Direct Effects of Delta Opioid Receptor Agonists on Invasion-associated Activities of HCT-8/E11 Colon Cancer Cells

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Abstract. *Background: Opioids and opioid receptors are an integral part of the tumour microenvironment and hence may influence tumour progression. Studies on direct effects of opioids on invasion-associated cellular activities are equivocal. We wanted to clarify these differences. Materials and Methods: The direct effects of the delta opioid receptor (DOR) agonists [D-Pen^2, D-Pen^5]-enkephalin (DPDPE), leu-enkephalin and [D-Ala^2, D-Leu^5]-enkephalin (DADLE) on invasion-associated activities of HCT-8 myc-DOR and HCT-8 FLAG-DOR colon cancer cells stably overexpressing DOR were studied. Results: The opioids showed a trend to stimulate invasion of single cells in collagen in one clone, while they did not influence invasion of the other clone. In other invasion assays, no effects were observed. They did not affect cell growth and homotypical cell–cell adhesion. DPDPE at 0.1 μM inhibited directional migration; the other opioids and concentrations tested were inefficient. Conclusion: Opioids differently influence invasion-associated cellular activities, depending on the expression level of DOR, experimental set-up, type and concentration of opioid.*

Opioids, in particular morphine, are mainly associated with cancer as analgesics. But it is becoming increasingly clear that opioids and their receptors are an integral part of the tumour microenvironment. Opioid receptors and endogenous opioid peptides were demonstrated in a wide variety of human tumours (1-3). Opioid peptides may be produced by cancer cells, especially in tumours of neuroendocrine origin (1), and cancer cells may contain enzymes to digest haemoglobin and caseins, thereby releasing the non-classical opioids haemorphins and casomorphins, respectively (4, 5). Alternatively, opioids may be supplied by the general circulation (6), produced by infiltrating leucocytes (7) or by nerve terminals and prostatic neuroendocrine cells in the tumour microenvironment (8).

Considerable evidence has been accumulated, from both *in vitro* and *in vivo* studies, indicating that opioids influence tumour progression. Morphine was shown to possess anti-tumour effects through relieving cancer pain (9). In contrast, other reports described tumour-promoting effects of opioids due to immunosuppression (10) or by promoting angiogenesis (11). In addition to these indirect effects, opioids can directly influence cancer cell invasion-associated activities such as proliferation and survival (12-20), motility and migration (5, 12, 14, 16, 17, 21), cell-substrate adhesion (12, 21) and invasion in Matrigel (12, 16, 21, 22) or native collagen type I (5). However, these studies are equivocal, reporting direct pro-invasive and anti-invasive effects of opioids as well as no direct effects on invasion-associated cellular activities.

To clarify these different results from literature, we investigated the direct effects of different delta opioid receptor (DOR) agonists on invasion-associated cellular activities in a defined model system. We established HCT-8 myc-DOR and HCT-8 FLAG-DOR cell lines stably overexpressing functional myc-DOR and FLAG-DOR, respectively. A radioligand binding experiment and Scatchard analysis revealed that HCT-8 myc-DOR cells contain almost twice as much DOR binding sites as HCT-8 FLAG-DOR cells, enabling us to evaluate the influence of the expression level of DOR on different responses. Cellular activities were studied in response to the synthetic DOR agonists [D-Pen^2, D-Pen^5]-enkephalin (DPDPE) and [D-Ala^2, D-Leu^5]-enkephalin (DADLE) and the endogenous DOR agonist leu-

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enkephalin to evaluate opioid-dependent effects. To evaluate concentration-dependent effects cells were exposed to a 100-fold concentration range of DPDPE.

Materials and Methods

Cell culture. HCT-8/E11 is an epithelial subclone from the human HCT-8 colon carcinoma cell line (23). HCT-8/E11 cells from the American Type Culture Collection (Rockville, MD, USA) were transfected with pcDNA3 vector containing myc-DOR or FLAG™-DOR construct (gift of G. Milligan, Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow, Scotland) by electroporation according to the manufacturers's instructions (Amaxa GmBH, Cologne, Germany). The HCT-8 myc-DOR and HCT-8 FLAG-DOR cell lines were obtained after selection in the presence of 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA). HCT-8 myc-DOR R is a subclone from HCT-8 myc-DOR selected for its round (R) morphotype as previously described (23, 23).

The cells were grown in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (Greiner Bio-One, Kremsmuenster, Austria) and 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and 2.5 μg/ml Fungizone (Bristol-Myers Squibb, New York, NY, USA) in a humidified atmosphere of 5% CO_2 in air at 37˚C.

Multicellular spheroid formation. A single-cell suspension (6 ml) containing 3×10^5 cells/ml culture medium was transferred to a 50 ml Erlenmeyer flask and incubated for 3 days on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, NJ, USA) at 70 rpm at 37˚C. The cultures were gassed with a mixture of 5% CO_2 in air.

Reagents. DPDPE, leu-enkephalin, DADLE, naloxone, transforming growth factor-α (TGF-α) and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Conditioned medium (CM) MRC5 was harvested from 6-day-old MRC5 human lung fibroblast cultures. CM was centrifuged at 2,562 × g for 15 min, filter sterilized through a 0.22 μm filter and stored at –20˚C.

