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Effects of Nitric Oxide-Releasing Nonsteroidal Anti-inflammatory Drugs (NONO-NSAIDs) on Melanoma Cell Adhesion

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Abstract

A new class of nitric oxide (NO•)-releasing nonsteroidal anti-inflammatory drugs (NONO-NSAIDs) were developed in recent years and have shown promising potential as NSAID substitutes due to their gentle nature on cardiovascular and gastrointestinal systems. Since nitric oxide plays a role in regulation of cell adhesion, we assessed the potential use of NONO-NSAIDs as anti-metastasis drugs. In this regard, we compared the effects of NONO-aspirin and a novel NONO-naproxen, to those exerted by their respective parent NSAIDs on avidities of human melanoma M624 cells. Both NONO-NSAIDs, but not the corresponding parent NSAIDs, reduced M624 adhesion on vascular cellular adhesion molecule-1 (VCAM-1) by 20-30% and fibronectin by 25-44% under fluid flow conditions and static conditions, respectively. Only NONO-naproxen reduced (~56%) the activity of β 1 integrin, which binds to α 4 integrin to form very late antigen-4 (VLA-4), the ligand of VCAM-1. These results indicate that the diazeniumdiolate (NO•)-donor moiety is critical for reducing the adhesion between VLA-4 and its ligands, while the NSAID moiety can impact the regulation mechanism of melanoma cell adhesion.

Keywords

NONO-NSAIDs; melanoma; cell adhesion; integrin

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Conflicts of Interest

No conflict of interest.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of inflammation and pain (Singh and Triadafilopoulos, 1999), including mild pain caused by cancer (Potter, 2005). Long-term use of NSAIDs can cause side-effects such as cardiovascular and gastrointestinal injuries (Lichtenberger *et al.*, 2006; Ng and Chan, 2010). A new class of nitric oxide (NO•)-releasing NSAIDs possessing a *N*-diazen-1-ium-1,2-diolate moiety (NONO-NSAIDs) were recently developed and have shown a reduction of harmful side-effects in comparison with normal NSAIDs (Velazquez *et al.*, 2005a; Velazquez *et al.*, 2007; Velazquez *et al.*, 2008; Abdellatif *et al.*, 2009; Chattopadhyay *et al.*, 2010). In addition to their anti-inflammatory profile, NONO-NSAIDs have shown potential for usage in cancer prevention and treatment (Chattopadhyay *et al.*, 2010; Flores-Santana *et al.*, 2012). The synthesis of NONO-NSAIDs was accomplished by attaching the pyrrolidin-1-yl-diazen-1-ium-1, 2-diolate (NONOate) moiety to the carboxylic acid group of two conventional NSAIDs through one-carbon methylene spacer and we report the synthesis of NONO-naproxen, a novel NONO-NSAID as described previously (Velazquez *et al.*, 2005a). In this report, we also describe the anti-metastasis potential of NONO-naproxen, NONO-aspirin and compared them to their corresponding parent NSAIDs.

Previous studies indicated that NO• plays a role in regulation of cancer cell distance migration, including melanoma cells (Tschugguel *et al.*, 1999; Shi *et al.*, 2000; Postovit *et al.*, 2004; Lahdenranta *et al.*, 2009; Baritaki *et al.*, 2010). Our previous studies indicated that very late antigen-4 (VLA-4), which is composed of $\alpha 4\beta 1$ integrins plays a critical role in melanoma cell adhesion to its ligand vascular cell adhesion molecule-1 (VCAM-1) under fluid flow conditions (Wang *et al.*; Liu and Wu, 2011). However, the effects of NONO-NSAIDs on metastatic potential, more specifically cancer cell adhesion and migration, remain unexplored. In this study, we demonstrated that both NONO-naproxen and NONO-aspirin can reduce melanoma adhesion to VLA-4 ligands - VCAM-1 or fibronectin under fluid flow conditions or static conditions respectively. The NONOate moiety on the NSAIDs is critical for their function in reducing the avidity of melanoma cells. These findings led us to hypothesize that NONO-NSAIDs could potentially be a new class of anti-metastasis drugs for cancer treatment.

2. Materials And Methods

2.1. Cell culture

The human melanoma cell line M624 was cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and with 5% carbon dioxide (CO₂). The cells were seeded in 100 mm cell culture dishes 24 h before experiment.

2.2. Reagents and Antibodies

Aspirin and (*S*)-naproxen were purchased from Sigma (St. Louis, MO). Anti-human $\beta 1$ mAb (4B7R) and anti-mouse IgG₁ (sc-3877) were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-human $\beta 1$ (activated) mAb (HUTS-4) was obtained from Millipore (Billerica, MA). Fluorescein isothiocyanate (FITC)-conjugated anti-human $\alpha 4$ (9F10) was obtained from BD Biosciences (Franklin Lakes, NJ). A FITC-conjugated secondary antibodies were also obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Fibronectin (0.1% solution) from human plasma was obtained from Sigma (St. Louis, MO). Recombinant human VCAM-1 was purchased from R&D Systems (Minneapolis, MN).

