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Selective NSAIDs Induce Thymosin β-4 and Alter Actin Cytoskeletal Organization in Human Colorectal Cancer Cells

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Nonstandard Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; NAG-1, NSAID-activated gene; Indo, Indomethacin; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their anti-inflammatory effects and have been shown to have chemopreventive effects as well. NSAIDs inhibit cyclooxygenase (COX) activity to exert their anti-inflammatory effects, but it is not clear whether their anti-tumorigenic ability is through COX inhibition. Using subtractive hybridization, we previously identified a novel member of the TGF-β superfamily that has anti-tumorigenic activity from indomethacin-treated HCT-116 human colorectal cancer cells. On further investigation of this library, we now report the identification of a new cDNA corresponding to the thymosin β-4 gene. Thymosin β-4 is a small peptide which is known for its actin-sequestering function, and it is associated with the induction of angiogenesis, accelerated wound healing, and metastatic potential of tumor cells. However, only selective NSAIDs induce thymosin β-4 expression in a time- and concentration-dependent manner. For example, indomethacin and SC-560 induce thymosin β-4 expression while sulindac sulfide does not. We show that selective NSAIDs induce actin cytoskeletal reorganization, a precursory step to many dynamic processes regulating growth and motility including tumorigenesis. This is the first report to link thymosin β-4 induction with NSAIDs. These data suggest that NSAIDs alter the expression of a diverse number of genes, and provide new insights into the chemopreventive and biological activity of these drugs.

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are most commonly used to treat various inflammatory diseases. NSAIDs are often exploited for their analgesic effects in alleviating swelling, redness, pain of inflammation, fever, and headache. The potent anti-inflammatory action of NSAIDs is widely known to be its inhibition of the cyclooxygenase (COX) enzymes which are responsible for synthesizing prostaglandins from arachidonic acid, causing inflammation (Hinz and Brune, 2002). Epidemiological studies, animal studies, and *in vitro* studies involving human colorectal cancer cells indicate NSAIDs possess anti-tumorigenic activity in colorectal cancer, and to a lesser extent, breast and esophageal cancer (Thun et al., 1993; Taketo, 1998; Gwyn and Sinicrope, 2002; Thun, 2003). While some data link NSAID chemopreventive activity to COX inhibition (Watson, 1998), other data indicate that such activity may be COX-independent. Early hypotheses centered on COX-2 inhibitory mechanisms. As these mechanisms were investigated, increasing evidence indicated possible prostaglandin-independent pathways, particularly with respect to the induction of apoptosis. For instance, inhibition of COX by NSAIDs may increase the cellular pool of free arachidonic acid (AA) by preventing its use as a substrate for prostaglandin synthesis, resulting in the induction of apoptosis (Chan, 1998; Cao et al., 2000). Furthermore, it has been shown that the R-enantiomer of the NSAID flurbiprofen, which does not inhibit COX, possesses chemopreventive activity in a mouse model of intestinal polyposis and prostate cancer (Wechter et al., 1997; Wechter et al., 2000). Non-COX-expressing colorectal cancer cells

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have been shown to undergo NSAID-induced apoptosis (Baek et al., 2001b). Thus, the pro-apoptotic activity of NSAIDs may act through both COX-dependent and COX-independent pathways.

Thymosin β-4 is a 4.9-kDa acidic polypeptide found to be ubiquitous in vertebrate cells (Low et al., 1981). Thymosin β-4 was discovered to be a major G-actin binding protein (Safer et al., 1991; Safer and Nachmias, 1994), whose function as a simple passive sequestering protein has been demonstrated *in vitro* (Sanders et al., 1992) and *in vivo* (Cassimeris et al., 1992). Thymosin β-4 stimulates tissue remodeling, cell and tissue healing after injury, and cell differentiation by mechanisms that have not been well defined (Grant et al., 1999; Philp et al., 2003). Recently, evidence has been presented implicating thymosin β-4 as a facilitator of tumor metastasis and angiogenesis. Overexpression of thymosin β-4 is associated with an increase in the expression of a known angiogenic factor, vascular endothelial growth factor (Cha et al., 2003). Other studies correlate elevated thymosin β-4 expression with metastasis in colorectal cancer cells (Wang et al., 2003) as well as non-small cell lung cancer (Ji et al., 2003). Thus, the expression of thymosin β-4 may stimulate tumor metastasis by activating cell migration and angiogenesis.

Our laboratory has studied NSAID-induced gene expression in cell culture to identify possible COX-independent mechanisms of action (Baek et al., 2001b). Using suppression subtractive hybridization, we recently reported the identification of a cDNA designated NSAID-activated gene (NAG-1) from an indomethacin-induced library of human colorectal cancer cells devoid of COX

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activity (Baek et al., 2001a; Baek et al., 2001b). Here we report another cDNA from same library, corresponding to the thymosin β-4 gene. We also demonstrate that thymosin β-4 is inducible by other NSAIDs including SC-560, and alters the cytoskeletal organization in human colorectal cancer cells, which may lead to tumorigenesis. We propose that varied expression of thymosin β-4 after treatment with different NSAIDs may help explain the efficacy of some NSAIDs versus others in regards to their chemopreventive effects.

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Materials and Methods

Cell lines and reagents: Cell lines in this study were purchased from American Type Culture Collection (Manassas, VA). HCT-116, human colorectal carcinoma cells, were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured at 37ºC under a humidified atmosphere of 5% CO_2 in air. PGE_2 was purchased from BIOMOL, (Plymouth Meeting, PA). Indomethacin, naproxen, ibuprofen, piroxicam, and diclofenac were purchased from Sigma-Aldrich (St. Louis, MO). Sulindac sulfide, SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole), and aspirin were purchased from Cayman Chemicals (Ann Arbor, MI). DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone) was obtained from Merck (Whitehouse Station, NJ). All NSAIDs were dissolved in dimethyl sulfoxide (DMSO) and stored at -20ºC prior to treatments.

