Original Research

Low-dose naltrexone targets the opioid growth factor–opioid growth factor receptor pathway to inhibit cell proliferation: mechanistic evidence from a tissue culture model

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Abstract

Naltrexone (NTX) is an opioid antagonist that inhibits or accelerates cell proliferation *in vivo* when utilized in a low (LDN) or high (HDN) dose, respectively. The mechanism of opioid antagonist action on growth is not well understood. We established a tissue culture model of LDN and HDN using short-term and continuous opioid receptor blockade, respectively, in human ovarian cancer cells, and found that the duration of opioid receptor blockade determines cell proliferative response. The alteration of growth by NTX also was detected in cells representative of pancreatic, colorectal and squamous cell carcinomas. The opioid growth factor (OGF; [Met^5]-enkephalin) and its receptor (OGFr) were responsible for mediating the action of NTX on cell proliferation. NTX upregulated OGF and OGFr at the translational but not at the transcriptional level. The mechanism of inhibition by short-term NTX required p16 and/or p21 cyclin-dependent inhibitory kinases, but was not dependent on cell survival (necrosis, apoptosis). Sequential administration of short-term NTX and OGF had a greater inhibitory effect on cell proliferation than either agent alone. Given the parallels between short-term NTX *in vitro* and LDN *in vivo*, we now demonstrate at the molecular level that the OGF–OGFr axis is a common pathway that is essential for the regulation of cell proliferation by NTX.

Keywords: low-dose naltrexone, LDN, opioid, cell proliferation, opioid antagonist, opioid growth factor, OGF, opioid growth factor receptor, OGFr, enkephalin, cyclin dependent inhibitory kinases

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Introduction

Naltrexone (NTX) is a general opioid receptor antagonist that is devoid of intrinsic activity, and blocks opioids from opioid receptors.[1–4] Opioid antagonist modulation of endogenous opioid systems has been used to decipher the function of opioid peptide–opioid receptor interactions in a number of biological processes and diseases.[5–15] One function of endogenous opioids is the regulation of growth through a tonically active inhibitory pathway.[16] Systemic exposure to a high dose of NTX (HDN) or a low dose of NTX (LDN), given multiple times each day, continuously blocks opioid receptors and accelerates cell proliferation and growth.[13,15,17–21] In contrast, intermittent or short-term opioid receptor blockade, achieved by daily administration of LDN or a low dose of naloxone, blocks opioid peptide–opioid receptor interactions for a short term each day (e.g. 4–6 h), and inhibits cell proliferation and growth in the interval when the opioid antagonist is no longer present.[6,15,18,22–24]

The opposing effects on growth observed with continuous and intermittent opioid receptor blockade are related to the pharmacological action of opioid antagonists. The response to opioid antagonist administration is a compensatory upregulation in the production of opioid peptides and opioid receptors.[20,25–27] Unlike continuous opioid receptor blockade, wherein the upregulated opioid peptides and receptors do not have the opportunity to interface, pharmacokinetic,[28] as well as nociceptive and functional,[15,18] studies have shown that blockade of opioids from opioid receptors for a short term (4–6 h) each day provides an 18–20 h window where the elevated opioids and opioid receptors can interact to elicit an exaggerated response (e.g. depression in cell proliferation).[23,29–31] In the case of neoplasia, for example, persistent blockade of opioid receptors from endogenous opioids has a profound effect on oncogenesis by accelerating tumor appearance and growth.[13,15,18,22] However, a temporary blockade of opioid receptors from native opioids markedly suppresses the onset and progression of carcinogenesis.[6,13,15,18,22,23]

One particular endogenous opioid–opioid receptor system that serves as a determinant of cell proliferation and growth, and is modulated by NTX, is the opioid growth factor (OGF) and its receptor, OGFr.[19,21] OGF, chemically termed [Met^5]-enkephalin, is a constitutively expressed native opioid peptide that is autocrine produced and secreted.[15] OGF interacts with OGFr (a non-classical opioid receptor) to delay the G_1/S phase of the cell cycle by modulating cyclin-dependent kinase inhibitory (CKI) pathways, and represses cell proliferation in normal and neoplastic cells.[16,32–35] An increase in OGF–OGFr activity in cancer cells by the addition of exogenous OGF,[35–43] treatment with imidazoquinoline compounds such as imiquimod and resiquimod[44] or transfection of sense cDNA for OGFr[45,46] depresses cell proliferation. In contrast, attenuation of the OGF–OGFr axis in cancer cells through disruption of peptide–receptor interfacing by continuous exposure to NTX,[35,37,39,43] neutralization of OGF by antibodies to this peptide[35,43] or a decrease in OGFr by antisense cDNA or siRNA[35,43,47] stimulates cell proliferation.

A number of lines of evidence suggest that NTX regulates growth through modulation of opioid peptide–opioid receptor interactions, and specifically through the OGF–OGFr axis. First, the effects of opioid antagonists on growth are stereospecific, indicating that the action of these agents is dependent on an opioid receptor.[24,48] Second, the duration of opioid receptor blockade, rather than drug dosage, is a determinant of the direction of growth effects, implying that these antagonists have an indirect action.[15] Third, OGF is the only opioid peptide that has been found to suppress cell proliferation.[35,49] Fourth, the influence of OGF is mediated by an opioid receptor.[16,35] Fifth, knockdown of OGFr blocks the effects of OGF, indicating that this opioid receptor is responsible for mediating peptide action.[35,43] Sixth, OGF and OGFr have been shown to be upregulated by treatment with NTX.[20,21] Although these observations provide a compelling argument that the OGF–OGFr axis is fundamental to NTX action with respect to growth, it may be argued that they are circumstantial rather than causal. To determine the mechanism of NTX's effects on growth, we have developed a tissue culture model of this opioid antagonist that parallels *in vivo* events with respect to LDN by exposing cells for six hours to NTX (i.e. short-term NTX) and examining the repercussions on cell proliferation. Such a tissue culture model removes the confounding influences introduced by systemic biology, and allows direct observation of mechanistic pathways related to LDN. We now show at a molecular level that LDN specifically targets the OGF–OGFr axis to regulate cell proliferation, and that opioid-based modulation of growth requires both OGF as well as OGFr.

Materials and methods

Cell culture

Human cancer cell lines, SKOV-3,[50] OVCAR-3,[51] MiaPaCa-2[52] and HCT-116,[53] were obtained from the American Type Culture Collection (Manassas, VA, USA), while SCC-1[54] was provided by Dr T Carey (Director of the University of Michigan Cancer Research Laboratory). Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C in the following media: Dulbecco's medium (MiaPaCa-2, SCC-1), RPMI 1640 medium (OVCAR-3, SKOV-3) and McCoy's 5a medium (HCT-116). All media were supplemented with 10% fetal calf serum, 1.2% sodium bicarbonate and antibiotics (5000 units/mL penicillin, 5 μg/mL streptomycin and 10 mg/mL neomycin), unless otherwise noted.

Cell growth

Log phase cells were plated and counted 24 h later (time 0) to determine seeding efficiency. Cultures were treated with NTX (10^{-5} mol/L) or an equivalent volume of sterile water. At the end of six hours, the media containing compound was removed and replaced with media either lacking NTX (short-term NTX) or containing NTX (continuous NTX). Media and compounds (when applicable) were replaced daily. All compounds were prepared in sterile water and dilutions represent final concentrations. An equivalent volume of sterile water was added to control wells. At designated times, cells were harvested with trypsin, stained with trypan blue to evaluate cell viability and counted with a hemacytometer. At least two aliquots per well and two or more wells/treatment/time point were sampled.

Antibody neutralization

Endogenous OGF was neutralized with a polyclonal antibody to this peptide (CO172, 1:200);[55] preimmune rabbit serum (IgG, 1:200) and sterile water-treated cells served as controls. Antibody, IgG and media were changed daily, and cells were counted after 72 h.

siRNA knockdown

Cells were transfected for 24 h with 20 nmol/L concentrations of one of the following siRNAs: MOR (mu opioid receptor), DOR (delta opioid receptor) or KOR (kappa opioid receptor) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), OGFr (Ambion, Austin, TX, USA), p16 or p21 (Santa Cruz Biotechnology), using Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were collected for growth curves or Western blotting to determine the level of protein knockdown 72 h after the start of transfection.[34] Two independent experiments were conducted.

Protein isolation and Western blotting

The level of protein knockdown resulting from siRNA transfection with MOR, DOR, KOR, OGFr, p16 or p21, as well as expression of OGFr following treatment with NTX, was determined by Western blotting following published procedures.[35] In brief, cells were solubilized in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche, Indianapolis, IN, USA). Protein (60 μg) was subjected to 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis, transferred to nitrocellulose and

probed with antibodies (1:200) against OGFr,[55] MOR, DOR, KOR or p16 (Santa Cruz Biotechnology) or p21 (BD PharMingen, San Diego, CA, USA). Optical densities were normalized to β-actin (1:5000; Sigma-Aldrich, St Louis, MO, USA), and the percent change in expression was calculated by dividing the normalized values of experimental samples to that of controls. Means and standard error (SE) were determined from two independent experiments.

