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Drug-induced alterations to gene and protein expression in IEC-6 cells suggest a role for calpains in the GI toxicity of non-steroidal anti-inflammatory agents

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Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; GI, gastro-intestinal; COX, cyclooxygenase ; DMSO, dimethyl sulfoxide; SC-560, (5-(4-chlorphenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole) ; NS-398, (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) ; SLR, signal log ratio ; ALLN, *N*-acetyl-Leu-Leu-Nle-CHO; ALLM, *N*-acetyl-Leu-Leu-methioninal ; IPTG, isopropyl-β-D-thiogalactopyranoside; IPA, Ingenuity pathway analysis ; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are used extensively as therapeutic agents, despite their well-documented gastrointestinal (GI) toxicity. Presently, the mechanisms responsible for NSAID-associated GI damage are incompletely understood. In this study, we used microarray analysis to generate a novel hypothesis about cellular mechanisms that underlie the GI toxicity of NSAIDs. Monolayers of intestinal epithelial cells (IEC-6) were treated with NSAIDs that either exhibit (indomethacin, NS-398) or lack (SC-560) inhibitory effects on IEC-6 migration. Bioinformatic analysis of array data identified the calpain cysteine proteases and their endogenous inhibitor calpastatin as potential targets of NSAIDs shown previously to retard IEC-6 migration. Accordingly, qRT-PCR and immunoblotting were performed to assess the effects of NSAIDs on the expression of mRNA and protein for calpain 8, calpain 2, calpain 1 and calpastatin. In treated IEC-6 monolayers, NS-398 decreased the expression of mRNA for calpain 2 and calpain 8. Both NS-398 and indomethacin decreased the protein expression of calpains 8, 2 and 1. None of the NSAIDs affected expression of calpastatin mRNA or protein. The calpain inhibitors, ALLM and ALLN, retarded IEC-6 cell migration in a concentration-dependant fashion, and these inhibitory effects were additive with those of indomethacin and NS-398. Our experimental results suggest that the altered expression of calpain proteins may contribute to the adverse effects of NSAIDs on intestinal epithelial restitution.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used extensively as therapeutic agents despite their well-documented gastrointestinal (GI) toxicity. Adverse gastrointestinal effects of NSAIDs in humans and other species include oral, gastric, duodenal, and colonic ulceration (Lichtenberger, 2001;Tomisato, et al., 2004). Despite exhaustive investigation, the mechanisms responsible for NSAID-associated GI damage are not completely understood. Evidence gathered to date suggests that NSAIDs may promote ulcer formation not only by inhibiting mucosal cyclooxygenase (COX) and decreasing cytoprotective prostaglandins (PG), but also by adversely influencing intestinal microflora, neutrophil recruitment, surface hydrophobicity and epithelial restitution (Lichtenberger, 2001;Little, et al., 2007). Whereas the inhibition of COX isoforms has received much attention and investigation as the basis of GI toxicity, it is acknowledged widely that COX inhibition cannot account for the complete spectrum of NSAID-induced toxicity seen clinically (Lichtenberger, 2001;Little, et al., 2007;Tomisato, et al., 2004).

The GI epithelium has several functions; the most important of which are uptake of nutrients and separation of the internal milieu from the external environment. Surface epithelial cells are continuously exposed to noxious agents and abrasive ingesta that may cause mucosal injury. Normally, superficial mucosal defects are repaired rapidly by migration of epithelial cells from proliferative zones into the wound. This process, mucosal restitution, represents a primary repair modality in the GI tract, and allows resealing of the epithelial barrier within minutes or hours via reformation of tight

junctions between cells (Dignass, 2001). Restitution is a well coordinated event that is dependent on cell migration, but independent of cell proliferation and differentiation (Dignass, 2001).

The migration of intestinal epithelial cells is modulated by a wide range of cytoplasmic effectors associated with a number of signaling pathways (Dignass, 2001;Pai, et al., 2001). Cellular components involved in the regulation of resting membrane potential, intracellular calcium dynamics, focal adhesion components and cytoskeletal integrity have been identified as common sites of action for interventions that impair intestinal restitution and inhibit ulcer healing (McCormack and Johnson, 2001;Pai, et al., 2001;Rao, et al., 2002). Unfortunately, there is a lack of knowledge about the global effects of NSAIDs on the components of these complex signal transduction networks. Our work presented here and elsewhere extends knowledge about the mechanisms underlying the adverse GI effects of NSAIDs. Recently, we have demonstrated that inhibition of cell migration by NSAIDs is related to drug effects on potassium channel expression and trafficking (Freeman, et al., 2007). The current study was conducted to characterize the effects of NSAIDs on gene expression and to identify novel mediators of NSAID toxicity.

To address these goals, IEC-6 cells were exposed to a series of NSAIDs with variable potential for GI toxicity and known effects on cell migration. Total RNA was isolated and transcriptional responses were evaluated using high density cDNA array analysis. Our results suggested that NSAIDs with adverse effects on epithelial restitution affect the

expression of genes associated with signaling pathways that influence the migration of intestinal epithelial cells. Specific targets of NSAID activity included the calpains, cysteine proteases involved in numerous cellular processes such as cell migration and invasion. The work presented herein demonstrates the power of microarray analysis to provide new insights into the molecular basis of NSAID toxicity.

Materials and Methods

Reagents

Cell culture medium was obtained from American Type Culture Collection (ATCC, Manassas, VA). Serum was obtained from Invitrogen (Calsbad, CA) and chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise. Matrigel basement membrane was purchased from BD Biosciences (Bedford, MA). SC-560 (5-(4-chlorphenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole) and NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) were obtained from Cayman Chemical (Ann Arbor, MI).

Treatment Protocols

We assessed the effects of NSAIDs with variable ulcerogenic potential on gene expression. Experimental treatments included: vehicle control (0.1% DMSO), indomethacin (10 or 100μM), NS-398 (10 or 100μM), or SC-560 (1μM). The conditions used in this investigation are based on previous work documenting predictable effects on cell migration at non-cytotoxic concentrations of drug.

Indomethacin is a non-selective COX-inhibitor that has been shown to induce ulcers in drug- treated animals (Kato, et al., 2001;Tavares, 2000;Peskar, et al., 2001).We have shown previously that chronic (72 h) exposure to 100 μM indomethacin decreases cell migration in wounded intestinal epithelial monolayers (Freeman, et al., 2007), and others have demonstrated that 250-500 μM indomethacin inhibits re-epithelialization of wounded gastric epithelial cell monolayers (Pai, et al., 2001). The highest concentration of indomethacin used in this study (100 μM) is consistent with plasma and tissue drug concentrations achieved after administration of therapeutic doses to rats and humans (Frey and El-Sayed, 1977;Kokoska, et al., 1998;Suzuki, et al., 1997). Moreover, this concentration of indomethacin has been shown to inhibit cellular PGE_2 synthesis *in vitro* without affecting cell viability or inducing apoptosis in monolayer cultures of epithelial cells (Kokoska, et al., 1998;Pai, et al., 2001;Tavares, 2000;Tomisato, et al., 2004).

NS-398 is a relatively selective inhibitor of COX-2 that is used experimentally (Kato, et al., 2001;Tavares, 2000). Administration of NS-398 alone does not induce gastric lesions in drug-treated animals (Tomisato, et al., 2004); however, this drug has been shown to delay ulcer healing (Brzozowski, et al., 2001;Peskar, et al., 2001). We have shown previously that chronic exposure (72 h) to 100 μM NS-398 decreases cell migration in wounded intestinal epithelial monolayers (Freeman, et al., 2007), and others have shown comparable inhibition of re-epithelialization of wounded gastric epithelial cell monolayers (Pai, et al., 2001). The highest concentration of NS-398 employed in the current investigation (100 μM) has been shown to inhibit synthesis of PGE2 *in vitro*

without affecting viability or inducing apoptosis in cultured epithelial cells (Pai, et al., 2001;Tavares, 2000;Tomisato, et al., 2004).