Reverse transcription (RT)-polymerase chain reaction (PCR). Two micrograms of total RNA, isolated according to the Qiagen RNeasy Mini protocol (Qiagen, Venlo, The Netherlands), were used for cDNA synthesis with Omniscript reverse transcriptase (Qiagen) and oligo-dT primers (Qiagen) according to the manufacturer's instructions. PCR mix contained PCR Buffer (Qiagen), 10 μl of cDNA, 200 μM of each dNTP (Qiagen), 1.25 U *Taq* DNA polymerase (Qiagen) and 10 pmol of each primer, with the forward primer 5'-GCCAAGCTGATCAACATCTG-3' and the reverse primer 5'-AAGCAGCGCTTGAAGTTCTC-3' (24), in a final volume of 50 μl. Cycling parameters were 40 cycles of 50 s at 95˚C, 90 s at 58˚C, and 5 min at 72˚C with an initial denaturation step (3 min at 95˚C) and a final prolongation step (10 min at 72˚C).

Radioligand binding experiment. Radioligand binding studies were performed as described (25). Briefly, cells were rinsed twice with DMEM/20 mM HEPES buffer, followed by 2 rinses with PBS/0.2% BSA before incubation for 30 min with [^{3}H]diprenorphine (PerkinElmer, Boston, MA, USA) (0.05-5 nM) in the presence or absence of 20 μM DPDPE to specifically target DOR or 20 μM naloxone to target all opioid receptors, in a final volume of 300 μl of 50 mM Tris-HCl, 1% BSA at pH 7.4. Cells were washed twice with PBS/0.2% BSA and once with PBS. Cells were harvested in 200 μl of 1 M NaOH, incubated for 30 min and neutralized with 200 μl of 1 M HCl. Three millilitres of a scintillation mixture (Insta-Gel Plus®, PerkinElmer) were added to 200 μl of the samples and radioactivity was counted (1500 Tri-Carb® Liquid Scintillation Analyzer, PerkinElmer). Each determination was carried out in duplicate. Scatchard analysis was performed using GraphPad Prism software (Graphpad Software, Inc., La Jolla, CA, USA).

ERK1/2 phosphorylation studies. Cells were serum-starved overnight before incubation with serum-free culture medium with or without (vehicle control) 0.1, 1 or 10 μM DPDPE, 1 μM leu-enkephalin or 1 μM DADLE for 5 min, unless indicated otherwise in the figure. For combination treatments with naloxone, the serum-starved cells were pre-treated for 1 h with 10 μM naloxone before incubation with the DOR agonist and 10 μM naloxone for 5 min. After rinsing in ice-cold PBS, cells were lysed with 2% CHAPS, 10 mM sodium phosphate pH 7.2, 1% sodium deoxycholate, 0.15 M sodium chloride, 1 mM sodium vanadate, 1 mM NaF, 2 mM phenylmethylsulphonylfluoride, 2.5 mg/ml $Na_4P_2O_7$, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Samples were prepared in Laemmli sample buffer and separated by SDS-PAGE, followed by Western blotting on Hybond-ECL nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Blots were stained with phospho-p44/42 MAP kinase antibody (Cell Signaling Technology, Beverly, MA, USA) and with p44/42 MAP kinase antibody (Cell Signaling Technology) or monoclonal Anti-α-Tubulin antibody (Sigma-Aldrich) after stripping. Proteins were visualized using ECL chemiluminescent Western Blotting Kit (GE Healthcare). Band intensities were determined with Quantity One software (Bio-Rad, Hercules, CA, USA).

Collagen invasion assay. A native collagen type I solution was prepared by mixing the following pre-cooled (at 4˚C) components on melting ice: 375 μl of native collagen type I (3.35 mg/ml) (BD, Franklin Lakes, NJ, USA) 90 μl of minimum essential medium (MEM) (10×) (Invitrogen), 430 μl of calcium- and magnesium-free Hanks' balanced salt solution pH 7.4, 90 μl of $NaHCO_3$ (0.25 M), 250 μl culture medium and 25 μl of NaOH (1 M); 1.25 ml was poured in a 6-well plate. After gelification, 1 ml culture medium with or without (vehicle control) 0.1, 1 or 10 μM DPDPE, 1 μM leu-enkephalin, 1 μM DADLE and/or 20 ng/ml TGF-α and containing 10^5 cells was added and cultures were incubated for 24 h. Invasion was evaluated by phase-contrast microscopy (Leica DMI3000 B, Leica, Solms, Germany): invasive cells were easily distinguished from non-invasive cells by their bipolar shape and the presence of cellular extensions inside the native collagen type I (Supplementary Figure 1). Invasive and non-invasive cells were counted in 5 randomly chosen microscopic fields/culture and 3 independent cultures were analyzed for each condition.