RNA isolation, Northern blotting and reverse transcriptase-polymerase chain reaction

To evaluate OGFr mRNA levels, Northern blotting was performed according to Zagon *et al.*[44] Total RNA was extracted using the Paris Kit (Ambion), separated on an agarose gel, transferred to a nylon membrane (Immobilon, Bio-Rad Laboratories, Hercules, CA, USA) and probed with ^{32}P-dCTP-OGFr cDNA or ^{32}P-dCTP-GAPDH cDNA. Optical densities were normalized to GAPDH and the percent change in expression was calculated by dividing the normalized values of experimental samples by that of sterile water-treated controls. Means and SE were ascertained from at least two independent experiments.

To determine preproenkephalin (PPE) mRNA expression levels, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. cDNA was synthesized from 0.7 μg denatured total cellular RNA and reverse transcribed in a final volume of 20 μL using the superscript III kit (Invitrogen). The cDNA equivalent of 0.2 μg total RNA was amplified by PCR in a final volume of 50 μL buffer containing 50 mmol/L KCl, 10 mmol/L Tris-HCl, 0.1% Triton X-100, 2 mmol/L $MgCl_2$, dNTPs (0.25 mmol/L each), 1 μM of each primer and 0.25 U Taq polymerase. PCR was carried out for 35 cycles in a PerkinElmer thermocycler (PerkinElmer, Foster City, CA, USA). The first cycle consisted of denaturation at 94°C for five minutes, annealing at 60°C for one minute, and primer extension at 72°C for 1 min. For the next cycles, the denaturation time was one minute, and during the last cycle, primer extension lasted 10 min. As a control, β-globulin was amplified under the same conditions as described for PPE. The 21-mer sense oligonucleotide GCGACGGTGAGGCCCTACGTC and 23-mer antisense oligonucleotide AGCCGGGTTCAGACACGACTCTA were used to amplify a 113 bp PPE fragment, while the 20-mer sense oligonucleotide ACACAACTGTGTTCACTAGC and 20-mer antisense CAACTTCATCCACGTTCACC were used to amplify a 100 bp β-globulin fragment. PCR products were run on a 2% agarose gel, visualized with ethidium bromide and the optical density of each band was determined and analyzed by QuickOne (Bio-Rad Laboratories). Each value was normalized to β-globulin. At least two samples were evaluated for each group, and means and SE were determined from three independent experiments.

Semiquantitative immunohistochemistry

To examine the distribution and relative levels of OGF and OGFr, log-phase cells grown on 22-mm round cover glasses were fixed and stained with antibodies to OGF and OGFr according to published procedures.[35,44] Polyclonal antibodies to OGF and OGFr were generated in the laboratory and have been fully characterized.[55] Controls included cells incubated only with secondary antibodies. Images were taken at the same exposure time with special care not to photobleach samples. The mean intensity of staining was determined for at least 100 cells/group, and three cover glasses/group. At least three cover glasses were examined.

Radioimmunoassay

To determine the levels of OGF secreted from cells into culture media, log phase SKOV-3 cells were treated for six hours with NTX or an equivalent volume of sterile water in serum-free media. At six hours, NTX-containing media was removed and replaced with serum-free media either lacking or containing NTX, as previously described. Media was not replaced from this time point onwards in the experiment so as to measure accumulated levels of secreted OGF. At designated times, 1 mL samples of media were collected and assayed for OGF using a radioimmunoassay kit from Peninsula Laboratories (San Carlos, CA, USA). Sterile serum-free media was also monitored as a control. At least two samples in duplicate were evaluated for each group.

OGFr binding assays

Receptor binding assays for OGFr were performed in log-phase cells treated with NTX (10^{-5} M) for either a short term or continuously using custom synthesized [^{3}H]-[Met^5]-enkephalin (PerkinElmer, Waltham, MA, USA; 52.7 Ci/mmol).[35,45,46] Non-specific binding was measured in the presence of unlabeled [Met^5]-enkephalin. Saturation binding isotherms were generated using GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA, USA), and independent assays were performed in duplicate at least three times.

DNA synthesis, apoptosis and necrosis

To evaluate DNA synthesis, cells were seeded on 22-mm diameter cover glasses in six-well plates and, at designated times, pulsed with 30 μM bromodeoxyuridine (BrdU; Sigma-Aldrich) for three hours. Preparations were fixed with 10% neutral-buffered formalin and stained with antibodies to BrdU (Invitrogen).[35] Using similar preparations, apoptosis was assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL; Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions.[35] Necrosis was ascertained by trypan blue exclusion staining.[35]

Chemicals

The following compounds were obtained from the indicated sources: [Met^5]-enkephalin (OGF), [Leu^5]-enkephalin (leu enk), [D-Pen^{2,5}]-enkephalin (DPDPE), [D-Ala^2,MePhe^4, Glyol^5]-enkephalin (DAMGO), β endorphin (β-endo), NTX, naloxone, dynorphin A1-8 (dynorphin), morphine sulfate (morphine), endomorphin 1 (endo-1), endomorphin 2

(endo-2), Sigma-Aldrich; U69,583, Upjohn Diagnostics (Kalamazoo, MI, USA).

Statistical analysis

All data were analyzed by means of GraphPad Prism software (GraphPad Software, Inc) using one-way analysis of variance, with subsequent comparisons made using Newman–Keuls tests.

Results

Short-term NTX treatment depresses the growth of cancer cells

To determine whether the duration of NTX treatment affects the growth of cancer cells, SKOV-3 cultures were treated with 10^{-5} M NTX, a concentration that is not toxic but has marked efficacy on growth, either (i) once for six hours (short-term NTX), (ii) six hours every 24 or 48 h; or (iii) continuously for 24 h on a daily basis (i.e. continuous NTX). A single application of short-term NTX, as well as exposure to short-term NTX every 48 h, inhibited cell number by 22–29% from control levels at 48–96 h (Figure 1a). In contrast, cultures treated with short-term NTX every 24 h had a comparable number of cells as controls at all time points evaluated. Continuous exposure to NTX increased cell number by 22–42% from control levels at 48–96 h.

To ascertain how long short-term NTX suppresses the proliferation of SKOV-3 cells, cultures were exposed only once for six hours with NTX or sterile water and monitored daily for cell number from 96–192 h. Cell number was reduced from control levels at 96 and 120 h. However, from 144 h onwards, cell number in short-term NTX-treated cultures was comparable with that of controls.

In a subsequent set of experiments, the growth effects of NTX given daily for a period of time shorter than six hours were assessed. Cultures treated daily for one, two or three hours and examined at 72 h were decreased by 24–33% from controls subjected to sterile water. Cultures treated with NTX on a daily regimen for four, five or six hours had comparable cell numbers to control levels.

Short-term opioid receptor antagonism with naloxone inhibits cancer growth

To determine whether another opioid antagonist administered for a short term alters growth, SKOV-3 cultures were treated with a single application of naloxone for only six hours. Administration of 10^{-3} and 10^{-4} M naloxone inhibited cell number by 28% and 25%, respectively, at 72 h (data not shown). Cultures subjected to 10^{-5}or 10^{-6} M naloxone, however, displayed no differences in cell number from controls receiving an equivalent volume of sterile water.

OGF is the endogenous opioid peptide specific for growth inhibition of cancer cells

To determine which opioid(s) is(are) responsible for the growth inhibition recorded with short-term NTX treatment, SKOV-3 cultures were exposed continuously for 72 h to 10^{-6} M concentrations of natural or synthetic opioid-related compounds, some specific for μ, δ and κ opioid receptors. OGF was the only opioid that had an effect on cell growth, depressing cell number by 32% compared with controls exposed to sterile water, a level equivalent to that recorded for short-term NTX (Figure 1b).

To test the specificity of short-term NTX on OGF with regard to cell proliferation, an antibody neutralization experiment was performed. At 72 h, in contrast to a reduction of 27% in cell number for cultures exposed to short-term NTX and treated with sterile water or IgG, cells treated with both short-term NTX and the antibody to OGF no longer exhibited a reduction in cell number (Figure 1c).

Silencing of OGFr, but not classical opioid receptors, blocks the inhibitory action of short-term NTX

The requirement of classical and/or non-classical opioid receptors for short-term NTX's action was evaluated at the molecular level using siRNA technology. Western blot analysis revealed that MOR, DOR, KOR or OGFr siRNA-transfected SKOV-3 cultures had reductions of 64 to 92% in these receptor protein levels relative to untransfected or scrambled siRNA transfected cultures (Figures 2a–e). Relative to untransfected or scrambled siRNA cultures, cells transfected with MOR, DOR or KOR siRNAs had an equivalent number of cells; however, cultures transfected with OGFr siRNA had 42% more cells (Figure 2e). The addition of short-term NTX inhibited cell number in cultures transfected with scrambled, MOR, DOR or KOR siRNA by 33% to 42% in comparison with cultures transfected with these same siRNAs and treated with sterile water. However, cell proliferation was not reduced by short-term NTX in cultures transfected with OGFr siRNA and, in fact, was 43% greater than untransfected cells exposed to sterile water. For comparative purposes, OGF depressed cell number in untransfected cells, as well as in cultures transfected with MOR, DOR, KOR or scrambled siRNAs, but not in preparations transfected with OGFr siRNA wherein cell number was 45% greater than in untransfected vehicle-treated cultures.