NONO-aspirin (Fig. 1A) was synthesized as previously described (Velazquez *et al.*, 2005a). NONO-naproxen (Fig. 1B) was synthesized as described in “Results” (Fig. 1C). ¹H-NMR spectra were acquired using a Varian Unity Inova spectrometer. Microanalyses were performed by Midwest Analytic (Indianapolis, IN) and were within ± 0.4% of theoretical values for all elements listed. Flash column chromatography was performed using Versapak® 23 × 110 mm cartridges (silica gel 20–45 μm). Nitric oxide gas was purchased from Matheson Gas Products (Montgomeryville, PA). (*S*)-naproxen sodium was purchased from the Sigma (St. Louis, MO). *O*²-(chloromethyl)diazene-1-ium-1,2-diolate was prepared according to a literature procedure (Tang *et al.*, 2001) except that the reaction of *O*²-sodium 1-(pyrrolidin-1-yl)diazene-1-ium-1,2-diolate with chloromethyl methyl sulfide was carried out in HMPA or DMSO at 25 °C for 48 h as previously reported (Velazquez *et al.*, 2005a).

2.3. Treatment

Aspirin/NONO-aspirin and naproxen/NONO-naproxen were dissolved in pure DMSO to obtain 0.5 M and 0.1 M stock solutions respectively and stored in -20 °C. The stock solutions were diluted with media to designated concentrations and immediately used for the experiments. Cells treated with the same amount of DMSO only were used as control.

2.4. Parallel Plate Flow Chamber Cell Adhesion Assays

Dynamic flow adhesion assays were performed using a parallel plate flow chamber in a laminar flow environment as described previously (Liu and Wu, 2010; Wang *et al.*, 2011). The flow chamber was placed on the stage of an inverted microscope and cell movement was monitored and recorded by iVision-Mac™ (BioVision Technologies, Inc, Exton, PA). 20 μg/ml of VCAM-1 proteins were placed on a 35 mm cell culture dish overnight at 4 °C followed by blockage of non-specific binding sites with 0.5% bovine serum albumin (BSA) in DPBS containing Ca⁺⁺/Mg⁺⁺ for 2 h at room temperature. After the treatment, melanoma cells (10⁶/mL cells suspended in DPBS flow buffer containing Ca⁺⁺/Mg) were pumped through the chamber on immobilized VCAM-1 protein at 0.48 dynes/cm² shear stress at room temperature, thereby mimicking the fluid mechanical environment of microcirculation and post-capillary venules (Jones *et al.*, 1996).

2.5. Static Cell Adhesion Assay

Fibronectin (25 μg/mL) from human plasma was coated onto 48-well culture plates at 37 °C for 1 h. The wells were then blocked with 1% (w/v) BSA in PBS for 45 min before the adhesion assay. The melanoma cells (2.5×10⁵), treated with DMSO (a control) or the drugs, were laid onto fibronectin (20 μg/mL)-coated plates and incubated at 37 °C for 1 h. After washing three times with PBS to remove unattached cells, the cells were fixed with 4% formaldehyde for 10 min, stained with crystal violet (0.5% in 2% Ethanol) for 5 min, washed once with water and allowed to air dry for 30 min. Following with 2% SDS solubilization, absorbance at 550 nm was measured using Spectra-max fluorescence plate reader (Molecular Devices Corp., Sunnyvale, CA).

2.6. Wound Healing Assay

A wound healing assay was conducted to measure the cell migration *in vitro*. Cells were cultured as confluent monolayers in 60-mm dishes for 24 h, and then a “wound” was made by manually scraping the middle of cell monolayers with a standard 200 μL pipette tip, washed with medium to remove debris and incubated in fresh complete medium with or without drugs as indicated. Images at five reference points along the scratch wound were visualized and photographed using an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) equipped with a digital camera system (iVision-Mac™, BioVision Technologies, Exton, PA) under 4× magnification at 0 and 35 h after wounding. The plates

were marked by razor on the outer bottom of the plates to ensure the pictures were taken at the same spot. After measuring the distances of the wound gaps, the relative gap distance for each group of samples was calculated using following equation:

$$\frac{\text{Gap Distance}_{\text{Drug}}(0\text{ h})}{\text{Gap Distance}_{\text{Drug}}(35\text{ h})} / \frac{\text{Gap Distance}_{\text{Vehicle}}(0\text{ h})}{\text{Gap Distance}_{\text{Vehicle}}(35\text{ h})}$$

2.7. Fluorocytometric Analysis

Surface protein expression on M624 cells was detected using immunofluorescence and flow cytometry. The cells were detached from plate by trypsin, and pelleted by centrifugation, washed twice with PBS, fixed with 4% formaldehyde at 37° C for 10 min and cooled on ice for 1 min. Cells were then blocked with 0.5% BSA in PBS for 10 min. Specific monoclonal antibodies against molecules of interest were first titrated to saturating concentrations. For each sample, cells were suspended in 50 µL saturating concentration of primary mAbs, or the isotype control anti-mouse IgG₁, for 30 min at room temperature, washed twice, and incubated with 50 µl of FITC-conjugated secondary antibodies and kept on ice for 30 min. Cells were washed twice, then resuspended in DPBS with Ca⁺⁺/Mg⁺⁺ for analysis on a FACSsort analyzer (BD Biosciences, Franklin Lakes, NJ).

To estimate cell apoptosis, M624 cells were detached from plates by trypsin at 1 h post-treatment after removing floaters. The cells were double stained with propidium iodide (PI) and annexin-V using the Apoptosis Detection Kit from BD Biosciences, followed by analysis on the FACSsort according to the manufacturer's instructions.

