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Title Page

# Naltrindole inhibits human multiple myeloma cell proliferation *in vitro* and in a murine xenograft model *in vivo*

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## Abstract

It has previously been demonstrated that immune cell activation and proliferation was sensitive to the effects of naltrindole, a non-peptidic δ-opioid receptor selective antagonist, therefore, we hypothesized that human MM would be a valuable model to study potential antineoplastic properties of naltrindole. [$^3$H]- naltrindole exhibited saturable, low affinity binding to intact human MM cells, however, the pharmacological profile of the binding site differed considerably from the properties of δ, κ and μ opioid receptors, and opioid receptor mRNA was not detected in MM cells by RT-PCR. Naltrindole inhibited the proliferation of cultured human U266 MM cells in a time- and dose-dependent manner with an $EC_{50}$ of 16 μM. The naltrindole induced inhibition of U266 cell proliferation was not blocked by a 10-fold molar excess of naltrexone, a non-selective opioid antagonist. Additive inhibition of MM cell proliferation was observed using a combination of naltrindole with the HDAC inhibitor, sodium valproate, the proteasome inhibitor, bortezomib, the glucocorticoid receptor agonist, dexamethasone, and the HMG CoA reductase inhibitor, simvastatin. Treatment of U266 cells with naltrindole significantly decreased the level of the active, phosphorylated form of the kinases, ERK and Akt, which may be related to its antiproliferative activity. The antiproliferative activity of naltrindole toward MM cells was maintained in co-cultures of MM and bone marrow derived stromal cells, mimicking the bone marrow microenvironment. *In vivo*, naltrindole significantly decreased tumor cell volumes using human MM cell xenografts in SCID mice. We hypothesize that naltrindole inhibits proliferation of MM cells through a non-opioid receptor-dependent mechanism.

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## Introduction

Multiple myeloma (MM) is an invasive plasma cell neoplasm of malignant cells that proliferate in the bone marrow. This incurable cancer is responsible for 10% of all hematological malignancies. MM is characterized by monoclonal gammopathy, destructive bone disease, renal failure, hypercalcemia, and hematogical dysfunction (Kyle and Rajkumar, 2004). The molecular pathogenesis of MM is complex. Gene expression profiling and deep genome sequencing has revealed that in many cases, chromosome translocations result in overexpression of growth regulatory genes via their juxtaposition to the immunoglobulin heavy chain locus, activation of the NF-*k*B pathway, activation of *MYC*, *FGFR3*, *KRAS*, *NRAS*, and loss of function mutations in the histone H3K27 demethylase gene *UTX* (Bergsagel and Kuehl, 2005; Annunziata et al., 2007; Keats et al., 2007; van Haaften et al., 2009; Chapman et al., 2011). The American Cancer Society estimated that in 2011, 11,400 men and 9,120 women were diagnosed with MM in the United States, and that 5,770 men and 4,840 women died of the disease. Despite the development of new treatment agents in the last decade (Lonial et al., 2011), including the immunomodulatory drugs, thalidomide and lenalidomide, and the proteasome inhibitor, bortezomib, the five-year relative survival rate for MM is approximately 40%. Obviously, there is great need for additional treatment options.

Naltrindole is a synthetic alkaloid with the pharmacological profile of a selective δ-opioid receptor antagonist (Portoghese et al., 1988). It contains an indole group, which mimics the phenyl group of phenylalanine[4] of enkephalin, attached to the morphinan base of naltrexone, a non-selective opioid antagonist. Naltrindole has also been reported to be a potent immunosuppressant. Similarly to cyclosporin A, naltrindole has been shown to suppress the allogeneic mixed lymphocyte reaction *in vitro* and to inhibit renal graft rejection *in vivo*

(Arakawa et al., 1992a; Arakawa et al., 1992b). Subsequently, it was reported that naltrindole and related δ-opioid receptor antagonists retain their immunosuppressive activity in δ-opioid receptor knockout mice and in triple μ/δ/κ-opioid receptor knockout mice, revealing a non-opioid receptor target for the immunosuppressant activity of naltrindole (Gaveriaux-Ruff et al., 2001). In this study we report that naltrindole inhibits the proliferation of human multiple myeloma cells *in vitro* and *in vivo* using a mouse xenograft model via a non- μ/δ/κ-opioid receptor signaling pathway.

## Methods

**Materials.** Opioid peptides were products of Multiple Peptide Systems (San Diego, CA) and salvinorin A was from Tocris Bioscience (Ellisville, MO). All other opioid ligands were obtained from the National Institute on Drug Abuse (Bethesda, MD). [$^3$H]-Naltrindole, supplied by NIDA, had a specific activity of 31.5 Ci/mmol. Bortezomib was provided by Millenium Pharmaceuticals (Cambridge, MA). Valproic acid, dexamethasone and simvistatin were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell culture**. We obtained the human U266 and RPMI 8226 multiple myeloma cell lines from American Type Culture Collection (TIB-196 and CCL-155, respectively). These cell lines were derived from biopsy samples from patients with multiple myeloma (Nilsson et al., 1970; Matsuoka et al., 1967 ). U266 and RPMI 8226 cells were cultured at 37°C in a humidified atmosphere containing 5% $CO_2$ in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate.

**Radioligand Binding Assays.** Homologous competitive binding assays were conducted in duplicate at room temperature using concentrations of [5,7-$^3$H]-naltrindole ranging from 5 nM to 200 µM. Samples containing tritiated naltrindole in the presence of excess unlabeled naltrindole (500 µM) were assayed to define non-specific binding, which was subtracted from total binding to obtain specific binding. Following incubation in a final volume of 0.1 ml for 30 min to reach equilibrium, binding assays were terminated by filtration through Whatman GF/B filters (VWR International, Buffalo Grove, IL). Filters were immersed in Ecoscint H liquid scintillation cocktail (National Diagnostics, Somerville, NJ) prior to determination of filter-bound radioactivity using a Beckman LS 1701 scintillation counter. Homologous competitive binding

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curves were analyzed by non-linear regression using Prism 3.0 (GraphPad Software, San Diego, CA) to determine $B_{max}$ and $K_d$ values.

For non-homologous competition analysis, whole MM cell assays were conducted as described above. Various concentrations of each ligand, including naltriben, SNC80, BNTX, naltrexone and morphine, ranging from 0.2 to 200 μM were assayed for displacement of [$^3$H]-naltrindole (5-10 nM). Binding assays were conducted as described above. $IC_{50}$ values were determined by nonlinear regression analysis of the displacement curves using Prism 3.0, and since the concentration of [$^3$H]-naltrindole used for competition assays was orders of magnitude below its $K_d$ value, the $IC_{50}$ approximated the $K_i$ values calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

**Reverse Transcriptase-Polymerase Chain Reaction**. Human U266 and RPMI-8226 multiple myeloma cells were harvested from dense cultures grown in 10 cm dishes in PBS and centrifuged at 1000 x *g* for 5 min. Total RNA was extracted and isolated from these cells using the RNeasy kit (Qiagen). PCR primer pairs, obtained from Eurofins MWG Operon (Huntsville, AL), were designed to selectively amplify δ, μ, and κ opioid receptor mRNAs, to yield the respective receptor cDNAs. Receptor-selective sense and antisense oligonucleotides were designed and chosen from adjacent receptor gene exons (that necessarily contained intervening introns) to ensure that any contaminating genomic DNA in the RNA preparation would not lead to false-positive PCR products. The primer sequences were (5' to 3'): human DOR fwd AGCGCCTCGTCCCTCGCCCTG, human DOR rev- GGTCGATGTCCACCAGCGTCC, human KOR fwd- CACATCTCCCCGGCCATCCCG, human KOR rev- GGGAGGTGCTCCCCAGAGCCT, human MOR fwd- GGCAGTCCCTCCATGATCACG, and human MOR rev- TGGGATTGTAACCAAGGCTTT. The ThermoScript two-step RT-PCR kit

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with Platinum Taq polymerase (Invitrogen Life Technologies) was used to perform the RT-PCR. cDNA synthesis was performed in the first step using 1 μg RNA and oligo(dT) primers in one cycle conducted at 50°C for 30 min followed by a denaturation step at 94°C for 2 min. In the second step, the PCR is performed in a separate thin-walled tube using 5 μl cDNA and opioid receptor gene specific primers. PCR amplification was carried out in 25 cycles, consisting of template denaturation at 94°C for 15 sec, primer annealing at 55°C for 30 sec, followed by polymerization at 72°C for 55 sec followed by a final extension for 1 cycle of 72°C for 10 min. Predicted PCR products were 747, 747, and 753 bp in length for human DOR, MOR and KOR, respectively. Human DOR, MOR and KOR plasmids, received from Dr. Lee-Yuan Liu-Chen, Temple University School of Medicine, were used as positive PCR controls.

**Sensitivity of naltrindole binding to the pH of the assay buffer.** U266 cells were incubated in PBS buffers adjusted to pH values from 4.5 to 8.5 as indicated for 30 min on ice. Subsequently, the "washed" samples were pelleted by centrifugation at 500 x *g* for 5 min at room temperature, resuspended in PBS (pH 7.4), centrifuged again, then resuspended in PBS (pH 7.4). Subsequently, specific [$^3$H]-naltrindole binding was assayed at 10 nM for the unwashed and washed cells following 30 min incubation at room temperature.

**Kinetics of association and dissociation of [$^3$H]-naltrindole binding.** Experiments to determine the association rate of [$^3$H]-naltrindole binding were conducted by assaying the specific binding of 10 nM [$^3$H]-naltrindole to human U266 multiple myeloma cells as described above at 22 ˚C and 4 ˚C at various times ranging from 1 to 50 min. To evaluate the kinetics of [$^3$H]-naltrindole dissociation, U266 cells were incubated with 10 nM [$^3$H]-naltrindole for 30 min to reach equilibrium, then 500 μM of unlabeled naltrindole was added and specific binding was

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determined at 22 ˚C and 4 ˚C at various times up to 50 min. Association and dissociation curves were analyzed by non-linear regression analysis using Prism 3.0.

**Effect of protein-modifying reagents and cycloheximide on [$^3$H]-naltrindole binding to U266 MM cells.** Intact U266 MM cells were treated with 50 mM dithiothreitol (DTT), 5 mM N-ethylmaleimide (NEM), or 1 mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) for 30 min at room temperature. Radioligand binding assays to assess the specific binding of 10 nM [$^3$H]-naltrindole were performed following a 30 min incubation at room temperature immediately following treatment with the protein-modifying reagents. Pilot experiments indicated that the results were similar whether the protein-modifying reagents were removed by washing and centrifugation or not, so for simplicity, binding assays were conducted in the presence of the protein-modifying reagents.

To gain insight into the turnover rate of the naltrindole binding site protein, U266 cells were incubated with the protein synthesis inhibitor, cycloheximide. U266 cells were incubated at 37˚C for varying lengths of time, ranging from 30 min to 9 h, in 5 μM cycloheximide in culture media. Cycloheximide was removed by washing the cells twice in PBS by centrifugation at 500 x *g* for 5 min, and were then resuspended in PBS. Specific binding assays were performed as usual following 30 min incubation with 10 nM [$^3$H]-naltrindole.

