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Inhibition of Cell Growth and DNA, RNA, and Protein Synthesis *in Vitro* by Fentanyl, Sufentanil, and Opiate Analgesics

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Abstract: We have studied the cytotoxic nature of two groups of narcotic analgesics. Group I consists of the opioids, morphine, codeine, hydromorphone, thebaine, and etorphine. Group II contains but two phenylpiperidine-type narcotics, fentanyl and sufentanil. To measure cytotoxicity, three different bioassays were employed using an established line of human cells. Specifically, the effects of narcotic analgesics on DNA, RNA, and protein synthesis were measured by following the uptake and incorporation of radiolabeled thymidine, uridine, and amino acids, respectively. Inhibition of cell growth also was studied by measuring population doubling times of logarithmically growing cells in the presence (or absence) of the test compounds. Lastly, cloning efficiencies of cells were determined in the presence of both groups of compounds. Group I compounds were significantly less inhibitory than Group II compounds by all three bioassays. Moreover, flow cytometric DNA analysis of cells treated with 100 and 320 μM etorphine HCl showed essentially no effects on cell cycle distribution. These *in vitro* results thus suggest that (1) fentanyl and sufentanil are inherently more cytotoxic than the opioid narcotics in Group I, and (2) the highly potent morphinoid drug etorphine HCl appears to have special promise as a transdermal narcotic to control pain.

As pain is the most common symptom for which patients seek medical relief (Chatton 1982), finding a reliable, convenient and safe route for the administration of analgesics to patients providing sustained symptomatic relief would be highly significant. In recent years there has been special interest in the transdermal delivery of narcotics in cancer patients (Lipman *et al.* 1980; Holley & Van Steenis 1984; Flynn *et al.* 1987). The transdermal delivery of narcotic analgesics may provide these advantages and have other clinical advantages such as (1) allowing patients to be ambulatory, (2) avoiding the pain of injections and, most importantly, (3) avoiding the peak and trough blood level phenomenon seen with other dosing. The transdermal route, however, exposes cells in the absorption pathway to unusually high concentrations of the permeating drug. This alone can preclude transdermal administration. In the light of this, it is of concern that no detailed studies of the cytotoxicity of these drugs against cells in culture have been published. The present study was undertaken to investigate the *in vitro* perturbations of cell growth and DNA, RNA, and protein synthesis in an established line of human cells by fentanyl, sufentanil, and the opioid analgesics codeine, etorphine, hydromorphone, morphine, and thebaine.

Materials and Methods

Chemicals. Fentanyl and sufentanil were gifts from Janssen Pharmaceutica (Piscataway, NJ, U.S.A.). Morphine sulfate, codeine phosphate and hydromorphone hydrochloride were obtained from The University of Michigan Hospital (Ann Arbor, MI). Etorphine hydrochloride was kindly supplied by both Dr. James H. Woods, Department of Pharmacology, The University of Michigan Medical School, and the National Institute on Drug Abuse (NIDA), Washington, DC. Thebaine was purchased from Sigma Biochemical Company (St. Louis, MO). [Methyl-^{3}H]thymidine (40 Ci/mmole), [5-^{3}H]Uridine (22 Ci/mmole), and ^{3}H-L-amino acid mixture (273 mCi/mg) were purchased from ICN Biochemicals, Inc. (Irvine, CA). Foetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT). Calf serum was purchased from Flow Laboratories, Inc. (McLean, VA). 4-(2-Hydroxyl)-1-piperazineëthane sulfonic acid (HEPES) was purchased from Calbiochem (La Jolla, CA).

Cell culture. KB cells, an established human cell line derived from an epidermal oral carcinoma, were routinely grown in Eagle minimum essential medium (MEM) with Hanks salts [MEM(H)] supplemented with 5% FBS. Cells were passaged according to conventional procedures using 0.05% trypsin plus 0.02% ethylenediaminetetraacetic acid (EDTA) in a HEPES-buffered salt solution (HBS) (Shipman 1969). In order to increase the likelihood of detecting bacterial and/or mixed bacterial and mycoplasmal contamination, antibiotics were never employed in the routine passage of cells (Hayflick 1973). Suspension cultures of KB cells were grown at 37° in MEM with spinner salts [MEM(S)] ($NaHCO_3$ reduced to 1.9 g/l) supplemented with 10% calf serum. Suspension grown KB cells had an average population doubling time of 24 hr. They were maintained between 2×10^5 and 3×10^5 cells/ml at a volume of between 250 and 300 ml in hanging bar-type 500 ml spinner flasks (Bellco Glass Co., Inc., Vineland, NJ).