2.8. Statistical Analysis

Data are expressed as the mean ± standard error. Statistical significance of differences between means was determined by two-tailed paired *t*-test. Statistical significance was defined as *p*<0.05.

3. Results

3.1 Synthesis and characterization of NONO-naproxen

(*S*)-Naproxen sodium (4.4 mmol, 1.1 g) was suspended in DMSO (17 mL) and mixed with a solution of *O*²-chloromethyl 1-(pyrrolidin-1-yl)diazene-1-ium-1,2-diolate (4.4 mmol, 0.8 g) (Velazquez *et al.*, 2005b) in DMSO (3 mL). This reaction mixture was stirred at 25° C for 3 h, following the progress by TLC; the complete disappearance of the chloromethyl diazeniumdiolate intermediate marked the end point. The resulting orange-brown liquid was poured into a beaker containing ice (100 mL). After shaking for a few minutes, the initial yellow powder became a sticky material, which had to be dissolved by addition of ethyl acetate (100 mL). This bilayer mixture was transferred to a separatory funnel where the organic layer was isolated and the water layer was washed with a second addition of ethyl acetate (50 mL). The combined organic phases were dried (Na₂SO₄) and the solvent evaporated under vacuum. The residue (about 1.8 g) was purified by flash column chromatography using a Versapack[®] column (23 g; 23 × 110 mm) and a mixture of Hex:EA (stepped gradient). Combined fractions containing the main spot from this reaction were evaporated under vacuum, yielding the novel NONO-naproxen as a bright yellow liquid (0.78 g, 47 % yield). ¹H-NMR (CHCl₃) δ 7.62-7.75 (m, 3H, naphtyl), 7.53 (dd, *J* = 8.5 Hz, 1.2 Hz, 1H, naphtyl), 7.31 (d, *J* = 2.4 Hz, 1H, naphtyl), 7.26 (dd, *J* = 8.5 Hz, 2.4 Hz, 1H, naphtyl), 5.71 (d, *J* = 7.2 Hz, 1H, OCHHO), 5.77 (d, *J* = 7.2 Hz, 1H, OCH'HO), 3.49 (m,

1H, CH), 3.45-3.50 (m, 4H, pyrrolidinyl H-2, H-5), 1.91-1.94 (m, 4H, pyrrolidinyl H-3, H-4), 1.62 (d, *J*: 6.72 Hz, 3H, CH₃). Anal. calc. C₁₉H₂₃N₃O₅ (C, H, N).

3.2. NONO-naproxen and NONO-aspirin reduce the avidity of M624 cells to VCAM-1

Integrins $\alpha 4\beta 1$ (VLA-4) play a critical role in adhesion between melanoma and endothelial cells via interaction with VCAM-1 (Wang *et al.*, 2011). To determine whether NONO-NSAIDs could potentially affect the avidity of melanoma to endothelial cells, parallel plate flow chamber cell adhesion assays were used to analyze the extent of effects of NONO-naproxen (CVM-16) and NONO-aspirin (CVM-01) on avidity of M624 cells to VCAM-1 under physiological flow conditions. Stable M624 tethering was optimal at 0.48 dyne/cm² wall shear stress. Under this shear stress, 40 \pm 3 cells/mm² were adhered to a VCAM-1 coated, but not to a 0.5% BSA coated surface, which indicated that M624 cells were specifically adhered to VCAM-1. After pretreating with indicated drugs or DMSO for 1 h, the cells were perfused through the chamber and the adhered cells were counted for 4 min. Our data showed that pretreatment with naproxen and aspirin have no statistically significant effect, but pretreatment with NONO-naproxen (0.1 mM) or NONO-aspirin (1.0 mM) reduced the avidity of M624 to VCAM-1 by 20.93 \pm 7.58% or 30.86 \pm 13.54%, respectively (Fig. 2A). These results suggest that NONO-NSAIDs can attenuate the avidity of M624 to VCAM-1, while NSAIDs themselves do not affect the adhesion significantly.

3.3. NONO-NSAIDs reduce adhesion of M624 cells to fibronectin under static conditions

Fibronectin plays an important role in cancer metastasis and while $\alpha 4\beta 1$ integrins are the receptors of fibronectin, they are also expressed on the surface of M624 cells (Juliano and Varner, 1993). Due to this relationship, the extent of effects of NONO-naproxen and NONO-aspirin on the avidity of M624 onto fibronectin were also analyzed using a static adhesion assay. Our static data showed that while naproxen and aspirin have no statistically significant effect, pretreatment with NONO-naproxen (0.1 mM) or NONO-aspirin (1.0 mM) for 1 h reduced the avidity of M624 to fibronectin by 25.32 \pm 2.98% or 44.29 \pm 2.23%, respectively (Fig. 2B). These results indicated that NONO-NSAIDs could potentially reduce the invasiveness of melanoma cells.

3.4. NONO-aspirin reduces cell migration by in vitro wound-healing assay

To determine whether the release of NO• has an impact on migration rate of the melanoma cells, we analyzed the effects of NONO-NSAIDs on cell migration used the *in vitro* wound-healing assay as previously described (Yang *et al.*, 2007). A concentration of 0.5 mM of NONO-aspirin or aspirin was used in the experiments due to toxicity of long-term (35 h) treatment of NONO-aspirin or aspirin at a concentration of 1.0 mM. Our data showed treatment of NONO-naproxen (0.1 mM) or naproxen (0.1 mM) did not statistically significantly change the migration rate of the melanoma cells (Fig. 3). However, treatment of NONO-aspirin (0.5 mM) or aspirin (0.5 mM) reduced the migration rate of the cells by 49.7 \pm 6.5% or 20.2 \pm 8.0% at 35 h post-wounding, respectively (Fig. 3). The results indicate that NONO-aspirin is more effective than aspirin in reducing the migration of melanoma cells.

