Depigmenting mechanism of NSAIDs on B16F1 melanoma cells

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1 NSAIDs down-regulate tyrosinase expression on murine B16F1 melanoma
2 cells.

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1 Keyword

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3 Non-steroidal anti-inflammatory drugs (NSAIDs), melanogenesis,
4 tyrosinase, microphthalmia-associated transcription factor (Mitf), B16
5 melanoma

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9 Abstract

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11 The aim of the present work was to clarify the anti-melanogenic
12 mechanism of non-steroidal anti-inflammatory drugs (NSAIDs).
13 Mefenamic acid, diclofenac and nimesulide were used in this study, and
14 these all drugs inhibit melanin synthesis on B16 melanoma cells. To
15 elucidate the anti-melanogenic mechanism of NSAIDs, we performed
16 Western blotting analysis for melanogenic proteins, such as tyrosinase,
17 TRP-1, TRP-2. NSAIDs inhibited tyrosinase expression.
18 Semi-quantitative RT-PCR analysis showed that the depigmentation effect
19 of NSAIDs might be due to the inhibition of tyrosinase gene transcription
20 but not the inhibition of microphthalmia-associated transcription factor
21 (Mitf). These results indicate that NSAIDs inhibit α -MSH-enhanced
22 melanin synthesis, and are candidate anti-melanogenic agents that they
23 might be effective in hyperpigmentation disorders.

1 Introduction

2
3 Non-steroidal anti-inflammatory drugs (NSAIDs) are known to act
4 by directly suppressing the activity of cyclooxygenase (COX), the key
5 enzyme catalyzing the biosynthesis of prostaglandins which induce
6 inflammation. Therefore, NSAIDs are generally used to treat pain,
7 inflammation and fever. NSAIDs were able to prevent the development
8 of cancer of the colon, breast, stomach, and lung [1-4]. These biological
9 effects of NSAIDs are the results of their anti-cancer ability, by inducing
10 apoptosis and cell cycle arrest [5, 6]. Acetylsalicylic acid is one of the
11 well-studied NSAIDs, also has been shown to be effective as an
12 antiplatelet drug in preventing heart attacks, stroke, and cerebral
13 thrombosis [7-9].

14 Human skin pigmentation is caused by melanin synthesis in UV
15 irradiated melanocytes. Tyrosinase is a key enzyme in melanin synthesis,
16 which can catalyze three different reactions; the hydroxylation of tyrosine
17 to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to
18 DOPAquinone and the oxidation of 5,6-dihydroxyindole (DHI) to
19 indole-quinone [10]. In the absence of thiols DOPAquinone changes to
20 DOPAchrome and then to DHI or indole 5,6-quinone 2-carboxylic acid
21 (DHICA). There are two other main enzymes in this melanogenic
22 pathway; tyrosinase-related protein-2 (TRP-2; DOPAchrome tautomerase),
23 which catalyzes the conversion of DOPAchrome to DHICA, and TRP-1
24 (DHICA oxidase) that catalyzes the oxidation of DHICA [11-15].

25 There are several signal pathways for enhancing melanin
26 production. The cAMP-mediated pathway is a well-known melanin
27 synthesis cascade. α -Melanocyte stimulating hormone (α -MSH),
28 prostaglandin E₂ (PGE₂), and adrenocorticotrophic hormone (ACTH)
29 activate the cAMP-mediated pathway [16-18]. The cGMP-mediated
30 pathway can also increase melanin production. This pathway is activated

1 by nitric oxide (NO) which is released by keratinocytes irradiated by
2 UV-B (19). In human melanocytes, Park et al. [20] reported protein
3 kinase C (PKC) can phosphorylate serine residues of tyrosinase at
4 positions 505 and 509 in the cytoplasmic domain, and that this
5 phosphorylation can enhance tyrosinase activity. UV light might affect
6 cell membranes and activate phospholipase C, releasing diacylglycerol
7 (DAG), which can activate PKC [21]. Microphthalmia-associated
8 transcription factor (Mitf) has a crucial role in the transcription of
9 melanogenic genes. Mitf is a transcription factor of the basic
10 helix-loop-helix leucine zipper (bHLH-LZ) family, which binds a highly
11 conserved motif termed the M-box to activate melanogenic gene
12 transcription factors such as tyrosinase, TRP-1 and TRP-2. Mitf
13 expression is regulated by several transcription factors.