SC-560 (1 μM,) inhibits COX-1 but not COX-2 activity *in vitro* (Smith, et al., 1998). SC-560 does not induce ulcers in animals treated with dosages sufficient to inhibit mucosal PGE_2 production (Peskar, et al., 2001;Tanaka, et al., 2001). We have shown previously that chronic exposure (72 h) to 1 μM SC-560 has no inhibitory effects on cell migration in wounded intestinal epithelial monolayers (Freeman, et al., 2007).

Cell Culture

The IEC-6 cell line, developed by Quaroni *et al.* (Quaroni, et al., 1979), was purchased from ATCC, (Manassas, VA). IEC-6 culture conditions were similar to those described previously (Freeman, et al., 2007). The basic culture medium consisted of DMEM supplemented with heat-inactivated fetal bovine serum (FBS, 5%), insulin (10 μg/ml) and gentamicin (50 μg/ml). Cells were maintained in 75 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell passages 16-20 were used for all experiments to minimize the effects of passage. Cells were seeded at a density of approximately 6.25 x 10^4 cells/cm^2 on 35, 60 or 100 mm plates thinly coated with Matrigel (BD Biosciences, Bedford, MA) and were grown to a confluent monolayer prior to the introduction of media containing experimental treatments. Cells were treated for 72 h with NSAIDs or the vehicle control prior either to harvesting RNA for microarray and qRT-PCR analysis or to lysing cells for immunoblot analysis.

Additional experiments were performed using IEC-Cdx2 cells (Suh and Traber, 1996). In this stably transfected cell line, the forced expression of the Cdx2 gene in IEC-6 cells induces a differentiated phenotype. The LacSwitch expression vector system (Stratgene, La Jolla, CA) is used to direct conditional expression of the Cdx2 gene, with isopropyl-β-D-thiogalactopyranoside (IPTG) serving as the inducer for gene expression. IEC-Cdx2 cell stocks were maintained in the same basic culture medium used for IEC-6, and grown in media supplemented with 4 mM IPTG for 16 days to induce the conditional expression of Cdx2 before experiments.

Affymetrix Gene Chip Analysis

RNA was isolated from treated IEC-6 cells using a commercially available kit (RNeasy Micro Kit, Qiagen, Germany). The quality and quantity of RNA were assessed by microfluidic electrophoresis (Bioanalyzer, Agilent Technologies, Palo Alto, CA) and a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE), respectively. Total RNA (10 μg) was processed and hybridized to separate high-density nucleotide gene chips for micro-array analysis according to manufacturer's standards. The gene chip used, Rat RAE 230-2.0 (Affymetrix, Santa Clara, CA), provides comprehensive coverage of the rat genome by interrogating over 31,042 transcripts and variants including more than 28,000 well-documented rat genes. Sample processing for RNA amplification, cDNA synthesis, labeling, and hybridization were carried out using the Small Sample Labeling Protocol vII developed by the Kansas University Medical Center-Microarray Facility: http://www2.kumc.edu/mrrc/microarray/Protocols.html. Probe arrays were scanned at a wavelength of 570 nm with a gene array scanner (Agilent,

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Microarray Core-KUMC, KS). All array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches on the chip surface. The methodology followed complies with the MIAME ("Minimum Information About a Microarray Experiment") guidelines which are provided at http://www.mged.org/Workgroups/MIAME/miame.html. The control and experimental conditions (DMSO vehicle control, indomethacin, NS-398, and SC-560) were repeated four times to ensure a robust analysis.

Signal intensities were quantified by pixel intensity, and expression signals were analyzed using the Affymetrix Data Acquisition Software, Gene Chip Operating Software1.4 (GCOS, Affymetrix). Statistical algorithms [detection, change call, signal log ratio (SLR)] were then used to identify differential gene expression in control and experimental samples. Data was imported into the Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems Inc., Redwood City, CA; http://www.ingenuity.com/products/pathways_analysis.html)

Probe sets were assigned as either present (P), absent (A) or marginal (M) based on detection *P* values (P, $P < 0.05$; M, $P = 0.05$-0.065; A, $P > 0.065$). Additional analysis was conducted if and only if three out of four samples in either the control or experimental groups were called present. Pair-wise comparisons between individual experimental and control arrays were made to generate an SLR value for each transcript. Student's t-test was done between the SLR values to determine the significance of these genes [using a *P*-value of 0.05]. Fold change was calculated from the median of the

SLRs; the cut-off for fold change was 1.5 for up- or down-regulated genes. This analysis by SLR cancels out the differences in individual intensities at the probe level.

IPA was used to analyze the data in the context of molecular mechanisms and to identify the signaling networks altered by NSAID treatment in an unbiased, gene by gene fashion. IPA utilizes a curated database and analysis system designed to determine how proteins work together to effect cellular changes. Using a global approach, IPA was used first to analyze common and distinct properties of all altered genes in relationship to one another. Identified genes were next mapped to the functional networks available in the IPA database and ranked by score. The score is the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. Initial results were mined further to detect alterations in the expression of mRNA transcripts encoding proteins related to the maintenance of cell migration.

Confirmation of Differential Gene Expression

Quantitative RT-PCR was used to confirm changes in mRNA expression levels detected by microarray analysis for selected genes. These included calpain 8, calpain 2, calpain 1, and calpastatin; 18S served as the internal standard. Primers were designed based on the reported rat sequences with the assistance of the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge MA). The primers used in these experiments (Invitrogen, Carlsbad, CA and Integrated DNA Technologies, Coralville, IA) are listed in Table 1.

All qRT-PCR reactions were run on a Cepheid SmartCycler (Sunnyvale, CA). Analyses of message for calpain 8, 2, 1 and 18S were performed in the presence of 0.2x SYBR green I (Molecular Probes, Eugene, OR) using RNA from treated cells and gene-specific primers (Table 2) Thermocycling protocols were as follows: 50°C for 30 min, 95°C for 15 min, 50 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and 78°C for 30 s. Melt curves were performed to ensure that only a single product was present. Gel electrophoresis in the presence of ethidium bromide confirmed that only single products of the appropriate size were obtained from each primer pair. Inclusion of a DNA ladder in gels permitted determination of amplicon sizes.

The SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, Carlsbad, CA), in combination with sequence specific primers and a dual labeled fluorogenic probe (Integrated DNA Technologies), was employed for the analysis of calpastatin message. The 18S primers were obtained from a commercially available kit (TaqMan, ribosomal RNA, Applied Biosystems, Foster City, CA), The real-time cycling protocol consisted of first-strand cDNA synthesis for 15 min at 50°C, initial activation for 2 min at 95°C, and then 40 cycles of denaturation for 15 s at 95°C, followed by annealing/extension for 30 s at 60°C. Single products of appropriate size were demonstrated by gel electrophoresis in the presence of ethidium bromide. All RT-PCR reactions were duplicated for each experimental condition from 3-4 separate RNA isolations.

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Prior to quantitative analysis, 10-fold dilutions of RNA were used as template for each primer pair to determine their amplification efficiency. The resulting Ct values were then fit by linear regression to a logarithmic scale. The slope was then used to determine primer efficiency using Equation 1: $E = 10^{\frac{-1}{(slope)}} - 1$, where E is the efficiency of the primer set and slope is the slope of the linear fit of the data.

Differences in the mRNA levels between experimental conditions were determined using the ΔCt values obtained from our qRT-PCR experiments in conjunction with the calculated efficiencies of each primer set. Using Equation 2: $FoldChange = \frac{(1+E_{GOI})^{-\Delta Ct_{GOI}}}{(1+E_{18S})^{-\Delta Ct_{18S}}}$, where Fold Change is the relative change in expression as compared to control, E_{GOI} is the efficiency of the primer set for the gene of interest, E_{18S} is the efficiency of the 18S primer set, ΔCt_{GOI} is the ΔCt for the gene of interest, and ΔCt_{18S} is the ΔCt for the 18S internal controls, we compared the relative expression levels of calpains 8, 2, 1, and calpastatin for the following treatment groups: DMSO vehicle control, indomethacin, NS-398, and SC-560; 18S served as the internal standard.