Repercussions of NTX treatment on transcription and translation of OGF

Studies on the expression of OGF and the gene that encodes this peptide, PPE, were evaluated in SKOV-3 cultures administered short-term NTX, continuous NTX or an equivalent volume of sterile water. PPE mRNA expression levels in cells were comparable regardless of treatment with NTX or sterile water at all time points examined (Figure 3a). Cellular levels of OGF monitored by semiquantitative immunohistochemistry, however, were increased in both the short-term and continuous NTX-treated cultures by 11% to 18% at 24 h and 22 to 32% at 72 h from sterile water-treated controls (Figure 3b). For all cultures, OGF was visible in the cytoplasm, and a speckling of immunoreactivity noted in cell nuclei. Cells processed only with secondary antibody showed no staining.

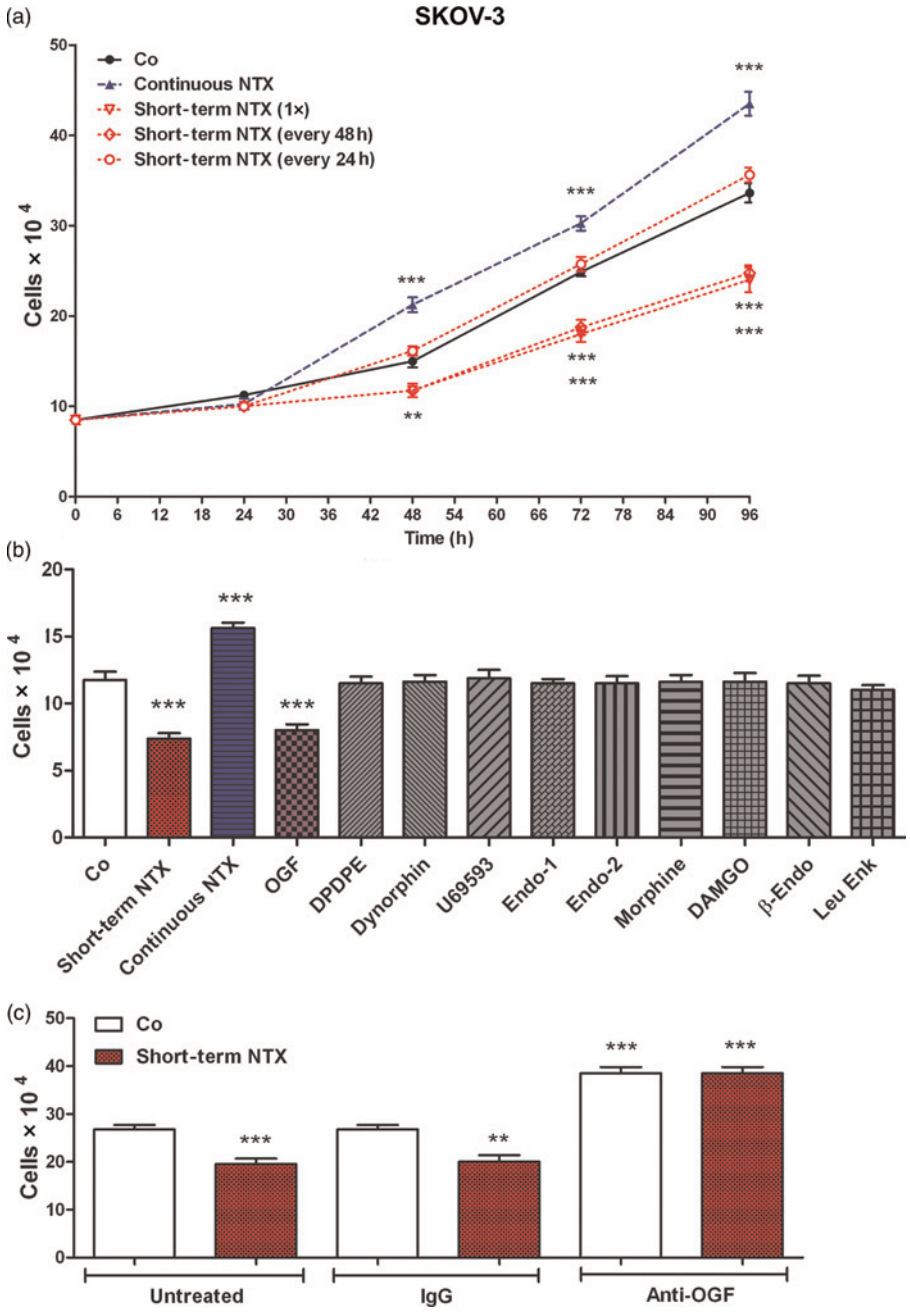

Figure 1 Short-term and continuous exposure to opioid agonists or antagonists and the growth of SKOV-3 cells. (a) Growth curves of cells subjected to short-term NTX (either once, every day or every other day), continuous NTX or an equivalent volume of sterile water (Co) over a 96 h period. (b) Cell number at 72 h in cultures exposed to a variety of opioid-related compounds or the opioid receptor antagonist NTX either once for a short term (6 h) or on a continual (daily) basis. (c) Cell number in cultures treated with NTX or an equivalent volume of sterile water for six hours. At the end of six hours, NTX-containing media was removed and replaced with media lacking NTX, and cultures were either administered a polyclonal antibody specific for OGF or pre-immune serum (IgG), or were not treated (untreated). Anti-OGF, IgG, compounds, and media were replaced daily unless otherwise noted, and cell number counted at 72 h. Data represent means ± SE from at least two aliquots/well and two wells/treatment group. Significantly different from sterile water-treated Co at respective times by $^{**}P < 0.01$ and $^{***}P < 0.001$. NTX, naltrexone; OGF, opioid growth factor; SE, standard error (A color version of this figure is available in the online journal)

To examine the effects of NTX treatment on the secretion of OGF from SKOV-3 cells, cultures were treated with this opioid antagonist for either a short-term or continuous duration; media was changed only at six hours. OGF levels in the media were similar in control, short-term and continuous NTX cultures at six hours (Figure 3c). However, by 24, 48 and 72 h, OGF levels were increased from control values by 23, 25 and 48%, respectively, in media from cultures treated with short-term NTX. In media sampled from cultures subjected to continuous NTX, OGF levels were increased 19% at 24 h, decreased at 48 h by 32% and similar to control levels at 72 h.

Repercussions of NTX treatment on transcription and translation of OGFr

OGFr mRNA levels were evaluated in SKOV-3 cultures administered short-term NTX, continuous NTX or an