Identification of Thymosin β-4 from an Indomethacin (INDO)-Induced cDNA Library in HCT-116 Cells: Isolation of mRNA from either indomethacin (100 μM) or vehicle treated (0.2% DMSO) cells was performed using a poly(A) spin mRNA isolation kit (New England BioLabs, MA). INDO(+) and INDO(-) cDNA libraries were constructed using the cDNA Subtraction Kit (CLONTECH, Palo Alto, CA) according to the manufacturer's protocol, as previously described (Baek et al., 2001b). A 170-bp fragment was isolated from the library and designated as INDO15. Homology searches were carried out using BLAST programs through e-mail servers at the National Center for Biotechnology Information. The sequence of this fragment matched identically with a sequence in the 3'

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untranslated region of thymosin β-4 mRNA (GenBank M17733). The cDNA corresponding to the full length mRNA sequence for thymosin β-4 was obtained by reverse-transcriptase PCR using the sense strand 5'-TCGTACTCGTGCGCCTCGCTTCGCTTTTCC-3' and the antisense strand 5'-CTGTCGTCCCACCCCACTTCTTCCACCCCAC-3' primers.

RNA Isolation and Northern Blot Analysis: When 70-80% confluence was obtained on 100 mm plates, the cells were treated at indicated concentrations and times with different compounds or DMSO in the absence of serum. Total RNAs were isolated using TRIzol reagents (Life Technologies, Rockville, MD) according to the manufacturer's protocol. For northern blot analysis, 10 μg of total RNA was denatured by incubating at 55ºC for 15 min, and then electrophoresed in a 1.4% agarose gel containing 2.2 M formaldehyde. RNA was subsequently transferred to a Hybond-N membrane (Amersham, Piscataway, NJ). After fixing the membrane by UV, the blots were pre-hybridized in hybridization solution (Rapid-hyb buffer, Amersham) for at least 1 h at 65ºC followed by hybridization with cDNA labeled with [α-^{32}P]dCTP by random primer extension (DECAprimeII kit, Ambion, Austin, TX). The probe used was either INDO15 or full-length thymosin β-4 fragment obtained by RT-PCR. After 1 h incubation at 65ºC, the blots were washed once with 1X standard saline sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature and 4X with 0.1X SSC/0.1% SDS at 65ºC. Equivalent loading of RNA samples was confirmed by 18S rRNA and mRNA abundance was estimated by intensities of

the hybridization bands of autoradiographs using Scion Image (Scion Image, Frederick, MD).

Analysis of Thymosin β-4 Peptide Content by High-Pressure Liquid Chromatography: HCT-116 cells were grown to 80% confluence in 150 mm plates and treated with different compounds or DMSO for 24 hrs in the absence of serum. Cells were washed 1X with cold PBS, scraped, and collected in 20 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (1 µg/ml leupeptin and pepstatin, 0.5 mM PMSF). Cells were sonicated and lysates were then centrifuged at 110,000 *g* for 60 min at 4ºC. 100 µl aliquots of the resulting supernatants were subjected to reverse-phase HPLC to determine cellular thymosin β-4 content. Analysis was performed using a C18 Ultrasphere ODS column (5µm; 4.6mm x 250mm; Beckman Coulter, Inc., Fullerton, CA). Solvent A was 0.1% trifluoracetic acid in water; solvent B was 0.08% trifluoroacetic acid in acetonitrile. The resolving gradient was 10-35% B in 12 min at 2 ml/min. Proteins were measured at 220 nm. Thymosin β-4 standard was generously provided by Dr. Ewald Hannappel (University Erlangen-Nuremberg, Erlangen, Germany).

Immunofluorescence: HCT-116 cells were seeded onto poly-lysine-coated glass coverslips in 35-mm sterile plastic dishes with 2 ml of media and 10% serum. After sufficient attachment, cells were treated with various NSAIDs for 24 hrs in serum-free media. After treatment, media was removed and cells washed twice with PBS. Cells were fixed with 3.5% formaldehyde, then rinsed twice with PBS, and permeabilized by treatment with 0.1% Triton X-100 for 2 min. For

actin-staining, cells were incubated with 1.5 μM TRITC-labeled phalloidin for 45 min and then washed twice with PBS. Fluorescent images were visualized using a Zeiss confocal microscope at 30X magnification.

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Results

Identification of Thymosin β-4 as an Indomethacin Induced Gene in HCT-116 Cells: To identify and isolate inducible genes by NSAIDs, we performed suppression subtractive hybridization utilizing the human colorectal adenocarcinoma cell line HCT-116, which does not express either COX-1 or COX-2 (Baek et al., 2002b). We have had success using this method to pinpoint altered levels of gene expression (Baek et al., 2001b). In this report, we have further analyzed this library and found several additional genes induced by indomethacin. As shown in Table 1, several genes were isolated which are induced by indomethacin. Among those, INDO15 was identified as thymosin β-4 and was of particular interest because it induces angiogenesis, which acts against the expected anti-tumorigenic effect of NSAIDs. Subsequently, the full-length mRNA of thymosin β-4 was isolated using RT-PCR as described in Methods. As shown in Fig. 1, INDO15 (black bar) represents a clone from the subtractive library. The open reading frame encoding the functional 43 amino acid peptide is shown in the grey bar.

Indomethacin Induces Thymosin β-4 mRNA in HCT-116 Cells in a Dose-dependent and Time-dependent Manner: To confirm our subtractive hybridization data, we measured thymosin β-4 expression after indomethacin treatment using Northern analysis. Both dose-response and time-course analyses were conducted. The thymosin β-4 mRNA expression was dependent on indomethacin concentration, with a significant increase in expression at 10 μM (Fig 2A), the highest increase in expression at 50 μM, followed by a decrease in

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expression at 100 μM. Thymosin β-4 expression increased with duration of indomethacin treatment (100 μM) with a 2-fold increase in expression at 1 hr. The highest mRNA expression was observed after 48 h of treatment (Fig. 2B). Thus, indomethacin induced thymosin β-4 mRNA in a concentration- and time-dependent manner.

Other NSAIDs Affect the Induction of Thymosin β-4 in HCT-116 Cells: After verifying thymosin β-4 induction via indomethacin treatment, HCT-116 cells were treated similarly with other NSAIDs to investigate thymosin β-4 induction. The other NSAIDs used were DFU(100 μM), sulindac sulfide (30 μM), SC-560 (25 μM), and aspirin (1000 μM), all over a 24 hr period (Fig. 3). Indomethacin and SC-560 induced thymosin β-4 mRNA expression while sulindac sulfide and aspirin did not affect thymosin β-4 levels. DFU (100 μM) treatment slightly downregulated thymosin β-4 expression (data not shown). SC-560 induced thymosin β-4 expression on the order of 3-4 fold and is significantly greater expression compared to indomethacin. We also examined NAG-1, a known anti-tumorigenic protein, induced by some NSAIDs (Baek et al., 2001b). As shown in Fig. 3, NAG-1 was induced by indomethacin, sulindac sulfide, and SC-560, which is consistent with previous results (Baek et al., 2002b). Interestingly, indomethacin and SC-560 induced both thymosin β-4 and NAG-1, whereas sulindac sulfide induced only NAG-1. Other NSAIDs (ibuprofen, diclofenac, piroxicam, and naproxen) were also examined for thymosin β-4 induction. It was found that ibuprofen significantly induced thymosin β-4 expression (2 fold), but not other NSAIDs (data not shown).