Western Blot Analysis

Whole cell lysates were collected using a commercially available isolation buffer (RIPA, Santa Cruz Biotechnology, Santa Cruz, CA) and protein content was determined by Micro BCA Protein Assay (Pierce, Rockford IL). Cell lysates were separated by SDS-PAGE, transferred to membranes, and probed sequentially with appropriate primary and secondary antibodies. Primary antibodies were obtained commercially (Triple Point

Biologic, Forest Grove, OR) and used at the following dilutions: calpain 8 (1:750); calpain 2 (1:500); calpain 1 (1:500); and calpastatin (1:750). Primary antibodies were diluted into the blocking buffer [5% non-fat milk in Tris-buffered saline (TBS) with Tween-20 (0.1%)]; TBS was made from a commercially available 10X stock (Bio-Rad (#170-6435). Membranes were incubated in primary antibody overnight at 4°C. Stabilized goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Pierce, Rockford, Illinois) was diluted 1:1000 into the blocking buffer, and membranes were exposed to secondary antibody for 1 h at room temperature. Immunocomplexes were visualized using an enhanced chemiluminescence detection system (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce).

Equal loading of protein was confirmed by stripping and reprobing membranes with antibody directed against actin (Sigma). Our previous work validated the use of actin as a loading control in experiments that included NSAID-treated IEC-6 monolayers (Freeman, et al., 2007). Densitometry was performed using imaging software (AlphaEaseFC, AlphaInnotech, San Leandro, CA). The expression of calpains 8, 2, 1 and calpastatin was normalized to actin for comparisons between treatment groups. Summary data shown is based on Western blots performed using cell lysates from at least three independent experiments employing all treatment groups.

Cell Migration Assay

The effect of calpain inhibition on IEC-6 cell migration was assessed using an established wounding assay (Freeman, et al., 2007). Briefly, cells were seeded on Matrigel basement

membrane until a confluent monolayer was established. A razor blade was used to sharply remove approximately one-third of the monolayer and create a border line (scratch line) after 24 h (calpain inhibitors) or 72 h (NSAIDs) of exposure to media containing either vehicle or a treatment. Micrographs (Nikon ACT-1) were captured after creation of this defect, and 4 h later. Cell Migration was measured in these captured images using analysis software to document restitution (Nova Prime, Bioquant, Nashville TN). A standardized rectangular region of interest (31,200 μm^2) was created and positioned at the scratch line; the percentage of this region occupied by migrating cells was measured over time. Two to three fields per replicate were examined and measured on three different monolayers per treatment.

The treatments used included the NSAIDs described previously and the calpain inhibitors ALLN (*N*-acetyl-Leu-Leu-Nle-CHO) and ALLM (*N*-acetyl-Leu-Leu-methioninal), alone and in combination at the concentrations indicated in the figure legends. The extent of migration post-wounding for various treatments was normalized to the appropriate drug-free control for presentation in order to facilitate comparisons. The vehicle control for the calpain inhibitors was 0.1% ethanol in experiments where these drugs were used alone, and 0.01% ethanol in experiments where the calpain inhibitors were used in combination with NSAIDs.

Statistical Analysis

Microarray experiments were performed in quadruplicate and statistical analyses were performed as described in the previous sections. The data resulting from qRT-PCR (fold

change), immunoblot analysis and migration assays are expressed as mean ± SE. Significant differences between treatment groups were identified by one-way ANOVA, and multiple comparisons were made using the LSD procedure (Statistix, Analytical Software, Tallahassee, FL). Differences were considered to be significant when $p < 0.05$. The numbers of replicates per treatment group and independent experiments associated with specific studies are provided in the figures or the accompanying legends. Data in graphs are presented as mean ± SEM.

Results

High-Density cDNA array analysis

The pair-wise Pearson correlations within each treatment (control, indomethacin, NS-398 and SC-560) revealed that all samples within each treatment had a high correlation coefficient, indicating that all samples proved fitful for further analysis. Cluster analysis of samples by using all present genes confirmed the conclusion based on Pearson correlations.

Ingenuity Pathway Analysis:

Genes identified as significantly influenced by NSAIDs were mapped to the functional networks available in the IPA database and ranked by score. The top network affected by indomethacin had a score of 17 and contained 11 focus genes that exhibited ±2.0-fold change (Supplemental Figure S1). The top three functions of this network were: cellular movement; cell morphology; cell-to-cell signaling and interaction. The top network influenced by NS-398 had a score of 18 and contained 12 focus genes that exhibited

±2.0-fold change (Supplemental Figure S2). The top three functions of this network were: lipid metabolism, DNA replication, recombination and repair; developmental disorder. The top network impacted by SC-560 had a score of 3 and contained 1 focus gene that exhibited ±2.0-fold change. The top three functions of this network were: cell-to-cell signaling and interaction; tissue development; cell morphology. The genes present in the top networks affected by indomethacin, NS-398 and SC-560 and the associated fold-changes are listed in Table 2.

There were a few genes common to the top networks influenced by the two NSAIDs known to inhibit intestinal cell migration. For example, microarray analysis showed ≥ -2.5 fold downregulation of transgelin and pleiotrophin and ≥ +2.5 upregulation of fatty acid binding protein 4 by both indomethacin and NS-398. It was also notable that the NSAIDs with inhibitory effects on cell migration altered the expression of genes involved in calpain-dependent signaling. NS-398 caused -3.2 fold change in the expression of calpain 8 and less dramatic downregulation of other calpains in the top network, calpain 2 and the calpain small subunit 1. Calpain 1 was not part of the NSAID affected networks identified by IPA; however, the calpain 1 gene was present and apparently down-regulated -1.3 fold by NS-398. Indomethacin was associated with a +1.4 fold change in the expression of calpastatin, the endogenous inhibitor of the calpain proteases.

Additional experiments were conducted to define further the effects of NS-398, indomethacin and SC-560 on the expression of calpain 8, calpain 2, calpain 1 and calpastatin. The decision to pursue this line of inquiry was influenced by our functional

data. Inhibition of caplains in migrating cells has been shown to produce distortions to the migrating front with retention of cellular protrusions at the rear of migrating cell (Franco, et al., 2004;Lokuta, et al., 2003). Interestingly, NSAID treatment induced this suite of morphological changes in migrating IEC-6 cells (Figure 1).

qRT-PCR:

The expression of calpain 8, calpain 2, calpain 1 and calpastatin mRNA was analyzed using qRT-PCR in an effort to confirm the microarray findings. Consistent with the microrarray data which indicated that treatment with NS-398 induced a -3.2 fold down-regulation of calpain 8 gene expression and a -1.2 fold downregulation of calpain 2 gene expression (Table 2), qRT-PCR showed that NS-398 significantly decreased the expression of calpain 8 (Figure 2a) and calpain 2 (Figure 2b) mRNA. Indomethacin and SC-560 had no effect on their expression.

The data obtained from qRT-PCR assay of calpain 1 mRNA expression exhibited more variability than those obtained for either calpain 8 or calpain 2. As a result, two series of experiments using different primer sets were performed (Table 1). Neither demonstrated a significant effect on NSAID treatment on calpain 1 expression; the results obtained using primer set 2 are featured in Figure 2C. None of the NSAIDs tested affected significantly the expression of calpastatin mRNA ($p > 0.05$, data not shown).

Western Blots:

In order to further characterize the influence of NSAIDs on the expression of the calpain family of proteins, we examined the relative expression of calpain 8, calpain 2, calpain 1, and calpastatin proteins in IEC-6 cells exposed to indomethacin, NS-398 and SC-560. NS-398 and indomethacin inhibited the protein expression of calpains 8 and 2 in IEC-6 cells when used at concentrations of either 100 μM (Figure 3) or 10 μM (supplemental Figure S3). Indomethacin inhibited expression of calpain 1 when used at 100 μM but not 10 μM, whereas NS-398 inhibited expression of calpain 1 when used at either concentration. There was no change in the protein expression of calpastatin after treatment with any NSAID at the highest concentration tested (Supplemental Figure S4).