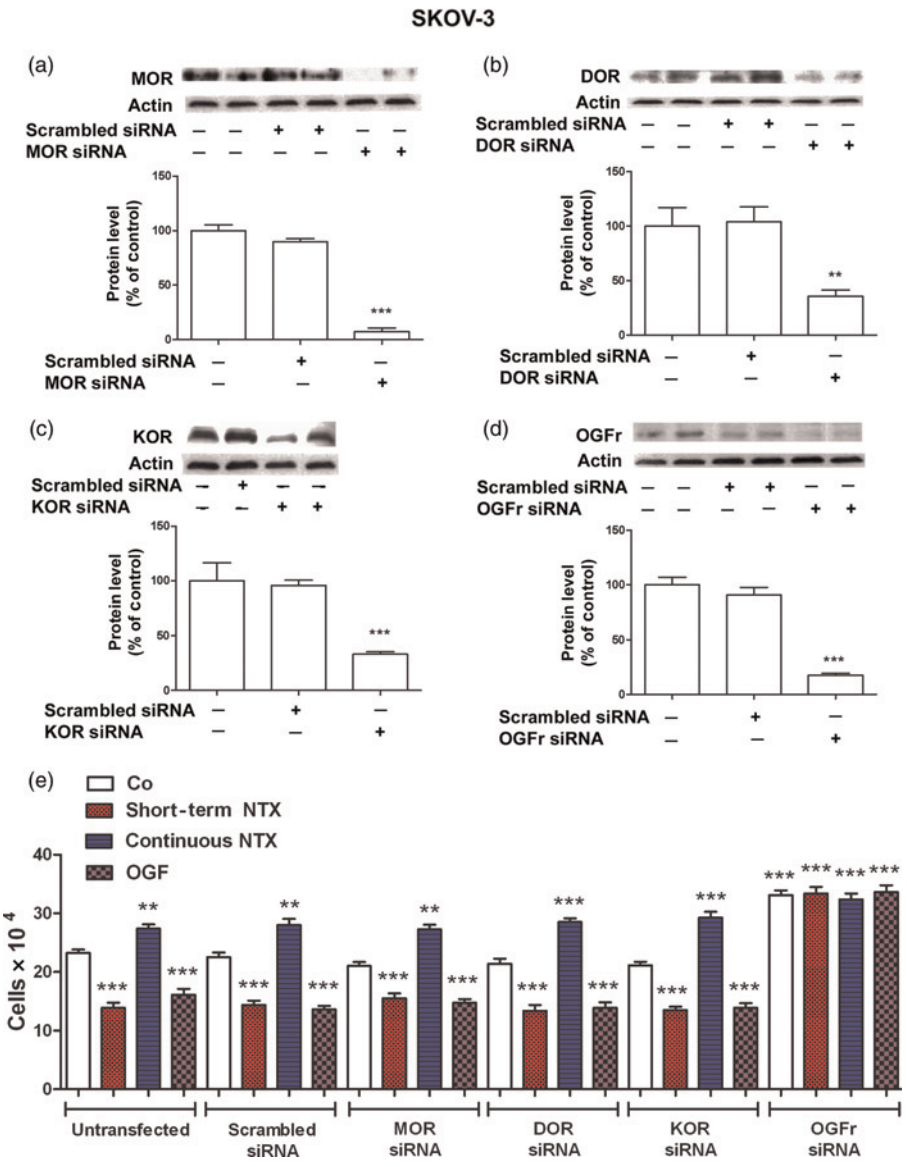

Figure 2 OGFr is required for short-term NTX and OGF's inhibitory action on the growth of SKOV-3 cells. (a–d) Western blot analysis and quantitative densitometry indicating the specificity and level of (a) MOR, (b) DOR, (c) KOR and (d) OGFr protein knockdown. Log-phase cells were transfected for 24 h with the indicated siRNAs; protein was isolated 72 h after the start of transfection. Data represent means ± SE for the percent of MOR, DOR, KOR, or OGFr relative to actin from two independent experiments. (e) Cell number at 72 h in cultures transfected with the indicated siRNAs. Six hours prior to termination of transfection, cultures were treated with either OGF, NTX or an equivalent volume of sterile water (Co). At 24 h, media containing transfection reagents and compounds was replaced with media lacking NTX (short-term NTX) or containing NTX (continuous NTX) or OGF. Compounds and media were replaced daily, except for the short-term NTX group where this opioid antagonist was only administered once for six hours. Values represent means ± SE from at least two aliquots/well and two wells/treatment. Significantly different from untransfected Co cultures at $^{**}P < 0.01$ or $^{***}P < 0.001$. OGF, opioid growth factor; OGFr, opioid growth factor receptor; NTX, naltrexone; SE, standard error; MOR, mu opioid receptor; DOR, delta opioid receptor; KOR, kappa opioid receptor (A color version of this figure is available in the online journal)

equivalent volume of sterile water. Expression of OGFr mRNA was comparable in cells treated with either regimen of NTX or sterile water (Figure 4a).

Cellular levels of OGFr as detected by semiquantitative immunohistochemistry were increased from control levels in both the short-term and continuous NTX cultures at 48 and 72 h by 13 to 28% (Figure 4b). For all cultures, OGFr was observed in the cytoplasm, with light-speckled staining noted in cell nuclei. Cells processed only with secondary antibody showed no staining. Western blot analysis revealed that cultures treated with short-term or continuous NTX were similar in OGFr levels at 6 and 24 h to that of cultures receiving sterile water, but increased 1.7- to 3.8-fold at 48 and 72 h (Figure 4c).

To further characterize OGFr, receptor binding analysis of nuclear fractions was performed. Binding affinities did not differ between cultures treated with short-term NTX, continuous NTX or sterile water at 72 h, with K_d values ranging from 4.9 to 5.4 nM (Figure 4d). Values for binding capacity of NTX-treated cells, however, were increased at 72 h compared with cultures receiving sterile water (5.8 ± 0.8), with increases of 109% for short-term NTX and 46% for continuous NTX recorded.

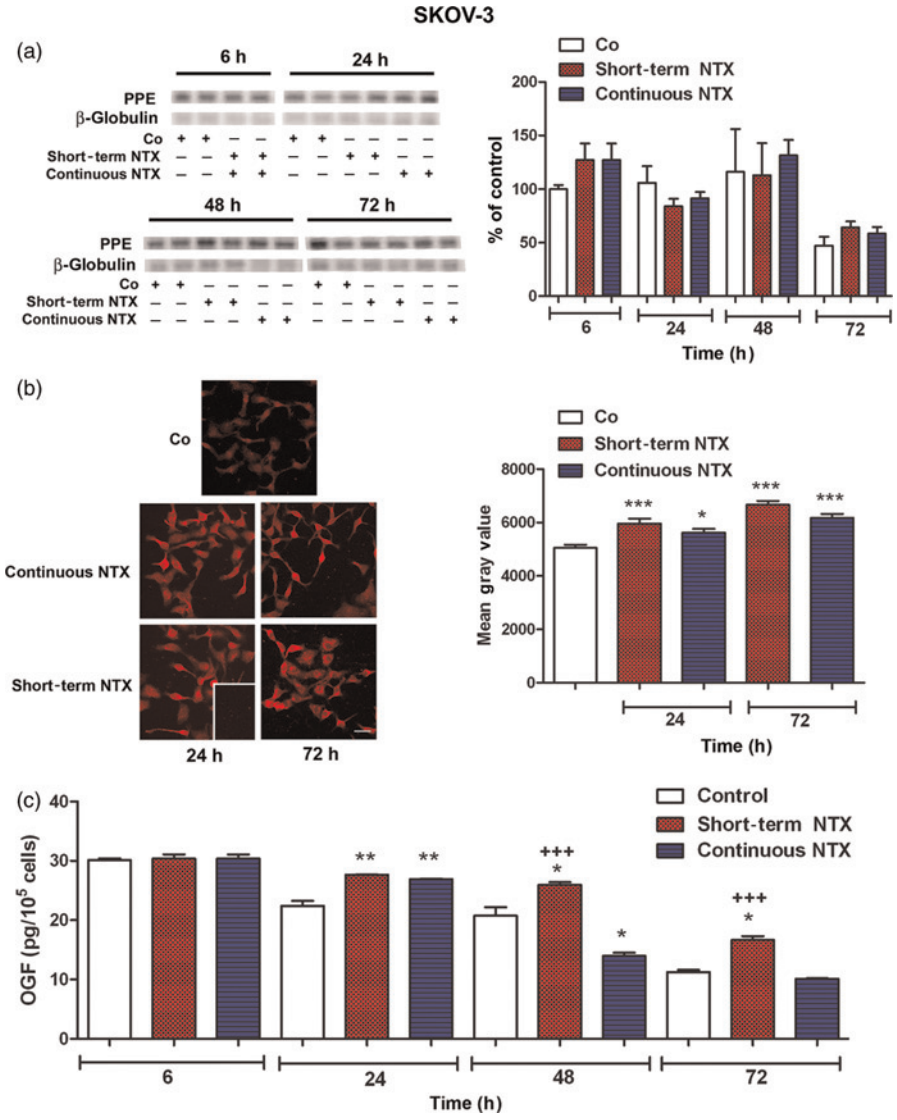

Figure 3 The effects of short-term and continuous NTX treatments on PPE mRNA and OGF. Cultures were administered NTX either once for six hours or continuously, or subjected to an equivalent volume of sterile water (Co). Media and compounds were replaced daily unless otherwise indicated. (a) Expression of PPE mRNA. RNA was isolated at the indicated times, reverse transcribed and the generated cDNA was amplified by PCR with primers for PPE and β-globulin. PCR products were separated on an agarose gel, stained with ethidium bromide, and analyzed by densitometry. Data represent means ± SE for the percent of the ratio of PPE relative to β-globulin from two wells/treatment group in three independent experiments. (b) Expression of cellular OGF. Photomicrographs of cells stained with a polyclonal antibody to OGF, and semiquantitative densitometry of staining intensity (mean gray value). Inset = secondary antibody only. Bar = 10 μm. Data represent means ± SE for at least 100 cells/cover glass and three cover glasses/treatment group. (c) Levels of secreted OGF in cultures treated with NTX in serum-free media either once for a short term (6 h) or continuously or subjected to an equivalent volume of sterile water (Co). At six hours, media containing compound was replaced with serum-free media; media was not changed after this time point. The continuous NTX group received this opioid antagonist on a daily basis. Media was collected at indicated times and subjected to radioimmunoassay. Data represent means ± SE from at least two wells/treatment group assayed in duplicate. Significantly different from Co at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, and from continuous NTX at $^{+++}P < 0.001$. NTX, naltrexone; SE, standard error; OGF, opioid growth factor; PPE, preproenkephalin (A color version of this figure is available in the online journal)

The effects of short-term NTX on cell proliferation are ubiquitous

To examine the ubiquity of short-term NTX on cell proliferation, human cancer cell lines representing another ovarian cancer (OVCAR-3), pancreatic cancer (Mia PaCa-2), squamous cell carcinoma of the head and neck (SCC-1), and colorectal cancer (HCT-116) were treated with short-term NTX and examined 72 h later. These cell lines receiving this regimen of NTX were reduced in cell number by 24–31% from their sterile water-treated controls, a result similar to that of cultures subjected to OGF (Figure 5). In contrast, continuous exposure of these cell lines to NTX increased cell number by 16–27% from sterile water controls.