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PGE_2 Effect on Thymosin β-4 Expression*:* The involvement of prostaglandins (PGs) in the development of human cancer has been known for long time. PGE_2 is the major AA metabolite of the COX pathway. We treated HCT-116 cells with PGE_2 and examine thymosin β-4 expression. As shown in Fig. 4, Northern analysis revealed that treatment with a broad range of PGE_2 concentrations did not alter thymosin β-4 expression, providing evidence that thymosin β-4 expression is prostaglandin independent. Next, different concentrations of PGE_2 were added to INDO treated-HCT-116 cells to examine if thymosin β-4 expression is altered in the presence of PGE_2. As shown in Fig. 4, thymosin β-4 is still induced by indomethacin, suggesting thymosin β-4 induction by NSAIDs is prostaglandin independent. We have previously shown that NAG-1 is not induced at this concentration of PGE_2 (Baek et al., 2002b). Taken together with previous data, PGE_2 did not alter thymosin β-4 or NAG-1 expression in HCT-116 cells.

Indomethacin and SC-560 Induces Thymosin β-4 Peptide in HCT-116 Cells: Upon verifying the upregulation of thymosin β-4 mRNA by selective NSAIDs, it was now of interest to determine if these NSAIDs had similar effects on thymosin β-4 protein levels. Due to the lack of a suitable antibody for reliable Western analysis, HPLC analysis was used to detect the presence of thymosin β-4 peptide. HCT-116 cells were treated with indomethacin (100 μM), SC-560 (25 μM), and vehicle (DMSO) for 24 hrs. The cells were lysed and ultracentrifuged. The supernatant was isolated and subjected to HPLC analysis. Thymosin β-4 standard was used to determine the retention time of approximately 8.1 minutes.

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Indomethacin-treated cells showed approximately 1.5 fold induction of thymosin β-4 peptide over vehicle levels (Fig. 5), while SC-560 treatment showed an induction of approximately 2.2 fold over vehicle peptide levels. These data are consistent with the levels of thymosin β-4 induction shown in our Northern analysis (Fig. 3).

NSAID Treatment Alters Actin Cytoskeleton Organization in HCT-116 Cells: Since thymosin β-4 is known to participate in the organization of the actin skeleton (Otto et al., 2002), we investigated the effects of NSAID treatment on the actin skeleton. HCT-116 cells were treated with two different NSAIDs, DFU and SC-560, the former of which slightly down-regulates thymosin β-4 (data not shown), and the latter of which significantly up-regulates both thymosin β-4 mRNA levels and peptide levels. Cells were stained for actin to visualize filaments and detect possible differences that may correlate with the observed changes in thymosin β-4 levels. SC-560 treated cells showed almost completely diminished actin staining in the cytoplasma of the cells, while there was greater intensity of staining in the plasma membrane region of the cells compared to vehicle. DFU appeared to increase actin staining as predicted since DFU appears to down regulate thymosin β-4 expression. The diminished staining in the cytoplasm of the cells with SC-560 may be attributed to higher levels of thymosin β-4 (Fig. 6).

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Discussion

Although NSAIDs are inhibitors of COX, one additional mechanism by which these drugs elicit their pharmacological and toxicological responses may be by altering gene expression. Our laboratory has used subtractive hybridization and micro-array techniques to identify genes altered by COX inhibitors (Baek et al., 2001b; Bottone et al., 2004). Since our major interest was related to the colon cancer prevention activity of NSAIDs, we used human colorectal cells in culture and indomethacin as a model drug to initially identify target genes. Indomethacin altered the expression of a large number of genes, but we chose to initially focus on a member of the TGF-β superfamily, NAG-1, since this protein indicated possible anti-tumorigenic activity. As a result of our studies, the anti-tumorigenic activity of this protein and its regulation by COX inhibitors and other anti-cancer chemicals has been extensively studied by this laboratory (Baek et al., 2001a; Baek et al., 2001b; Baek et al., 2002a; Baek et al., 2002b; Bottone et al., 2002; Baek et al., 2003; Wilson et al., 2003; Baek et al., 2004). In this report, we have further characterized the genes altered by indomethacin in human colorectal cells and identified thymosin β-4 as a new target for indomethacin.

We present evidence that only selective NSAIDs induce the thymosin β-4 and in contrast to NAG-1, thymosin β-4 appears to be a pro-tumorigenic gene. Thymosin β-4 is a 43-amino acid peptide mainly known for its actin monomer-sequestering function. Thymosin β-4 has recently been associated with angiogenesis (Malinda et al., 1997), accelerated wound healing (Frohm et al., 1996; Malinda et al., 1999), and the metastatic potential of tumor cells

(Yamamoto et al., 1993; Clark et al., 2000; Huff et al., 2001; Otto et al., 2002), although little is known about the mechanisms by which thymosin β-4 promotes metastasis. The induction of thymosin β-4 mRNA and peptide by selective NSAIDs may result in altered actin cytoskeleton organization. We utilized TRITC-phalloidin staining of filamentous actin in HCT-116 cells after treatment with various NSAIDs. In DFU-treated HCT-116 cells, a slight downregulation of thymosin β-4 (data not shown) was observed and the actin staining was brighter than vehicle-treated cells, suggesting a slight increase in actin concentration due to lower expression of thymosin β-4. However, in SC-560 treated cells, in which strong induction of thymosin β-4 is observed, very significant changes in actin expression and localization were detected. There was very little staining throughout the cytoplasma of the cells. Areas near the plasma membrane showed very intense staining. The lack of staining in the body is consistent with the notion that increased levels of thymosin β-4 would sequester a greater amount of actin in its monomeric form. These data support earlier findings of thymosin β-4 expression and F-actin organization (Kobayashi et al., 2002). The intense staining near the plasma membrane is not well understood, but suggests that thymosin β-4 plays a role in cell surface dynamics through actin cytoskeleton reorganization. Understanding this role may be central in understanding how thymosin β-4 promotes metastasis.