Multicellular spheroid invasion in native collagen type I. A total of 1.25 ml native collagen type I solution was poured in a 6-well plate. Multicellular spheroids were formed as described above and 1 ml of the multicellular spheroid suspension was transferred to a 15 ml tube and allowed to settle by gravity. This pellet was mixed with 1.25 ml native collagen type I solution and gently poured onto the pre-formed native collagen type I gel. After gelification, 1 ml culture medium with or without (vehicle control) 0.1, 1 or 10 μM DPDPE, 1 μM leu-enkephalin, 1 μM DADLE and/or 100 ng/ml

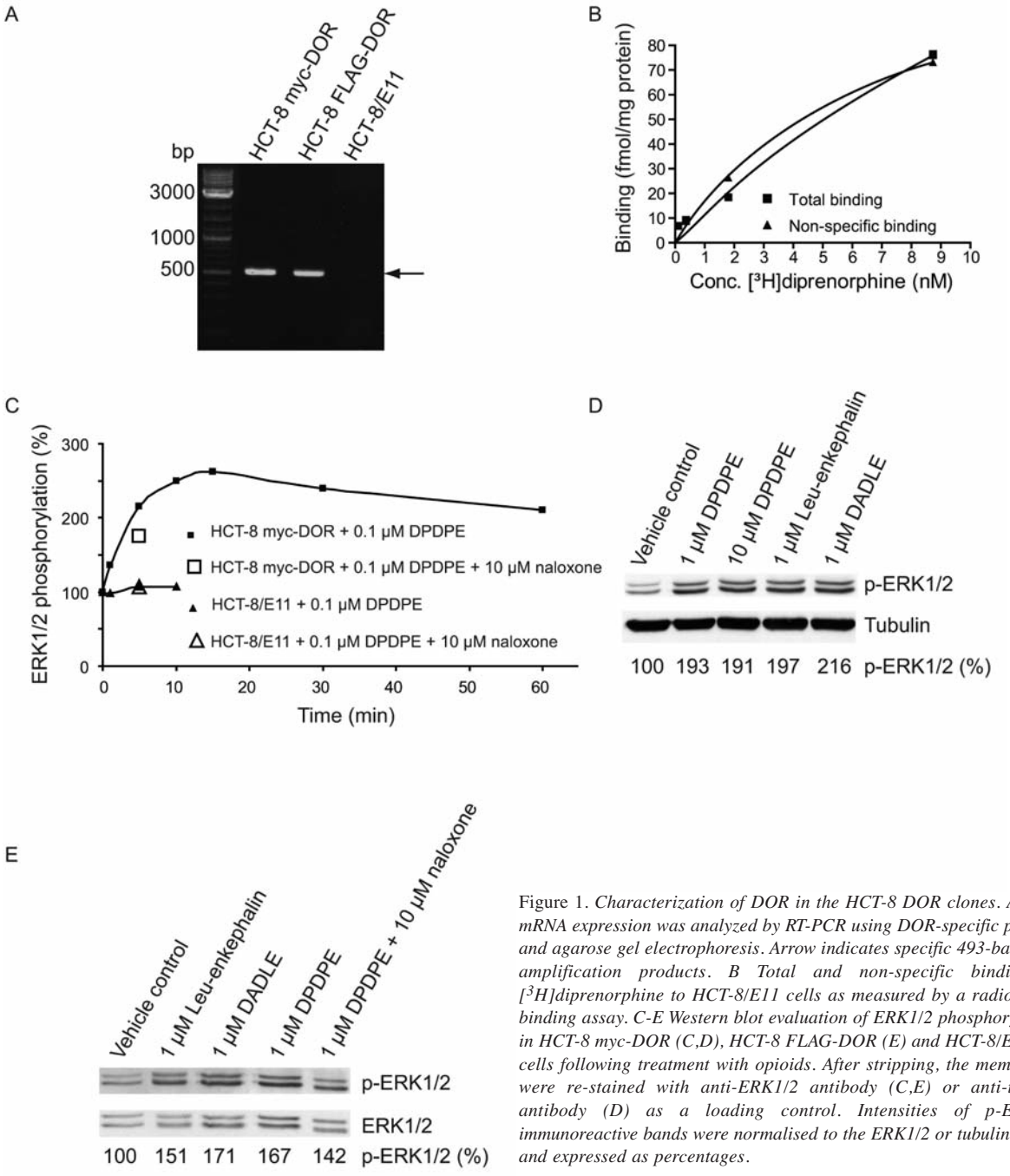

Figure 1. *Characterization of DOR in the HCT-8 DOR clones. A DOR mRNA expression was analyzed by RT-PCR using DOR-specific primers and agarose gel electrophoresis. Arrow indicates specific 493-base pair amplification products. B Total and non-specific binding of [^{3}H]diprenorphine to HCT-8/E11 cells as measured by a radioligand binding assay. C-E Western blot evaluation of ERK1/2 phosphorylation in HCT-8 myc-DOR (C,D), HCT-8 FLAG-DOR (E) and HCT-8/E11 (C) cells following treatment with opioids. After stripping, the membranes were re-stained with anti-ERK1/2 antibody (C,E) or anti-tubulin antibody (D) as a loading control. Intensities of p-ERK1/2 immunoreactive bands were normalised to the ERK1/2 or tubulin values and expressed as percentages.*

TGF-α was added and cultures were incubated for 3 days. Invasive activity was evaluated by phase-contrast microscopy.