14 NSAIDs were well-studied, and reported on various aspects, but
15 studies on melanogenesis were rarely. Recently, we found acetylsalicylic
16 acid (aspirin) inhibit tyrosinase protein level subsequent melanogenesis on
17 B16 melanoma cells [22]. Therefore, it is important to investigate the
18 effect of other NSAIDs on melanogenesis. In the present study, we
19 investigated the further effects of NSAIDs on aspects of melanogenic
20 related gene transcription and protein expression.

Materials and Methods

Cell culture

B16F1 murine melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Cell proliferation

NSAIDs (mefenamic acid, diclofenac and nimesulide) were purchased from Sigma) was dissolved in dimethyl sulfoxide (DMSO). NSAIDs were added to the cell cultures at final concentrations of 50 and 100 µM for 5 days, respectively. All culture dishes had α-MSH (Sigma) added to them at final concentrations of 10 nM. Cells were collected with trypsin / ethylenediaminetetraacetic acid after treatment, and were counted with a Fuchs-Rosenthal cytometer.

Measurement of melanin content

Melanin content was measured as described previously [23]. The cells were treated with NSAIDs (50 and 100 µM) and α-MSH (10 nM) for 5 days. After treatment, the cells were detached by incubation in trypsin/ ethylenediaminetetraacetic acid. After precipitation, cell pellets containing a known number of cells were solubilized in boiling 2 M NaOH

1 for 20 min. Spectrophotometric analysis of melanin content was
2 performed at 405 nm absorbance. To estimate the effect of
3 anti-melanogenic effect of NSAIDs, we also observed melanoma under
4 microscopy at final concentration of 150 μ M.

5 6 7 Tyrosinase assay

8
9 Tyrosinase assay was performed as reported by Masamoto et al.
10 with slight modifications [24]. First, 300 μ l of 2.5 mM
11 3,4-dihydroxyphenylalanine (L-DOPA) solution, 50 μ l of DMSO with or
12 without a sample chemical, and 1.1 ml of 0.1 M phosphate buffer (pH 6.8)
13 were mixed. The mixture was preincubated at 25°C for 10 min before
14 50 μ l of 1380 units/ml tyrosinase in aqueous solution was added, and the
15 reaction was monitored at 475 nm. A control reaction was conducted
16 with DMSO alone. The percentage of activity of tyrosinase was
17 calculated as follows: $100 - [(A - B)/A \times 100]$, where A represents the
18 difference in the absorbance of the control sample between an incubation
19 time of 0.5 and 1.0 min, and B represents the difference in the absorbance
20 of the test sample over the same incubation period. The optical density
21 (O. D.) was measured by a UV spectrophotometer at 475 nm.

22 23 24 DOPA staining of gels

25
26 The DOPA staining assay was performed as described previously
27 [25]. B16 melanoma cells which were stimulated by α -MSH (10 nM)
28 were treated with or without NSAIDs (50 and 100 μ M) for 5-days. To
29 identify the active form of tyrosinase, samples lysed in 0.1 M sodium
30 phosphate buffer (pH 6.8) containing 1% Triton-X-100, 1mM PMSF, 10

1 $\mu\text{g}/\text{ml}$ aprotinin, and $10\ \mu\text{g}/\text{ml}$ leupeptin (but without mercaptoethanol or
2 heating) were resolved by 7.5% SDS-polyacrylamide gel electrophoresis.
3 Gels containing tyrosinase bands were placed in a flat-bottomed container
4 with 200 ml of rinse buffer ($0.1\ \text{M}\ \text{NaH}_2\text{P}\ \text{O}_4$, pH 6.8) and equilibrated at
5 room temperature with gentle shaking. After 30 min, the rinse buffer was
6 drained from the gels and replaced with fresh buffer. After repeating the
7 rinse procedure once more, the gels were transferred into 200 ml of a
8 staining solution which contained the rinse buffer supplemented with 5
9 mM L-DOPA, and incubated in a dark place for 3 h at 37°C . Protein
10 bands that contained tyrosinase activity were visualized in the gels as dark
11 bands.