The Effect of Calpain Inhibition on Cell Migration:

Concentration-response relationships were established for NS-398, indomethacin and two calpain inhibitors, ALLM (also known as calpain inhibitor II) and ALLN (also known as calpain inhibitor I). Concentration-dependent inhibition of cell migration was observed in IEC-6 monolayers exposed to either ALLN or ALLM (Figure 4). Moreover, migrating cells in the monolayers exposed to these calpain inhibitors appeared to have retained cell membrane protrusions at the rear of the migrating front suggestive of inhibition of adhesion complex turnover (Figure 4). As expected from our previous work (Freeman, et al., 2007), 72 h of exposure to either indomethacin or NS-398 also inhibited IEC-6 cell migration in a concentration-dependent manner (Figure 5).

Interestingly, the inhibition of intestinal cell migration was greater when cells were exposed to the combination of Indo or NS-398 and a calpain inhibitor than when cells

were exposed only to a calpain inhibitor or only to one of these two NSAIDs. For example, combined treatment with calpain inhibitors and NS-398 or Indo at the highest concentrations used resulted in complete inhibition of cell migration (data not shown), as expected from the dramatic inhibition shown in Figures 4 and 5, respectively, for treatment with either 100 μM calpain inhibitors or 100 μM NSAIDs. Moreover, 10 μM NS-398 inhibited migration by 25% and 10 μM ALLN inhibited migration by 27%, whereas the combination of 10 μM NS-398 and 10 μM ALLN inhibited migration by 52%. In contrast, the effects on IEC-6 migration of SC-560 in combination with either ALLM or ALLN were not significantly different than those of the calpain inhibitors alone.

Comparison of IEC-6 to IEC-cdx2 cells

IEC-6-Cdx2 cells exhibit the ultrastructural characteristics of differentiated villus enterocytes, and an increased rate of migration in response to wounding compared to the parent IEC-6 cell line (Rao, et al., 2002). We have shown previously that NSAIDs affect cell migration similarly in IEC-6 and IEC-Cdx2 cells (Freeman, et al., 2007). We were interested in ascertaining if NSAIDs also influence the expression of calpain proteases in these more-differentiated intestinal epithelial cells. To this end, immunoblot analysis was used to assess the expression of calpain protein in IEC-Cdx2 monolayers exposed to NSAIDs for 72 h. As shown in Figure 6, indomethacin and NS-398, but not SC-560, decreased the expression of calpains 8, 2 and 1 by IEC-Cdx2 cells.

Discussion

NSAIDs are prescribed commonly to relieve pain and control inflammation, despite their significant adverse GI side effects (Lichtenberger, 2001). Efforts to develop less toxic NSAIDs led to the introduction of COX-2 inhibitors with decreased GI complications; however, epidemiological data show that the GI benefits of these drugs may be offset by the increased cardiovascular toxicity associated with selective inhibition of COX-2 (Motsko, et al., 2006). The development of safe, effective NSAIDs will remain elusive until the multi-factorial basis for the drugs' GI toxicity is better understood. To this end, we used a multi-step functional genomic strategy to identify novel signaling pathways that contribute to NSAID-inhibition of GI epithelial cell migration.

We employed a series of techniques to identify changes in mRNA and protein expression induced in IEC-6 monolayers by three NSAIDs with variable pharmacodynamic profiles and established effects on epithelial migration (Freeman, et al., 2007). First, high density microarray analysis was used to screen for novel effects of NSAIDs on gene expression. Specifically, we sought to identify changes in gene expression associated with exposure to indomethacin and NS-398, two NSAIDs that inhibit epithelial migration *in vitro* and contribute to ulcer persistence *in vivo*, but unassociated with exposure to SC-560, an NSAID without adverse effects on GI epithelial migration. IPA facilitated the interpretation of the microarray findings by enabling data analysis in context of biologically relevant signaling pathways. Microarray analysis was followed by qRT-PCR and immunoblotting to confirm changes in the mRNA and protein expression of selected

genes. Finally, pharmacological antagonism of candidate genes provided additional support for their functional significance.

The IEC-6 cell line was employed for a number of reasons. First, these non-transformed cells have been used extensively to study intestinal epithelial cell migration and there is abundant knowledge about the signal transduction pathways involved in their migration (Freeman, et al., 2007; Guo, et al., 2002;McCormack and Johnson, 2001;Rao, et al., 2002). In addition, the IEC-6 cell line has been employed previously by laboratories investigating wound healing through microarray analysis, and, as a result, genomic alterations have been linked to protein expression and/or to phenotypic change (Hafner, et al., 2005;Liu, et al., 2005). Finally, we have documented previously the effects of NSAIDs on IEC-6 cell migration under experimental conditions identical to those employed here (Freeman, et al., 2007).

The high density microarray and IPA revealed three genes common to the top networks influenced by indomethacin and NS-398: pleiotrophin (≥ -2.5 fold downregulation); transgelin (≥ -2.5 fold downregulation); and fatty acid binding protein 4 (≥ +2.5 fold upregulation). These genes have not been identified in previous analyses of wound-healing associated genes in IEC-6 cells (Hafner, et al., 2005;Liu, et al., 2005). However, there is some functional data implicating these proteins in cell migration, invasion and wound healing (Gunnersen, et al., 2000; Ohlsson, et al., 2005; Martin, et al., 2006). Although pleiotropin, transgelin and fatty acid-binding protein 4 represent potential drug

targets, we did not investigate their roles in NSAID-inhibition of intestinal epithelial migration.

We were more intrigued by the changes induced by NSAIDs in the expression of genes encoding the calpain proteases and their endogenous inhibitor calpastatin. The calpain family of cysteine proteases regulates many cellular processes, including migration (Franco and Huttenlocher, 2005). Calpains are involved in cell migration specifically through directing the disassembly/reassembly of cytoskeletal elements and cell-cell adhesions, thereby permitting cells to flatten, spread, and detach rear adhesions (Franco and Huttenlocher, 2005). Microarray analysis suggested that NS-398 induced a 3-fold down-regulation of calpain 8, and less dramatic down regulation of other calpains and the calpain small subunit 1. Indomethacin had no apparent effect on the expression of calpain mRNAs, but did upregulate the expression of calpastatin by +1.4 fold.

We attempted to validate drug-induced changes in the expression of calpains 8, 2 and 1 as well as calpastatin using qRT-PCR and immunoblotting to assess expression of mRNA and protein. We focused on those signaling molecules, either because of their expression in the gastro-intestinal tract or because of their association with signal transduction in migrating cells (Franco and Huttenlocher, 2005;Hata, et al., 2006). The results of qRT-PCR validated our microarray findings that NS-398 induced downregulation of calpain 8 and calpain 2 mRNA. However, we were unable to demonstrate either downregulation of calpain 1 mRNA by NS-398 or upregulation of calpastatin mRNA by indomethacin using this technique.

Many factors may contribute to inconsistencies between the results obtained by microarray vs. qPCR. In a systematic study of these factors, lower correlations between microarray and qPCR data were found for genes exhibiting low levels of change (<1.4 – fold) than for those showing more significant up- or downregulation (Morey, et al., 2006). This may have contributed to our inability to correlate qRT-PCR data with microarray data for calpain 1 and calpastatin, given that the microarray analysis indicated only –1.3 fold downregulation of calpain 1 by NS-398 and +1.4 fold upregulation of calpastatin by indomethacin. The contribution of other factors cannot be ruled out, although care was taken to minimize problems associated with experimental variables such as RNA quality and primer efficiency.

Western blotting was used subsequently to assess the effects of NSAIDs on the protein expression of calpain and calpastatin, because determination of mRNA levels by either microarray or qPCR analysis may not accurately predict protein expression. Large scale studies that have examined the correlation between mRNA and proteins across gene products and cell line have shown significant discrepancies that do not result from measurement errors, but rather reflect the biology of gene expression (Tew, et al., 1996;Tian, et al., 2004). Protein expression is regulated not only at transcription, but also post-transcription, at translation and post-translation.