Chick heart invasion assay. The assay was performed as described by Bracke *et al*. (26). Briefly, pre-cultured 9-day-old embryonic chick heart fragments (PHF) were confronted with HCT-8 myc-DOR multicellular spheroids or with HCT-8 myc-DOR R cells scraped from a culture flask on semi-solid agar medium. After 24 h, the culture pairs were transferred to 5 ml Erlenmeyer flasks and cultured on a Gyrotory shaker at 120 rpm at 37˚C for 4 days. The cultures were gassed with a mixture of 5% CO_2 in air. The culture medium was supplemented with or without (vehicle control) 0.1 μM DPDPE. After fixation in Bouin-Hollande's solution, cultures were embedded in paraffin, serially sectioned and stained with hematoxylin and eosin.

Sulforhodamine B (SRB) assay. Cells were cultured in 96-well tissue culture dishes in 200 μl culture medium with or without (vehicle control) 0.1, 1 or 10 μM DPDPE, 1 μM leu-enkephalin, 1 μM

DADLE or 1 μg/ml 5-FU for 3 days. Cells were fixed by addition of 50 μl 50% trichloroacetic acid (Sigma-Aldrich) in distilled water and incubation at 4˚C for 1 h. Cells were rinsed with water, dried and stained with sulforhodamine B (0.4% in 1% acetic acid) (Sigma-Aldrich) for 30 min at room temperature. Cells were washed 4× with 1% glacial acetic acid and dried. Protein-bound dye was dissolved in 10 mM Tris buffer, pH 10.5. Optical density was determined at 490 nm with a plate reader (Vmax Microplate Reader; Molecular Devices, Palo Alto, CA, USA).

Slow aggregation assay (SAA) on agar. Tissue culture dishes (96-well) were coated with 50 μl semi-solid agar medium consisting of 100 mg agar (Bacto™ Agar; BD) in 15 ml sterile Ringer's salt solution (Ringer B. Braun, Melsungen, Germany) that was sterilized through boiling 3× for 10 s. After gelification, a single-cell suspension of 2×10^4 cells in 100 μl medium (culture medium containing 5% serum) was seeded onto the agar. Another 100 μl medium with or without (vehicle control) 0.2, 2 or 20 μM DPDPE, 20 μM leu-enkephalin, 20 μM DADLE (final concentrations were respectively, 0.1, 1 and 10 μM DPDPE, 10 μM leu-enkephalin and 10 μM DADLE) or 100 μl CM MRC5 were added and cultures were incubated for 48 h. Aggregate formation was evaluated by bright field microscopy (Leica DMI3000 B, Leica).

Directional migration of multicellular spheroids. Multicellular spheroids were explanted in 24-well plates. After attachment to the substrate, 1 ml culture medium with or without (vehicle control) 0.1, 1 or 10 μM DPDPE, 1 μM leu-enkephalin, 1 μM DADLE or 1 ml CM MRC5 was added and cultures were incubated for 24 h. Images were acquired at 0 h and 24 h with a phase-contrast microscope, equipped with a camera. The mean diameter of the circular area covered by the migrating cells was calculated from 4 measurements in directions differing by 45 degrees. Migration after 24 h was calculated as the mean diameter at 24 h after subtraction of the mean diameter at 0 h.

Statistical analysis. Statistical evaluation of the data was performed with ANOVA using GraphPad InStat3 software (Graphpad Software, Inc.). The logistic regression method was applied for the collagen invasion assay.

Results

Characterization of DOR in HCT-8 myc-DOR and HCT-8 FLAG-DOR cells. DOR transcripts were absent in the parental cell line HCT-8/E11, whereas specific 493-base pair amplification products were detected in the HCT-8 DOR clones (Figure 1A). Radioligand binding studies and Scatchard analysis revealed the presence of 426.9 fmol DOR binding sites/mg protein (B_{max}) and a dissociation constant (K_d) of [^{3}H]diprenorphine of 1.362 nM in HCT-8 myc-DOR cells. In HCT-8 FLAG-DOR cells, B_{max} was 234.7 fmol/mg protein and K_d was 1.139 nM. In HCT-8/E11 cells, total binding equaled non-specific binding measured in the presence of the non-selective opioid naloxone, confirming the absence of opioid receptors (Figure 1B).

Since opioids were shown to activate ERK1/2 (27) we studied ERK1/2 phosphorylation in the HCT-8 DOR clones to verify whether the exogenous DORs were functional. DPDPE stimulated ERK1/2 phosphorylation within 5 minutes of treatment and for at least 1 hour in HCT-8 myc-DOR cells (Figure 1C). The antagonist naloxone reduced DPDPE-stimulated ERK1/2 phosphorylation (Figure 1C), showing that DPDPE signals *via* DOR. In contrast, ERK1/2 phosphorylation was not stimulated in HCT-8/E11 cells after treatment with DPDPE (Figure 1C). Stimulation of ERK1/2 phosphorylation in HCT-8 myc-DOR cells was also observed with 1 μM and 10 μM DPDPE and with leu-enkephalin and DADLE (Figure 1D). HCT-8 FLAG-DOR cells also responded to DPDPE, leu-enkephalin and DADLE by stimulation of ERK1/2 phophorylation (Figure 1E), which could be reduced by naloxone (Figure 1E).