In our study of thymosin β-4 induction, several NSAIDs of varying structures including indomethacin, sulindac sulfide, aspirin, and SC-560 were examined for induction of thymosin β-4. Indomethacin and SC-560 showed very

strong induction of thymosin β-4, while sulindac sulfide and aspirin showed very little to no induction. In contrast, indomethacin, sulindac sulfide, SC-560 and aspirin stimulated the expression of NAG-1. Thus, the pattern of gene expression by NSAIDs is not the same for all NSAIDs and appears to depend on the NSAID under study. This conclusion may help explain the differences in chemopreventive effectiveness of the different NSAIDs. For example, sulindac sulfide and aspirin are well recognized for their chemopreventive activity with colorectal tumors in several *in vivo* animal models, and are very potent inducers of NAG-1. However, they do not increase the expression of thymosin β-4. Thus, the gene expression profile for sulindac sulfide and aspirin favors anti-tumorigenic activity. In contrast, SC-560, which also has some controversial chemopreventive properties (Cheuk et al., 2002), increases the expression of both NAG-1 and thymosin β-4. Its effectiveness as a preventive drug could be compromised by the expression of thymosin β-4. Thus, differences in gene expression by NSAIDs may influence the different biological activity and could also contribute to differences in the toxic side effects of the different NSAIDs.

NSAIDs are primarily used for their anti-inflammatory activity and recent data show that thymosin β-4 also possesses anti-inflammatory effects (Young et al., 1999; Sosne et al., 2002). The induction of thymosin β-4 by selective NSAIDs suggests a possible additional mechanism for NSAID anti-inflammatory activity.

In summary, we present the first report linking thymosin β-4 expression with NSAID treatment. Selective NSAIDs induce thymosin β-4, an actin-sequestering protein that is implicated in angiogenesis and tumor metastasis and

its expression may compromise the chemopreventive activity of NSAIDs mediated by NAG-1 and other genes. We propose that NSAIDs have dual targets in cells. First is the well established inhibition of prostaglandin formation by targeting cyclooxygenases, while the second target is alteration in gene expression (Baek et al., 2002b). Although we have focused on changes in gene expression that occur independent of COX inhibition, we cannot exclude the possibility that changes in gene expression can also be dependent on the formation of prostaglandins and thereby altered by inhibition of COX activity. By advancing the knowledge of NSAID mechanisms, it is our hope that NSAIDs can be more effectively used as therapeutics.

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References

Baek SJ, Horowitz JM and Eling TE (2001a) Molecular cloning and characterization of human nonsteroidal anti-inflammatory drug-activated gene promoter. Basal transcription is mediated by Sp1 and Sp3. *J Biol Chem* **276**:33384-33392.

Baek SJ, Kim JS, Nixon JB, DiAugustine RP and Eling TE (2004) Expression of NAG-1, a transforming growth factor-beta superfamily member, by troglitazone requires the early growth response gene EGR-1. *J Biol Chem* **279**:6883-6892.

Baek SJ, Kim KS, Nixon JB, Wilson LC and Eling TE (2001b) Cyclooxygenase inhibitors regulate the expression of a TGF-beta superfamily member that has proapoptotic and antitumorigenic activities. *Mol Pharmacol* **59**:901-908.

Baek SJ, Wilson LC and Eling TE (2002a) Resveratrol enhances the expression of non-steroidal anti-inflammatory drug-activated gene (NAG-1) by increasing the expression of p53. *Carcinogenesis* **23**:425-434.

Baek SJ, Wilson LC, Hsi LC and Eling TE (2003) Troglitazone, a peroxisome proliferator-activated receptor gamma (PPAR gamma) ligand, selectively induces the early growth response-1 gene independently of PPAR gamma. A novel mechanism for its anti-tumorigenic activity. *J Biol Chem* **278**:5845-5853.

Baek SJ, Wilson LC, Lee CH and Eling TE (2002b) Dual function of nonsteroidal anti-inflammatory drugs (NSAIDs): inhibition of cyclooxygenase and

induction of NSAID-activated gene. *J Pharmacol Exp Ther* **301**:1126-1131.

Bottone FG, Jr., Baek SJ, Nixon JB and Eling TE (2002) Diallyl disulfide (DADS) induces the antitumorigenic NSAID-activated gene (NAG-1) by a p53-dependent mechanism in human colorectal HCT 116 cells. *J Nutr* **132**:773-778.

Bottone FG, Jr., Martinez JM, Alston-Mills B and Eling TE (2004) Gene modulation by Cox-1 and Cox-2 specific inhibitors in human colorectal carcinoma cancer cells. *Carcinogenesis* **25**:349-357.

Cao Y, Pearman AT, Zimmerman GA, McIntyre TM and Prescott SM (2000) Intracellular unesterified arachidonic acid signals apoptosis. *Proc Natl Acad Sci U S A* **97**:11280-11285.

Cassimeris L, Safer D, Nachmias VT and Zigmond SH (1992) Thymosin beta 4 sequesters the majority of G-actin in resting human polymorphonuclear leukocytes. *J Cell Biol* **119**:1261-1270.

Cha HJ, Jeong MJ and Kleinman HK (2003) Role of thymosin beta4 in tumor metastasis and angiogenesis. *J Natl Cancer Inst* **95**:1674-1680.

Chan TA, Morin, P.L., Vogelstein, B., Kinzler, K.W. (1998) Mechanism underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proc Natl Acad Sci U S A* **95**:681-686.

Cheuk BLY, Chew SBC, Fiscus RR and Wong PYD (2002) Cyclooxygenase-2 Regulates Apoptosis in Rat Epididymis Through Prostaglandin D2. *Biol Reprod* **66**:374-380.

Clark EA, Golub TR, Lander ES and Hynes RO (2000) Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **406**:532-535.

Frohm M, Gunne H, Bergman AC, Agerberth B, Bergman T, Boman A, Liden S, Jornvall H and Boman HG (1996) Biochemical and antibacterial analysis of human wound and blister fluid. *Eur J Biochem* **237**:86-92.