We observed NSAID-induced changes in protein expression that were both congruent and incongruent with the measurements of mRNA. Most significantly, we found that

indomethacin and NS-398, the NSAIDs that inhibit epithelial cell migration, decreased the expression of calpain 8, calpain 2 and calpain 1 proteins in IEC-6 cells. In contrast, SC-560, the NSAID without adverse effects on migration, had no effect on the expression of calpain protein by IEC-6 cells. Similar results were obtained using the more differentiated intestinal epithelial cell line IEC-Cdx2. Functional data were also consistent with decreased expression of calpain protein in cells treated with either NS-398 or indomethacin, as both drugs also induced morphologic changes suggestive of retained adhesions at the rear of migrating IEC-6 cells. This phenomenon was documented previously in fibroblasts and neutrophils after inhibition of calpain expression or activity (Franco, et al., 2004;Lokuta, et al., 2003).

We demonstrated that pharmacological inhibition of calpains retarded IEC-6 migration in a fashion similar to that seen following exposure to indomethacin and NS-398. The calpain inhibitors ALLM and ALLN decreased IEC-6 cell migration in a concentration-dependent manner. Moreover, the morphological changes associated with calpain inhibition were similar to those seen with NS-398 and indomethacin. When IEC-6 cells were treated with ALLM or ALLN in combination with either NS-398 or indomethacin, the drug effects on cell migration were additive, as anticipated from the mechanisms of action. NS-398 and indomethacin affect calpain activity by decreasing the expression of calpain proteins. ALLM and ALLN antagonize calpain activity by binding to the active site on the protease domain of expressed protein. The combined effects of NSAIDs and calpain inhibitors reported in this investigation are consistent with but do not prove the

hypothesis that calpain inhibition may contribute to NSAID antagonism of GI epithelial migration.

To our knowledge, this is first report demonstrating decreased calpain expression in intestinal epithelial cells treated with NS-398 and indomethacin. However, at least three previous studies have described decreases in calpain expression, release or activity after treatment with NSAIDs. In studies using chondrocytic cells, NS-398 suppressed TNF-α-stimulated expression and release of calpain, whereas indomethacin suppressed TNF-α-stimulated release of calpains (Fushimi, et al., 2004). In that investigation, the mechanism whereby the NSAIDs stimulated calpain release was presumed to depend on COX inhibition, because PGE2 and EP2 agonists were shown to accelerate calpain release. The mechanisms underlying NSAID suppression of TNF-α-stimulated calpain expression were not addressed experimentally (Fushimi, et al., 2004). In another, unrelated investigation, indomethacin was shown to reduce the activity of spinal cord calpains *in vitro* (Banik, et al., 2000). In that work, the concentrations of indomethacin required to suppress calpain activity were in the millimolar range, and thus higher than the concentrations typically needed to inhibit COX. Interestingly, indomethacin showed similar concentration-dependent inhibition of purifed calpain activity (Banik, et al., 2000). Recent work has demonstrated NSAID-inhibition of COX-2 cleavage through COX-independent mechanisms likely to involve cysteine proteases (Mancini, et al., 2007). Taken together, the results from these three investigations suggest that the mechanisms that underlie NSAID effects on calpains are complex.

Our findings are consistent with the hypothesis that NSAID-effects on calpains can contribute to the drugs' pharmacodynamic and toxicodynamic profiles. However, the experiments we have performed to date are insufficient to implicate a cellular mechanism of action for NSAID inhibition of calpain expression in intestinal epithelial cells. Clearly, this will be an important future focus for our laboratory.

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Footnotes

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Legends for Figures

Figure 1. Photomicrographs of migrating IEC-6 cells exposed to vehicle control or specific NSAIDs (SC-560, 1 μM; Indomethacin, 100 μM; NS-398, 100 μM) for 72 h prior to wounding of the monolayer. Images were captured 4 h post-wounding. Large arrowheads indicate the scratch lines for the wounding assays. Small arrowheads highlight the distortion of cell morphology and retained cellular contacts at the rear of the cells treated with either indomethacin or NS-398.

Figure 2. NSAID effects on calpain gene expression in IEC-6 cells exposed to indomethacin (Indo, 100 μM), NS-398 (100 μM), SC-560 (1 μM) or vehicle control (Con). qRT-PCR results are presented as a fold-change in expression of mRNA for calpain 8 (panel A), calpain 2 (panel B) and calpain 1 (panel c). Results based on n = 4 independent isolations of RNA. Asterisks indicate significant differences from control at $p < 0.05$.

Figure 3. Calpain protein expression in IEC-6 cells exposed to indomethacin (Indo, 100 μM), NS-398 (100 μM), SC-560 (1 μM) or vehicle control (Con). Exemplar immunoblots (right-hand panels) show specific detection of the calpain proteins at $M_r \cong 80$. Summary data (left-hand panels) depict calpain expression normalized to the actin loading control using densitometry for calpain 8 (panel A, n = 4), calpain 2 (panel B, n = 3) and calpain 1 (panel C, n = 4). Asterisks indicate significant differences from control at $p < 0.05$.

Figure 4. Effects of calpain inhibitors on IEC-6 cell migration. A) Photomicrographs of migrating IEC-6 cells exposed to the vehicle control or the calpain inhibitor ALLM for 24 h prior to wounding of the monolayer. Images were captured 4 h post-wounding. Large arrowheads indicate the scratch lines for the wounding assays. Small arrowheads highlight the distortion of cell morphology and retained cellular contacts at the rear of the treated cells. B and C) Migration was measured as the fraction of a defined rectangular area (31,200 μM^2) occupied at 4 h post-wounding by migrating cells in monolayers exposed to various concentrations of ALLM (panel B) and ALLN (panel C). The data are normalized to the migration response observed in time-concurrent vehicle controls. Asterisks (*) indicate significant differences from control as determined by analysis of variance followed by a post hoc test for multiple comparisons ($p < 0.05$).

Figure 5. NSAID effects on IEC-6 migration, in the presence and absence of calpain inhibition. Epithelial migration was measured as described for Figure 4 in IEC-6 monolayers exposed to indomethacin (Indo), NS-398 or SC-560 for 72 h and calpain inhibitors (ALLM, ALLN) for 24 h prior to wounding. The data are normalized to the migration response observed in time-concurrent vehicle controls. Significant differences were determined by analysis of variance followed by a post hoc test for multiple comparisons ($p < 0.05$). A) Indomethacin (Indo) and NS-398 inhibit IEC-6 migration in a concentration-dependent fashion (* indicates significant differences from control). B) Combined treatment of IEC-6 monolayers with an NSAID plus a calpain inhibitor inhibited epithelial migration to a significantly greater extent than treatment with Indo, NS-398 or a calpain inhibitor alone (* indicates significant differences from control,

whereas + indicates significant differences from both control and single drug treatment). C) The effects on IEC-6 migration of combined treatment with SC-560 plus a calpain inhibitor were not significantly different than the effects of the calpain inhibitors alone (* indicates significant differences from control).

Figure 6. Calpain (Capn) protein expression in IEC-Cdx2 cells exposed to indomethacin (Indo, 100 μM), NS-398 (100 μM), SC-560 (1 μM) or vehicle control (Con). Exemplar immunoblots (right-hand panels) show specific detection of the calpain proteins at $M_r \cong 80$. Summary data (left-hand panels) depict Capn expression normalized to the actin loading control using densitometry for Capn 8 (panel A, n = 4), Capn 2 (panel B, n = 3) and Capn 1 (panel C, n = 4). Asterisks indicate significant difference from control at $p < 0.05$.