Cell counts. Total cell counts were made with a model Z_F Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) equipped with a 100 μm orifice. Viable counts were determined by means of trypan blue dye exclusion. Population doubling times were calculated by means of a least squares program fitting the exponential portion of the growth curve.

Plating efficiencies. Log-phase KB cells from suspension cultures were diluted with medium and planted in plastic tissue culture dishes

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(150 × 25 mm, Falcon, Oxnard, CA) at a final concentration of 500 to 650 cells per dish. Growth medium consisted of MEM(E) supplemented with 10% FBS. After incubation for 12 to 14 days at 37° in a humidified 4% CO_2-96% air atmosphere, the medium was gently aspirated from the plates. Clones were rinsed with HBS, fixed *in situ* with absolute methanol for 1–2 min., stained with 0.1% crystal violet in 20% methanol and enumerated. Clones ≥ 1 mm in diameter were scored.

Radiolabeled precursors incorporation assays. Cytotoxicity of narcotic analgesics was determined in part by measuring inhibition of DNA, RNA, and protein synthesis. Cells were planted using a Costar Transplate-96 (Costar, Cambridge, MA) in 96-well dishes at a concentration of 1.2×10^4 cells per well suspended in 200 µl of growth medium. After incubation for 20–24 hr at 37° in a humidified atmosphere of 4% CO_2 −96% air, 150 µl of medium was removed per well. One hundred µl of medium containing drugs at twice their final concentrations were added to each well using a Titertek Multichannel pipette (Flow Laboratories, Inc., McLean, VA). Fifty µl of medium containing radioactive precursors then was added to each well to give final concentrations of 2 µCi/ml of [^{3}H]dThd and 3 µCi/ml of [^{3}H]Urd or ^{3}H-amino acids. In all experiments [^{3}H]dThd and [^{3}H]Urd were diluted with unlabeled dThd and Urd (final concentration of 2 µM) to ensure that sufficient precursor was available for uptake during the labeling period. Following addition of drugs and labeled precursors in triplicate, plates were incubated for an additional 16–24 hr. Logarithmic cell growth occurred during this time with continual uptake of labeled precursors. At the end of the incubation period, cells were harvested from each well using a Skatron Cell Harvester (Skatron, Inc., Sterling, VA). Cultures from individual wells were harvested onto filter paper and washed free of unincorporated label with sequential washes with 5% trichloroacetic acid and distilled water using the Skatron unit. Filters were dried, circles from individual cultures punched from the filter mat and placed into mini-vials. Liquid scintillation solution was added and radioactivity was determined in a Beckman model LS 8100 liquid scintillation spectrometer (Turk *et al.* 1987).

Cell growth rate. KB cells were planted in 6-well plastic tissue culture dishes (Costar, Cambridge, MA) at a concentration of 1×10^5 cells per well and incubated for 20–24 hr. Medium was decanted, the cell monolayer was rinsed once with HBS, and fresh growth medium containing various concentrations of drugs was added. After additional periods of incubation at 37°, medium was removed and cells were harvested with 0.05% trypsin-0.02% EDTA in HBS. Cells were enumerated using a Coulter Counter as described above. The numbers of cells at each time point and concentration were plotted as a function of time to construct growth curves.

Data analysis. Dose-response relationships were constructed by linearly regressing the percent inhibition of DNA, RNA, and protein synthesis against log drug concentrations. The 50% inhibitory concentrations (IC50) were calculated from regression lines (Goldstein 1964). A positive control (vidarabine) was used in all assays. Results of sets of assays were rejected if inhibition by the positive control deviated from its mean response by more than 1.5 standard deviations.

Flow cytometry. The exact procedures used for the handling and staining of cells and subsequent flow cytometric evaluation have been described in a previous publication (Nassiri *et al.* 1990).