**Subcellular Fractionation Studies.** To elucidate the cellular localization of the naltrindole binding site in multiple myeloma cells, radioligand binding assays were conducted to compare the binding activity of intact U266 MM whole cells, cell homogenates, a membrane preparation and the cytosolic fraction, and to compare the properties of the U266 binding site labeled by $^3$H-naltrindole to the delta opioid receptor expressed in HEK 293 cells and labeled with $^3$H-diprenorphine. Human U266 MM cells and human HEK 293 cells stably expressing the Flag-

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tagged delta opioid receptor (DOR) (Yadav et al., 2007) were harvested in phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 500 x *g* for 5 min. Whole cell pellets were resuspended in PBS and centrifuged as above. The cell pellets were resuspended in PBS, pH 7.4, aliquots were removed for binding, and the remainder was homogenized with a Tekmar tissuemizer (Cincinnati, OH), aliquots were set-aside for binding, and a membrane fraction was prepared by ultracentrifugation of the homogenate at 100,000 x *g* for 30 min. The membrane pellet was resuspended by homogenization in PBS, pH 7.4, and the resulting supernatant represented the soluble cytoplasmic fraction. The protein concentration of the membrane preparations was determined using the $D_c$ protein assay (BioRad, Hercules, CA) with bovine serum albumin as the standard. Radioligand binding assays were conducted in a final volume of 0.1 ml, using whole cells, cell homogenates, the cell membrane preparation and the soluble cytoplasmic supernatant. Binding assays were conducted in duplicate at room temperature using 3 nM [15,16-$^3$H]-diprenorphine (specific activity 50.0 Ci/mmol, Perkin Elmer, Boston, MA) as the radioligand for binding to HEK 293 DOR fractions or 7 nM [5,7-$^3$H]-naltrindole (specific activity 31.5 Ci/mmole, National Institute on Drug Abuse Drug Supply Program) for binding to U266 fractions. Samples containing tritiated diprenorphine in the presence of excess unlabeled diprenorphine (30 μM) and samples containing tritiated naltrindole in the presence of excess unlabeled naltrindole (500 μM) were assayed to define non-specific binding, which was subtracted from total binding to obtain specific binding. Following incubation for 30 min to reach equilibrium, the binding assays were terminated by filtration through Whatman GF/B filters (VWR International, Buffalo Grove, IL), and were washed twice with 4 ml of ice-cold PBS. Saturation curves and competitive displacement curves were analyzed by non-linear regression using Prism 3.0 (GraphPad Software, San Diego, CA) to determine $B_{max}$, $K_d$, and $IC_{50}$

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values. For radioligand binding assays to the soluble cytosolic fractions, a polyethylene glycol-precipitation method was employed (Howells et al., 1982). Following incubations as described above, 0.5 ml of 0.1 % γ-globulin and 0.5 ml of 25 % polyethylene glycol were added to the 0.1 ml assay tubes, vortexed, then filtered through GF/B filters and washed twice with 4 ml of 7.5 % polyethylene glycol. Filters were immersed in Ecoscint H liquid scintillation cocktail (National Diagnostics, Somerville, NJ) prior to determination of filter-bound radioactivity using a Beckman LS 1701 scintillation counter. [$^3$H]-Naltrindole binding to the soluble cytosolic fraction was also assayed using gel filtration. Following incubation of duplicate 0.1 ml aliquots of the cytosolic fraction with 10 nM [$^3$H]-naltrindole alone (to assess total binding), or with 10 nM [$^3$H]-naltrindole in the presence of 500 μM unlabeled naltrindole (to determine non-specific binding), samples were applied to 7 ml mini-columns of Sephadex G-25 packed in serological pipets and 0.5 ml fractions were collected and eluted with 1 x PBS, pH 7.4. Fractions corresponding to the void volume (determined by elution of Blue Dextran), where the [$^3$H]-naltrindole/binding site protein complex should elute, were counted using a Beckman LS 1701 scintillation counter to determine binding activity in the total and non-specific binding samples.

*Cell fractionation following hypotonic lysis.* Further attempts to determine the subcellular locale of the $^3$H- naltrindole binding site were made following hypotonic lysis of the MM cells, adapted from a protocol using a murine thymoma cell line, BW5147 (Merker and Handschumacher, 1984). Human U266 MM cells were resuspended at 5 x $10^7$ cells/ml in cold 10 mM Tris HCl, 1 mM $MgCl_2$, pH 7.45. After 10 min at 0 ˚C, tonicity was established with 10x Hanks' balanced salt solution and the cell suspension was ruptured by forcing it three times through a syringe fitted with a 26 gauge needle. This procedure causes approximately 90% of the cells to rupture, as determined by counting cells with trypan blue in a hemocytometer. The suspension was

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centrifuged at 800 x *g* for 20 min and the resulting sediment was designated the low speed pellet and contained the cell nuclei and unbroken cells. The supernatant was recentrifuged at 100,000 x *g* for 60 min and the resulting sediment was designated the high speed pellet, and the resulting supernatant was labeled the cytosol. Aliquots of the fractions, including whole cells, the low and high speed pellets and the cytosol were assayed for $^{3}$H- naltrindole binding. Binding activity displayed by whole cells and the low and high speed pellets was assayed using the GF/B filtration assay, while binding to the cytosol was assayed by LH-20 gel filtration chromatography as described by Merker and Handschumacher (1984) and by precipitation of the $^{3}$H-naltrindole/binding site complex in 90% ammonium sulfate followed by filtration through Whatman GF/B filters.

*$^{3}$H- naltrindole binding to intact mitochondria.* The method of Frezza et al. (2007) was employed to isolate intact mitochondria from U266 MM cells. U266 cells grown in five 10 cm diameter dishes were centrifuged at 600 x *g* for 5 min and resuspended in 7 ml of IBc buffer containing 10 mM Tris/MOPS, 1 mM EGTA/Tris and 0.2 M sucrose, pH 7.4. One ml of the cell suspension was set-aside for whole cell binding, and the remainder was homogenized using a Teflon pestle in a fitted glass vessel using 10 up/down strokes while rotating at 1600 rpm. One ml of the homogenate was removed for binding and the rest was centrifuged at 600 x *g* for 10 min at 4 ˚C. The resulting P1 pellet, which consisted largely of nuclei and any remaining unbroken cells, was resuspended in 1ml IBc buffer, and the supernatant was centrifuged at 7000 x *g* for 10 min at 4 ˚C. Pellet (P2), which consists largely of the mitochondrial fraction, was resuspended in 1ml IBc. $^{3}$H- naltrindole binding was compared in the whole cell, homogenate, P1 and P2 fractions, as was the protein content, determined by the BioRad $D_c$ method.

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**Western Blotting.** U266 cells were incubated in the absence or presence of naltrindole (50 μM) for varying lengths of time. Whole cell lysates were prepared by solubilization of cells with 1X lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO4$, 1 μg/ml leupeptin) and a protease/phosphatase inhibitor cocktail (1:100, Thermo Scientific). The detergent lysate was centrifuged at 16,000 x *g* for 20 min, and the supernatant was recovered for immunoblot analysis. The protein concentration of each lysate was determined using the $D_c$ protein assay (BioRad, Hercules, CA) with bovine serum albumin as the standard. Protein (40 μg per lane) was resolved using 12% SDS/PAGE at 120 V for approximately 1.5 h and transferred to Immobilon P polyvinylidene difluoride membranes (Millipore, Bedford, MA) at 100 V for 1 h. Immunoblots were incubated in blocking buffer (50 mM Tris-HCl, pH 7.5, 1 M glucose, 10% glycerol, 0.5% Tween-20, 3% non-fat dry milk, 1 mM $CaCl_2$ and 0.005% Thimerosol) for 30 min at room temperature and then incubated overnight with either a rabbit phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody that detects endogenous levels of phosphorylated p44 and p42 MAP Kinase (1:1000, #9101 Cell Signaling) or with a phospho-Akt (Ser473) Rabbit mAb that detects endogenous levels of Akt only when phosphorylated at Ser473 (1:1000, #4060 Cell Signaling). Immunoblots were washed 3 times with TBST (200 mM Tris-HCl, pH 7.5, 1% Tween-20, 150 mM NaCl, 1 mM $CaCl_2$) for 5 min each and incubated with secondary antibody conjugated to horseradish peroxidase in blocking buffer (goat anti-rabbit, 1:3000, Jackson Immunoresearch). Membranes were washed 3 times with TBST for 10 min each and developed using ECL Western blotting substrate (Pierce Biotechnology, Rockford, IL) and HyBlot CL autoradiography film (Denville Scientific, Metuchen, NJ). Blots were quantitated using Image J softwere. These blots were stripped for 30 min at 50 °C in stripping buffer (62.5

mM Tris-HCl, pH 6.5, 2% SDS and 100 mM β-mercaptoethanol) prior to incubation with either p44/42 MAPK (Erk1/2) antibody that detects endogenous levels of total (phosphorylated and non-phosphorylated) p44/42 MAP kinase (1:1000, #9102, Cell Signaling) or with Akt (pan) (C67E7) Rabbit mAb that detects endogenous levels of total Akt protein (1:1000, #4691, Cell Signaling).

**WST-1 Cell Proliferation Assay.** U266 cells were plated in 96-well plates at 2,000 cells per well in 100 µl of RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate. Cells were incubated in quadruplicate in the presence of the various anti-neoplastic agents to construct dose-response curves, alone or in combination with various doses of naltrindole. U266 MM cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for 72 h. At the end of the incubation 10 µl of WST-1 cell proliferation reagent (Roche Applied Science, Indianapolis, IN) was added to each well and the plates were returned to the incubator for 1 h. Absorbance was then measured at 450 nm using a Synergy HT plate reader (BIO-TEK Instruments, Winooski, VT). Cell proliferation data was analyzed using Prism 3.0 (GraphPad Software, San Diego, CA).

**VI-CELL DETERMINATION OF CELL PROLIFERATION.** The Beckman Coulter Vi-Cell instrument is an automated cell counter and viability analyzer, which uses trypan blue exclusion staining, combined with imaged-based analysis to determine the total number of viable cells and viability percentages. U266 cells were cultured using 6 cm dishes incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ in 3 ml RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate. Cell media was collected and duplicate 1 ml aliquots were removed for the Vi-cell readings.

**Caspase 3 assay as a marker for apoptosis.** Western blotting was performed to detect caspase-3 activation in MM cells, using an anti-caspase 3 antibody (Transduction Labs., Lexington, KY) that recognizes the inactive form of caspase-3 at 32 KD. An apoptotic signal induces intracellular cleavage of Caspase-3 from inactive proform to active form p17 and p12 (Erhardt et al., 1996, Armstrong et al, 1996). RPMI 8226 and U266 MM cells were grown at 37 ˚C and incubated for 0, 24, 48, 72 and 96 h with or without 10, 30 and 50 µM naltrindole in RPMI culture medium and cell lysates were prepared to perform western blotting experiments. MG132 (25 µM), a proteosome inhibitor, was used as a pro-apoptotic positive control.

**FITC-Annexin V fluorescence activated cell sorting as a screen for apoptosis.** Apoptosis was monitored using FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen ™, San Diego, CA). Cells undergoing apoptosis expose the phospholipid, phosphatidylserine (PS), on their outer leaflet of the plasma membrane. Annexin V is a 35-36 kDa phospholipid-binding protein that has a high affinity for PS, and can be conjugated to fluorochromes including FITC. This complex can be detected by flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, propidium iodide (PI) dye is used along with FITC Annexin V staining. PI binds to DNA, only in the cells which have lost their plasma membrane integrity and are in late stage of apoptosis (Vermes et al, 1995). RPMI 8226 and U266 MM cells were grown at 37 ˚C and incubated for 0, 24, 48, 72 and 96 h with or without 10, 30 and 100 µM naltrindole in RPMI culture medium. The cells were washed with PBS twice and then resuspended in 1X Binding Buffer at a concentration of $1x10^6$ cells/ml. 100 µl of this solution was transferred to a 5ml culture tube and 5 µl of FITC Annexin-V and 5 µl PI was added to it. Cells were then vortexed and incubated for 15 min at RT in the dark. 400 of 1X Binding Buffer was added to each tube and FACS analysis was performed using a BD

Biosciences FACSCalibur instrument. MG132 (25 μM), a proteasome inhibitor, was used as a pro-apoptotic positive control.

**Co-culture of human U266 MM cells with human HS-5 bone marrow stromal cells.** We obtained the human U266 MM and human HS-5 cell lines from American Type Culture Collection. The U266 MM cell line is derived from biopsy samples from a 53-year-old man that died of MM. HS-5 cells are derived from a 30-year-old male and are bone marrow stromal cells transformed with human papilloma virus E6/E7 genes. U266 cells were cultured at 37°C in a humidified atmosphere containing 5% CO2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. HS-5 cells were cultured similarly but with Dulbecco's Modified Eagle's Medium (DMEM).

*Naltrindole effects on U266 and HS-5 co-cultured cell lines*

Media was aspirated from a 10-cm dish containing HS5 cells and the adherent cells were washed with phosphate buffered saline. Trypsin/EDTA solution was added to the dish and allowed to incubate at 22°C for ten min. The contents of the dish along with 5 ml DMEM were transferred to a 15 ml centrifuge tube and centrifuged for 5 min at 300 x *g*. The supernatant was aspirated and the pellet was resuspended in 28 ml of DMEM medium. 1 ml was taken from this HS5/DMEM mixture and placed into a sample cup to determine cell concentration with the Vi-Cell XR. 3 ml of the HS5/DMEM cell suspension was added to 8 3-cm culture dishes. The dishes were allowed to incubate at 37˚C for one h.

Medium was pipetted from a 10-cm dish containing U266 cells into a 15 ml centrifuge tube. Cells were centrifuged for 4 min at 300 x *g* and the supernatant was discarded. The pellets were resuspended in 28 ml RPMI. 1 ml was taken from this U266/RPMI mixture and placed into a sample cup for Vi-Cell analysis. The effect of naltrindole at 0, 5, 20 and 50 μM was tested

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following a 72 h incubation in culture with U266 cells alone, and U266 cells co-cultured with HS-5 cells, all grown in RPMI media. In co-cultures, the concentration of U266 cells added was 3 times that of the concentration of attached HS5 cells. After 72 h, the culture medium was transferred to a Vi-Cell cuvette prior to analysis of 1 ml from each sample in duplicate with the Vi-Cell instrument. Dose response curves were analyzed by non-linear regression analysis using Prism 5.0 (GraphPad, San Diego, CA).