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Table 1: Primer Sequences

Target	Primer Sequences	Amplicon Size (BP) / Efficiency (%)
18S	F: 5'-gaggttcgaagacgatcaga R: 5'-tcgctccaccaactaagaac	316 100.29
calpain 8	F: 5'-agaagatggcgagttctgga R: 5'-ccagtacgtgcctgggtagt	190 102.00
calpain 2	F: 5'-tgccccagctggaacacg R: 5'-caggtgagggtgtccgg	149 95.25
calpain 1 set 1	F: 5'-aggaggtggatgacgcagac R: 5'-ggacctggtacactgcaaaac	141 97.50
calpain 1 set 2*	F*: 5'-tatcctccaccgagtggttc R*: 5'-tccttggtgggtagcaaatc	129 89.35
calpastatin	F: 5'-tgcaggagtgaccagaagtg R: 5'-gcttgcctaagatggctctg P: 5'-56-FAM-aagccctgcaggctctgtcagattc-TAM	144 102.72

F, Forward; R, Reverse; P, Probe; *primer set responsible for data in Figure 2

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Table 2. Genes in the top networks altered by indomethacin (Indo), NS-398 and SC-560, identified by Ingenuity Pathway Analysis

NSAID	Genes in Network
Indo	***Down-regulated:***
	PTN (pleiotrophin),-4.0
	TAGLN (transgelin), -3.6
	NID (nidogen), -2.2
	CXCL2 (chemokine (C-X-C motif) ligand 2), -2.1
	H19 (H19 fetal liver mRNA), -2.1
	CALD1 (caldesmon 1), -1.4
	FST (follistatin), -1.2
	SDC1 (syndecan 1), -1.1
	PTMA (prothymosin alpha), -1.1
	TNC (tenascin C), -1.1
	SDC4 (syndecan 4), -1.0
	Unchanged:
	DNMT1 (DNA (cytosine-5-)-methyltransferase 1)
	FN1 (fibronectin 1)
	ROCK1 (Rho-associated coiled-coil forming kinase 1)
	INHBA (inhibin, beta A)
	LGALS3BP (galectin-3 binding protein)
	Up-Regulated:
	BMP7 (bone morphogenetic protein 7), +2.4
	CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1), +2.3
	CSPG2 (chondroitin sulfate proteoglycan 2), +2.0
	FABP4 (fatty acid binding protein 4), +6.5
	INHBE (inhibin, beta E), +2.1

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CAST (calpastatin), +1.4

GSN (gelsolin), +1.3

CASP3 (caspase 3), +1.1

IER3 (immediate early response 3), +1.1

MYC (myelocytomatosis viral oncogene), +1.1

RELA (v-rel reticuloendotheliosis viral oncogene homolog A), +1.1

NS-398 ***Down-regulated:***

TAGLN (transgelin), -3.7

STX5A (syntaxin 5),-3.4

CAPN8, (calpain 8), -3.2

GPAM (glycerol-3-phosphate acyltransferase, mitochondrial), -2.8

PTN (pleiotrophin), -2.5

MAP3K12 (MAP kinase kinase kinase 12), -2.3

PIK3CA (phosphoinositide-3-kinase, catalytic, alpha polypeptide), -2,2

IGFPB6 (IGF binding protein 6), -2.1

RIN1 (Ras and Rab interactor 1), -2.1

PIAS2 (protein inhibitor of activated STAT, 2), -1.9

NRC31(nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)), -1.6

SPARC (secreted protein, acidic, cysteine-rich (osteonectin)), -1.5

Scd2 (stearoyl-Coenzyme A desaturase 2), -1.3

CAPN2 (calpain 2), -1.2

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CAPN5 (calpain 5), -1.2

FMR1 (fragile X mental retardation syndrome 1 homolog), -1.2

MTPN (myotrophin), -1.2

PIK3CB (phosphatidylinositol 3-kinase, catalytic, beta polypeptide), -1.2

CAPNS1 (calpain small subunit), -1.1

EDN1 (endothelin 1), -1.1

FASN (fatty acid synthase), -1.1

NRAS (neuroblastoma ras oncogene), -1.1

SREBF1 (sterol regulatory element binding factor 1), -1.1

Unchanged:

CAPN10 (calpain 10)

RAF1 (v-raf-1 murine leukemia viral oncogene homolog 1)

Up-regulated:

FABP4 (fatty acid binding protein 4), +2.6

IL6 (interleukin 6), +2.2

VEGF (vascular endothelial growth factor A), +2.0

MIF (macrophage migration inhibitory factor), +1.5

VCAM1 (vascular cell adhesion molecule 1), +1.3

MYC (myelocytomatosis viral oncogene), +1.2

PPID (peptidylprolyl isomerase D (cyclophilin D)), +1.1

RPS7 (ribosomal protein S7), +1.1

S100A6 (calcyclin), +1.1

SC-560 *Down-regulated:*

ITGB8 (integrin beta 8), -2.9

ITGAV (integrin, alpha V), -1,1

Unchanged:

FN1

Up-regulated:

None

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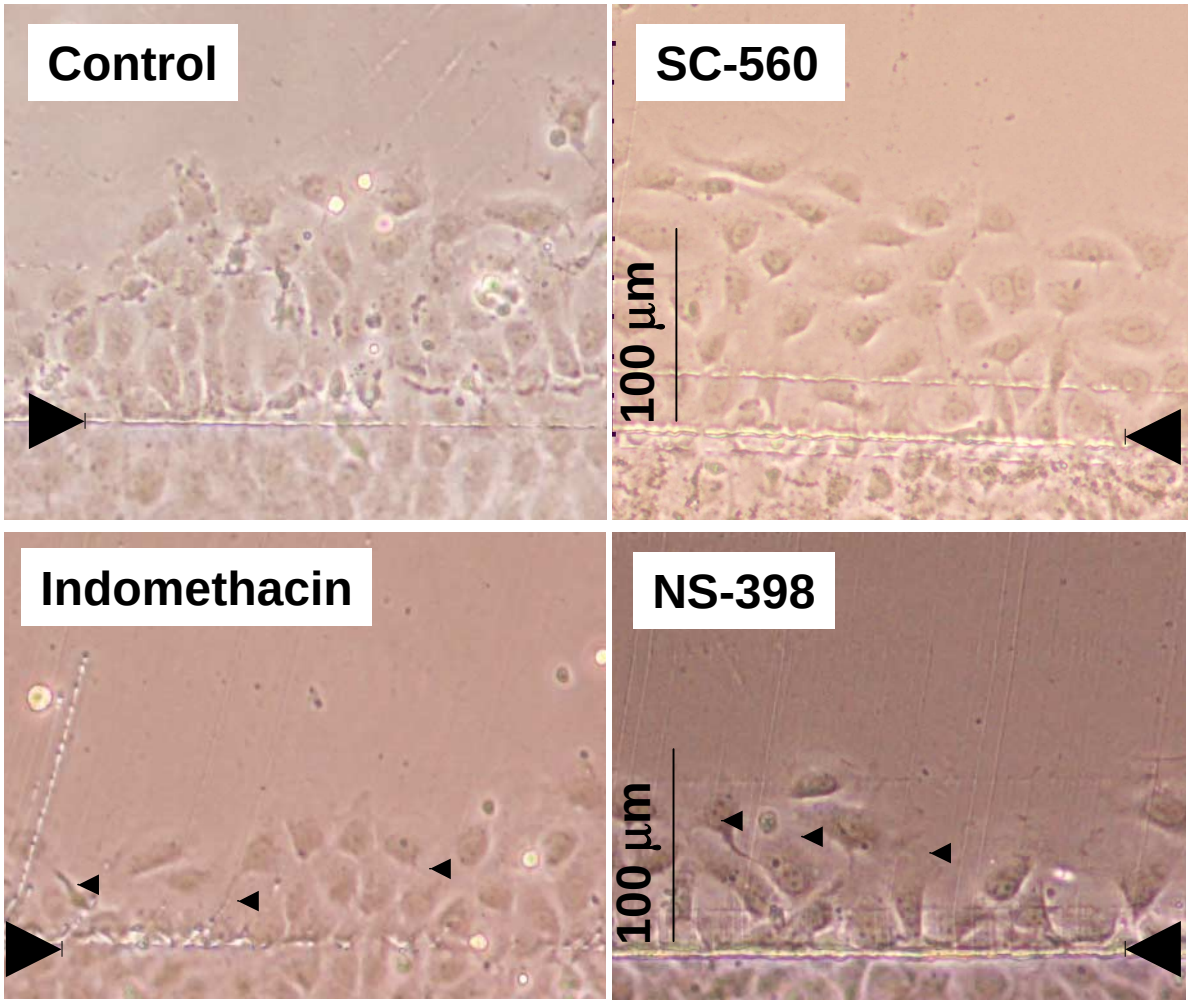