Grant DS, Rose W, Yaen C, Goldstein A, Martinez J and Kleinman H (1999) Thymosin beta4 enhances endothelial cell differentiation and angiogenesis. *Angiogenesis* **3**:125-135.

Gwyn K and Sinicrope FA (2002) Chemoprevention of colorectal cancer. *Am J Gastroenterol* **97**:13-21.

Hinz B and Brune K (2002) Cyclooxygenase-2--10 years later. *J Pharmacol Exp Ther* **300**:367-375.

Huff T, Muller CS, Otto AM, Netzker R and Hannappel E (2001) beta-Thymosins, small acidic peptides with multiple functions. *Int J Biochem Cell Biol* **33**:205-220.

Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, Thomas M, Berdel WE, Serve H and Muller-Tidow C (2003) MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene* **22**:8031-8041.

Kobayashi T, Okada F, Fujii N, Tomita N, Ito S, Tazawa H, Aoyama T, Choi SK, Shibata T, Fujita H and Hosokawa M (2002) Thymosin-beta4 regulates

motility and metastasis of malignant mouse fibrosarcoma cells. *Am J Pathol* **160**:869-882.

Low TL, Hu SK and Goldstein AL (1981) Complete amino acid sequence of bovine thymosin beta 4: a thymic hormone that induces terminal deoxynucleotidyl transferase activity in thymocyte populations. *Proc Natl Acad Sci U S A* **78**:1162-1166.

Malinda KM, Goldstein AL and Kleinman HK (1997) Thymosin beta 4 stimulates directional migration of human umbilical vein endothelial cells. *Faseb J* **11**:474-481.

Malinda KM, Sidhu GS, Mani H, Banaudha K, Maheshwari RK, Goldstein AL and Kleinman HK (1999) Thymosin beta4 accelerates wound healing. *J Invest Dermatol* **113**:364-368.

Otto AM, Muller CS, Huff T and Hannappel E (2002) Chemotherapeutic drugs change actin skeleton organization and the expression of beta-thymosins in human breast cancer cells. *J Cancer Res Clin Oncol* **128**:247-256.

Philp D, Huff T, Gho YS, Hannappel E and Kleinman HK (2003) The actin binding site on thymosin beta4 promotes angiogenesis. *Faseb J* **17**:2103-2105.

Safer D, Elzinga M and Nachmias VT (1991) Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. *J Biol Chem* **266**:4029-4032.

Safer D and Nachmias VT (1994) Beta thymosins as actin binding peptides. *Bioessays* **16**:473-479.

Sanders MC, Goldstein AL and Wang YL (1992) Thymosin beta 4 (Fx peptide) is a potent regulator of actin polymerization in living cells. *Proc Natl Acad Sci U S A* **89**:4678-4682.

Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H and Hazlett LD (2002) Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. *Exp Eye Res* **74**:293-299.

Taketo MM (1998) Cyclooxygenase-2 inhibitors in tumorigenesis (part I). *J Natl Cancer Inst* **90**:1529-1536.

Thun MJ (2003) NSAIDs and esophageal cancer: ready for trials but not yet broad clinical application. *Gastroenterology* **124**:246-248.

Thun MJ, Namboodiri MM, Calle EE, Flanders WD and Heath CW, Jr. (1993) Aspirin use and risk of fatal cancer [see comments]. *Cancer Res* **53**:1322-1327.

Wang WS, Chen PM, Hsiao HL, Ju SY and Su Y (2003) Overexpression of the thymosin beta-4 gene is associated with malignant progression of SW480 colon cancer cells. *Oncogene* **22**:3297-3306.

Watson AJ (1998) Chemopreventive effects of NSAIDs against colorectal cancer: regulation of apoptosis and mitosis by COX-1 and COX-2. *Histol Histopathol* **13**:591-597.

Wechter WJ, Kantoci D, Murray ED, Jr., Quiggle DD, Leipold DD, Gibson KM and McCracken JD (1997) R-flurbiprofen chemoprevention and treatment of intestinal adenomas in the APC(Min)/+ mouse model: implications for prophylaxis and treatment of colon cancer. *Cancer Res* **57**:4316-4324.

Wechter WJ, Leipold DD, Murray ED, Jr., Quiggle D, McCracken JD, Barrios RS and Greenberg NM (2000) E-7869 (R-flurbiprofen) inhibits progression of prostate cancer in the TRAMP mouse. *Cancer Res* **60**:2203-2208.

Wilson LC, Baek SJ, Call A and Eling TE (2003) Nonsteroidal anti-inflammatory drug-activated gene (NAG-1) is induced by genistein through the expression of p53 in colorectal cancer cells. *Int J Cancer* **105**:747-753.

Yamamoto T, Gotoh M, Kitajima M and Hirohashi S (1993) Thymosin beta-4 expression is correlated with metastatic capacity of colorectal carcinomas. *Biochem Biophys Res Commun* **193**:706-710.

Young JD, Lawrence AJ, MacLean AG, Leung BP, McInnes IB, Canas B, Pappin DJ and Stevenson RD (1999) Thymosin beta 4 sulfoxide is an anti-inflammatory agent generated by monocytes in the presence of glucocorticoids. *Nat Med* **5**:1424-1427.

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Legends for Figures

Table 1. Indomethacin (INDO)-Induced Genes from HCT-116 Cells.

Messenger RNAs were isolated from INDO-treated (100 μM) or vehicle-treated (0.2% DMSO) HCT-116 cells. Suppression subtractive hybridization was used to construct the INDO(+) and INDO(-) libraries. The clone "INDO15" is homologous with the sequence for human thymosin β-4.

Figure 1. Schematic Diagram for Full-length Thymosin β-4 mRNA. The grey bar indicates the open reading frame encoding the functional 43 amino acid peptide. The black bar labeled INDO15 indicates the fragment identified by subtractive hybridization from HCT-116 cells.

Figure 2. Northern Analysis of Thymosin β-4 Expression in Indomethacin-treated HCT-116 Cells. (A) Dose-response of thymosin β-4 expression. HCT-116 cells were treated with varying doses of indomethacin for 24 hrs. (B) Time-point northern analysis. HCT-116 cells were treated with 100 μM indomethacin for various time-points. In both (A) and (B), total RNA (10 μg) was loaded in each lane, transferred onto a nylon membrane, hybridized with thymosin β -4 probe. Equal amount of total RNAs were shown in the bottom.