The combination of short-term NTX and OGF diminishes cancer cell number to a greater extent than either agent alone

To ask whether exogenous OGF enhances the repressive effect of short-term NTX, OGF was added to SKOV-3 and OVCAR-3 cells six hours following a short-term exposure to NTX at the time when this opioid antagonist was removed from cultures; OGF and media were

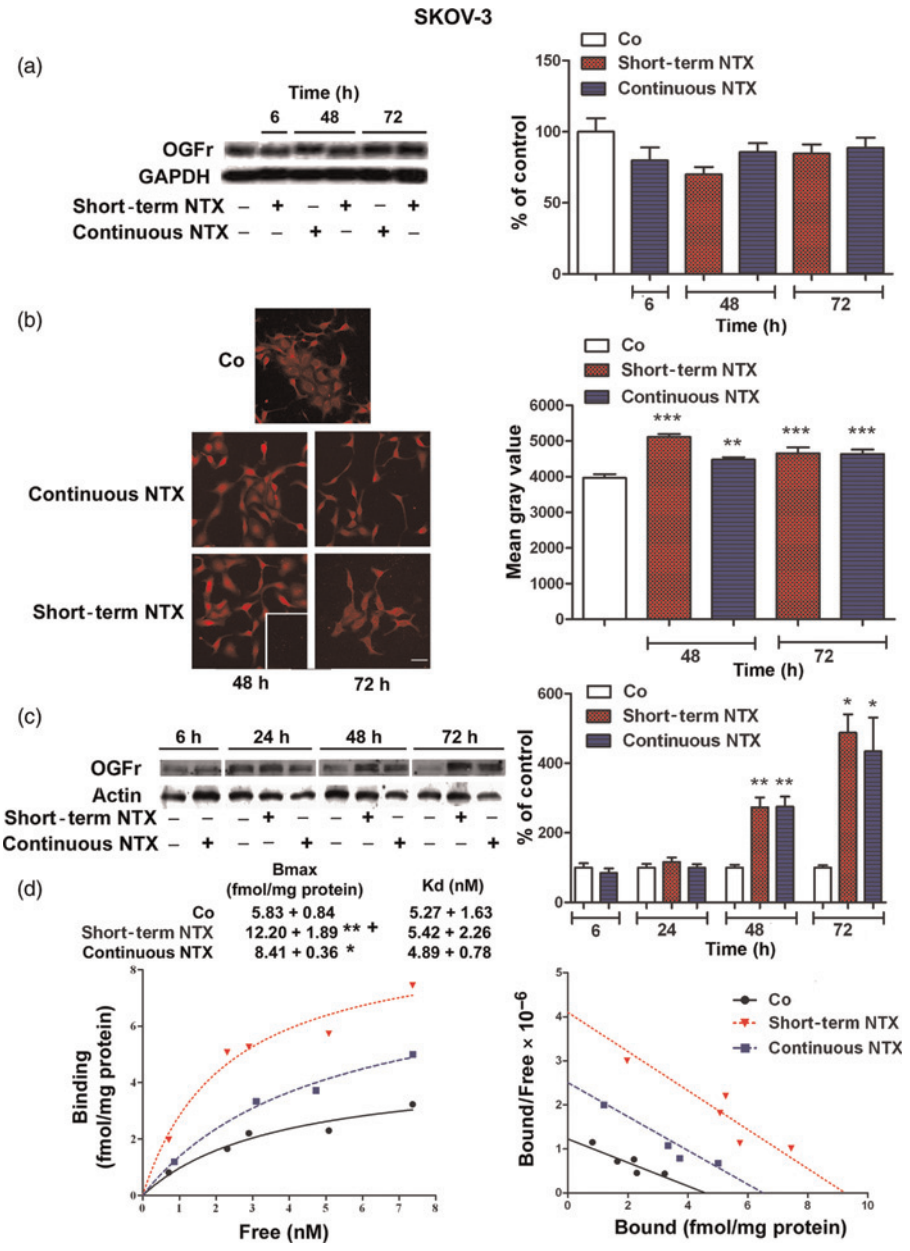

Figure 4 The effects of short-term and continuous NTX treatments on OGFr mRNA and protein. Cultures were treated with NTX either once for a short term (6 h) or continuously, or with an equivalent volume of sterile water (Co). (a) OGFr mRNA levels measured by northern blot and densitometric analysis at the indicated times. Data represent means ± SE for the percent of OGFr relative to GAPDH from two independent experiments. (b) OGFr expression assessed by immunohistochemistry. Photomicrographs of cells stained with a polyclonal antibody to OGFr and evaluated by semiquantitative densitometry (mean gray value). Bar = 10 μm. Data represent means ± SE for 100 cells/cover glass and three cover glasses/treatment group. (c) OGFr expression measured by Western blot analysis. Total proteins were isolated at the indicated times, probed with antibodies specific to OGFr or actin, and measured by quantitative densitometry. Data represent means ± SE of the percent of OGFr relative to actin from two independent experiments. (d) Representative saturation isotherms and Scatchard plots of specific binding of [^{3}H]-[Met^5]-enkephalin to nuclear homogenates of SKOV-3 cells at 72 h. Means ± SE for binding affinity (K_d) and binding capacity (B_{max}) determined from at least three independent assays performed in duplicate. Significantly different from Co at $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, and from continuous NTX at $^+P < 0.05$. OGFr, opioid growth factor receptor; NTX, naltrexone; SE, standard error (A color version of this figure is available in the online journal)

replaced daily. Administration of either OGF or short-term NTX reduced the number of ovarian cancer cells by 20–33% from control levels at 72 h (Figure 6). However, the sequential treatment with short-term NTX and OGF depressed the number of SKOV-3 and OVCAR-3 cells by 35 and 61%, respectively, from sterile water-treated controls. In contrast to SKOV-3 cells treated with either NTX or OGF, the combination of these agents reduced cell number by 13–19%. With regard to OVCAR-3 cells, exposure to both NTX and OGF decreased cell number by 41 to 43% relative to cells treated with just one compound.

Short-term NTX alters DNA synthesis but not apoptosis or necrosis

To evaluate the mechanism by which short-term NTX inhibits human cancer cell growth, as well as to

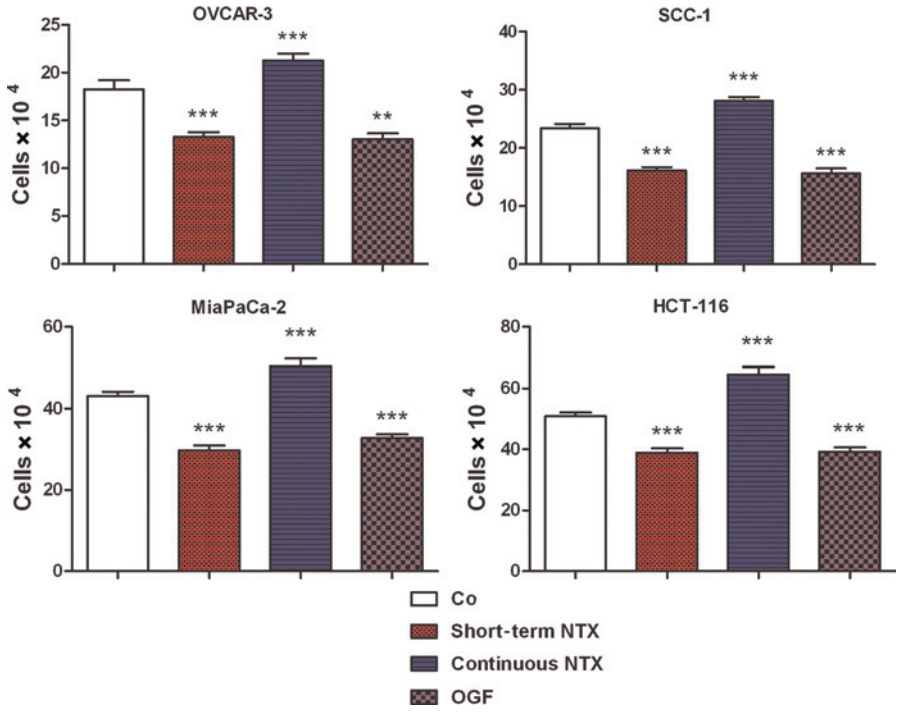

Figure 5 The effects of NTX and OGF are ubiquitous. Cell number at 72 h in OVCAR-3, SCC-1, MiaPaCa-2, and HCT-116 cells exposed to NTX for either a short-term or continuous duration, OGF, or an equivalent volume of sterile water (Co). Data represent means ± SE. Significantly different from Co at $^{**}P < 0.01$ or $^{***}P < 0.001$. NTX, naltrexone; OGF, opioid growth factor; SE, standard error (A color version of this figure is available in the online journal)