Invasion assays. We first investigated the effect of the DOR agonists on the invasive activity of the HCT-8 DOR clones in native collagen type I gels, the main interstitial matrix component. Although not statistically significant for all opioid treatments separately, the DOR agonists showed a trend to stimulate invasion of HCT-8 myc-DOR cells in native collagen type I, whereas the invasive activity of HCT-8 FLAG-DOR cells was not changed by the DOR agonists (Figure 2a). Because transforming growth factor-α (TGF-α) strongly stimulated invasion of both clones, combination treatments of TGF-α and the opioids were analyzed to evaluate anti-invasive effects (Figure 2b). The DOR agonists showed a trend for an additional pro-invasive effect on invasive HCT-8 myc-DOR cells; they neither stimulated, nor inhibited invasion of HCT-8 FLAG-DOR cells.

Invasive growth of HCT-8 DOR multicellular spheroids in native collagen type I was evaluated after 3 days of treatment with or without the DOR agonists, alone or in combination with TGF-α. All conditions showed invasion into collagen with fingering margins inside the collagen (Figure 3A and Supplementary Figure 2). However, the invasion area of TGF-α–treated spheroids was larger than that of vehicle controls and TGF-α–treated spheroids showed an irregular shape in contrast to the rounder shape of vehicle controls. Moreover, TGF-α stimulated the formation of cellular extensions invading into collagen. This phenotype correlates with pro-invasive signalling by TGF-α (28). The DOR agonists did not change the invasive properties of HCT-8 DOR spheroids since opioid-treated spheroids behaved like vehicle controls. Combination treatments of TGF-α and the opioids revealed that the DOR agonists did not change pro-invasive signalling by TGF-α.

In the chick heart invasion assay HCT-8 myc-DOR cells were non-invasive and formed an epithelioid layer around the chick heart tissue (PHF), whereas the round variant HCT-8 myc-DOR R invaded into the PHF (Figure 3B). Treatment with 0.1 μM DPDPE did not change the invasive activities of the cell lines (Figure 3B), demonstrating that it neither induces invasion of HCT-8 myc-DOR cells, nor inhibits invasion of HCT-8 myc-DOR R cells into PHF.

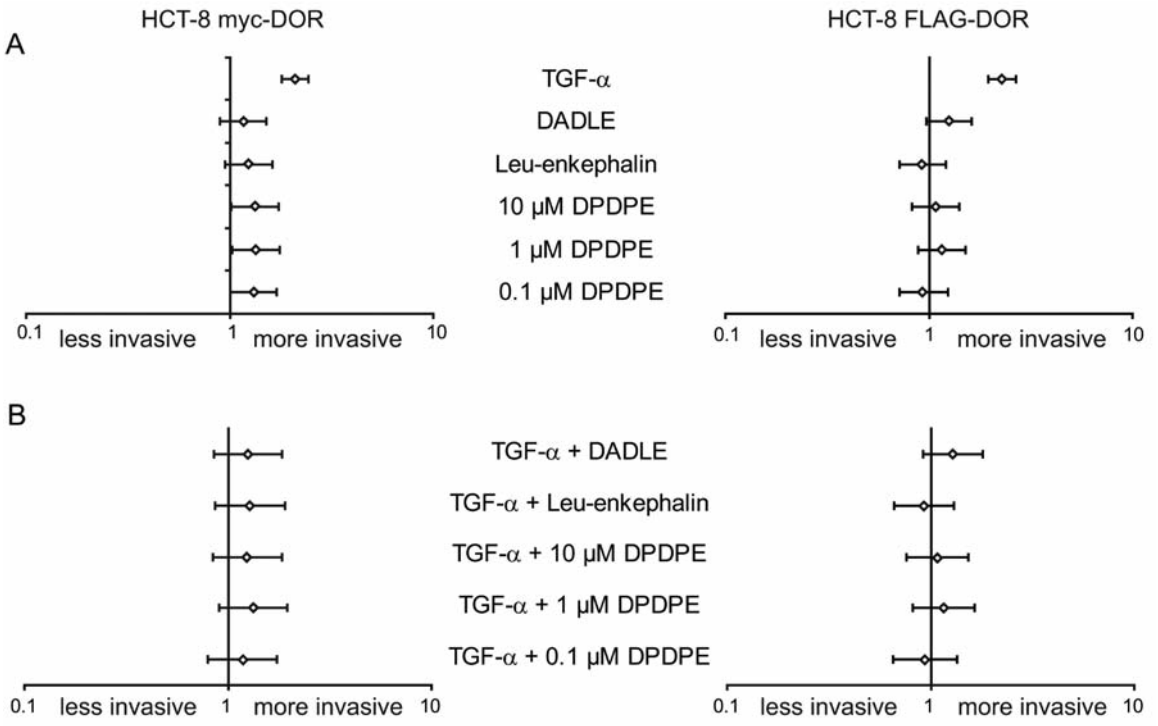

Figure 2. *Invasion of HCT-8 myc-DOR and HCT-8 FLAG-DOR single cells in native collagen type I. The cells were incubated with DOR agonists and/or TGF-α on top of a native collagen type I gel. Data show odds ratios for invasion with 95% confidence intervals comparing invasion of opioid-treated cultures versus vehicle control (A) or TGF-α- + opioid-treated cultures versus TGF-α-treated culture (B).*

Cell growth. To examine whether the DOR agonists affect cell growth, we performed a sulforhodamine B (SRB) assay to measure cellular protein content. The HCT-8 DOR clones were cultured in the presence of vehicle, DPDPE, leu-enkephalin, DADLE or the growth inhibitor 5-FU (29). In contrast to 5-FU, no opioid had an effect on cell growth that differed significantly from the vehicle control after 3 days of treatment (Figure 4A).