Figure 3. Northern Analysis of Thymosin β-4 and NAG-1 Expression in HCT-116 Cells after Treatment with Various NSAIDs. HCT-116 cells were grown to 80 % confluence in 10-cm plates and treated with either vehicle (0.2%

DMSO) or NSAIDs in serum-free media at the indicated concentration. Isolated RNAs were electrophoresed on a 1.4 % agarose gel and transferred to a nitrocellulose membrane. Full length mRNA sequences for thymosin β-4 and NAG-1 were used as probes.

Figure 4. **Effect of PGE_2 on Thymosin β-4 expression.** HCT-116 cells were treated with various concentrations of PGE_2, or a combination of PGE_2 and indomethacin. Northern analysis was performed and thymosin β-4 full length cDNA was used for probe. Equal amount of total RNAs were shown in the bottom.

Figure 5. Reversed-phase HPLC Analysis for Thymosin β-4 in HCT-116 Cells Treated with NSAIDs. Thymosin β-4 standard was dissolved in 0.1% trifluoroacetic acid in water. HCT-116 cells were treated with Indomethacin (100 μM) and SC-560 (25 μM) for 24 hours. Lysates were ultracentrifuged, and the supernatant was subjected to HPLC as described in Materials and Methods. Thymosin β-4 standard was used to calculate the retention time of thymosin β-4 (approximately 8.1 minutes).

Figure 6. F-Actin Staining in HCT-116 Cells Treated with NSAIDs. HCT-116 cells grown to 50 % confluence and treated with vehicle (0.2% DMSO), DFU (100 μM), or SC-560 (25 μM) for a period of 24 hrs in serum-free media. The cells

were then fixed and stained with TRITC-labeled phalloidin for F-actin staining and observed by confocal laser microscopy at 30X magnification. Hue has been manually changed from red to green for more sensitive visualization, maintaining relative intensities constant. In the changing process, relative staining intensities remain unchanged.

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Table 1. Indomethacin (INDO)-Induced Genes from HCT-116 Cells

Clones	Size (bp)	Highly Homologous Sequences (GenBank #)
INDO12	300	Unknown
INDO13	250	Unknown
INDO14	500	Human bHLHZip transcription factor (XM_032817.3)
INDO15	170	Human thymosin β4 (BC022857.1)
INDO18	600	Human bromodomain-containing 7 (XM_003122.6)
INDO22	250	Unknown
INDO27	195	Human hypothetical protein FLJ20546 (XM_041599)
INDO28	314	Human cytochrome c oxidase subunit IV (BC021236)
INDO29	190	TGF-beta (U18242): NAG-1
INDO33	320	Human heat shock cognate 70 (Y00371)
INDO34	250	Unknown
INDO44	180	Unknown

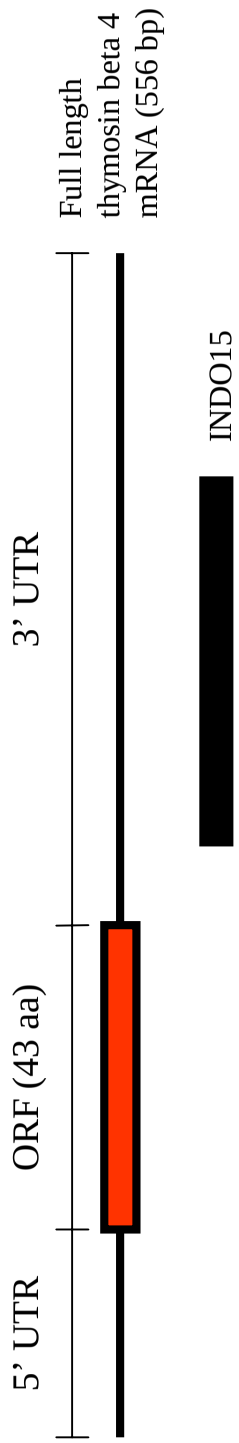
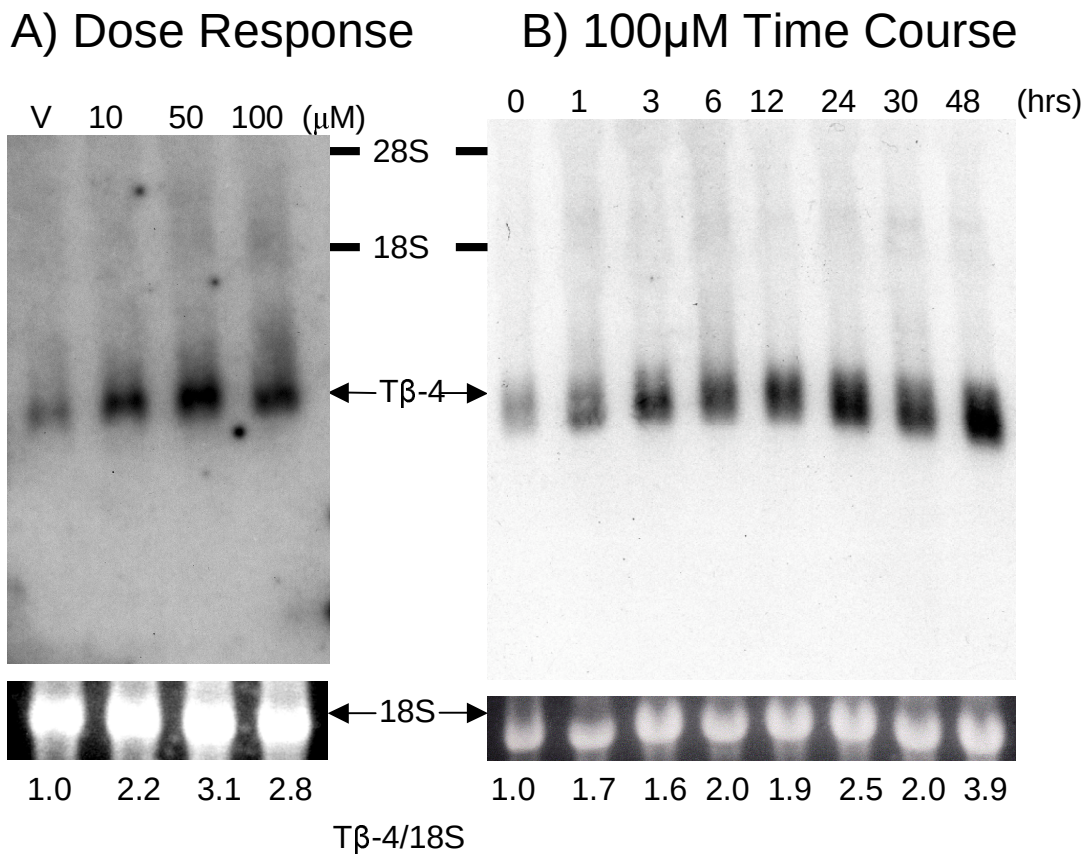


Figure 1.