3.5. Effects of NONO-NSAIDs on integrin on the surface of M624

To explore the mechanism underlying the NONO-NSAIDs reduced avidity of melanoma to VCAM-1 and fibronectin, we analyzed the extent of effect of NONO-naproxen and NONO-aspirin on the cell surface expression of integrins $\alpha 4$ and $\beta 1$ using flow cytometry (Fig. 4A, Table 1). Our data indicated that the naproxen and NONO-naproxen had no statistically significant effect on the expression of $\alpha 4$ and $\beta 1$ integrins on the cell surface (Fig. 4B, Table 1). However, while naproxen had no effect, NONO-naproxen reduced the

amount of the activated $\beta 1$ integrin (recognized by HUTS-4 mAb) by $56.36 \pm 11.42\%$ (Fig. 4B, Table 1). In contrast, aspirin reduced the total amount of $\beta 1$ integrin by $16.58 \pm 2.16\%$ (recognized by anti- $\beta 1$ mAb) (Fig. 4C, Table 1). It appeared that NONO-aspirin also reduced the expression of $\beta 1$ integrin by $17.50 \pm 10.52\%$, but it was not statistically significant (Fig. 4C, Table 1).

3.6 Effects of NONO-NSAIDs on apoptotic death of M624

Since avidity of cells could be reduced due to endocytosis of cell surface integrin when undergoing apoptosis (Tsai *et al.*, 2008; Liu and Wu, 2010), we determined whether the reduction of cell adhesion by NONO-NSAIDs was due to the death of M624 cells. The cells were treated under adhesion assay treatment conditions and cell apoptosis was analyzed by annexin V-FITC/PI double staining followed by flow cytometry. Our data indicated that pretreating cells with NONO-NSAIDs or NSAIDs either had no effect (NONO-naproxen) or slightly reduced (NONO-aspirin, naproxen and aspirin) the amount of apoptotic cells without statistic significance (Fig. 5). These results indicate that the reduced avidity of M624 cells to VCAM-1 or fibronectin is not due to an increased amount of apoptotic death of the cells after the treatment.

4. Discussion

Aspirin and NONO-aspirin have been shown to alter hematologic cell adhesion (Wallace *et al.*, 1995; Pillinger *et al.*, 1998). NSAIDs have also recently been shown to reduce adhesion of breast cancer cells to endothelial cells (Bischofs *et al.*, 2012). Our previous studies indicated that the interaction between VLA-4 ($\alpha 4\beta 1$ integrins) and VCAM-1 is critical for adhesion between human melanoma (M624) and endothelial cells (HUVEC) (Wang *et al.*, 2011) and the activities of Akt and NOSs play important roles in regulation of VLA-4-mediated melanoma cell adhesion to endothelial VCAM-1 (Liu and Wu, 2011). In this study, the effects of NSAIDs and NONO-NSAIDs, – specifically aspirin, naproxen, NONO-aspirin and NONO-naproxen – on melanoma cell adhesion and VLA-4 surface expression/activation were determined. Our data showed that while naproxen (0.1 mM) and aspirin (1.0 mM) did not have a statistically significant effect, both NONO-naproxen (0.1 mM) and NONO-aspirin (1.0 mM) reduced melanoma adhesion on either VCAM-1 or fibronectin (Fig. 2). The use of 0.1 mM solutions of naproxen and NONO-naproxen was due to the lower solubility of NONO-naproxen in aqueous media. A higher concentration of aspirin (1.0 mM) was used due to the fact that the IC_{50} 's of aspirin are approximately 30 and 5 times higher than the IC_{50} 's of naproxen for COX-1 and COX-2 respectively (Noreen *et al.*, 1998). It appears that the release of NO• by NONO-NSAIDs is the major factor for reducing the dynamic and static adhesion of melanoma to VCAM-1 and fibronectin, considering that the parent NSAIDs alone did not have any statistically significant effect on the cell adhesion (Fig. 2). The release of NO• can also reduce the migration rate of melanoma cells, as NONO-aspirin (0.5 mM) was much more effective reducing the migration of M624 cells in the wound-healing assay (Fig. 3). The ineffectiveness of NONO-naproxen (0.1 mM) on M624 wound-healing assay could be due to its lower concentration, which would generate less NO•. The reduction of the migration rate was not correlated with the reduction of avidity of the cells as NONO-naproxen (0.1 mM) was more effective in reducing the avidity of M624 than NONO-aspirin (0.5 mM) was for both dynamic and static adhesion assays (Fig. 2 vs. S1).