Results

Cytotoxicity measurement using labeled precursors.

Incorporation of radioactive precursors into DNA, RNA, and protein was determined in KB cells; IC50 values are shown in table 1. Concentrations of narcotics were tested in the range of 10 µM to 10 mM for the incubation period of 20–24 hr. As seen in table 1, in group I narcotics, the IC50 values for nucleic acids and protein were > 1 mM without exception, considerably higher than seen within group II. Additionally, cells treated with group I narcotics appeared normal at all drug concentrations when observed microscopically. Group II narcotics inhibited the incorporation of labeled precursors into cellular DNA, RNA, and protein at concentrations < 0.5 mM. Using morphine as a reference, fentanyl and sufentanil were ≥ 10 times as toxic as the opioid compounds in group I. When the inhibitory concentration values in group II were averaged to give a general indication of cytotoxicity, sufentanil was 2.6-fold more inhibitory than fentanyl.

Table 1.

Effect of narcotic analgesics on incorporation of radiolabeled precursors into DNA, RNA, and protein.

		IC50 (mM)[a]		
Group	Drug	DNA	RNA	Protein
I	Codeine	> 1.0[b]	> 1.0[b]	> 1.0[b]
	Hydromorphone	> 1.0[b]	> 1.0[b]	> 1.0[b]
	Morphine	2.50	4.21	3.42
	Thebaine	> 1.0[b]	> 1.0[b]	> 1.0[b]
	Etorphine	> 1.0[b]	> 1.0[b]	4.15
II	Fentanyl	0.47 (0.16 − 15.00)[c]	0.25 (0.09–4.3)	0.30 (0.15–0.93)
	Sufentanil	0.08 (0.02–0.56)	0.20 (0.06–4.0)	0.11 (0.04–2.0)

a Calculated from the regression lines of the dose-response relationship.
b Less than 15% inhibition observed at highest concentration (1.0 mM) tested.
c 95% confidence interval given in parenthesis.

Cell growth rates. Treatment of KB cells in culture for up to 72 hr with various concentrations of group I narcotics, excluding etorphine, resulted in no suppression of cell growth (fig. 1). Etorphine inhibited cell growth moderately at the higher concentrations. Cells treated with 100 µM etorphine had a similar population doubling time (PDT) to that observed in untreated cells during the first 48 hr of incubation. Within the last 24 hr of treatment, the PDT of treated cells was prolonged, but not significantly. At a concentration of 320 µM etorphine, however, cell growth was slowed with an increase in PDT from 22 hr (untreated cells) to 35 hr.

Group II narcotics showed significant cell growth suppression when applied in high concentrations. As seen in fig. 2, both fentanyl and more exaggeratedly sufentanil, affected the growth of KB cells over the first 24 hr incubation period. Three hundred twenty µM fentanyl increased the PDT to 33 hr whereas the same concentration of sufentanil resulted in a state of negative cell growth. Direct microscopic examination of sufentanil-treated cell cultures revealed that significant cell death was occurring at a concentration of 320 µM.