Figure 1

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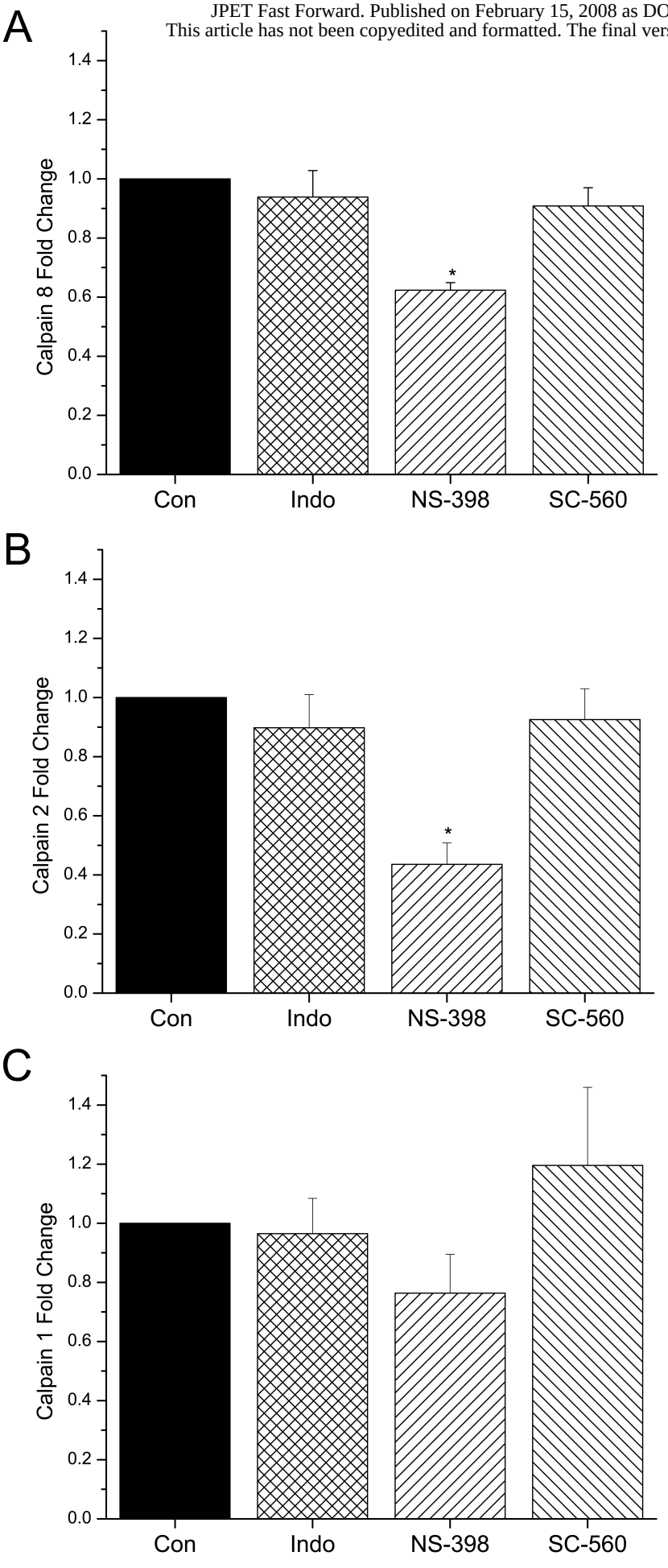
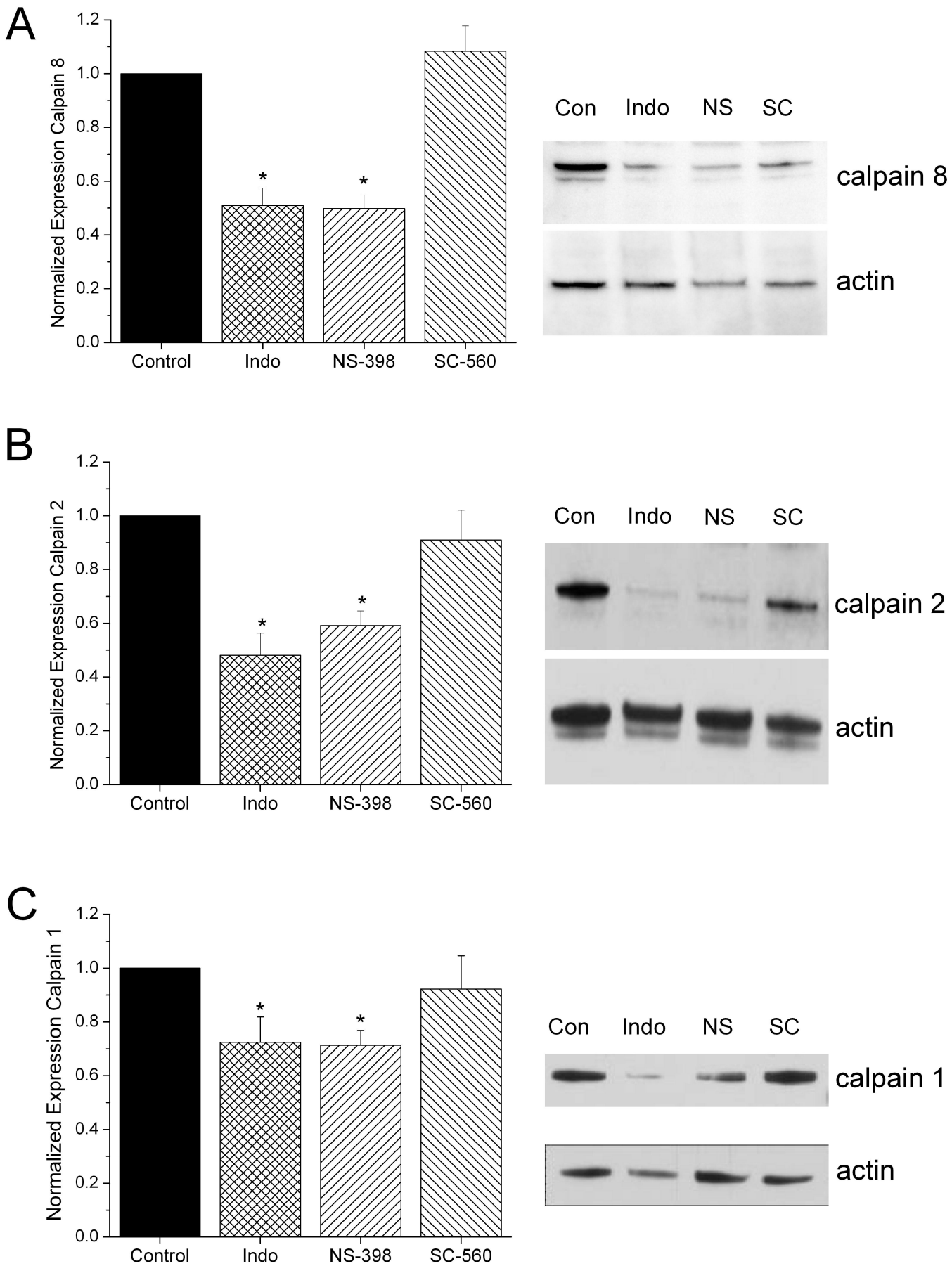
Figure 2

