucidated. Selectivity of morphinans towards murine cells might be the result of some mechanism of drug resistance present in the human cell lines. Development of resistance to drugs may be related to failure of the cells to undergo apoptosis in response to the drug, failure of the drug to reach and/or affect its intracellular target, intracellular drug transport resistance mechanisms, subcellular redistribution, drug interaction, enhanced DNA repair, and failure to apoptose[37, 38].

Novel non-peptide opioids could potentially be utilized in many different clinical situations where immunosuppression is undesirable, as shown for μ-selective ligands such as morphine[39]. Because of their effects on immune function, μ-opioid agonists might not be optimal for management of moderate to severe pain following a variety of surgical procedures, cancer, and other related traumatisms. However opioid derivatives, such as morphinans, may have not only the potential to stimulate the immune system, but also have the capacity to inhibit tumor cell growth. Thus making these compounds potentially suitable in treating not only pain, but also enhancing the immune status of immunocompromised individuals against cancer and infectious diseases. Understanding structure and function relationship will allow the design, synthesis and use of non-immunosuppressive or immunostimulating opioids as adjuvants in the therapy of disease-associated pain in the individual with immune dysfunction.

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