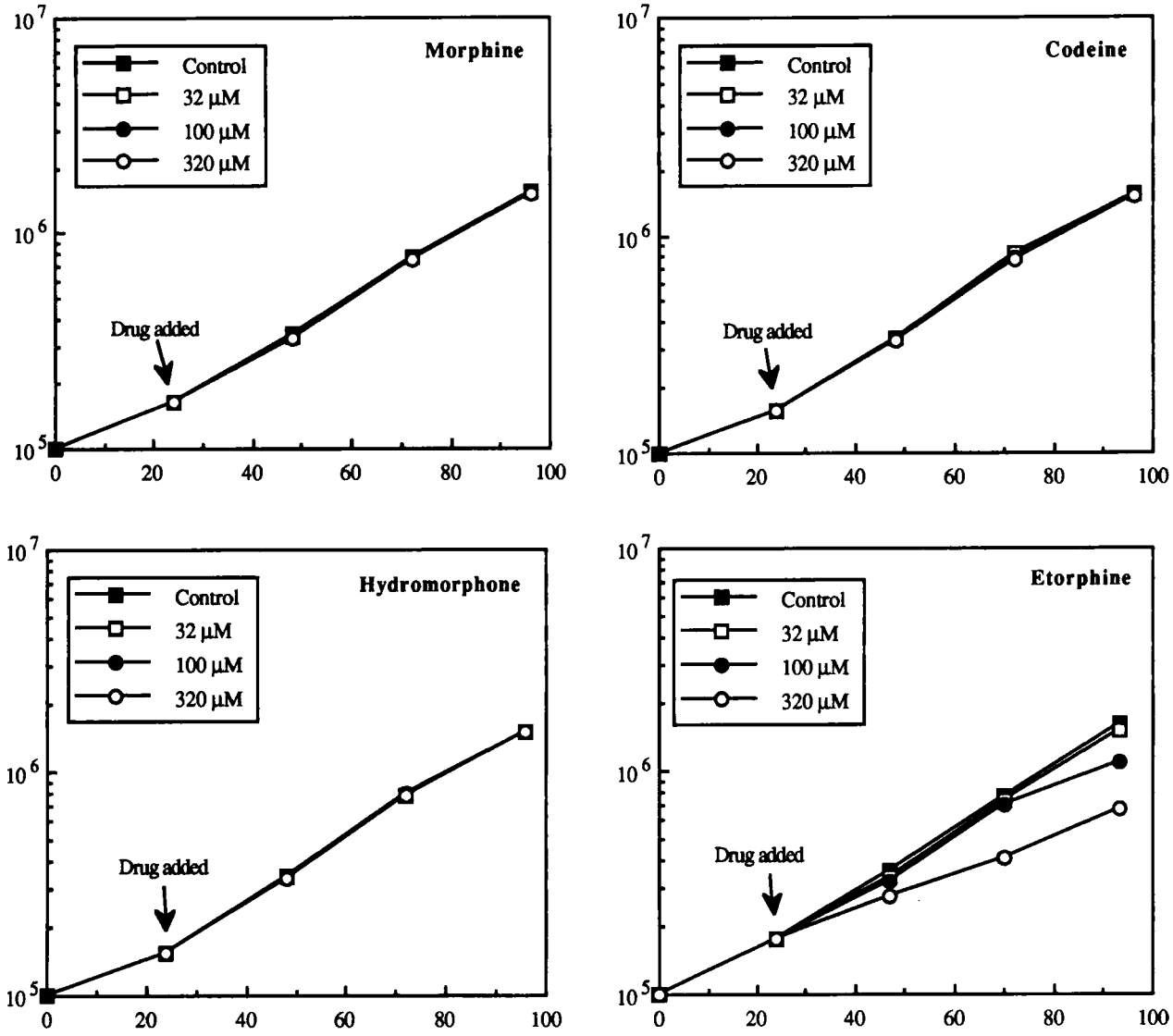

Fig. 1. Effect of group I narcotics on cell growth. KB cells were planted at 1×10^5 per well in 6-well cluster dishes and incubated for approximately 24 hr. At the time indicated by arrows, the medium was decanted and fresh medium containing selected concentrations of drug was added. At subsequent times, cells were harvested and enumerated.

Cloning efficiencies. The ability of KB cells to clone in the presence and absence of the various concentrations of narcotics was examined. The results for group I and II narcotics are presented in table 2. Fifty percent inhibitory doses were compared using Scheffe's method of multiple comparison. Among these two groups, morphine had the highest IC50 value of 1.86 mM, and, in contrast, sufentanil had the lowest IC50 value of 0.1 μM. Based on analysis of variance, morphine was significantly less inhibitory than any other drug. Etorphine, thebaine, hydromorphone, and codeine as a group were significantly less inhibitory than the group consisting of thebaine, hydromorphone, codeine, fentanyl and sufentanil. Etorphine was significantly less inhibitory than either fentanyl or sufentanil.

Effects on cell cycle distribution. The potential cytotoxicity of etorphine HCl in cell cultures was also determined by using flow cytometry. Fig. 3 illustrates the DNA histograms of untreated and etorphine-treated cells at 100 and 320 μM concentrations. There was no difference of DNA content between untreated and 100 μM treated cultures. At 320 μM etorphine, a slight effect was seen resulting in fewer cells in the G_2/M phase of treated cells when compared to the control histogram. This observation correlated well with the slight prolongation of population doubling times seen in the cell growth experiments. Prior to cell preparation and staining for flow cytometry, all cultures were examined microscopically. Neither cell death nor any morphological abnormalities was noted in cells exposed to either concentration of etorphine.