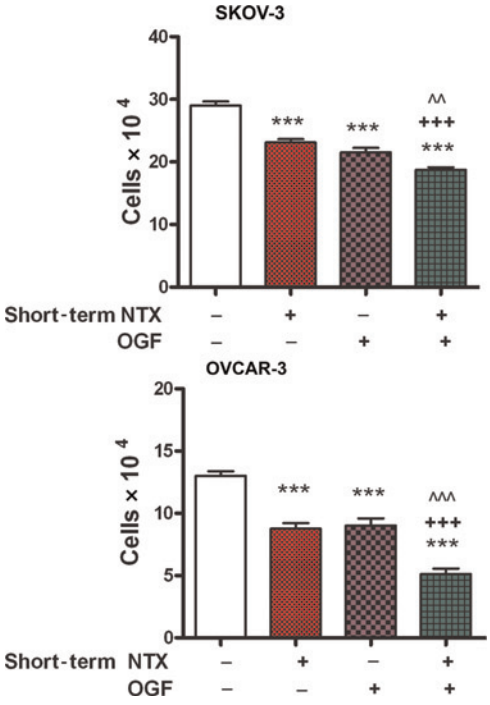

Figure 6 The combination of short-term NTX followed by daily OGF treatment provides an additive inhibitory effect on reducing cell number in SKOV-3 and OVCAR-3 cultures. Cell number at 72 h in cultures treated with NTX once for six hours (short-term NTX), OGF, the combination of short-term NTX followed by OGF or an equivalent volume of sterile water (Co). Media was replaced daily, as were compounds except for the short-term NTX group. Values represent means ± SE for at least two aliquots/well and two wells/treatment group. Significantly different from Co at $^{***}P < 0.001$, from OGF at $^{\wedge\wedge}P < 0.01$ and $^{\wedge\wedge\wedge}P < 0.001$, and from short-term NTX at $^{+++}P < 0.001$. NTX, naltrexone; OGF, opioid growth factor; SE, standard error (A color version of this figure is available in the online journal)

determine the duration of opioid receptor blockade, DNA synthesis (as measured by BrdU incorporation) in SKOV-3 cultures exposed at 0 h to short-term NTX was monitored. The proportion of BrdU-labeled cells in cultures receiving short-term NTX was increased by 50%, 49% and 18% at 6, 9 and 12 h, respectively, relative to control levels, whereas at 15 h, the proportion of BrdU positive cells was comparable to that of the control group (Figure 7a). From 18 to 72 h, the proportion of BrdU-labeled cells in cultures that were given short-term NTX was decreased by 34–48% relative to control values.

To compare the effects of short-term NTX, continuous NTX and OGF treatment on DNA synthesis, SKOV-3 cultures were subjected to short-term NTX, continuous NTX, OGF or an equivalent volume of sterile water and pulsed with BrdU at designated times. BrdU incorporation in cells treated with continuous NTX was increased by 10–37% from 6 to 72 h compared with control levels (Figure 7b). In contrast, DNA synthesis in short-term NTX-treated cultures was increased by 37% at six hours, but decreased by 39%, 45% and 32% at 24, 48 and 72 h, respectively, relative to controls at these time points. The degree of inhibition on DNA synthesis seen with short-term NTX was similar at 24, 48 and 72 h to that recorded in cultures receiving continuous OGF.

Examination of apoptosis (TUNEL) and necrosis (trypan blue staining) at 72 h in SKOV-3 cells treated with short-term NTX revealed less than 0.1% positive cells for apoptosis and necrosis, and these data were comparable with that obtained with cells subjected to sterile water.

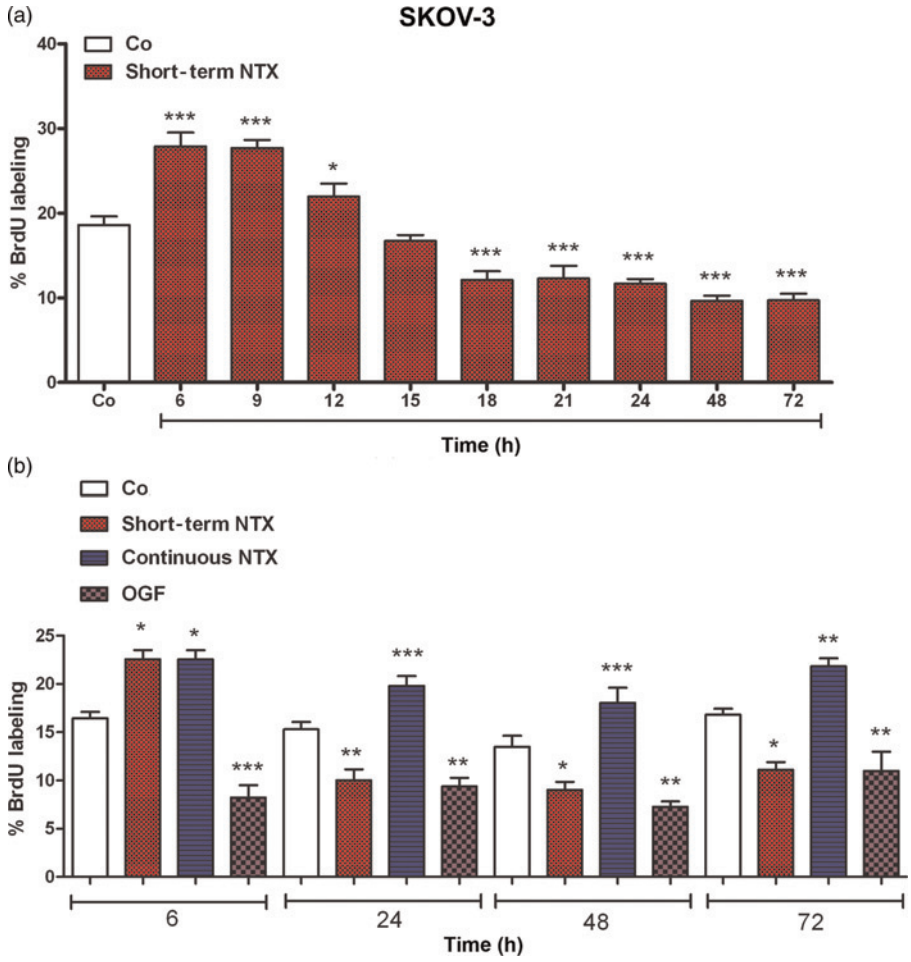

Figure 7 Effect of short-term NTX, continuous NTX, or OGF on DNA synthesis. (a) DNA synthesis in cells treated with one six hour exposure to NTX (short-term NTX) or an equivalent volume of sterile water (Co), and incubated with BrdU three hours prior to fixation at the indicated times. Treatments were initiated so that all groups were harvested at 72 h. (b) DNA synthesis in cells treated with one six-hour application of NTX (short-term NTX), continuous NTX, OGF or an equivalent volume of sterile water (Co), and incubated with BrdU three hours prior to fixation at the indicated times. Data represent means ± SE. Significantly different from Co by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. NTX, naltrexone; OGF, opioid growth factor; SE, standard error; BrdU, bromodeoxyuridine (A color version of this figure is available in the online journal)

p16 and/or p21 are required for short-term NTX-induced growth inhibition

To evaluate whether the mechanism of short-term NTX's effects on cell proliferation requires the p16 and/or p21 CKI pathways, SKOV-3 and OVCAR-3 cells were transfected with p16, p21, both p16 and p21 or scrambled siRNA and treated with short-term NTX. At 72 h, Western blot analysis revealed that p21 expression in SKOV-3 cells transfected with p21 siRNA was reduced 67% from untransfected cells – no expression of p16 was recorded (Figure 8a). OVCAR-3 cells, however, transfected with p16 and/or p21 siRNA had significant reductions in expression of p16 (up to 52%) and p21 (up to 54%), relative to untransfected control cultures (Figure 8c).

Short-term NTX treatment in SKOV-3 cells (which lack p16) reduced cell number from control levels by up to 40% when cells were either untransfected or transfected with scrambled or p16 siRNA (Figure 8b). Cell number in cultures with a knockdown of p21 and treated with short-term NTX was comparable to control values. A similar pattern of cell alterations was noted with exposure to OGF. Continuous NTX treatment accelerated cell replication by up to 32% regardless of transfection with p16 and/or p21 siRNA.

Using OVCAR-3 cells (which contain p16 and p21), short-term NTX repressed cell number up to 43% in untransfected cultures or cells transfected with either scrambled, p16 or p21 siRNAs (Figure 8d). However, when both p16 and p21 were knocked down in combination, the number of cells at 72 h in cultures exposed to short-term NTX or OGF was comparable to that of sterile water controls. In contrast, continuous exposure to NTX accelerated cell replication up to 63% from control levels, regardless of transfection with p16 and/or p21 siRNAs.