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Indo
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Con
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calpain 8
actin
B
Normalized Expression Calpain 2
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Control
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SC
calpain 2
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Normalized Expression Calpain 1
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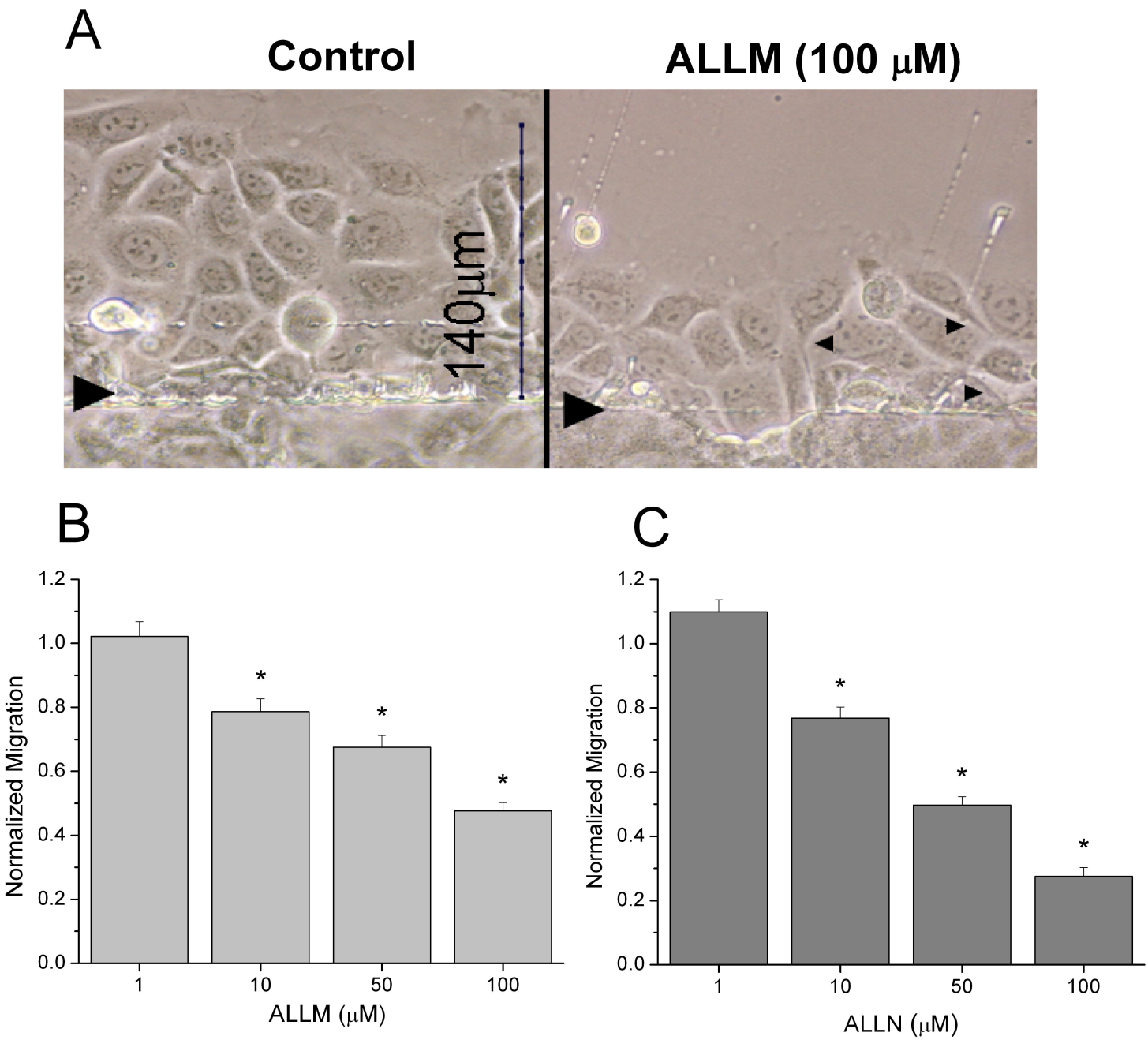
SC
calpain 1
actin

Figure 3


A
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10
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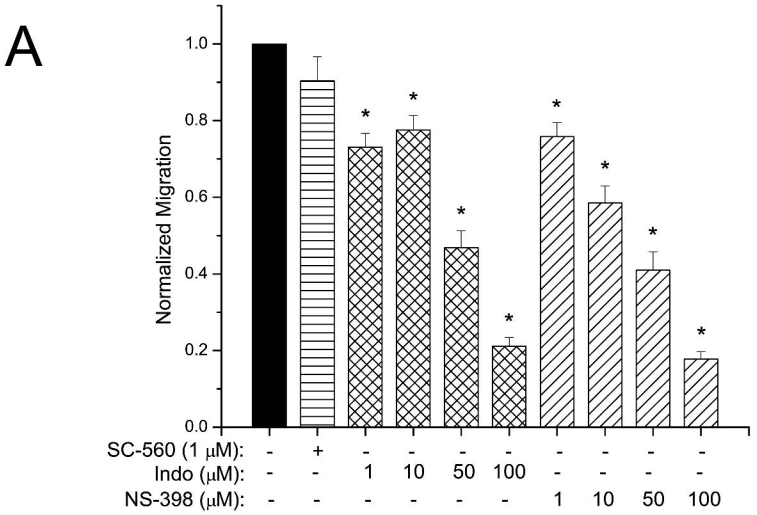
ALLN (μM)

Figure 4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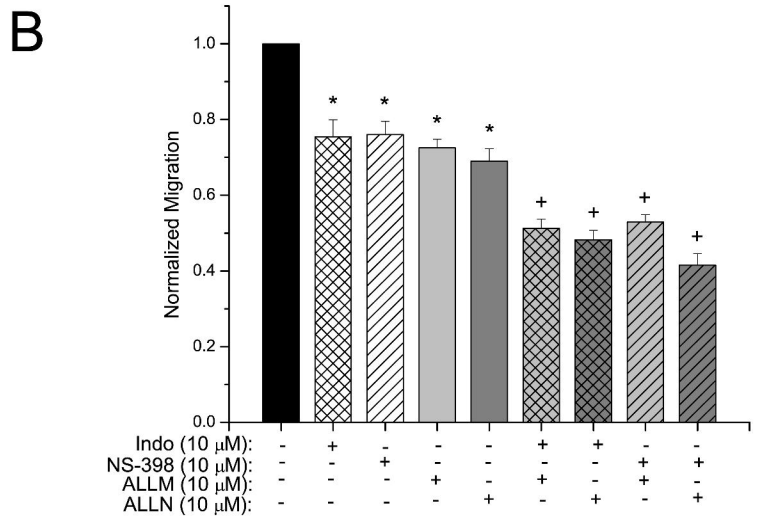
NS-398 (μM): - - - - - - 1 10 50 100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Indo (10 μM): - + - - - + + - -
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ALLM (10 μM): - - - + - + - + -
ALLN (10 μM): - - - - + - + - +

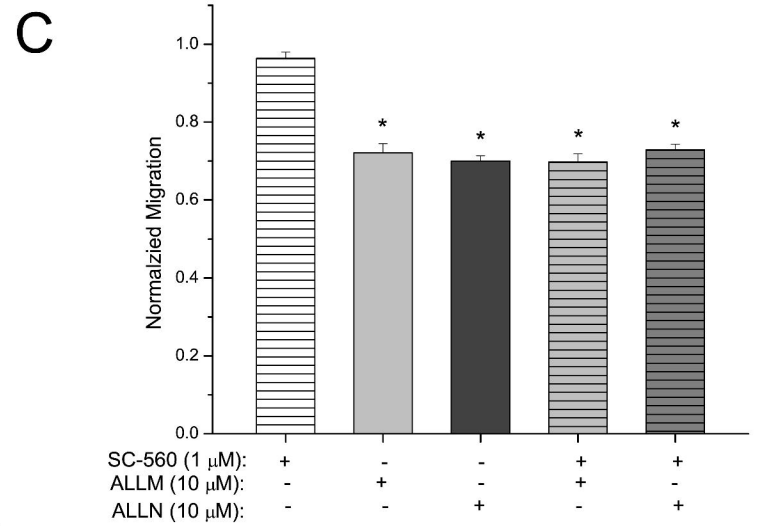
C
Normalzied Migration
1.0
0.8
0.6
0.4
0.2
0.0
SC-560 (1 μM): + - - + +
ALLM (10 μM): - + - + -
ALLN (10 μM): - - + - +

Figure 5

Figure 6