To further elucidate the mechanism of the NONO-NSAID-induced alternation of melanoma adhesion, the surface expression and activation of $\alpha 4$ and $\beta 1$ integrins were analyzed post-treatment, since the interaction VLA-1 ($\alpha 4\beta 1$ integrins) plays a critical role in the melanoma adhesion (Wang *et al.*, 2011). Our data showed that while naproxen had no statistically significant effect on the expression and activation of $\alpha 4$ and $\beta 1$ integrins, NONO-naproxen

significantly reduced the activity of $\beta 1$ integrin without altering the expression of the integrins (Fig. 4B, Table 1). The reduced $\beta 1$ activity is expected to decrease the activity of $\alpha 4\beta 1$ integrins, since the other VCAM-1 binding protein, $\alpha 4\beta 7$ integrin, is not present in the cell surface due to the lack of $\beta 7$ expression (Wang *et al.*, 2011). In contrast to NONO-naproxen, NONO-aspirin appeared to reduce the expression of $\beta 1$ integrin (although it was not statistically significant), without altering the activity of the protein (Fig. 4C, Table 1). However, this is not likely to be the cause of NONO-aspirin-reduced melanoma cell adhesion, because aspirin exerted a similar reduction in the expression of $\beta 1$ (Fig. 4C, Table 1) without altering the avidity of the cells (Figs. 2). However, the NONO-NSAID-dependent reduction of melanoma cell adhesion was cell line dependent; neither NONO-naproxen (0.1 mM) nor NONO-aspirin (0.5 mM) reduced the avidity of melanoma A375 cells, which express a much lower level of $\alpha 4$ integrin than M624 (data not shown), to VCAM-1 or fibronectin (Fig. S2). In contrast, NONO-aspirin (0.5 mM) increased the adhesion of A375 cells to VCAM-1 under flow condition (Fig. S2). These results suggest that while NONO-NSAIDs have potential to be used as anti-metastasis drugs, a pre-diagnosis of the adhesion properties of melanoma might be required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- NONO-naproxen, a novel nitric oxide-releasing NSAID, was synthesized.
- NONO-NSAIDs, but not their parent NSAIDs, reduced melanoma adhesion.
- NONO-naproxen, but not NONO-aspirin and NSAIDs, reduced activity of $\beta 1$ integrin.

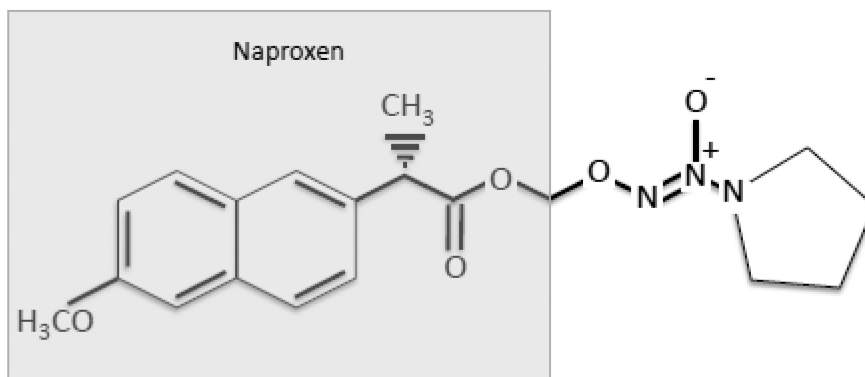
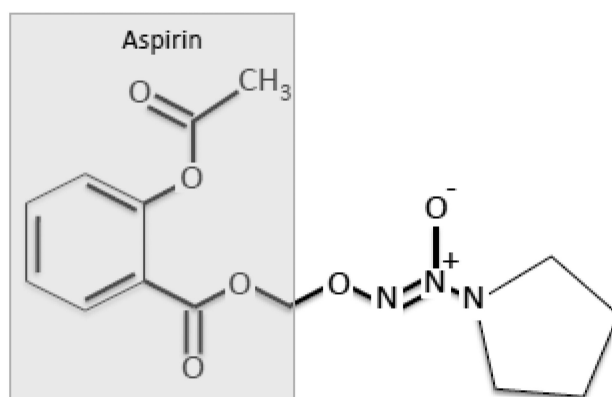
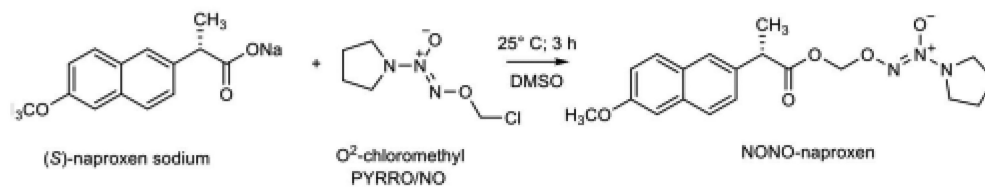
A. NONO-Naproxen**B. NONO-Aspirin****C. Scheme of NONO-Naproxen synthesis**

Fig. 1. Chemical structures of NONO-Naproxen (A) and NONO-Aspirin (B). The boxed shadow part is the structure of naproxen or aspirin. C. The reagents and conditions for NONO-naproxen synthesis.