Cell–cell adhesion. Homotypical cell–cell adhesion normally counteracts the escape of cells into surrounding tissues, and invasion is thus often associated with reduced homotypical cell–cell adhesion (30). We examined the effects of the DOR agonists on aggregation of the HCT-8 DOR clones in an SAA on agar. HCT-8 myc-DOR cells spontaneously formed irregular, compact aggregates that partly fused (Figure 4B). DPDPE, leu-enkephalin and DADLE did not change the aggregation pattern. Similarly, they did not influence aggregation of HCT-8 FLAG-DOR cells (Figure 4B). In contrast, smaller aggregates were formed in the presence of conditioned medium (CM) of MRC5 fibroblasts (Figure 4B). MRC5 fibroblasts secrete hepatocyte growth factor/scatter factor (HGF/SF) that was shown to induce cell–cell dissociation (31). In order to investigate whether the opioid concentration affects the response, DPDPE was tested at concentrations ranging from 0.1 μM to 10 μM, but did not result in changes of the aggregation patterns (Supplementary Figure 3).

Migration. Directional migration of HCT-8 DOR multicellular spheroids on tissue culture plastic was studied in response to the DOR agonists. CM MRC5, containing HGF/SF, was used as a positive control for migration (32). Directional migration of the HCT-8 myc-DOR spheroids was significantly inhibited after 24 h of treatment by DPDPE at 0.1 μM, while concentrations of 1 and 10 μM had no effect (Figure 4C). Leu-enkephalin and DADLE were also ineffective. Directional migration of HCT-8 FLAG-DOR multicellular spheroids was not influenced by the DOR agonists (Figure 4C).

Discussion

Opioids and opioid receptors were found in colon tumours (1,33), as well as in colon cancer-derived cell lines such as human HT-29 cells (34) and mouse 26-L5 cells (12). Oliveira *et al*. (5) showed the presence of DOR in HCT-8/E11 cells by Western blot analysis. In contrast, our HCT-8/E11 cells do not express DOR as we demonstrated by RT-