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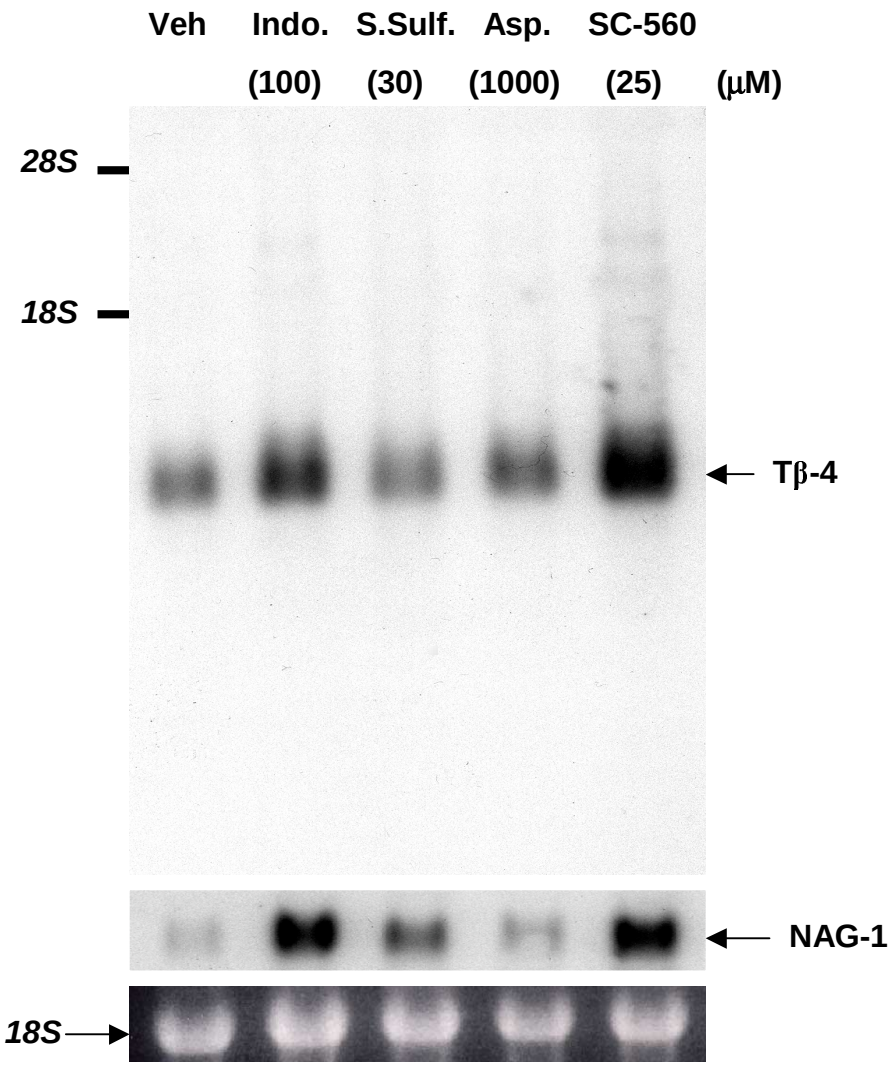

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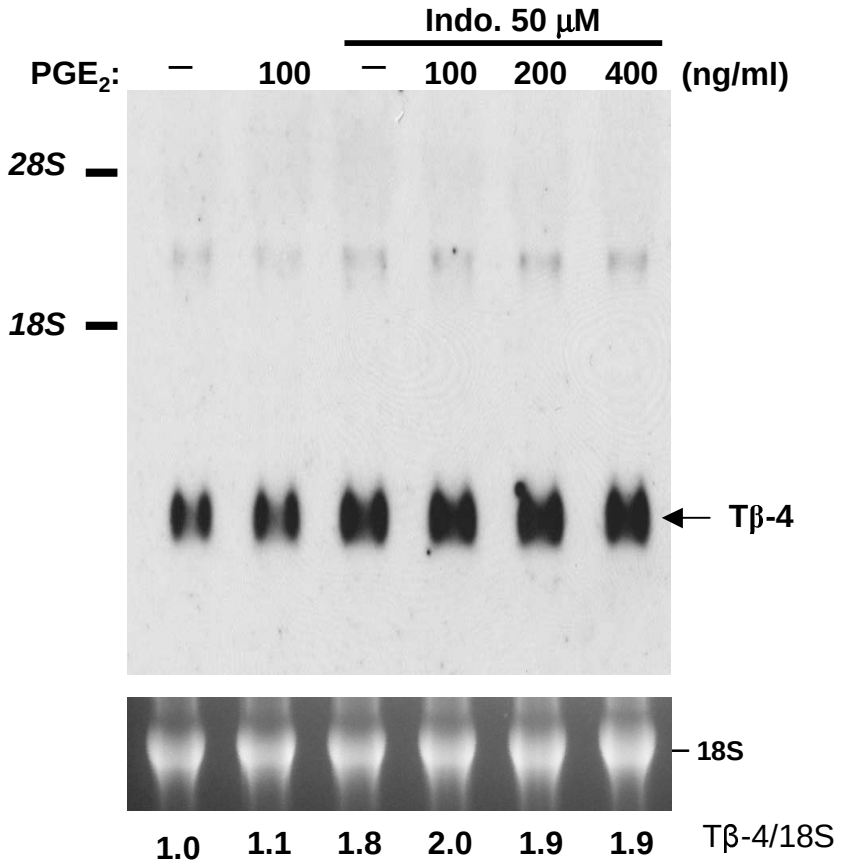