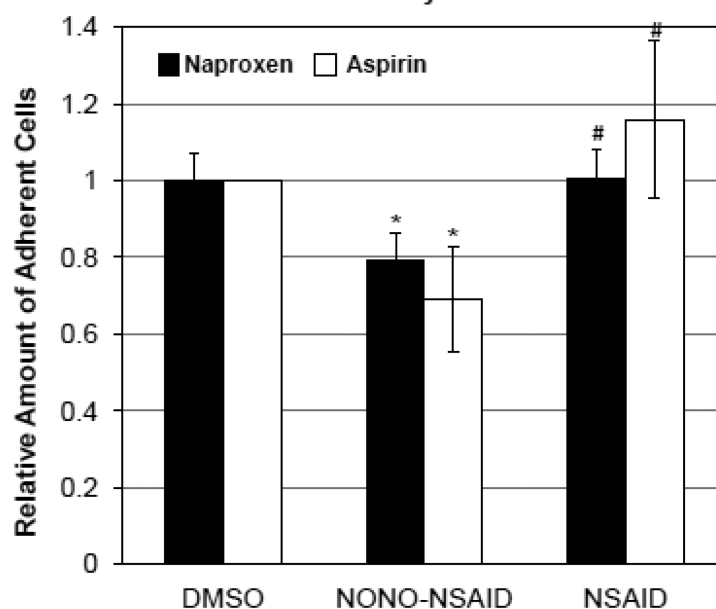
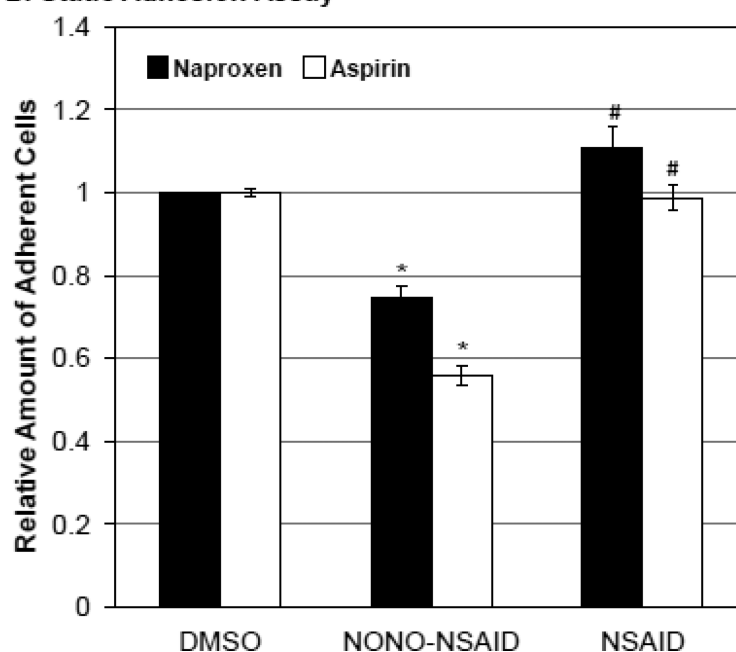
A. Flow Chamber Adhesion Assay**B. Static Adhesion Assay**

Fig. 2. NONO-NSAIDs reduce adhesion of M624 melanoma cells to VCAM-1 and fibronectin. A: M624 cells were treated with DMSO, NONO-NSAID or NSAID for 1 h and then perfused over immobilized soluble VCAM-1 at a physiologic shear stress of 0.48 dyne/cm² using a parallel plate flow chamber. The number of cells adhering in a single field of view was counted after 4 min of perfusion. B: M624 cells were treated with DMSO, NONO-NSAID or NSAID for 1 h and then incubated in a fibronectin-coated plate at 37 °C for 1 h. Data is normalized by DMSO and expressed as the mean \pm SE from four independent experiments. * p <0.05 vs. DMSO group. # p >0.05 vs. DMSO group.