**Naltrindole binding and anti-proliferative effects on freshly isolated peripheral blood mononuclear cells, red blood cells and platelets.** Blood was drawn from a volunteer into 5 heparinized Vacutainer tubes, and centrifuged in a 50 ml sterile conical tube at 1000 x *g* for 20 min with the brake off. Buffy coat cells (10 ml) were obtained and diluted in an equal volume of complete RPMI 1640 medium, then layered over 2 15 ml conical tubes containing 5 ml of Ficoll Histopaque (Sigma) and centrifuged at 540 x *g* for 30 min. An aliquot of the upper layer containing platelets, and peripheral blood mononuclear cells obtained from the Ficoll interphase, as well as red blood cells from under the Ficoll phase were diluted with RPMI medium and centrifuged at 282 x *g* for 10 min with the brake off. Platelets, RBCs and PBMC cell pellets were washed 2 more times with RPMI complete medium. Aliquots of these cell suspensions were centrifuged again and the pellets were resuspended in PBS to assay $^{3}$H- naltrindole binding (10 nM) to whole cells, in comparison with binding to human U266 multiple myeloma cells. PBMC and RBC cell suspensions (3 ml) were plated onto 6 cm dishes and the effect of naltrindole at 0, 5, 20 and 50 μM on cell proliferation was assayed with the Vi-cell instrument following a 72 h incubation.

**Efficacy of naltrindole to inhibit tumor growth in a xenograft model of human RPMI 8226 multiple myeloma cells injected into SCID mice.** This study was done in collaboration with

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Washington Biotechnology, Inc. (Columbia, MD). Female SCID mice, 4-5 weeks old were from Harlan Sprague-Delaney (Indianapolis, IN) and housed four mice per cage with filter tops and autoclaved bedding. Human RPMI 8226 multiple myeloma cells (passage 8 from ATCC) were inoculated subcutaneously into both flanks of SCID mice (ten million cells per flank). After 8 days, 12 mice were divided into 2 groups of 6 mice each: vehicle injected and naltrindole injected (30 mg/kg). Animals were dosed daily for 36 d and body weights and xenograft tumors were measured twice a week with a digital caliper (tumor volume = length x width x width x ½). Naltrindole was dissolved in distilled water to make a 3 mg/ml solution and mice were injected with 10 ml/kg daily.

**PROTEIN ASSAY.** The protein concentration of samples was determined using the $D_c$ protein assay (BioRad, Hercules, CA) with bovine serum albumin as the standard. Sample concentrations were determined by interpolation of the best-fit line generated by linear regression analysis using Prism 5.0 (GraphPad Software, San Diego, CA).

**STATISTICAL ANALYSIS.** The statistical significance of mean values was determined using one or two way analysis of variance (ANOVA), including Tukey's multiple comparison test.

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## Results

### Characterization of the naltrindole binding site in U266 multiple myeloma cells:

[$^3$H]-Naltrindole interacts in a saturable manner with micromolar affinity to an abundant binding site in intact human U266 multiple myeloma cells. Specific [$^3$H]-naltrindole binding (i.e. displaceable with 500 μM unlabeled naltrindole) was readily observed when using 5-10 nM of the radioligand. However, competition analysis, using concentrations of non-radiolabeled naltrindole ranging from 0.5 to 200 μM to displace 10 nM [$^3$H]-naltrindole, indicated that its $IC_{50}$ was approximately 15 μM. The low affinity of the naltrindole binding site made typical saturation analysis with increasing concentrations of [$^3$H]-naltrindole prohibitive, therefore, we transformed the homologous competitive binding analysis data to calculate the amount of naltrindole bound at each concentration based on the dilution factor of the radioisotope. Homologous competitive binding analysis indicated that the apparent dissociation constant, $K_D$, was 20 ± 4 μM, and the maximum number of binding sites, $B_{max}$, was 1.8 ± 0.1 nmoles/mg protein (Fig. 1). It should be noted that the affinity of naltrindole for this binding site is considerably lower than its affinity for the classical δ-opioid receptor, where the $K_D$ for naltrindole is 1.5 nM (Chaturvedi et al., 2000). The *inset* of Fig. 1A displays [$^3$H]-naltrindole binding to the U266 cells in the low nanomolar range; the binding is linear rather that saturating as it would be for [$^3$H]-naltrindole binding to the delta opioid receptor. Conversely, the $B_{max}$ for naltrindole in the U266 cells is exceptionally high. For comparison, we created a human embryonic kidney 293 cell line that expressed δ-opioid receptor cDNA driven by the strong cytomegalovirus promoter that displayed a $B_{max}$ of 10 pmoles/mg protein (Christoffers et al., 2005).

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Naltrindole interacted with a binding site expressed in another human multiple myeloma cell line, RPMI 8226 cells, in a similar fashion. The $K_D$ for naltrindole derived from homologous competitive binding analysis was 32 ± 7 μM and the $B_{max}$, was 2.4 ± 0.6 nmoles/mg protein (Fig. 1B). The concentration of naltrindole required to displace 50% of [$^3$H]-naltrindole binding was 16 ± 4 μM (Fig. 1C). Given the unusual pharmacological properties of the naltrindole binding site expressed in the human multiple myeloma cell lines, we looked for expression of delta, mu and kappa opioid receptor mRNA in RPMI 8226 cells using reverse transcriptase-polymerase chain reactions with opioid receptor-specific oligonucleotide primer pairs (Fig. 1D). When RNA from the RPMI-8226 cells was used as RT-PCR template, the results were negative. The positive control reactions yielded products of the expected size (747, 747, and 753 bp for the δ, μ, and κ opioid receptors, respectively. Although a faint PCR product approximately 1000 bp in length was observed using kappa opioid receptor primers, DNA sequence analysis indicated that the band was a PCR artifact and had no sequence homology to human kappa opioid receptor mRNA.

The optimal pH of the 1 x phosphate-buffered saline used for binding assays was found to be at neutral to slightly alkaline pH (Fig. 2A). [$^3$H]-Naltrindole binding was increased by 25% at pH 8.5 relative to the binding at pH 7.4. The $pK_a$ of the amino nitrogen of morphine is approximately 8; assuming that the $pK_a$ of naltrindole is similar, the pH profile suggests that naltrindole binding is favored slightly when the basic nitrogen is uncharged. At pH values below neutrality (pH 6.5, 5.5 and 4.5), binding declines to 20-25% of the binding at pH 7.4. When cells were incubated in acidic pH buffers or slightly alkaline buffer, then washed and re-equilibrated at pH 7.4, binding activity was restored back to the level assayed at pH 7.4, with the exception of cells incubated at the lowest pH, 4.5. In that case, binding activity was not recovered, presumably due to non-reversible denaturation of the binding site at pH 4.5.

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Experiments were conducted to examine the kinetics of association and dissociation of naltrindole binding to U266 cells at 22 ˚C and at 4 ˚C. The rate of binding was rapid at both temperatures, being half-maximal at 1 to 2 min, and binding reached equilibrium at both temperatures at approximately 20 min (Fig. 2B). At 22˚C, the association rate constant, calculated by non-linear regression analysis of the association curve using Prism 3.0, was 2650 +/- 650 $M^{-1}$ $s^{-1}$. The dissociation rate was also rapid at both temperatures (Fig. 2C). At 4 ˚C, the half-life of dissociation was approximately 10 min, while at 22 ˚C it was 26 +/- 6 seconds. The dissociation rate constant at 22˚C was 0.031 +/- 0.008 $s^{-1}$. The apparent dissociation constant calculated from the ratio $k_{off}$ / $k_{on}$ was 11.5 μM, which was in excellent agreement with the dissociation constant of 10 - 20 μM calculated by homologous competition analysis.

Naltrindole binding to U266 MM cells was inhibited following incubation of viable cells with several protein-modifying reagents. Naltrindole binding was inhibited by incubation with dithiothreitol (DTT), which reduces disulfide bonds to yield free cysteine residues, to N-ethylmaleimide (NEM), which alkylates reduced cysteine residues and to 5,5'-dithio-bis(2-nitrobenzoic acid (Ellman's reagent, DTNB), which also alkylates free sulfhydryl groups (Fig. 3A). These results strongly implicate a critical proteinaceous component of the naltrindole binding site, and suggest that both a disulfide bond(s) and a free cysteine residue(s) are present that are critical for the maintenance of the naltrindole binding site crevice or for the overall active conformation of the naltrindole binding site protein. It is widely accepted that DTNB is not cell-permeable, due to its negative charge (Laragione et al., 2003) therefore, the inhibition mediated by this reagent implies that a critical cysteine residue or residues is/are accessible to the reagent from the extracellular aspect of the plasma membrane that is required for naltrindole binding.

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The proteinaceous character of the naltrindole binding site was further confirmed by incubating viable U266 cells with the protein synthesis inhibitor, cycloheximide, which inhibited [$^3$H]-naltrindole binding in a time-dependent manner. Nonlinear regression analysis of the decay curve for specific binding indicated that the naltrindole binding site had an apparent half-life of 7.1 h (Fig. 3B). While cycloheximide is certainly cytotoxic, we did not observe extensive cell death at earlier time points up to the half-life of the naltrindole binding site.

We observed that naltrindole binding to MM cell lines was maximal when radioligand binding assays were conducted using whole cells (Fig. 4A). [$^3$H]-Naltrindole binding to U266 multiple myeloma cells was decreased by 60% when cells were disrupted by homogenization prior to radioligand binding assays. This was not the case for [$^3$H]-diprenorphine binding to the δ opioid receptor expressed in human embryonic kidney 293 (DOR HEK293) cells, in which essentially all of the binding activity was retained in the homogenate (Fig. 4A). Furthermore, when binding assays were conducted using a membrane fraction obtained by centrifuging the U266 homogenate at 100,000 x *g*, [$^3$H]-naltrindole binding was decreased by 75% relative to whole cells, while [$^3$H]-diprenorphine binding activity to DOR HEK293 cells was fully recovered in the membrane fraction from DOR HEK293 cells relative to whole cells or the homogenate. Thus, unlike opioid ligand binding to delta, mu and kappa opioid receptors, it appears that the [$^3$H]-naltrindole binding site on MM cells is not localized to membrane fractions. [$^3$H]-Naltrindole binding was also assayed using the soluble supernatant 100,000 x *g* cytosolic fraction from U266 cells by Sephadex G25 gel filtration assays and polyethylene glycol precipitation methods (Howells et al., 1982), however, no specific binding was detected (data not shown). At the present time, the fate of the [$^3$H]-naltrindole binding activity subsequent to cell homogenization is unclear.

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We also conducted subcellular fractionation studies following hypotonic lysis of myeloma cells, with the notion that this method may be less disruptive to cells than homogenization with the Tekmar tissuemizer. Once again, the binding activity within the low and high speed pellet fractions was decreased significantly relative to intact cells, and no specific binding was observed in the cytosolic fraction when assayed either by LH-20 gel filtration chromatography (Merker and Handschumacher, 1984) or by precipitation with 90% ammonium sulfate (Fig. 4B).

Subcellular fractionation of cells following homogenization using a Teflon pestle/glass vessel in an isotonic sucrose buffer yielded similar results (Fig. 4C). Disrupting the cells by homogenization decreased [$^3$H]-naltrindole binding by 50% relative to the binding activity of intact cells, and binding to the P1 nuclear fraction and the P2 mitochondrial fraction was about 50% of the binding expressed as fmol/mg protein relative to intact U266 cells. In our opinion, residual binding in subcellular fractions subsequent to homogenization in Fig. 4 is probably due to the presence of intact cells, since more thorough homogenization at the outset of fractionation further reduces naltrindole binding in subcellular fractions.

The pharmacological profile of the naltrindole binding site expressed in U266 MM cells was investigated using competitive binding assays with naltrindole (Portoghese et al., 1988), naltriben (Portoghese et al., 1990), SNC80 (Bilsky et al., 1995), 7-benzylidenenaltrexone (BNTX, Portoghese et al., 1992), naltrexone and morphine (Fig. 5). The rank order of potency for inhibition of [$^3$H]-naltrindole binding (with $IC_{50}$ values in parentheses) was naltrindole (7.5 μM) > naltriben (20 μM) > SNC80 (25 μM) > BNTX (60 μM) > naltrexone (300 μM) > morphine (660 μM). In addition, the following opioid compounds exhibited negligible affinity (less than 20% inhibition of [$^3$H]-naltrindole binding at 100 μM) for the naltrindole binding site

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expressed in U266 MM cells: salvinorin A, naloxone, dynorphin (1-13), β-endorphin, DSLET, DAMGO and DADL.