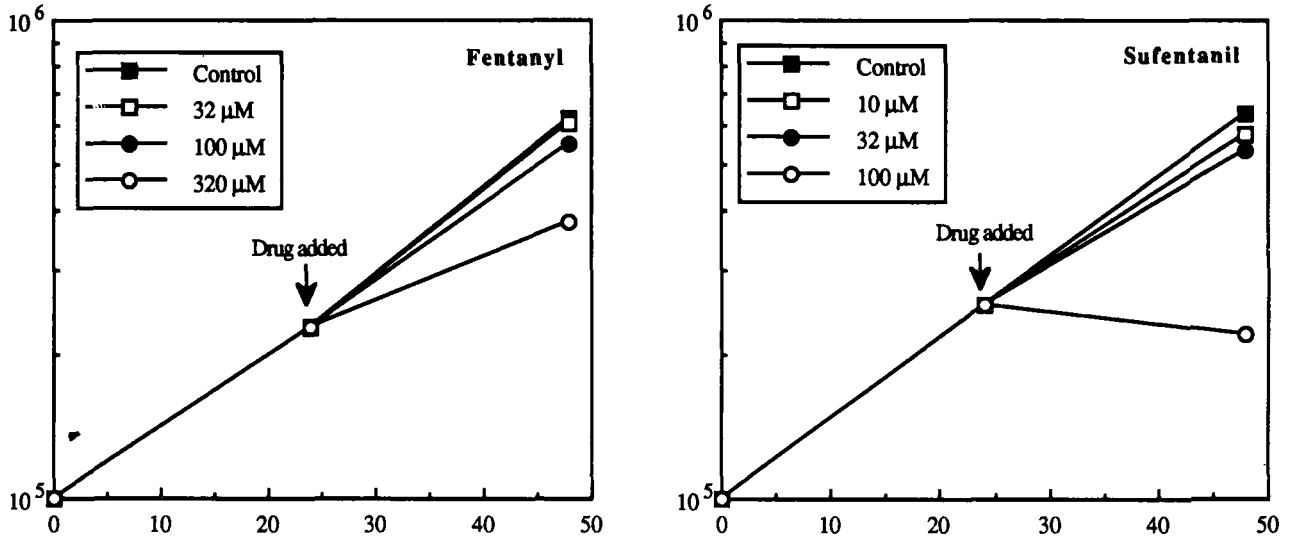

Fig. 2. Effect of group II narcotics on cell growth. Procedures were identical to those described in the legend to fig. 1.

Discussion

A series of narcotic analgesics was tested in three distinct bioassays for their cytotoxic effects in an established line of human cells. Experiments using radioactive precursors revealed that opioid narcotics did not significantly affect DNA, RNA, or protein biosynthesis at concentrations below 1.0 mM. In contrast, fentanyl, and to a greater extent, sufentanil, markedly decreased the rate of synthesis of nucleic acids and protein.

We examined also the ability of narcotics to affect cell growth. Results indicated that codeine, morphine, and hydromorphone had no effect at the highest doses tested (320 μM), whereas fentanyl and etorphine had a measurable impact on growth at 320 μM. Sufentanil started inhibiting cell growth at 32 μM and completely inhibited growth at a

Table 2.

Effect of narcotic analgesics on the relative plating efficiency of KB cells.[a]

	Drug	n[b]	IC50 (mM)[c]	S.D.
*	Sufentanil	3	0.10	±0.001
	Fentanyl	3	0.20	±0.038
	Codeine	3	0.34	±0.171
*	Hydromorphone	3	0.52	±0.286
	Thebaine	2	0.74	±0.097
	Etorphine HCl	2	0.98	±0.061
	Morphine	3	1.86	±0.348

a Approximately 500 to 650 cells were seeded per dish. After incubation for 12 to 14 days at 37°, clones were fixed and stained with crystal violet. Clones ≥ 1 mm in diameter were scored.
b Number of experiments.
c Arithmetic mean of concentration of drug required to produce a 50% reduction in the number of clones developing in the absence of drug.
* Arithmetic means within bracketed groups were not significantly different (> 0.05) using Scheffe's method of multiple comparisons.