Discussion

This study shows for the first time in a tissue culture model that brief exposure to the opioid antagonists NTX or naloxone suppresses cell proliferation, and that the effects are mediated by opioid peptide–opioid receptor pathways

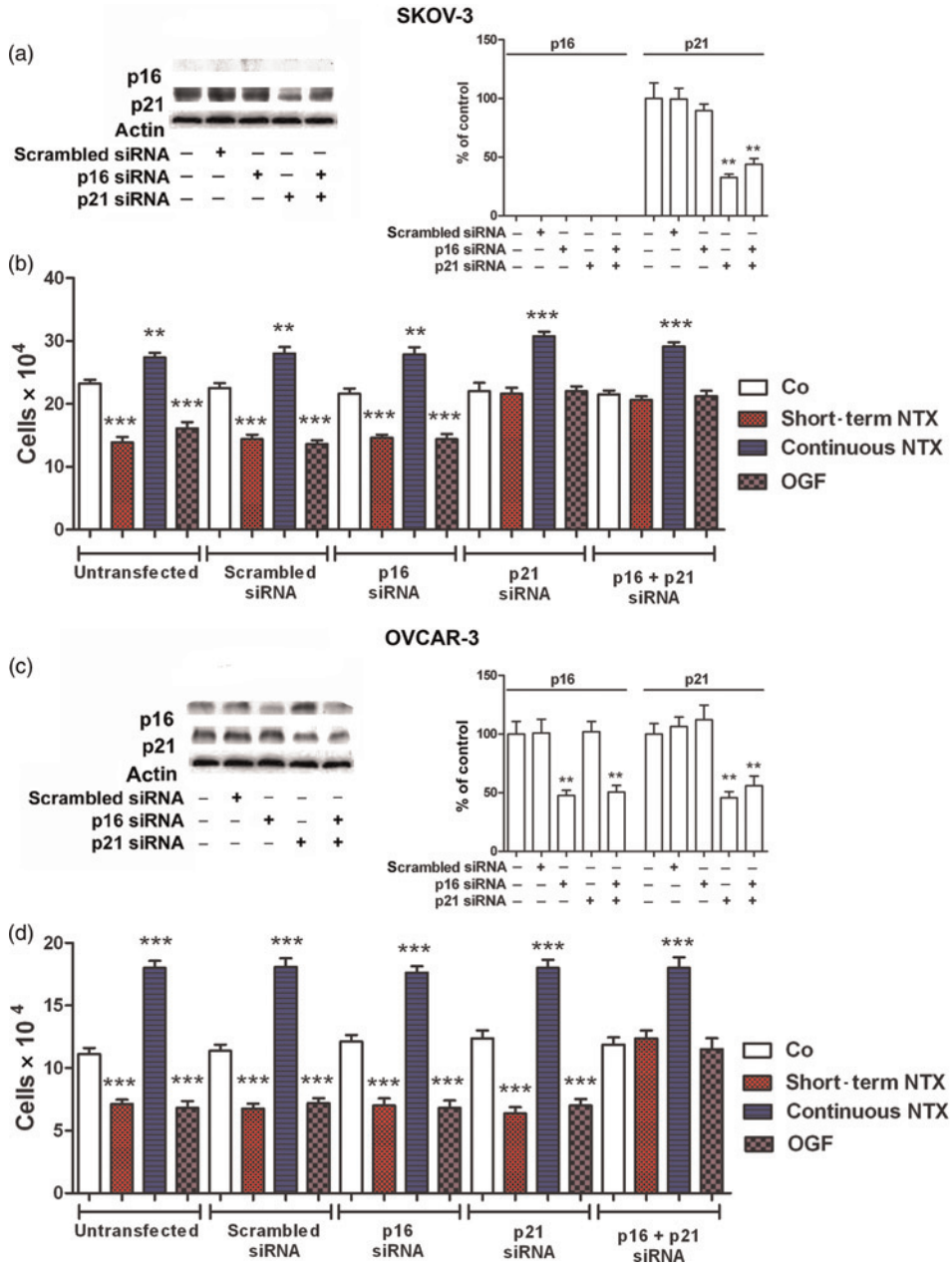

Figure 8 Inhibition of cell proliferation by short-term NTX treatment requires p16/p21 pathways. (a, c) Western blot analysis demonstrating the specificity and knockdown of p16/p21 in (a) SKOV-3 and (c) OVCAR-3 cells. Cells were transfected for 24 h with p16/p21 or scrambled siRNAs, total proteins isolated 72 h after the start of transfection, preparations probed with antibodies specific to p16, p21 or actin, and blots measured by quantitative densitometry. Data represent means ± SE for the percent of p16 or p21 relative to actin from two independent experiments. (b, d) Cell number at 72 h in (b) SKOV-3 and (d) OVCAR-3 cells transfected with the indicated siRNAs and treated with NTX either for a short term or continuously, OGF or an equivalent volume of sterile water (Co). Values represent means ± SE for two aliquots/well and two wells/treatment group. Significantly different from untransfected Co cultures at $^{**}P < 0.01$ and $^{***}P < 0.001$. NTX, naltrexone; SE, standard error, OGF, opioid growth factor (A color version of this figure is available in the online journal)

and independent of systemic processes. A number of key observations emerged from this *in vitro* investigation with respect to opioid antagonist action on growth. First, the duration of opioid receptor blockade determines response in terms of the effects on cell proliferation and not the opioid antagonist itself, explaining what appear to be paradoxical repercussions in our tissue culture studies. Hence, the same concentration of drug utilized for varying periods of time resulted in different outcomes. Cells subjected to NTX for six hours once or every two days, but not daily, depressed cell number, suggesting that a sufficient interval is required for a rebound of opioid action to be observed. However, consistent with previous *in vitro* reports,[35,37,38,41–43] continuous blockade of opioid peptides from opioid receptors accelerated cell proliferation, indicating that opioid–receptor interactions function as an inhibitory influence on the cell cycle. Second, opioid peptide–opioid receptor interfacing is tonically active, and critical to the regulation of cell number, as demonstrated by continuous opioid peptide–receptor blockade and escalated cell proliferation. Third, the inhibitory effects of short-term NTX exposure on growth are not permanent,

indicating that opioid antagonist modulation of cell proliferation is temporary and devoid of toxicity. Fourth, the influence of these agents on growth processes is not related to one specific opioid antagonist, with both NTX and naloxone exhibiting modulatory capabilities under *in vitro* conditions. Fifth, the inhibition of cell proliferation by opioid antagonists observed in tissue culture is ubiquitous, and was recorded in four diverse cancers. Sixth, opioid antagonist inhibition of growth is not reliant on systemic factors, as the present studies were conducted in a tissue culture setting. Thus, these findings *in vitro* reveal that opioid antagonist action on growth is not directly related to these agents themselves, but rather is targeted to the interaction of opioid peptides and receptors.

Although it was determined that opioid peptides are tonically active in regulating cell number through a receptor-mediated inhibitory pathway, the peptide(s) and receptor(s) involved required identification. Using a variety of opioids, some with high affinity to classical or non-classical opioid receptors, the pentapeptide OGF was discovered to be the only opioid to alter cell number, a result consonant with previous findings.[35,37,42,49] While the inhibitory action of OGF was similar in magnitude to that of short-term NTX, the relationship of OGF to short-term NTX and its effects on growth mandated examination. When this regimen of opioid antagonist treatment was tested in the face of neutralization of OGF by antibodies, cells were no longer inhibited by exposure to short-term NTX. In fact, cell number was found to be greater than in control cultures, supporting the contention that the opioid peptide (i.e. OGF) involved with short-term NTX was constitutively expressed and tonically active. To determine which opioid receptor functions in short-term NTX action, siRNA technology was used to knockdown the expression of classical and non-classical opioid receptors and these cells were challenged by short-term NTX treatment. The results revealed that the loss of only OGFr eliminated the repressive action of short-term NTX. Therefore, these data demonstrate that suppression of cell number by short-term NTX is determined by a singular endogenous opioid–opioid receptor pathway – OGF–OGFr.