**Inhibition of proliferation of multiple myeloma cells by naltrindole**

Naltrindole was tested for its effect on proliferation of human U266 MM cells, and it was observed that the antiproliferative activity correlated well with the $IC_{50}$ for displacement of [$^3$H]-naltrindole binding to the cells. Using the Wst-1 tetrazolium assay, following 72 h of treatment, naltrindole inhibited the proliferation of U266 multiple myeloma cells in a concentration-dependent manner with an $EC_{50}$ of 16 ± 0.1 μM (Fig. 6A). Regarding the time course of its antiproliferative activity, naltrindole caused a decrease in the number of viable cells in a time-dependent manner, being maximal at 72 h (Fig. 6B). As expected, the number of viable cells in control, untreated U266 cell samples increased with time in culture, while naltrindole at 50 μM displayed a cytostatic effect on the cells, effectively inhibiting the time-dependent increase in viable cell number (Fig. 6B). It was also apparent from Fig. 8B that naltrindole was not inducing cell death, when one compares the number of viable cells in the naltrindole treated samples to the number of viable cells in the 24 h control sample. Using the Wst-1 assay, it was observed that treatment of U266 cells for 72 h with the opioid antagonist, naltrexone, at 100 μM actually slightly stimulated the proliferation of U266 cells slightly relative to control, untreated cells (although this increase was not statistically significant), however, the anti-proliferative effect of 10 μM naltrindole was not blocked by the ten-fold higher concentration of naltrexone (Fig. 6C), suggesting, once again, that the effect of naltrindole was not due to activation or inhibition of the delta opioid receptor. Pharmacological intervention for the treatment of a variety of diseases often utilizes a combination of drugs that have multiple targets, and this is the case for treatment of multiple myeloma. We tested the antiproliferative activity of drugs that target histone

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deacetylase (sodium valproate, 200 μM), the glucocorticoid receptor (dexamethasone, 5 μM), the proteasome (bortezomib, 10 nM), and HMG-CoA reductase (simvastatin, 1 μM) alone and in combination with 10 μM naltrindole following 72 h treatment of U266 MM cells using the Wst-1 tetrazolium assay. As displayed in Fig. 6D, at the concentrations indicated, all therapeutic agents inhibited U266 cell growth by 20-25%. The decrease in the number of viable cells induced by valproic acid and bortezomib was statistically significant ($p<0.05$); the decrease in the number of viable cells induced by naltrindole alone, dexamethasone alone, and simvastatin alone did not quite reach statistical significance ($p>0.05$). Combined drug treatment with naltrindole and the other chemotherapeutic agents at these sub-maximal concentrations resulted in a statistically significant, additive effect on cell proliferation compared with control untreated cells, as well as with cells treated with the single drugs alone. For instance, the combination of naltrindole and bortezomib resulted in a statistically significant decrease in the number of viable cells compared with cells treated with naltrindole alone, as well as cells treated with bortezomib alone.

To investigate more directly whether naltrindole was inducing programmed cell death the MM cells, a biochemical assay to detect caspase-3 cleavage and activation was employed. Caspase-3 is a pro-apoptotic protease that is activated in the programmed cell death signaling pathway, and its activation involves proteolytic cleavage of an inactive 32 kDa precursor protein. As shown in Fig. 7A, western blot analysis using an antibody that recognizes the inactive caspase-3 proenzyme indicated that 24 h treatment of RPMI 8226 or U266 MM cells with 10, 30, or 50 μM naltrindole had no effect on caspase-3 cleavage and activation, nor did treatment with 50 μM naltrindole for 48 h or 72 h. In contrast, as a positive control, treatment of the MM cell lines with 25 μM MG132, a proteasome inhibitor, for 24 h, caused a marked decrease in the level of the caspase-3 proenzyme, with no effect on α-tubulin, used as a loading control.

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Further confirmation that naltrindole does not induce programmed cell death in MM cells was obtained utilizing fluorescence-activated cell sorting with annexin V as a marker for apoptosis. Treatment of RPMI 8226 MM cells with 10, 30, or 50 μM naltrindole for 24 h had little effect of the fraction of cells that were annexin V-positive and propidium iodide-negative (the lower right quadrant) corresponding to cells undergoing early stages of apoptosis, as compared to control untreated cells (Fig. 7B, *left panel*). In contrast, treatment of RPMI 8226 cells with 25 μM MG132 for 24 h caused a dramatic increase in the population of cells that were annexin V-positive and propidium iodide-negative. Even a more prolonged treatment of RPMI 8226 cells for 48 and 72 h with 50 μM naltrindole failed to induce apoptosis as indicated by the minimal effects on the population of annexin V-positive, propidium iodide-negative cells (Fig. 7B, *right panel*).

Multiple myeloma cells home to the bone marrow, and interactions between MM cells and normal cells within the bone marrow microenvironment can attenuate the anti-tumor activity of drugs used to treat MM (Anderson and Carrasco, 2011), therefore, we investigated the anti-proliferative effect of naltrindole using U266 MM cells co-cultured with human HS-5 bone marrow stromal cells. Radioligand binding assays demonstrated that human HS-5 bone marrow stromal cells express the naltrindole binding site, and the $IC_{50}$ of naltrindole was 36 μM in these cells (Fig. 8A). In this set of experiments, non-linear regression analysis of naltrindole concentration-response curves using the Vi-cell assay generated an $EC_{50}$ of 20 μM for inhibition of U266 cell proliferation. The $EC_{50}$ for inhibition of HS-5 cell proliferation was 1.75-fold higher (35 μM), while the $EC_{50}$ for inhibition of U266 cell proliferation grown in the presence of HS-5 cells was intermediate, at 29 μM (Fig. 8B). Thus, naltrindole displayed a cytostatic effect

against U266 MM cells even in the protective microenvironment established in co-culture with bone marrow stromal cells.

The phosphatidyl inositol 3-kinase/Akt pathway is another signal transduction pathway utilized by multiple myeloma cells to proliferate and escape apoptosis. Treatment of U266 cells with 50 μM naltrindole also decreased the levels of phosphorylated Akt/PKB (protein kinase B) in a time-dependent manner. As shown in Fig. 9A, levels of phospho-Akt, normalized to total Akt, were decreased significantly by 20-50% following exposure to naltrindole for 20, 30, 60, 120 min and 24 h as compared to untreated control cells.

The MAP kinase pathway downstream of activated Ras is a major signaling cascade that leads to cell proliferation, therefore, it was of interest to determine whether the anti-proliferative effects on naltrindole on MM cells were correlated with inhibition of this pathway. We found that naltrindole treatment decreased phosphorylation, and hence activation, of ERK (extracellular signal-regulated kinase) as determined by western blotting using a phospho-ERK-specific antibody (Fig. 9B). Phosphorylated ERK levels decreased by 35-40% at 10, 30, 60 and 120 min following treatment of U266 cells with 50 μM naltrindole, but were unchanged relative to untreated control cells after a 5 min incubation. Phosphorylated ERK levels for all samples were normalized to the corresponding total ERK levels prior to calculating the effect of naltrindole relative to untreated control cells.

It was also of interest to examine naltrindole binding to normal human blood cells and to test their sensitivity to the anti-proliferative effects of naltrindole. As shown in Fig. 10 A, specific naltrindole binding (assayed using 7 nM [$^3$H]-naltrindole) was detected using normal human peripheral blood mononuclear cells and platelets, but human red blood cells did not exhibit any detectable naltrindole binding. Concentration-response analysis indicated that peripheral blood

mononuclear cells were approximately 5-fold less sensitive to the anti-proliferative effects of naltrindole following a 72 h incubation relative to U266 multiple myeloma cells, while the number of viable red blood cells remained the same in the absence and presence of naltrindole, as expected from their lack of specific naltrindole binding (Fig. 10 B).

Based on these very promising *in vitro* experiments that clearly demonstrated the anti-proliferative activity of naltrindole against MM cell lines, we sought to evaluate the *in vivo* efficacy of naltrindole using a murine SCID/human RPMI 8226 MM xenograft model. SCID mice were subcutaneously inoculated bilaterally in the hind flanks with human RPMI 8226 MM cells. When palpable tumors were present, mice were injected with vehicle or naltrindole ip daily at 30 mg/kg, for 36 days. Tumor volumes in the control and drug-treated mice were measured bi-weekly. As shown in Fig. 11 A, naltrindole significantly decreased the tumor volume in mice from day 21 onward. Body weights differed among the control and naltrindole-treated groups at days 34, 36, and 39, however, it is not known how much the increased tumor burden in the control animals contributes to their increased body weight (Fig. 11 B).

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## Discussion

Morphine and other opioids have been used for pain relief, and a variety of other indications for centuries, and opioid analgesics remain the most powerful and widely used therapeutic agents for pain control in current medical practice (Niscola et al., 2006). Studies have also demonstrated the importance of endogenous and exogenous opioids in modulating the proliferation of immune cells and neoplastic cells. Phagocytes, T-cells, B-cells, and NK cells have been demonstrated to be affected by opioids (McCarthy et al., 2001). Inhibitory effects of opioids on cell proliferation have been well characterized in several human hematological malignancies, including promyelocytic leukemia (Takeuchi et al., 2006) and erythroid leukemia (Mernenko et al., 1996).

Several investigators reported that naltrindole has immunosuppressive and anti-proliferative activity mediated through a non-opioid receptor mechanism (House et al., 1995; Gaveriaux-Ruff et al., 2001; Chen et al., 2004). Based on our data, we conclude that the naltrindole binding site expressed in U266 multiple myeloma cells is not the δ-opioid receptor (or the μ- or κ-opioid receptor). The $K_D$ and the $B_{MAX}$ values for naltrindole and the affinities of other opioids that we have studied are significantly different than those reported for the classical δ, κ and μ opioid receptors. Substantial loss of detectible binding after cell homogenization is also not observed for the δ, κ and μ opioid receptors. In addition, we can detect specific [$^3$H]-naltrindole binding, but not [$^3$H]-diprenorphine binding (a non-selective opioid antagonist with high affinity for δ, κ and μ opioid receptors) to U266 and RPMI 8226 multiple myeloma cells.

The pharmacological profile of the naltrindole binding site differs significantly from the classical δ-opioid receptor, which binds Met-enkephalin, D-Ala$^2$, D-Leu$^5$-enkephalin, D-Ser$^2$-Leu-enkephalin-Thr$^6$, etorphine and diprenorphine with $K_D$'s in the low nanomolar range, as opposed to the poor affinity of these compounds for the naltrindole binding site. The pharmacological

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profile of the naltrindole binding site also differs from the properties of the μ-opioid receptor, which binds morphine and the synthetic peptide, D-Ala$^2$-N-methyl Phe$^4$-Gly$^5$-ol-enkephalin with nanomolar affinity, and of the κ-opioid receptor, which binds dynorphin A (1-13), salvinorin A, and U69,693, with high affinity (see Raynor et al., 1994 and Mansour et al., 1995 for ligand affinity and selectivity).

Zagon and colleagues have suggested that an opioid growth factor receptor and its purported ligand, Met-enkephalin, influence cellular proliferation through non-opioid mechanisms (Zagon et al., 2002). It appears, however, that the opioid growth factor receptor is not a target for naltrindole. Met-enkephalin is reported to bind to this receptor with high affinity and to inhibit murine neuroblastoma cell proliferation at nanomolar concentrations. We found, however, that Met-enkephalin has no effect on the proliferation of multiple myeloma cells at concentrations up to 200 μM, and does not bind to the naltrindole binding site with high affinity (data not shown). The identity of the naltrindole binding site in multiple myeloma cells is currently unknown. This laboratory has purified and identified μ, δ, and κ opioid receptors using mass spectrometry (Christoffers et al., 2003; Christoffers et al., 2005; Wannemacher et al., 2008), however, our efforts to purify and identify the naltrindole binding site in multiple myeloma cells are compromised by the loss of binding activity in subcellular fractions. Investigations into developing binding assays in subcellular fractions continue, along with efforts to identify the binding site by expression cloning or miRNA knockdown.

Multiple growth factors affecting many intracellular signaling pathways, and many interactions between myeloma cells with the bone marrow environment are involved in development and progression of multiple myeloma. Some of the most important signals regulating cell growth arise from the PI3K/Akt, NFκB, MEK/ERK, p38 MAPK, SAPK/JNK, JAK/STAT pathways.

The diversity of survival and anti-apoptotic mechanisms available to multiple myeloma cells contributes to the development of resistance to chemotherapy and radiation - a major problem in management of the disease. It is clear that targeting multiple pathways with combination therapies is beneficial for improving outcomes in patients with multiple myeloma. In recent years, with better understanding of multiple myeloma pathology, many promising drug targets have been identified and are currently being evaluated for use in therapy (Hwang et al., 2006; Lentzsch et al., 2004; Piazza et al., 2007; Podar et al., 2005). We hope that investigating the mechanisms through which naltrindole exerts its growth inhibitory effects will establish a role for naltrindole in combination anti-neoplastic therapy.