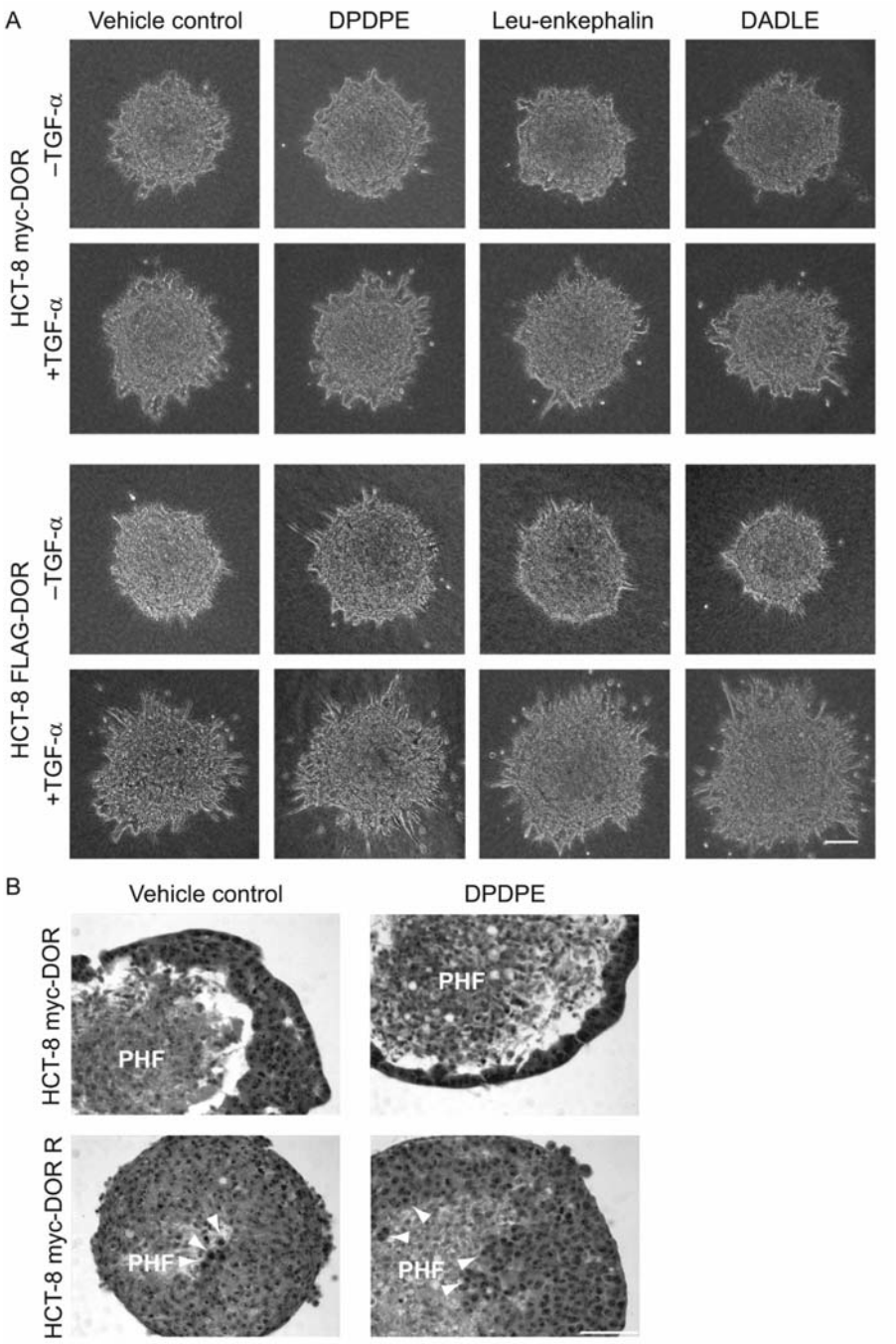

Figure 3. *A Invasion of HCT-8 myc-DOR and HCT-8 FLAG-DOR multicellular spheroids in native collagen type I. Phase-contrast images of spheroids embedded in collagen type I and incubated with DOR agonists at 1 μM and/or TGF-α. Scale bar: 100 μm. B Light micrographs of hematoxylin-eosin stained sections from confronting cultures of pre-cultured embryonic chick heart fragments (PHF) with HCT-8 myc-DOR multicellular spheroids or HCT-8 myc-DOR R cells in the absence or presence of DPDPE. Arrowheads show HCT-8 myc-DOR R cells invading the PHF. Scale bar: 100 μm.*

PCR, a radioligand binding experiment and absence of stimulation of ERK1/2 phosphorylation by DPDPE. HCT-8/E11 cells may have lost the ability to express DOR during cell culture. Indeed, HCT-8/E11 is a subclone from the HCT-8 cell line that was shown to be genetically unstable due to a mutated mismatch repair gene (35). Therefore, to ensure expression of DOR, we established the HCT-8 DOR clones that stably overexpress functional DOR as evidenced by the stimulation of ERK1/2 phosphorylation in response to the DOR agonists.