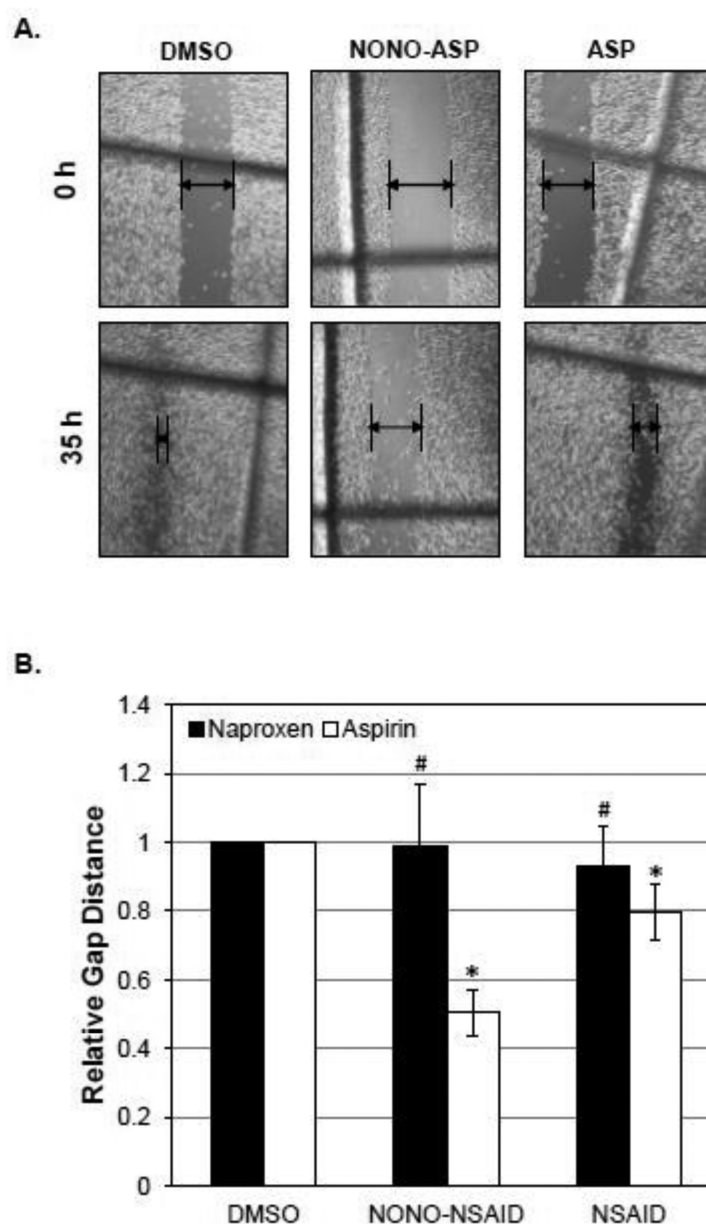
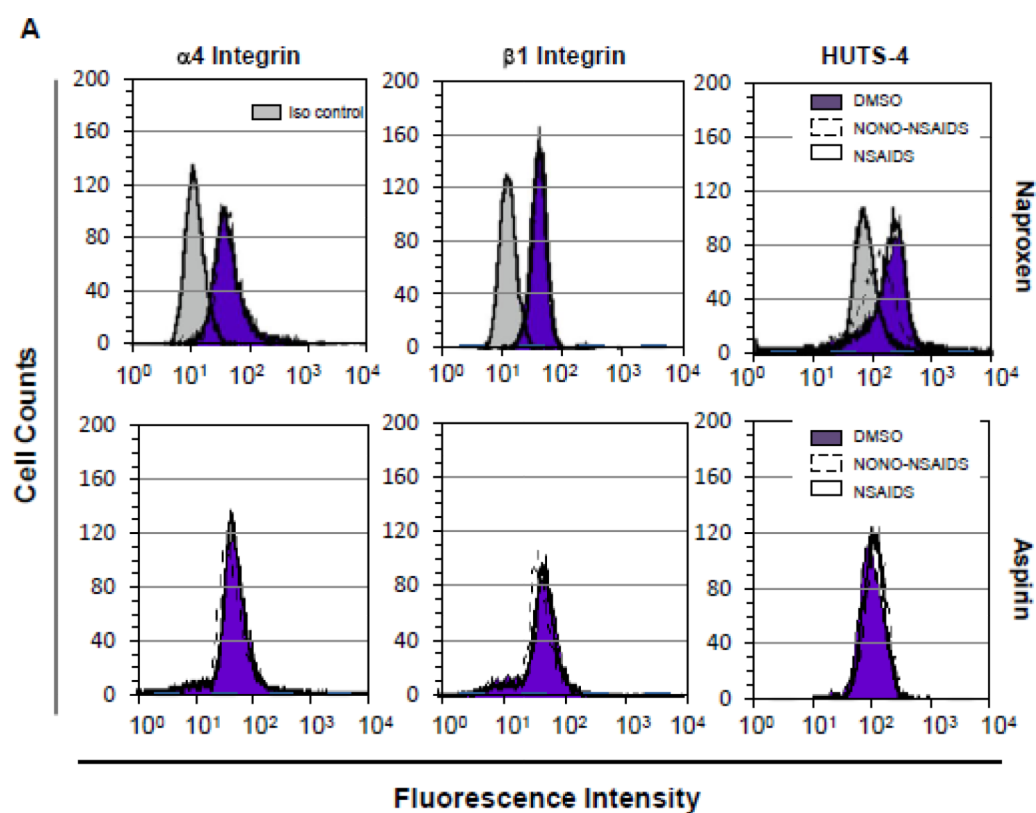
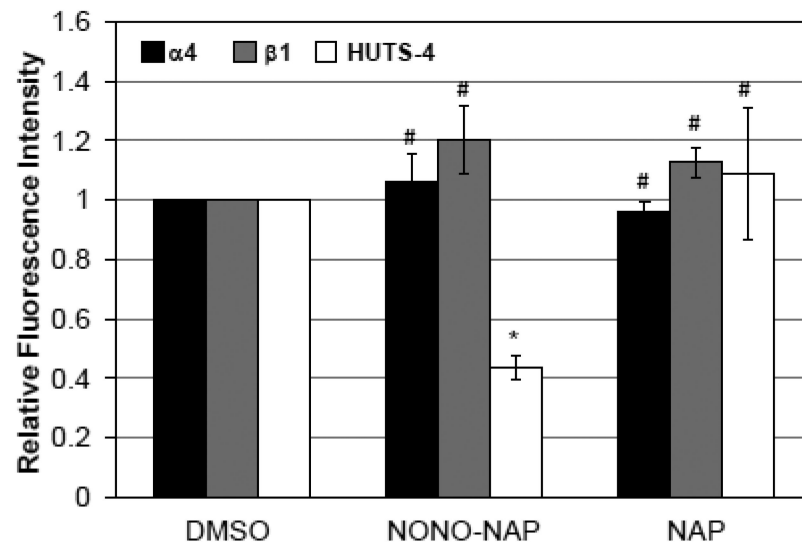


Fig. 3. Effect of NONO-NSAIDs/NSAIDs on M624 cell migration *in vitro* by wound-healing assay. The monolayer of M624 cells was scratched with a 200 μ L plastic pipet tip and then fed with fresh media containing DMSO, NONO-NSAID or NSAID for 35 h. A: Representative phase-contrast images (4 \times magnification) of the wounds at 0 and 35 h post-wounding. B: Relative gap distance. Data represents an average from three independent experiments, and expressed as mean \pm SE. * $p < 0.05$ vs DMSO, # $p > 0.05$ vs. DMSO.