Several signaling pathways have been implicated in the anti-proliferative effects of naltrindole. IL-6 has many important roles in the regulation of immune responses and inflammation; it acts as a growth factor and as an anti-apoptotic factor in multiple myeloma (Bommert et al., 2006; Nishimoto and Kishimoto, 2006). Human U266 multiple myeloma cells produce IL-6, thus inhibition of IL-6 secretion or interference with its signaling is a possible mechanism for the growth inhibition by naltrindole that we observed in those cells. IL-6 signaling activates the JAK/STAT pathway, with downstream activation of MAPK, and Akt pathways, which are among the major growth promoting mechanisms in multiple myeloma. Chen et al. (2004) reported that naltrindole inhibited constitutive phosphorylation of Akt and induced apoptosis in small cell lung cancer cells. We have observed that naltrindole inhibits both Akt and ERK phosphorylation, and hence activation, in human U266 multiple myeloma cells, and this effect may underlie its anti-proliferative activity.

Targeting of growth or survival promoting pathways is important for successful treatment of multiple myeloma, thus we investigated synergy of naltrindole with other multiple myeloma

antiproliferative agents, including dexamethasone, simvastatin, bortezomib and valproic acid. NF-κB activation occurs via Akt pathway activation, and inhibition of NF-κB causes apoptosis of multiple myeloma cells, which is a mechanism of action of the proteasome inhibitor, bortezomib, approved for the treatment of multiple myeloma (Montagut et al., 2006; Piazza et al., 2007). We have observed that bortezomib and naltrindole act in an additive fashion in inhibiting proliferation on U266 cells. In addition, we have also observed additivity in the antiproliferative effects of the HDAC inhibitor, valproic acid, and naltrindole on U266 cells. Histone modifications are key components of the chromatin remodeling machinery. Regulation of gene expression by chromatin remodeling plays a key role in cell cycle progression and cell survival; interfering with this process by HDAC inhibitors is detrimental to many neoplastic cells, including multiple myeloma. Clinical trials to evaluate HDAC inhibitors for the treatment of multiple myeloma are ongoing (Gallinari et al., 2007; O'Connor et al., 2006). The short chain fatty acid, valproic acid, is a widely used anticonvulsant. It has also been shown to be an HDAC inhibitor and to have anti-neoplastic properties, including activity against multiple myeloma cell lines (Blaheta et al., 2005; Kaiser et al., 2006; Schwartz et al., 2007). In this study we have also demonstrated that naltrindole increases the anti-proliferative activity of the widely used anti-multiple myeloma agent, dexamethasone, a glucocorticoid agonist, and the HMG CoA reductase inhibitor, simvastatin. Statins are prescribed for hypercholesterolemia, but also display antineoplastic activity. Simvastatin has been shown to inhibit multiple myeloma cell proliferation, perhaps due to its inhibition of Ras farnesylation and synthesis of IL-6 (Gronich et al., 2004). We hope that these studies will stimulate further investigation of the combined use of naltrindole with other antineoplastic agents in human clinical trials to treat multiple myeloma. Opioid receptors undergo agonist-induced desensitization and downregulation, causing lower

receptor levels and diminished response to agonists, contributing to the development of tolerance (Corbett et al., 2006). Tolerance to opioid analgesics is a problem in management of chronic pain. Inactivation of the δ receptor by pharmacological or genetic methods results in attenuation of morphine tolerance. δ-Opioid receptor knock-out mice do not develop analgesic tolerance to morphine (Nitsche et al., 2002; Zhu et al., 1999). Similar effects were seen using antisense oligodeoxynucleotides targeted to the δ-opioid receptor gene (Kest et al., 1996). Naltrindole was also able to attenuate development of morphine-induced tolerance and acute physical dependence in rodents without blocking morphine analgesia when 10 pmoles was injected intracerebroventricularly (Abdelhamid et al., 1991), or when administered either at 1mg/kg subcutaneously or at 10 μg/mouse intracerebroventricularly (Hepburn et al., 1997). These are doses in which naltrindole maintains its delta receptor selectivity. Although naltrindole is not currently in clinical use, its parent compound, naltrexone, is used extensively and has a good safety profile. Naltrindole is, however, widely used in animal studies. Naltrindole has been administered without overt toxicity at concentrations of 1-30 mg/kg (Drower et al., 1991; Stevenson et al., 2003). Based on our *in vivo* studies reported herein, it is important to test the antiproliferative activity of naltrindole in xenograft models using lower doses and with less-frequent treatment regimens. We propose that naltrindole, or other structurally similar compounds, may be clinically useful in attenuating the development of tolerance to opioid analgesics in multiple myeloma patients, provided that the delta receptor selectivity can be maintained, in addition to its non-opioid receptor mediated anti-neoplastic properties. This compound could prove to be especially valuable in hematological malignancies, such as multiple myeloma, because of the sensitivity of immune cells to naltrindole and severe pain related morbidity associated with this disease.

**Authorship Contributions**

Participated in research design: Mundra, Terskiy, and Howells
Conducted experiments: Mundra, Terskiy, and Howells
Contributed new reagents or analytical tools**:** Howells
Performed data analysis: Mundra, Terskiy, and Howells
Wrote or contributed to the writing of the manuscript: Mundra, Terskiy, and Howells

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## Footnotes

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## Figure Legends

**Fig. 1.** Naltrindole binding sites in human U266 and RPMI 8226 multiple myeloma cells. A, Saturation curve of [$^3$H]-naltrindole binding to human U266 multiple myeloma cells derived from homologous competitive binding analysis as described in the text. The *inset* displays the linear increase in [$^3$H]-naltrindole specific binding using radioligand concentrations ranging from 1 – 40 nM. The $K_D$ for [$^3$H]-naltrindole was 20 μM, and the $B_{max}$ for U266 cells was 1.8 nmoles/mg protein. B and C, Saturation curve derived from homologous competitive binding analysis of naltrindole displacement of [$^3$H]-naltrindole binding using the radioligand at 10 nM using RPMI 8226 cells, shown in B and C. D, Lack of evidence for expression of δ, μ, and κ opioid receptor mRNA in human RPMI 8226 multiple myeloma cells. RNA was isolated from human RPMI 8226 cells and amplified by RT-PCR using selective opioid receptor primer pairs. Twenty five cycles of PCR were conducted and analyzed on 1.2% agarose gels, following staining with 1 μg/ml ethidium bromide. Lane M, size marker DNA standards (the prominent mid-gel DNA standard is 1000 bp). Lane C, RT-PCR reaction that lacked the Thermoscript reverse transcriptase as a negative control. Lanes labeled "Plasmid; δ, μ, and κ" were positive controls in which 1 nag each of the human opioid receptor plasmids were used as PCR templates. Lanes labeled "RPMI 8226 Cells; δ, μ, and κ" correspond to opioid receptor RT-PCR products derived from amplification of RNA isolated from human RPMI 8226 cells using receptor-selective primer pairs. Data are derived from 3 or more independent experiments.

**Fig. 2.** pH dependence and kinetic analysis of [$^3$H]-naltrindole binding to human U266 multiple myeloma cells. A, Sensitivity of $^3$H-naltrindole specific binding to varying the pH of the assay

buffer. Human U266 multiple myeloma cells were harvested from culture dishes, centrifuged at 167 x *g* for 5 min, then resuspended in 1X PBS at pH 8.5, 7.4, 6.5, 5.5 or 4.5. Binding assays, conducted in duplicate, were incubated at 24 ˚C for 30 min with [$^3$H]-naltrindole at 7.5 nM, and non-specific binding was determined in the presence of 500 μM unlabeled naltrindole. Other samples, labeled "restored to pH 7.4" in the Figure, were incubated in buffers at the various pH values for 30 min, centrifuged at 167 x *g* for 5 min, resuspended in 1X PBS, pH 7.4, centrifuged again, then the cell pellets were resuspended in 1X PBS at pH 7.4 prior to conducting the radioligand binding assays. B and C, Kinetic analysis of association rates and dissociation rates of [$^3$H]-naltrindole specific binding to human U266 multiple myeloma cells. Association rate experiments were conducted in 1X PBS buffer, pH 7.4, at 22 ˚C and 4 ˚C at the indicated time points, using [$^3$H]-naltrindole at 10 nM. Dissociation rates were calculated by first incubating intact U266 cells for 30 min at 22 ˚C and 4 ˚C in 1X PBS, pH 7.4 with 10 nM [$^3$H]-naltrindole in the absence and presence of 500 μM unlabeled naltrindole to determine specific and non-specific binding at the zero minute time point, then unlabeled naltrindole at 500 μM was added to a sample containing 10 nM [$^3$H]-naltrindole, and 100 μl aliquots were assayed for specific binding at the time points indicated. Curves were generated by non-linear regression analysis of the data using GraphPad Prism software, version 5.0c. Data are derived from 3 or more independent experiments.

**Fig. 3.** Effect of protein-modifying reagents and the protein synthesis inhibitor, cycloheximide, on specific [$^3$H]-naltrindole binding to human U266 multiple myeloma cells. A, sensitivity of [$^3$H]-naltrindole specific binding to U266 cells after treatment with various protein-modifying

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reagents. Intact U266 cells were incubated for 30 min at 24 ˚C with 50 mM dithiothreitol, 5 mM N-ethylmaleimide, or 1 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent). [$^3$H]-Naltrindole binding assays using 10 nM of the radioligand in the absence and presence of 500 μM unlabeled naltrindole were performed for 30 min at 24 ˚C immediately following the treatments. B, To assess the turnover rate of the naltrindole binding site, cycloheximide was added to U266 cell cultures at 120 μg/ml (or water for vehicle controls) for the indicated lengths of time, cells were centrifuged at 375 x *g*, then resuspended in 1X PBS to measure specific binding using 10 nM [$^3$H]-naltrindole. The data were fitted to a first order decay curve using non-linear regression analysis (GraphPad Prism, version 5.0c); the half-life of the naltrindole binding site was calculated to be 7.1 h. Data are derived from 3 or more independent experiments.

**Fig. 4.** Subcellular fractionation studies of the nalrtrindole binding site in human U266 multiple myeloma cells. In panel A, [$^3$H]-naltrindole binding to U266 cell fractions was compared to [$^3$H]-diprenorphine binding to HEK 293 cells expressing the δ-opioid receptor. Cell homogenates were prepared by homogenizing the cells using a Tekmar tissuemizer (Cincinnati, OH) in 1X PBS. Membranes were isolated by ultracentrifugation (100,000 x *g* for 30 min) of the homogenates. [$^3$H]-Naltrindole specific binding was assayed at 7 nM using comparably equal volumes of cells or cell fractions. U266 homogenate and membrane binding was significantly decreased compared to whole-cell binding, while [$^3$H]-diprenorphine binding (3 nM) to the δ-opioid receptor expressed in HEK 293 cells was similar in whole cells, homogenates, and membrane fractions. B, Specific [$^3$H]-naltrindole binding (8 nM) to U266 subcellular fractions

following hypotonic lysis compared to whole cell binding. C, Subcellular fractionation of U266 using an isotonic sucrose buffer according to Frezza et al. (2007), as described in the Methods section. Specific $^{3}$H- naltrindole binding was compared in whole cells, homogenates, and P1 and P2 fractions. Data are derived from 3 or more independent experiments.

**Fig. 5.** Competition analysis of $^{3}$H-naltrindole binding to human U266 multiple myeloma cells by select opioid compounds. Dose-response curves for displacement of specific [$^{3}$H]-naltrindole binding (7.5 nM) are shown using unlabeled naltrindole, naltriben, SNC 80, BNTX, naltrexone and morphine. Data are derived from 3 or more independent experiments.

**Fig. 6.** Naltrindole inhibits multiple myeloma cell proliferation. A, Concentration-dependent anti-proliferative activity of naltrindole following incubation for 72 h with human U266 multiple myeloma cells, using the Wst-1 assay. B, Time-dependent anti-proliferative activity of naltrindole using the Vi-Cell assay of the number of viable U266 cells (one way ANOVA, using Turkey's multiple comparison test, $F(5, 18) = 30.41$, $p < 0.01$). C, Naltrindole (10 μM) inhibited the proliferation while naltrexone (100 μM) slightly stimulates the proliferation of human U266 multiple myeloma cells, although neither of these effects quite reached statistical significance. Naltrexone (100 μM) at a ten-fold molar excess relative to naltrindole, had no effect on the anti-proliferative effects of naltrindole (10 μM) on U266 cells implying that a non-opioid receptor-mediated mechanism was involved. D, Effect of anti-multiple myeloma agents on U266 cell proliferation, alone and in combination with naltrindole. Nti, naltrindole; VPA, valproic acid;

Dex, dexamethasone; BT, bortezomib; SMSgt, simvastatin. Data are derived from 3 or more independent experiments (one way ANOVA, using Turkey's multiple comparison test, $F(9, 20) = 17.88$, $p < 0.01$).