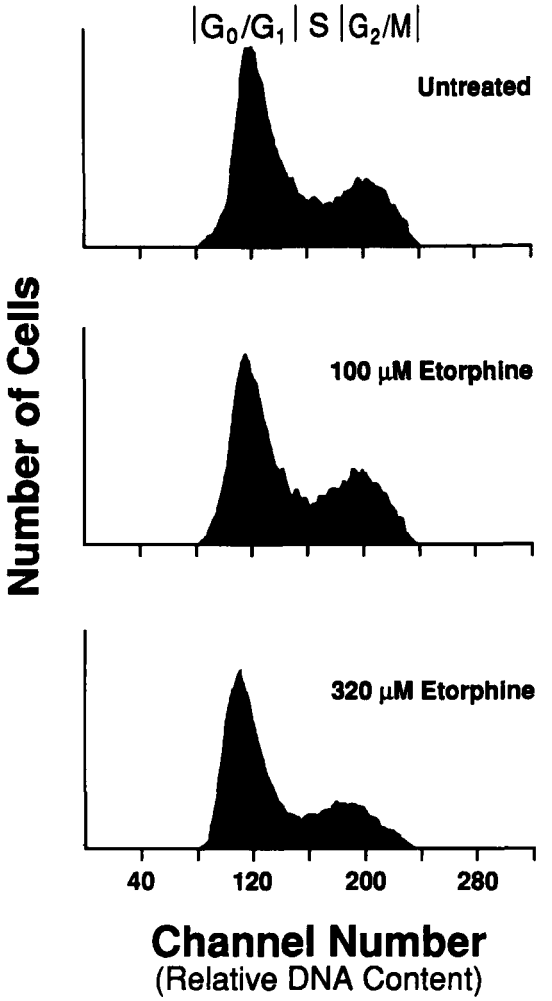

Fig. 3. Flow histograms illustraing the response of KB cells to 100 and 320 μM etorphine HCl. In all samples, 10^4 nuclei were analyzed by flow cytometry.

concentration of 100 μM. In order to ensure that the results observed were not peculiar to the transformed cell line used, similar cell-growth experiments were carried out with human foreskin fibroblasts. Virtually identical results were obtained (data not shown).

The ability of cells to clone in the presence of drugs was measured to further evaluate the cytotoxic effects of the narcotics. Cloning efficiency is a sensitive method to examine cell growth and viability and lends credibility to estimates of cytotoxicity as measured either by the uptake of radiolabeled precursors or by cell growth. Such experiments confirmed that fentanyl and sufentanil were more cell suppressive than the morphine-like analgesics evaluated. When tested at the highest concentration used in this set of experiments (1 mM), both fentanyl and sufentanil suppressed clonal growth completely. The exact mechanism(s) by which the narcotic analgesics inhibit macromolecular biosynthesis and cell growth is not understood.

Flow cytometry technology has been widely used in both clinical and research fields of medicine, especially in antineoplastic (Laerum & Farsund 1981; Barlogie *et al.* 1983) and antiviral (Nassiri *et al.* 1990; Schols *et al.* 1990) drug screening to monitor cellular toxicity. Utilizing this procedure, we were able to confirm the non-cytotoxic nature of etorphine HCl. The flow cytometric results showed that etorphine in relatively high concentrations did not significantly perturb the cell cycle.