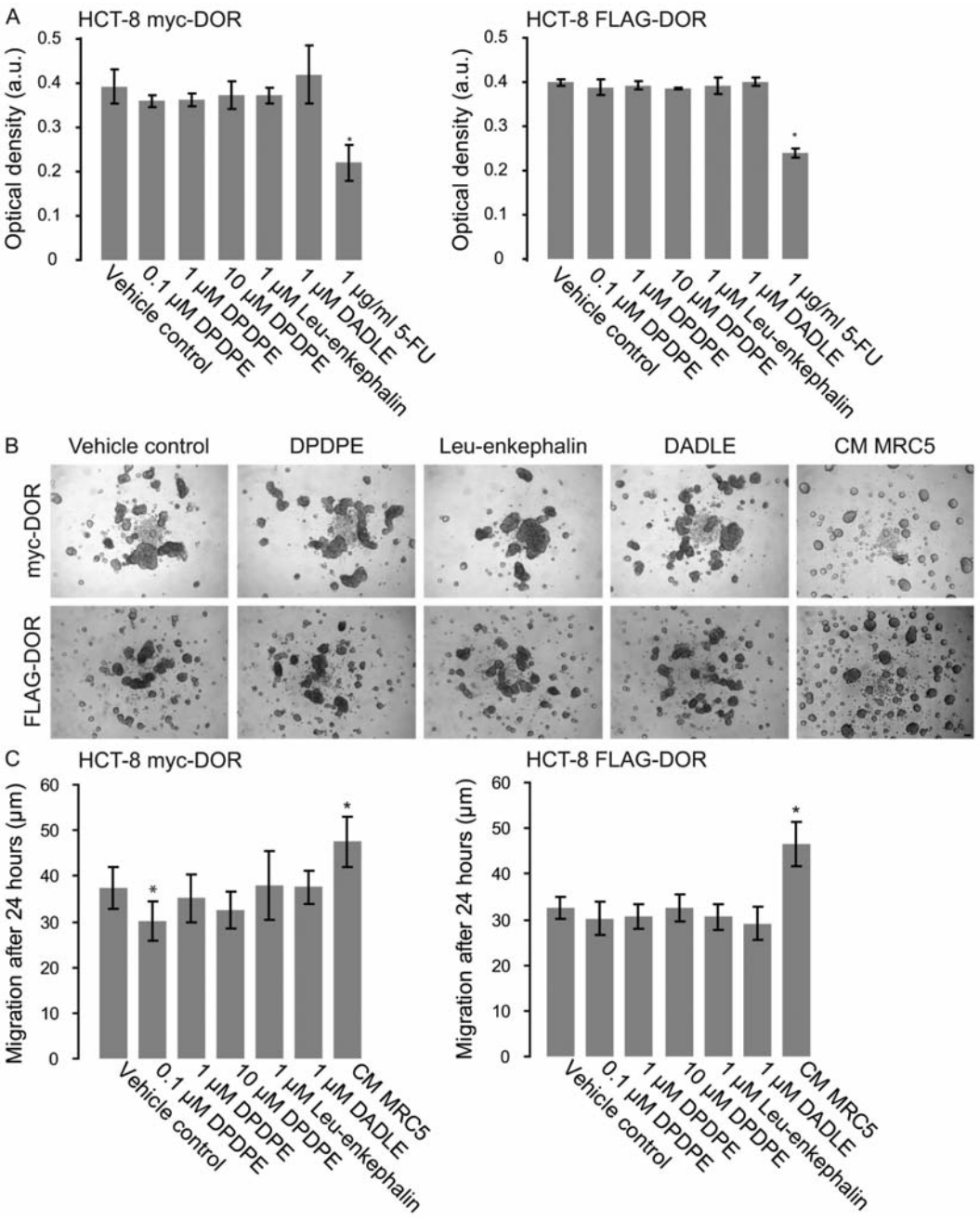

Figure 4. *DOR agonists do not affect cell growth of the HCT-8 DOR clones. A SRB assay was performed on cultures that were treated with DOR agonists or 5-FU. Cellular protein content was measured as optical density at 490 nm. Bars represent mean optical density±standard deviation calculated from 5 cultures. *Significantly different from the vehicle control (p-value<0.01). B Homotypical cell–cell adhesion is not affected by DOR agonists in the HCT-8 DOR clones as evidenced by a SAA. Bright field images of cultures that were treated with DOR agonists at 1 µM or CM MRC5. Scale bar: 100 µm. C Directional migration of the HCT-8 DOR clones on tissue culture plastic in response to DOR agonists or CM MRC5. Bars represent mean migration after 24 h calculated from at least 5 cultures, with error bars for standard deviation. *Significantly different from the vehicle control (p-value<0.05).*

The present study shows that invasion-associated cellular activities of the HCT-8 DOR clones are differently modulated by the DOR agonists and that several factors may account for these differences. In the HCT-8 myc-DOR clone, the DOR agonists showed a trend to stimulate invasion of single cells into native collagen type I, whereas in the HCT-8 FLAG-DOR clone, they did not influence invasion. Scatchard analysis revealed that HCT-8 myc-DOR cells contain more DOR-

binding sites than HCT-8 FLAG-DOR cells (B_{max} values were 426.9 and 234.7 fmol/mg protein, respectively), suggesting that the expression level of DOR may determine the opioid response. Similarly, the DOR agonists stimulated ERK1/2 phosphorylation to a larger extent in HCT-8 myc-DOR cells than in HCT-8 FLAG-DOR cells. The pro-invasive trend of the effect of DOR agonists on the HCT-8 myc-DOR clone was not observed in the other invasion assays, multicellular spheroid invasion in native collagen type I or the chick heart invasion assay. These assays may be less sensitive, but the invasion assays also differ in utilizing single cells *versus* multicellular spheroids and the use of cell-free native collagen type I *versus* living chick heart tissue as a matrix. These differences may account for the different outcomes, illustrating the influence of the experimental set-up on the response to opioid. A growing number of studies have shown that opioid receptors may exist in multiple active states that differ in signalling properties following activation by different agonists (36). We found that DPDPE, but not DADLE and leu-enkephalin, were able to inhibit directional migration of HCT-8 myc-DOR multicellular spheroids. Moreover, these results also show that the opioid concentration may be a factor determining the response to opioid since 0.1 μM DPDPE significantly inhibited directional migration of HCT-8 myc-DOR multicellular spheroids, but DPDPE had no significant effect at concentrations of 1 and 10 μM. These different results are in line with the ambiguous reports of direct opioid effects on invasion-associated cellular activities in literature. Ogasawara *et al*. (16) found that leu-enkephalin reduced the invasive potential of mouse 26-L5 colon carcinoma cells in Matrigel in a concentration-dependent manner, with increasing inhibition of invasion with concentrations ranging from 10^{-10} to 10^{-6} M, whereas Nagakawa *et al*. (22) reported no effect of leu-enkephalin in the same concentration range on invasion of human PC-3 prostate cancer cells in Matrigel. However, the lack of effects on PC-3 cells might be due to the absence of DOR (15). Zagon *et al*. (21) studied the influence of a variety of natural and synthetic opioids, including leu-enkephalin at 10^{-6} M μM, on invasion in Matrigel of a variety of human cancer cell lines. None of the opioids tested altered invasion. These studies clearly demonstrate that the choice of cell line may be responsible for the different responses. Apart from different expression levels of the opioid receptors, cell line-specific effects may be determined by the signalling pathways and effector systems that are present in the cell line (37). Different effects of opioids on cell growth (38) were also reported. Migration of MCF7 cells was inhibited by αs1 casomorphin, a kappa opioid receptor (KOR) agonist, (14), whereas a β-casein derived peptide stimulated migration of HCT-8/E11 cells, possibly *via* DOR signalling (5). Besides the differences in cellular context, activation of different opioid receptor subtypes may here account for the different outcomes. In the present study we found that cell aggregation of the HCT-8 DOR clones was unaffected by the DOR agonists as assessed in an SAA on agar, whereas Debruyne *et al*. (39) found that morphine and other mu opioid receptor (MOR) agonists stimulated aggregation of HEK FLAG-MOP cells stably overexpressing MOR on agar. These different results show again that the cellular context is a major determinant of the observed opioid effect. In addition, different opioids were used that signal *via* different opioid receptor subtypes.

In conclusion, the present study shows that opioids can influence cellular activities associated with the invasion phenotype, but their effects are determined by various factors such as cellular context, opioid receptor expression level, type of opioid, opioid concentration and experimental set-up.

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