OGF and OGFr were detected in ovarian cancer cells in this report as well as in earlier studies,[35,43] indicating the ubiquitous nature of this axis and its availability for modulation by short-term NTX. In examining the repercussions of NTX on the OGF–OGFr axis, we discovered that transcription of neither PPE (the gene giving rise to OGF) nor OGFr were altered by this opioid antagonist (Figure 9a). However, both OGF and OGFr were upregulated in cells exposed to NTX, signifying that opioid receptor blockade results in a compensatory increase at the translational level (Figure 9a). As long as NTX is present, the upregulated OGF and OGFr cannot interact, thereby allowing cells to escape the regulatory influence of the OGF–OGFr axis (Figure 9b). In the case of short-term NTX, the upregulated peptide and receptor can interface and elicit an exaggerated physiological response – inhibition of cell proliferation in the interval when NTX is no longer present (Figure 9b). Our findings that either a decrease in OGF (i.e. antibody neutralization) or OGFr (i.e. siRNA) can eliminate the inhibitory effect of short-term NTX and increase cell

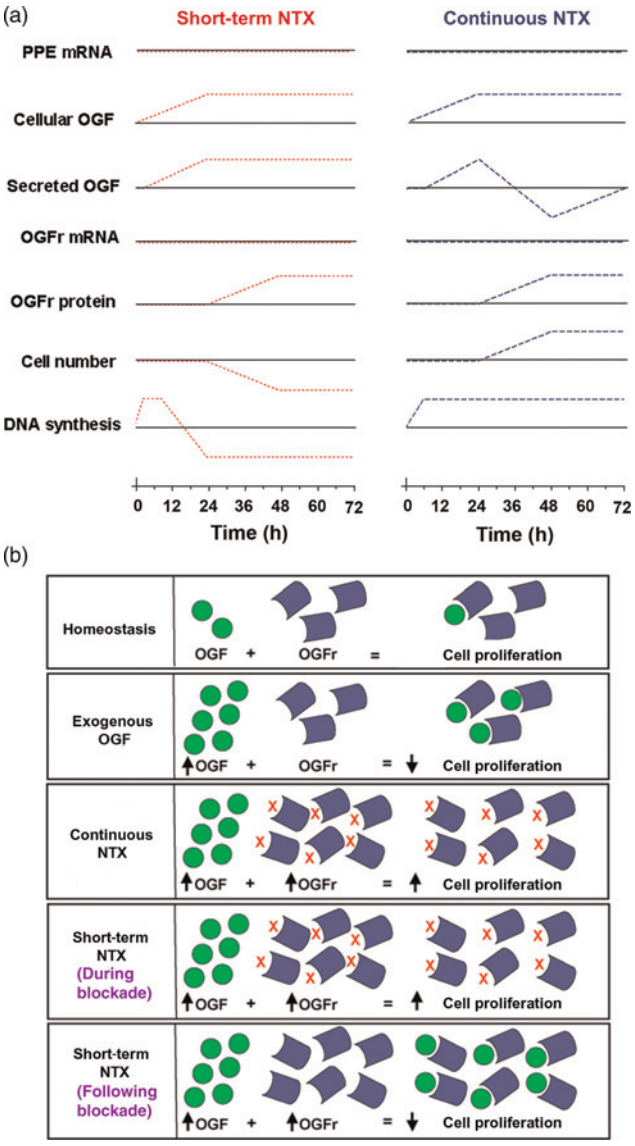

Figure 9 Schematic representation of the effects of NTX administered for either a short-term or continuous duration on the OGF–OGFr axis. (a) Effects of a single six hour application of short-term NTX (dotted line), continuous NTX (dashed line) or equivalent volume of sterile water (solid line) on the levels of PPE mRNA, cellular OGF, secreted OGF, OGFr mRNA, OGFr protein, cell number and DNA synthesis. (b) Schematic illustrating the action of short-term NTX, continuous NTX or exogenous OGF in the regulation of cell proliferative events. OGF, opioid growth factor; NTX, naltrexone; OGFr, opioid growth factor receptor (A color version of this figure is available in the online journal)

number also indicates that the peptide and the receptor are in a self-regulatory autocrine loop which maintains homeostatic equilibrium of cell proliferative processes.

The mechanism of short-term NTX's inhibitory action on cell number was found to be related to DNA synthesis, and not to alterations in pathways of cell survival (apoptosis, necrosis). Although the outcome of short-term NTX treatment was a reduction in cell number, studies using BrdU as a marker for DNA synthesis showed that brief exposure to NTX resulted in a biphasic response (Figure 9a). Thus, DNA synthesis was increased for up to 12 h after the initiation of short-term NTX treatment (i.e. 6 h after termination of

NTX), but was reduced from control levels from 18 h up to three days after initiating opioid antagonist treatment. Moreover, the magnitude of reduction in DNA synthesis in cells exposed to short-term NTX was similar to that of OGF. As expected, based on previous findings,[35,56] DNA synthesis in cells subjected to continuous NTX was increased throughout the three-day period of drug exposure. Additional experimentation with respect to the molecular target of short-term NTX on the cell cycle indicated that p16 and p21 were responsible for NTX's effects. Thus, knockdown of p16 and/or p21 in cancer cells eliminated the inhibitory influence of short-term NTX, as well as OGF as reported earlier,[32–35] thereby providing molecular proof that short-term NTX is dependent on CKI pathways.

The results of this study explored the adaptive response to continuous and discontinuous opioid receptor blockade on physiological processes in a tissue culture environment, and have compelling parallels with the action of LDN and HDN in animals, as well as our overall understanding of the OGF–OGFr axis. These include: (i) LDN and HDN lead to an overall decrease and increase, respectively, in DNA synthesis,[13,20] just as short-term and continuous NTX in tissue culture display dissimilar responses on cell replication; (ii) LDN[13,19] and short-term NTX have a biphasic effect on DNA synthesis, initially elevating cell proliferation for a brief interval followed by a marked decrease for the remaining period of time; (iii) LDN[15,18,24] and short-term NTX are both dependent on the duration of opioid receptor blockade, and not drug dosage; (iv) LDN[15,18,24,48] and short-term NTX do not work directly, but rather indirectly through endogenous opioids and opioid receptors; (v) short-term NTX and OGF[32–35] are both dependent on p16 and/or p21 CKI pathways; (vi) NTX upregulates OGF and OGFr both under *in vitro* and *in vivo*[20,21] conditions; (vii) the effects of LDN[6,15] and short-term NTX are neither toxic nor related to cell survival; and (viii) LDN[6,13,15,17–20] and short-term NTX modulate the growth of a wide variety of cells and tissues. Thus, we submit that a tissue culture model of LDN action *in vivo* has been established, allowing an understanding of the mechanism of LDN in a paradigm that is not confounded by systemic interactions.

Our discovery of LDN and HDN with regard to growth in 1983[17,18] has led to the transition of opioid antagonists and opioid agonists from the bench to the bedside. LDN has been shown to be non-toxic in a phase I clinical trial,[9] and to have efficacy in improving clinical and inflammatory activity, as well as in promoting mucosal healing, in subjects with active Crohn's disease.[57] With our understanding in the present study, the mechanism of LDN now can be seen to be dependent on the OGF-OGFr axis, resulting in depression of cell proliferative processes. Particularly important, is that both T- and B-lymphocyte proliferation have been reported to be suppressed by the OGF–OGFr axis,[58,59] implying that LDN can serve as a means for modulating autoimmune diseases through this native pathway. In fact, both LDN and OGF prevent and diminish expression of experimental autoimmune encephalomyelitis in preclinical studies,[14,60,61] providing novel therapeutic implications for utilization of these agents in patients with multiple sclerosis. OGF also has been found to be non-toxic,[62] and efficacious in the treatment of patients with advanced pancreatic cancer,[63] suggesting that LDN may be effective in utilizing this peptide–receptor axis in the treatment of neoplasia. Taking together the results of the present study in tissue culture showing that the mechanism of short-term NTX is dependent on the OGF–OGFr pathway, and the similarities of this *in vitro* model to that of LDN *in vivo*, the action of LDN with regards to cell proliferation can be explained by the targeting of this opioid antagonist to the OGF–OGFr axis. Thus, OGF and LDN function to regulate cell proliferative processes through a common pathway – the OGF–OGFr axis (Figure 9).

In summary, the results of this study contribute to a better understanding of the regulatory mechanisms of cell proliferation. The clinical implications of this investigation are that LDN may serve as a treatment for a wide range of conditions that involve impairment/disregulation of cell proliferation and inflammatory activity. At present, the OGF–OGFr axis has been found to be a physiological determinant of diverse human neoplasias[43] and OGF has been successful in Phase II trials with respect to prolonging survival of patients with advanced pancreatic cancer.[63] This would suggest that leveraging of the OGF–OGFr system with LDN may provide an advantageous therapy in the treatment of some cancers. Moreover, LDN reverses inflammation in patients with Crohn' disease,[9,57] providing the promise that other autoimmune diseases such as multiple sclerosis, Crohn's, diabetes mellitus type 1, celiac, and systemic lupus erythematosus also may be responsive to the modulation of endogenous opioid systems. In a more speculative manner, taking advantage of the OGF–OGFr axis in diseases of the immune system (HIV/AIDS, chronic granulomatous), infections, hypersensitivity (e.g. allergies, contact dermatitis) and neurodegeneration (e.g. Alzheimer's disease, Parkinson's disease), which involve cell proliferation also could benefit from therapeutic manipulation of endogenous opioid peptides and receptors with agents such as LDN.

Author contributions: All authors (RND, PJM, ISZ) participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. RND performed the experiments, and RND, PJM and ISZ wrote the manuscript.

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