Preliminary studies in our laboratories (Jolicoeur *et al.*, personal communication) suggest that etorphine HCl diffuses through the skin at rates consistent with the transdermal use of this drug. A permeability coefficient of 3.5×10^{-3} cm/hr was obtained using human cadaver skin. Thus, amongst all compounds tested and based on considerations of high potency and low cytotoxicity, it appears that etorphine and etorphine derivatives may have considerable potential as agents for the transdermal control of pain (Shipman *et al.* 1990). Blane *et al.* (1967) have reported that etorphine HCl, given subcutaneously, was 1,000–80,000 times more potent than morphine, depending on the test situation. Although resembling the action of morphine in rodents, cats, dogs, and monkeys by causing analgesia, catatonia, and respiratory depression, etorphine, in contrast, fails to cause emesis in the dog. Research conducted by the U.S. Army (Hydro 1973) demonstrated that etorphine HCl is 36,000 times more effective in tranquilizing mice than in causing their death. Blane & Robbie (1970) administered etorphine HCl tablets sublingually to cancer patients (more than 5,000 treatments). Nausea and vomiting were not seen and the only untoward side effect was occasional slight sedation. Although further experimentation with this interesting compound obviously is necessary, it does appear that etorphine is sufficiently non-toxic and potent to be delivered transdermally at safe and effective concentrations.

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References

Barlogie, B., M. N. Raber, J. Johnnes, T. S. Johnson, B. Drewinko, D. W. Swartzendruber, W. Gohde, M. Andreef & E. J. Freireich: Flow cytometry in clinical cancer research. *Cancer Res.* 1983, **43**, 3982–3997.

Blane, G. F., L. A. Boura, A. E. Fitzgerald & R. E. Lister: Actions of etorphine hydrochloride (M99). *Brit. J. Pharmacol. Chemotherap.* 1967, **30**, 11–22.

Blane, G. F. & D. S. Robbie: Trial of etorphine (M99 Reckitt) in carcinoma pain: preliminary report. *Proc. Brit. Pharmacol. Soc.* 1970, **39**, 252–253.

Chatton, M. J.: Pain. In: *Current medical diagnosis and treatment.* Eds.: M. A. Krupp and M. J. Chatton. Lange Medical Publishers, Los Altos, California, 1982, pp. 9–13.

Flynn, G. L., W. M. Smith & T. A. Hagen: In vitro transport. In: *Transdermal delivery of drugs.* Eds.: A. F. Kydonieus and B. Berner. CRC Press, Boca Raton, Florida, 1987, Vol. 1, pp. 45–59.

Goldstein, A.: *Biostatistics, an introductory text.* MacMillan Co., New York, 1964, pp. 156–161.

Hayflick, L.: Screening tissue cultures for mycoplasma infections. In: *Tissue culture: Methods and applications.* Eds.: P. F. Kruse, Jr. and M. K. Patterson. Academic Press, New York, 1973, pp. 722–728.

Holley, F. O. & C. Van Steenis: Transdermal administration of fentanyl for postoperative analgesia. *Anesthesiol.* 1984, **65**, A548.

Hydro, W. R.: *Process of purifying oripavines.* U.S. Patent No. 3,763,167, issued October 2, 1973.

Laerum, D. O. & T. Farsund: Clinical application of flow cytometry: a review. *Cytometry* 1981: **2**, 1–13.

Lipman, A. G.: Drug therapy in cancer pain. *Cancer Nurs.* 1980, **3**, 39–46.

Nassiri, M. R., J. L. Hudson, J. S. Pudlo, G. M. Birch, L. B. Townsend & J. C. Drach: Flow cytometric evaluation of the cytotoxicity of novel antiviral compounds. *Cytometry* 1990, **11**, 411–417.

Schols, D., R. Pauwels, J. Desmyter & E. De Clercq: Flow cytometric method to monitor the destruction of CD^+ cells following their fusion with HIV-infected cells. *Cytometry* 1990, **11**, 736–743.

Shipman, C. Jr.: Evaluation of 4-(2-hydroxyethyl)-1-piperazineëthane sulfonic acid (HEPES) as a tissue culture buffer. *Proc. Soc. Exp. Biol. Med.* 1969, **130**, 305–310.

Shipman, C. Jr., M. R.Nassiri & G. L. Flynn: U.S. Patent *Transdermal delivery of the narcotic analgesics etorphine and analogs,* No. 4,891,377, issued Jan. 2, 1990.

Turk, S. R., C. Shipman, Jr., M. R. Nassiri, G. Genzlinger, S. Krawczyk, L. B. Townsend & J. C. Drach: Pyrrolo [2,3-*d*] pyrimidine nucleosides as inhibitors of human cytomegalovirus. *Antimicro. Agents Chemotherap.* 1987, **31**, 544–550.