**Fig. 7.** Naltrindole does not cause activation of caspase-3, a marker for apoptosis, in multiple myeloma cells. A, Naltrindole treatment of RPMI 8226 cells (*left panel*) or U266 cells (*right panel)* for 24 h at 10, 30, or 50 μM, or treatment with 50 μM naltrindole for 24, 48, or 72 h, did not elicit caspase-3 cleavage and activation, whereas treatment of the cells with 25 μM MG132, a proteasome inhibitor, resulted in caspase-3 activation and apoptosis. B, FACS analysis of RPMI 8226 cells confirmed that naltrindole did not trigger apoptosis of the cells under these conditions. *Left panel*: Naltrindole treatment of RPMI 8226 cells for 24 h at 10, 30, or 50 μM did not cause an increase in the population of cells that were annexing V-positive and presidium iodide-negative, while the positive control, MG 132, clearly induced apoptosis. *Right panel*: Incubation of RPMI 8226 cells with 50 μM naltrindole for 24, 48, or 72 h did not induce apoptosis, while MG 132 did. Data are derived from 3 or more independent experiments.

**Fig. 8.** The anti-proliferative activity of naltrindole toward human U266 multiple myeloma cells is maintained when they are co-cultured with human HS-5 bone marrow stoma cells. A, Naltrindole binds to human HS-5 bone marrow stoma cells with an $IC_{50}$ of 36 μM. B, To mimic the bone marrow microenvironment, co-culturing U266 with HS-5 cells resulted in a slight shift to the right in the dose-response curve for naltrindole following incubation for 72 h, changing the

anti-proliferative $EC_{50}$ to 29 μM from 20 μM. Data are derived from 3 or more independent experiments.

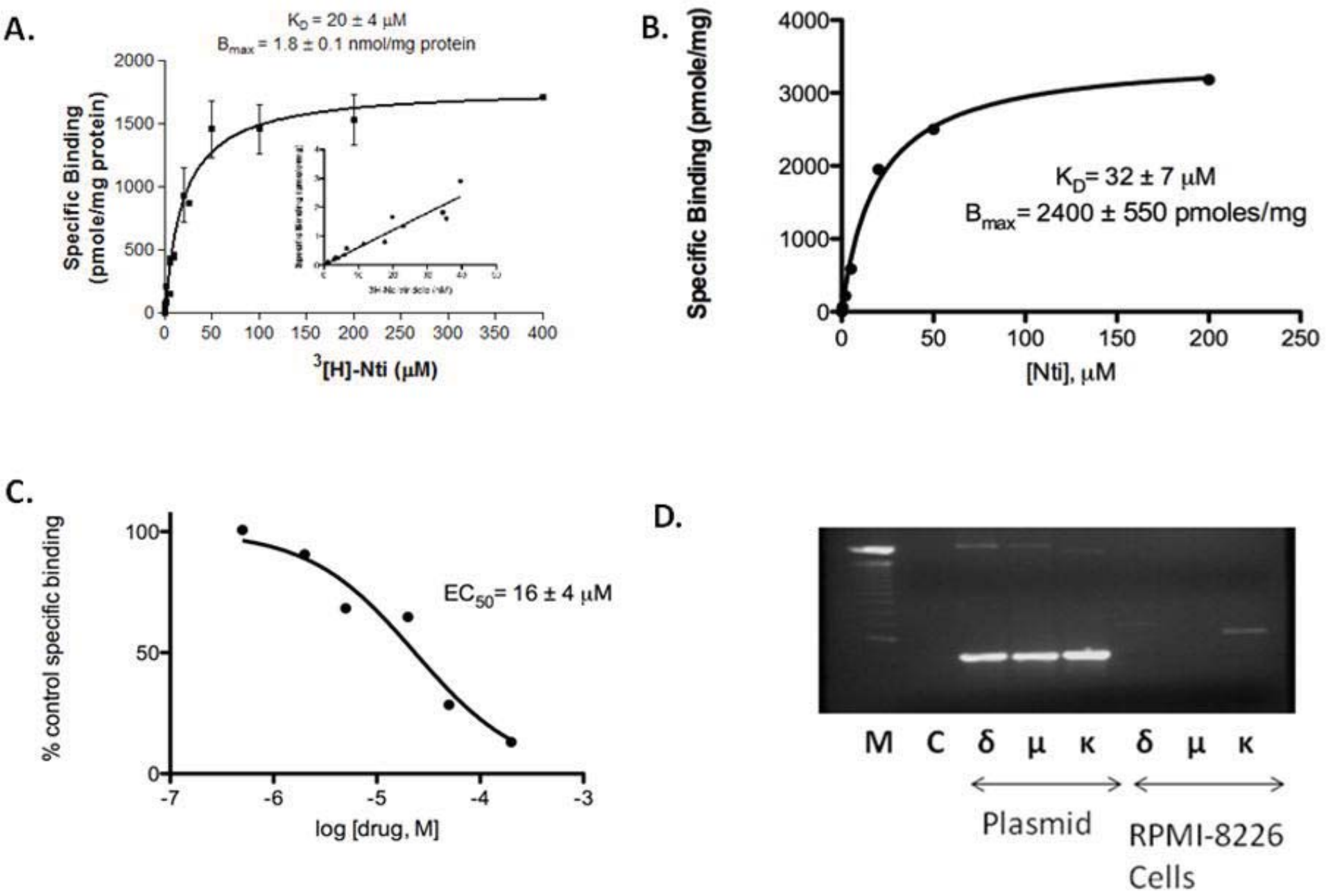
**Fig. 9.** Inhibition of the extracellular signal-regulated kinas (ERK) and Akt pathways by naltrindole in U266 cells. A, *Left panels*: Inhibition of the Akt (protein kinas B) pathway by naltrindole in U266 cells. *Left panels*: Western blot analysis of the time-dependent decrease in phosphorylated, activated Akt in response to 50 μM naltrindole, while total Akt levels are unaltered. *Right panel*: quantification of the level of phospho-Akt normalized to the level of total Akt; data are derived from 3 independent experiments, and statistical analysis revealed that the mean phospho-Akt levels in the 2 h and 24 h naltrindole-treated samples were significantly decreased relative to the control (one way ANOVA, using Tukey's multiple comparison test, $F(5, 15) = 9.5$, $p < 0.05$). B, Western blot analysis of the time-dependent decrease in phosphorylated, activated ERK in response to 50 μM naltrindole, while total ERK levels were unaltered. *Right panel*: quantification of the level of phospho-ERK normalized to the level of total ERK; data are derived from 3 independent experiments, and statistical analysis revealed that the mean phospho-ERK levels in naltrindole treatment groups at 10 min, 30 min, 1 h and 2 h were significantly decreased compared to the control untreated group (one way ANOVA, using Tukey's multiple comparison test, $F(5, 11) = 17.6$, $p < 0.001$).

**Fig. 10.** Naltrindole binding and anti-proliferative activity to freshly isolated human peripheral blood mononuclear cells, red blood cells and platelets compared to human U266 multiple

myeloma cells. A, Specific binding of 6 nM [$^3$H]-naltrindole to freshly isolated human peripheral blood mononuclear cells, red blood cells and platelets compared to human U266 multiple myeloma cells. B, Dose-response curve of the efficacy of naltrindole to decrease the number of viable cells following a 72 h incubation with human peripheral blood mononuclear cells and red blood cells compared with U266 multiple myeloma cells. Data are derived from 3 or more independent experiments.

**Fig. 11.** Effect of naltrindole on multiple myeloma tumor growth in a murine SCID/human RPMI 8226 multiple myeloma xenograft model. As described in the Methods section, human RPMI 8226 multiple myeloma cells were inoculated subcutaneously into both flanks of SCID mice (ten million cells per flank). After 8 days, 12 mice were divided into 2 groups of 6 mice each: vehicle injected and naltrindole injected (30 mg/kg). Animals were dosed daily for 36 d and size of xenograft tumors (A) and body weights (B) were measured twice a week with a digital caliper. Multiple myeloma tumor volumes were significantly decreased relative to vehicle controls from day 21 onward (one way ANOVA, using Tukey's multiple comparison test, $F(27, 109) = 90$, $p < 0.0001$), and body weights in naltrindole-treated animals were significantly different from vehicle-treated controls at days 34, 36 and 39 (one way ANOVA, using Tukey's multiple comparison test, $F(27, 136) = 6.7$, $p < 0.0001$).

# Figure 1



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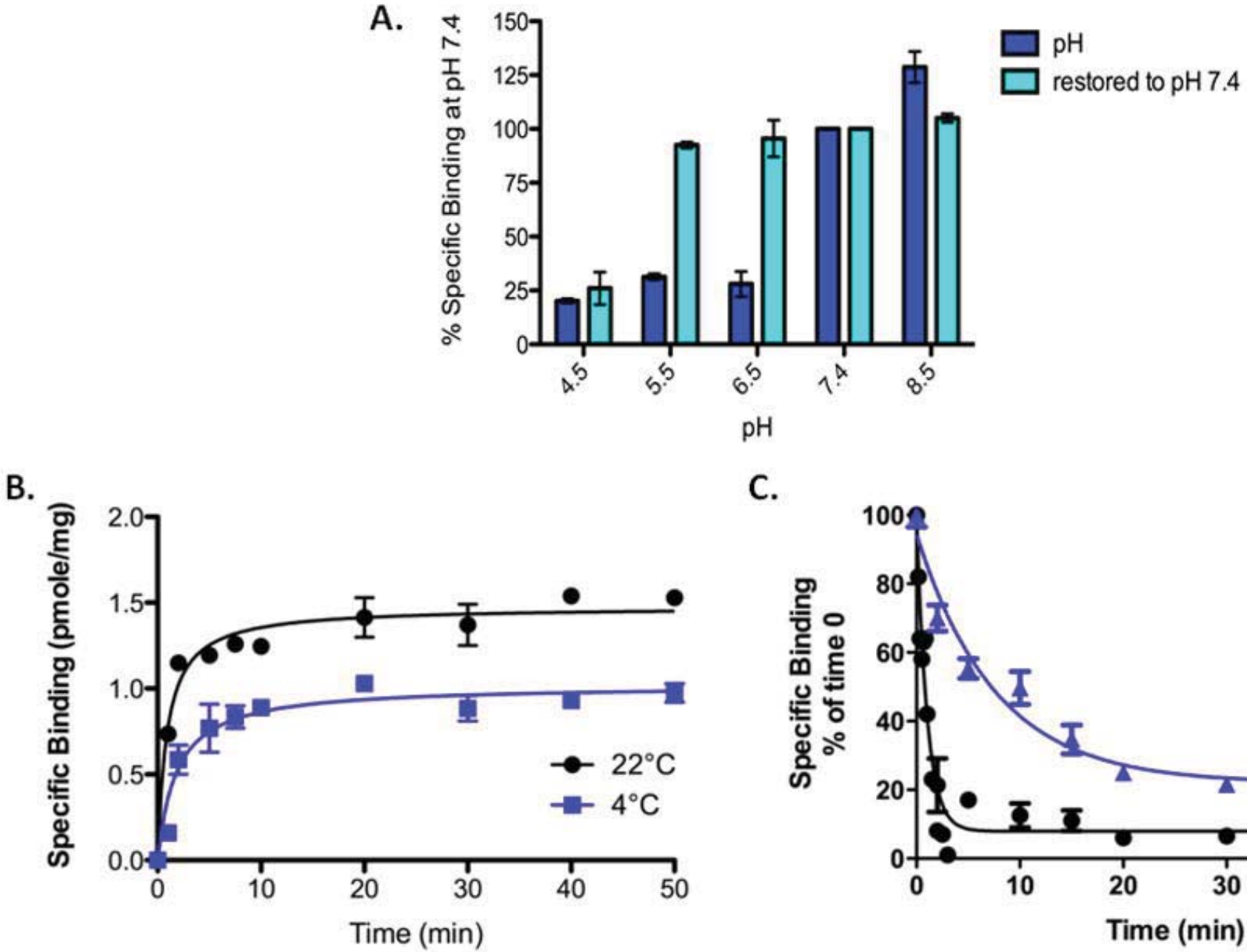