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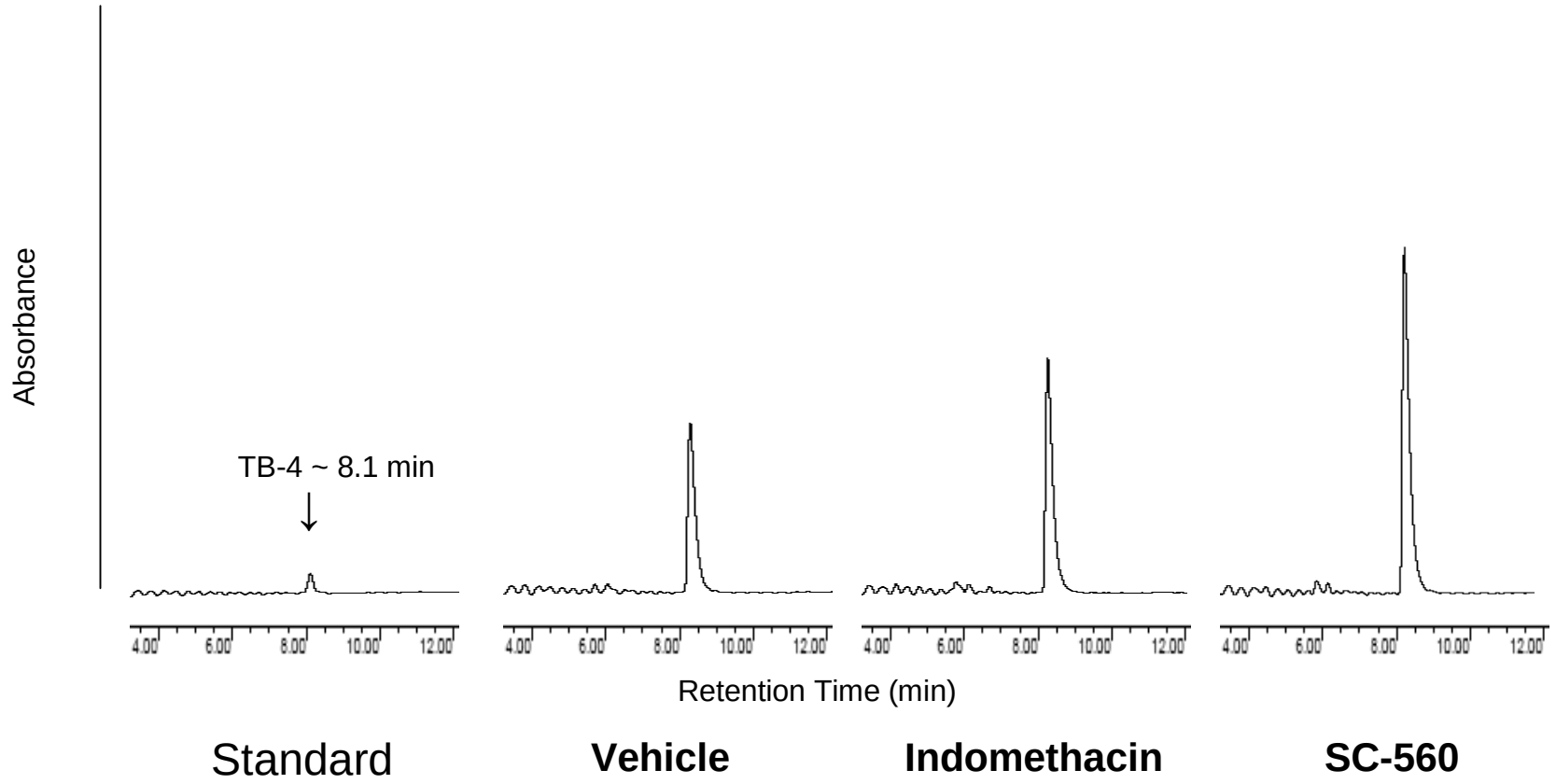

Figure 5.

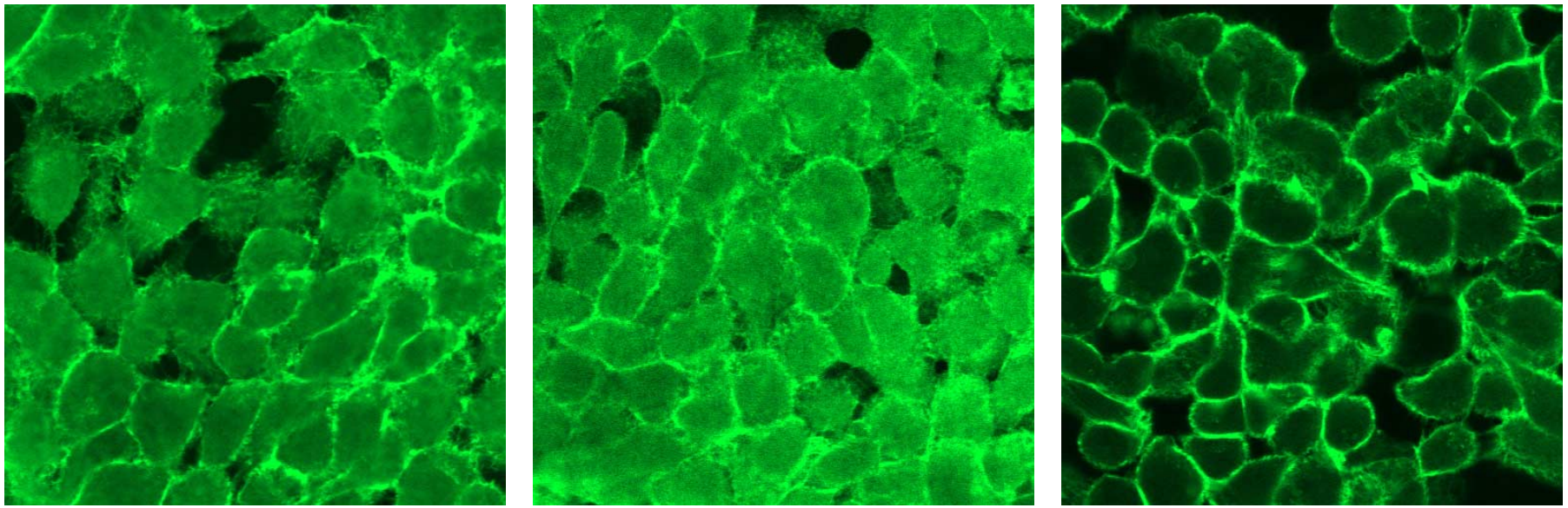

Figure 6.