B. The Effect of NONO-naproxen and naproxen on expression of the integrins



C. The Effect of NONO-aspirin and aspirin on expression of the integrins

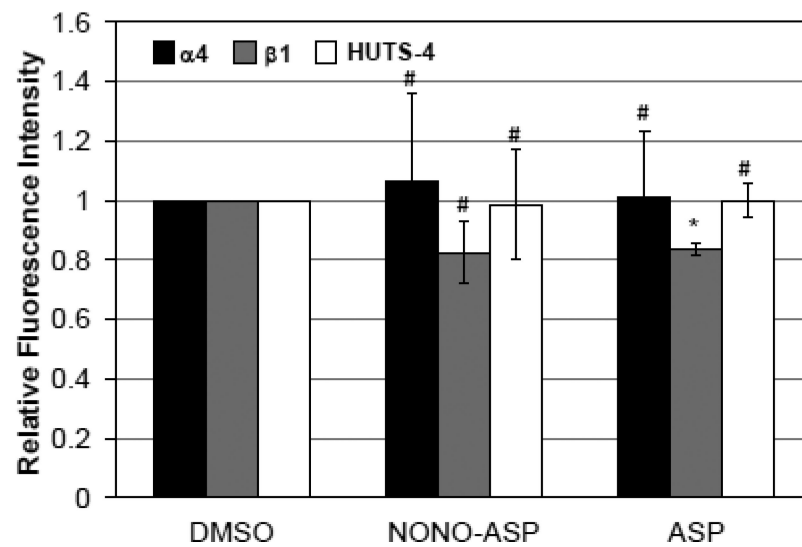


Fig. 4.

Flow cytometry analysis of the effect of NONO-NSAIDs/NSAIDs on surface expression of integrins on M624 cells. The cells were treated with DMSO, NONO-NSAID or NSAID for 1 h and the surface expression of $\alpha 4$, $\beta 1$ and activated $\beta 1$ integrins were determined by flow cytometry. A. Representative histograms. B-C. The relative levels of surface expression of $\alpha 4$, $\beta 1$ and activated $\beta 1$ integrins. Data is normalized by DMSO and expressed as the mean \pm SE from three independent experiments. * $p < 0.05$ vs. DMSO group. # $p > 0.05$ vs. DMSO group.

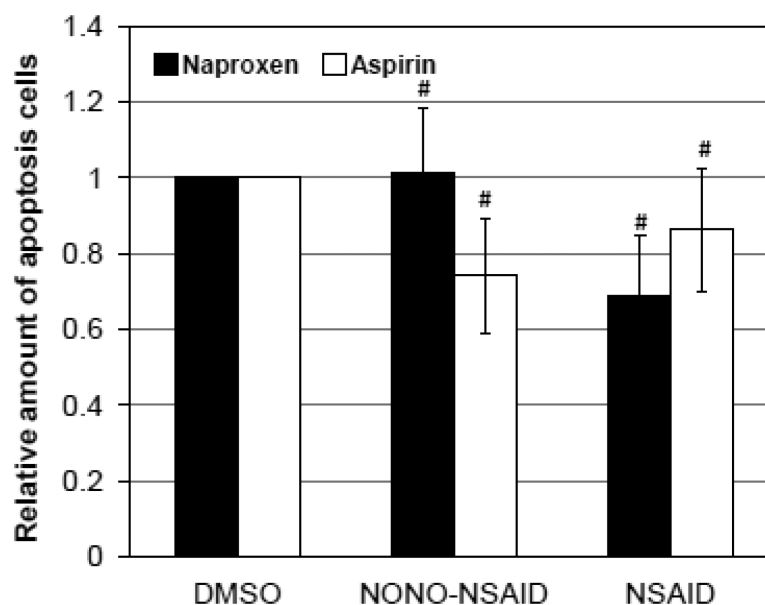


Fig. 5.

Flow cytometry analysis of the effect of NONO-NSAIDs/NSAIDs on apoptotic death of M624 cells. The cells were treated with DMSO, NONO-NSAID or NSAID for 1 h and double-stained with FITC-conjugated annexin-V and PI. Cells that were stained with both FITC and PI were counted as apoptotic cells. Data is normalized by DMSO and expressed as the mean \pm SE from three independent experiments. [#] $p > 0.05$ vs. DMSO group.

Table 1

Surface expression of $\alpha 4$, $\beta 1$ and activated $\beta 1$ integrins.

	$\alpha 4$ Integrin	$\beta 1$ Integrin	Active $\beta 1$ Integrin
Aspirin	1.01 \pm 0.23	0.83 \pm 0.02	1.00 \pm 0.06
NONO-Aspirin	1.06 \pm 0.29	0.82 \pm 0.11	0.99 \pm 0.19
Naproxen	0.96 \pm 0.03	1.20 \pm 0.11	1.09 \pm 0.22
NONO-Naproxen	1.06 \pm 0.10	1.13 \pm 0.05	0.44 \pm 0.04

The M624 cells were treated with naproxen (0.1 mM), NONO-naproxen (0.1 mM), Aspirin (1 mM), NONO-Aspirin (1 mM) and DMSO for 1 h. Data is normalized by DMSO and expressed as the mean \pm SD from three independent experiments.