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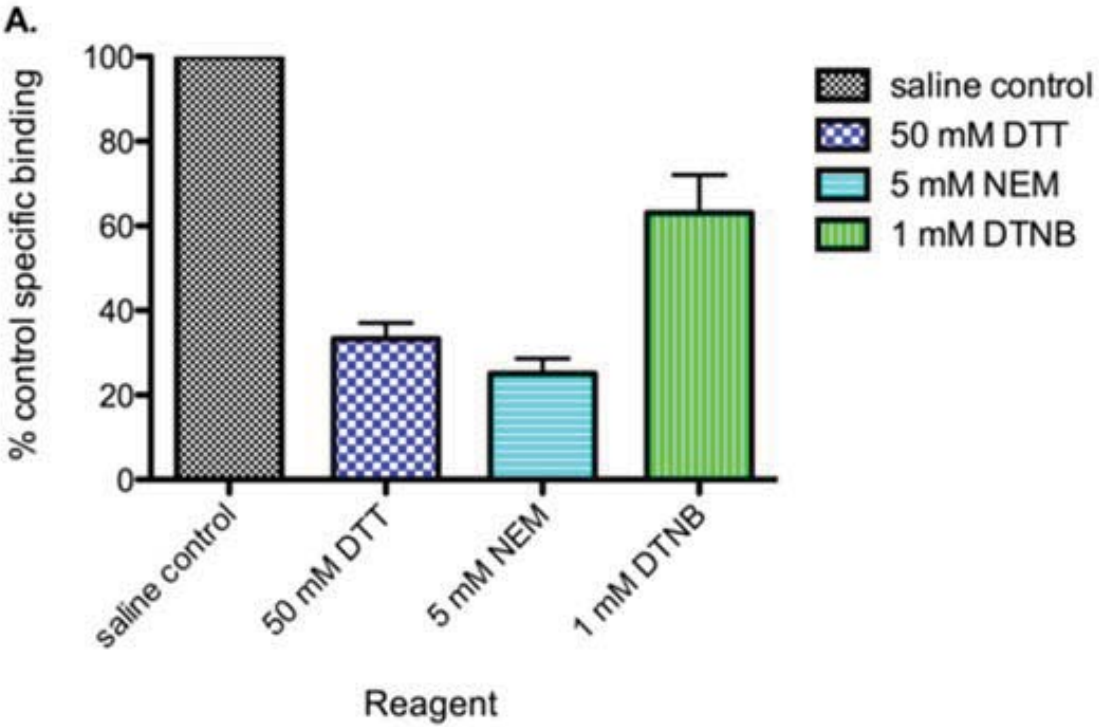


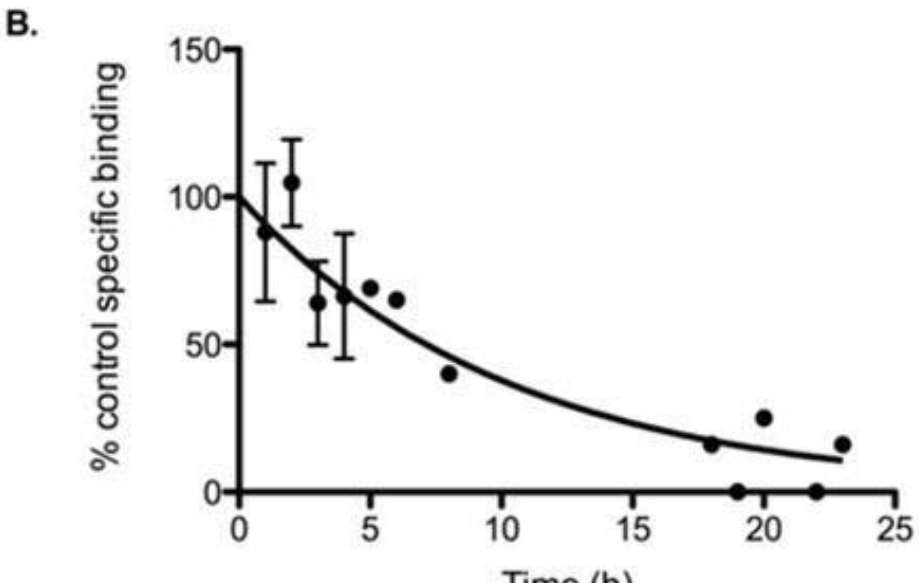

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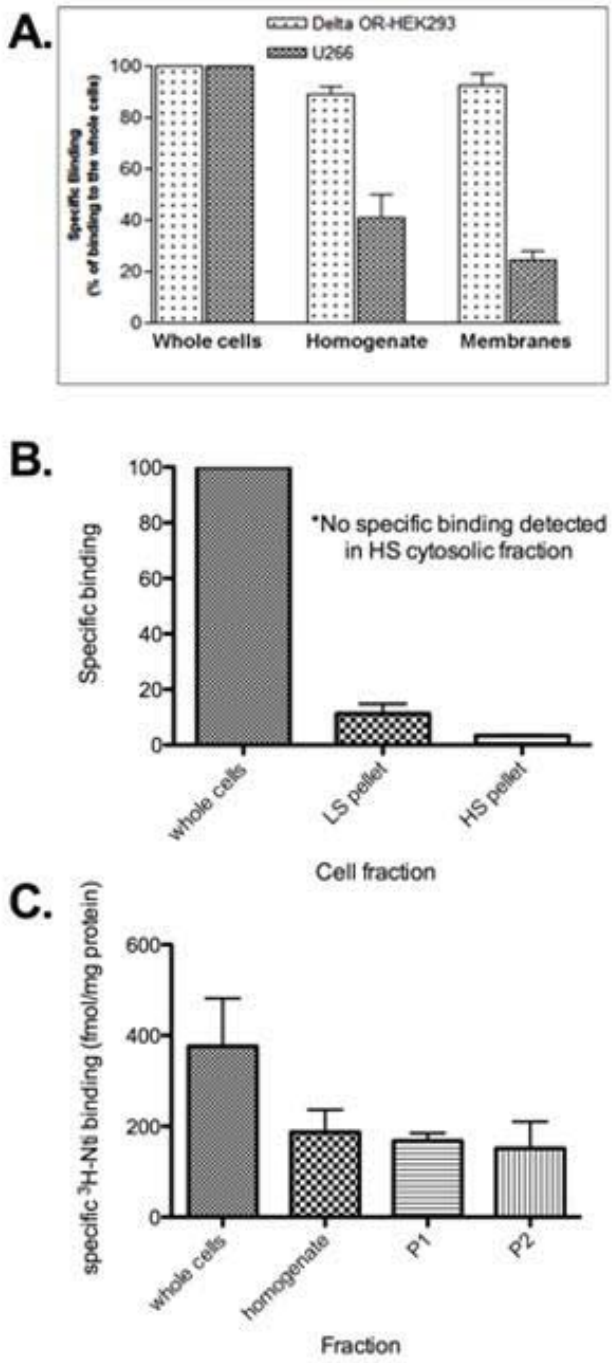
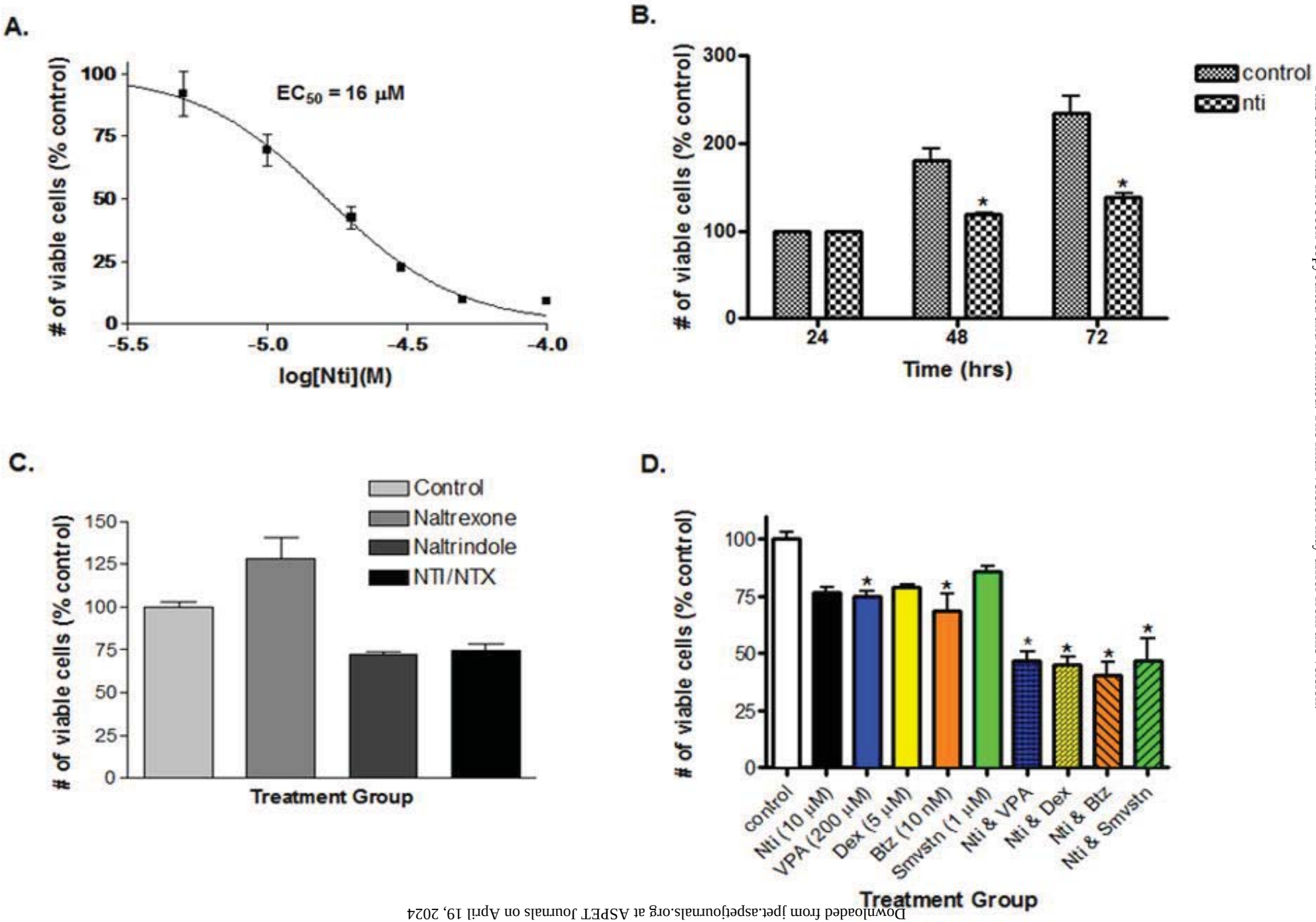


Figure 5

# Figure 6



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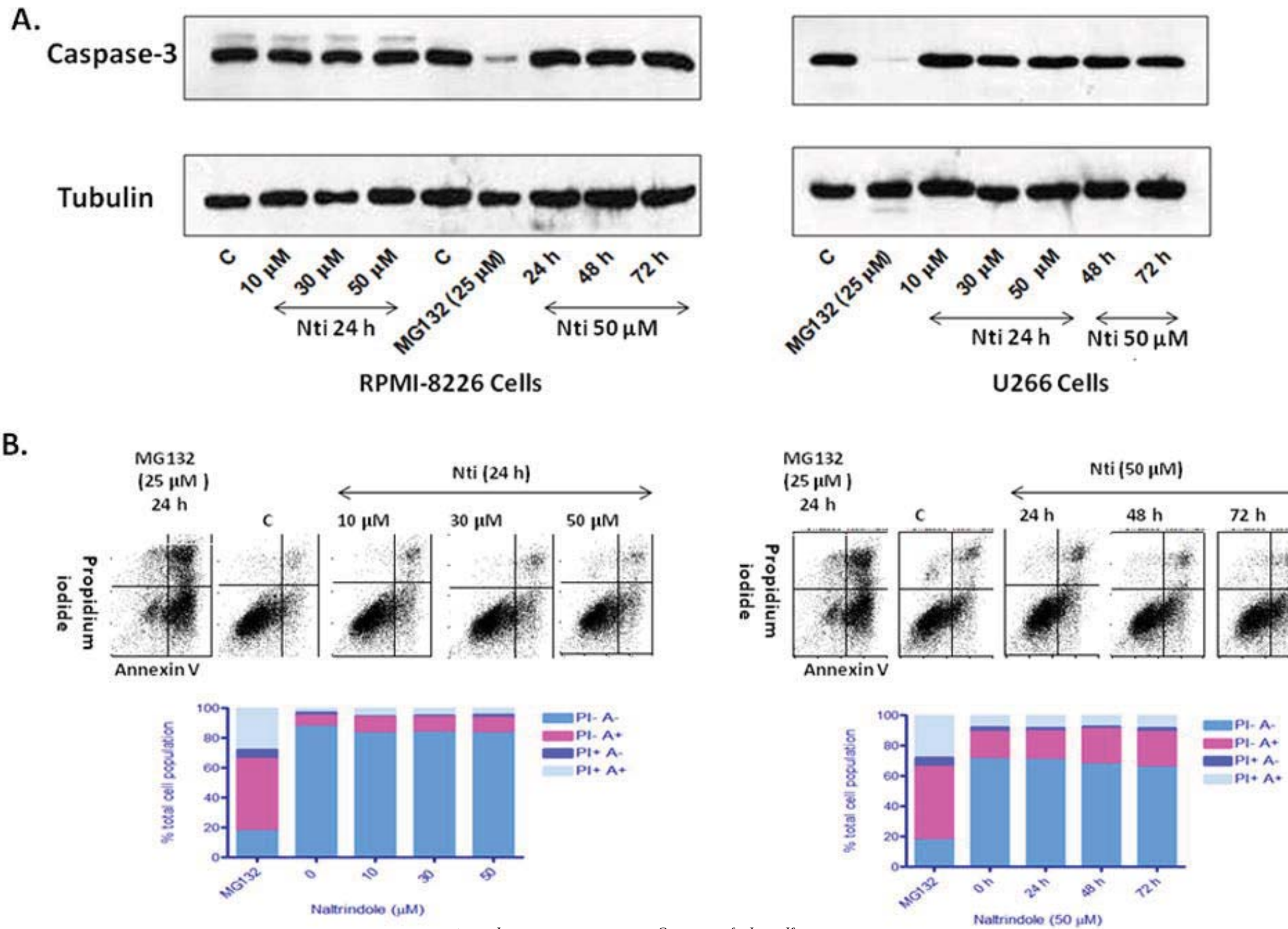
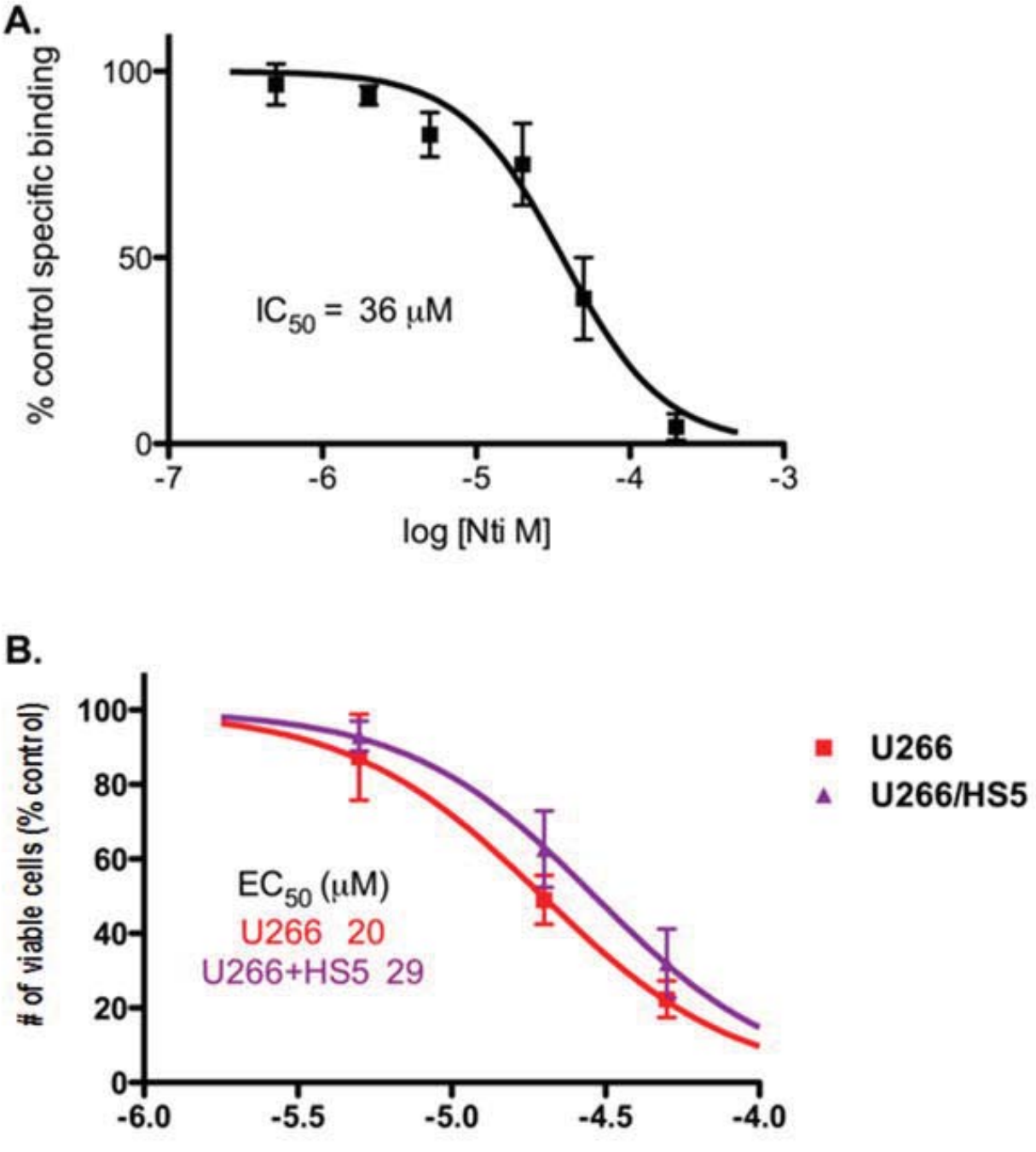
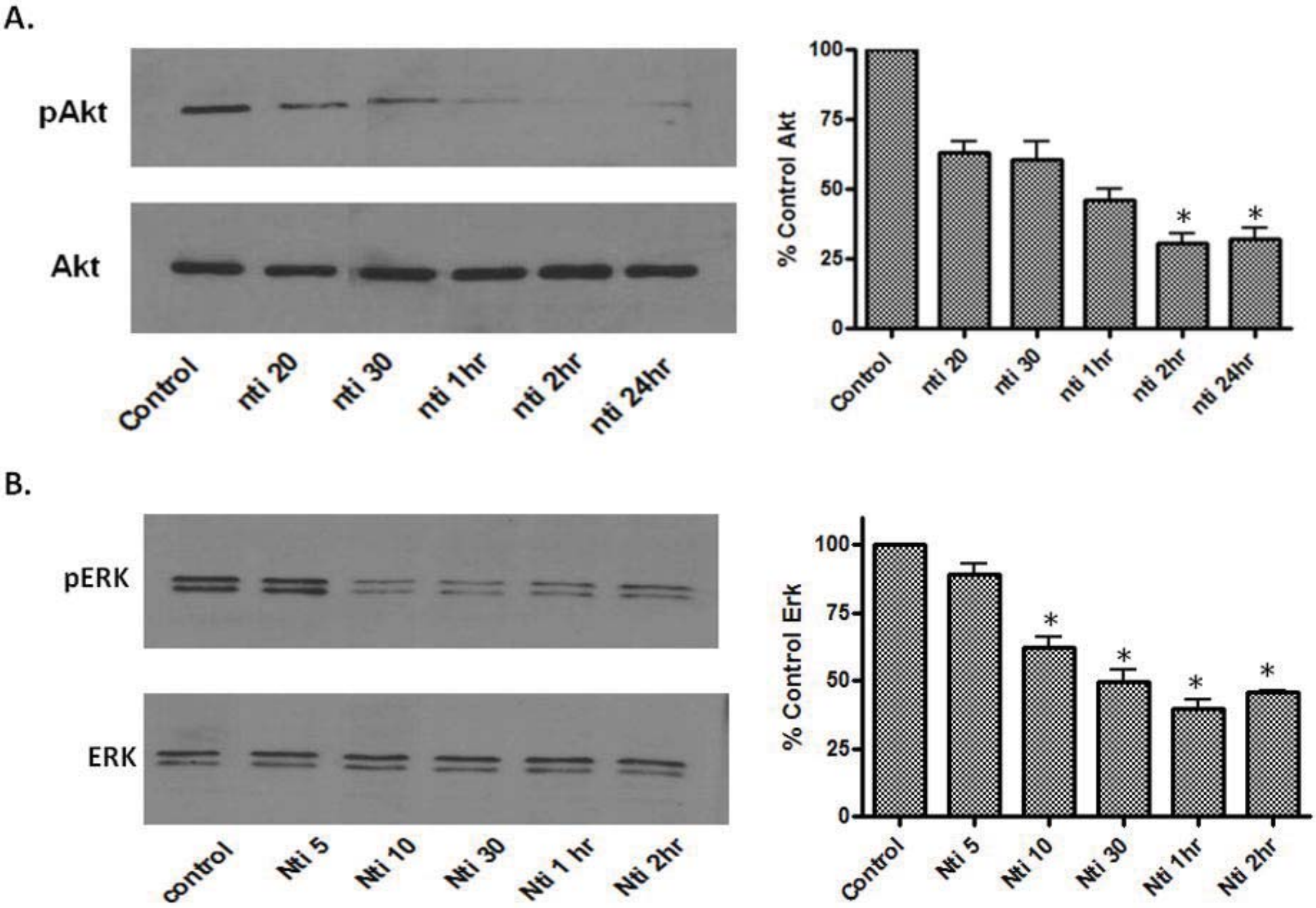


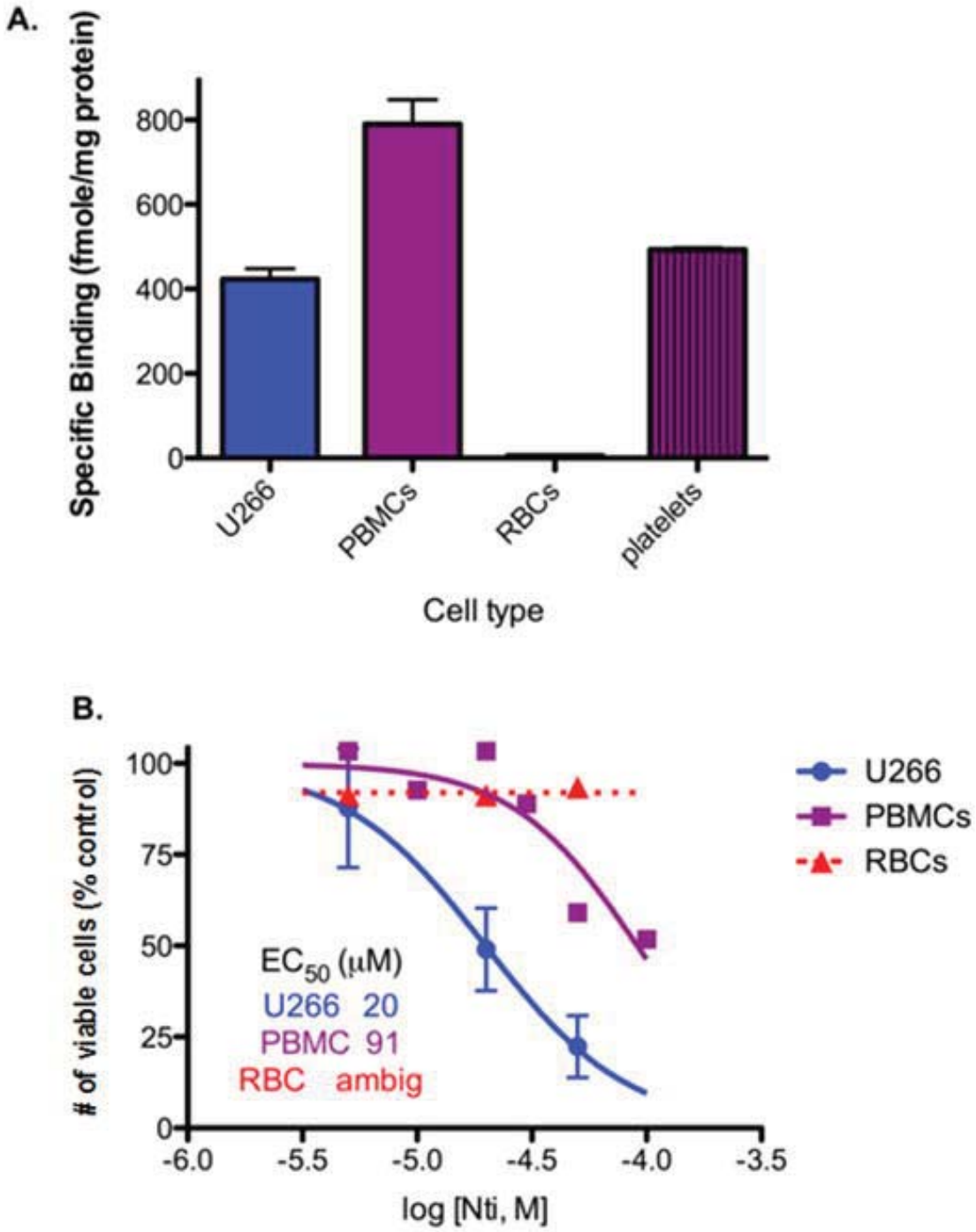
# Figure 8



# Figure 9



# Figure 10



# Figure 11