14 Western blotting

16 To determine the amount of tyrosinase and tyrosinase-related protein
17 expression, western blotting analysis was performed. B16 melanoma
18 cells which had been stimulated by α -MSH were treated with NSAIDs
19 (50 - $150\ \mu\text{M}$) for 5-days. After treatment, the cells were collected and
20 lysed with cell lysis buffer [$50\ \text{mM}$ Tris-HCl (pH6.8), 2% SDS, 6%
21 β -mercaptoethanol, 1% glycerol]. Whole cell lysates (5×10^4 cells
22 equivalents per lane) were separated by 7.5% SDS-polyacrylamide gel
23 electrophoresis and transferred to a nitrocellulose membrane. The
24 membrane was blocked with 5% skimmed milk in phosphate-buffered
25 saline containing 0.05 % Tween 20. Tyrosinase, TRP-1, and TRP-2
26 bands were detected with the rabbit polyclonal anti-tyrosinase antibody
27 (dilution 1:1000), rabbit polyclonal anti-TRP-1 antibody (dilution 1:1000),
28 and rabbit polyclonal anti-TRP-2 antibody (dilution 1:500), respectively,
29 which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA,
30 USA), and then further incubated with horseradish peroxidase-conjugated

1 anti-rabbit IgG antibody at a 1:1500 dilution. Bound antibodies were
2 detected using an enhanced chemiluminescence kit (Amersham) following
3 the manufacturer's instructions. Loading control was assessed using
4 anti- β -actin antibody.

5 6 7 Semi-quantitative reverse transcription-polymerase chain reaction 8 (RT-PCR)

9
10 To investigate the effect of NSAIDs on melanogenic gene
11 expression, semi-quantitative reverse transcription-polymerase chain
12 reaction (RT-PCR) analysis was performed. B16 melanoma cells were
13 treated with or without NSAIDs (200 μ M) for 3-days. After treatment,
14 total RNA was extracted from the cells using ISOGEN (NIPPON GENE,
15 Japan). Reverse transcription and cDNA amplification were carried out
16 with 1 μ g of isolated total RNA using the TaKaRa RT-PCR kit (TaKaRa).
17 All procedures were conducted following the manufacturer's instructions.
18 The oligonucleotide primers used for the PCR were as follows: tyrosinase
19 upstream 5'-GGC CAG CTT TCA GGC AGA GGT-3'; downstream 5'-
20 TGG TGC TTC ATG GGC AAA ATC-3', TRP-1 upstream 5'-GCT GCA
21 GGA GCC TTC TTT CTC-3'; downstream 5'-AAG ACG CTG CAC
22 TGC TGG TCT-3', TRP-2 upstream 5'-GGA TGA CCG TGA GCA ATG
23 GCC-3'; downstream 5'-CGG TTG TGA CCA ATG GGT GCC-3', Mitf
24 upstream 5'-CCC GTC TCT GGA AAC TTG ATC G-3'; downstream
25 5'-CTG TAC TCT GAG CAG CAG GTG-3', β -actin upstream 5'-TGG
26 AAT CCT GTG GCA TCC ATG AAA C-3'; downstream 5'-TAA AAC
27 GCA GCT CAG TAA CAG TCC G-3'. The reaction was cycled 25 times
28 for 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C. After amplification, fifty
29 percent of the reaction mixture was analyzed by electrophoresis on 1.5%
30 agarose gels and stained with ethidium bromide. In order to check the

1 reproducibility of the results, each experiment was carried out more than
2 three times. Specific primers for β -actin were used as controls.

3 4 5 Statistical analysis

6
7 All statistical calculations were carried out using the SPSS software
8 (14.0 for windows) program. Data were analyzed using the Dunnett's
9 test.

Results

The Effect of NSAIDs on Cell Proliferation

To test the effect of NSAIDs on cell proliferation, B16 melanoma cells were incubated with mefenamic acid, diclofenac and nimesulide (50 or 100 μ M) for 5 days. Cell number were decreased when melanoma were stimulated with α -MSH (Fig. 1). In α -MSH stimulated melanoma cells, mefenamic acid enhanced cell growth in a dose-dependent manner (approximately 120% at 50 μ M, 170% at 100 μ M versus α -MSH alone treated cells) (Fig. 1). Diclofenac and nimesulide had no significant effect on cell proliferation.

Melanin formation

To determine the effects of NSAIDs on melanogenesis, B16 melanoma cells stimulated with α -MSH were cultured in the presence of mefenamic acid and diclofenac at 50 or 100 μ M for 5 days. The melanin content of the B16 melanoma cells that had not been treated with stimulants such as α -MSH was very low (Fig. 2A). Melanin formation by B16 melanoma cells were inhibited significantly either mefenamic acid (approximately 46% at 50 μ M and 22% at 100 μ M) or diclofenac treatment (approximately 61% at 50 μ M and 22% at 100 μ M). Nimesulide also inhibit melanogenesis at 100 μ M treatment (37% of control). As shown in Fig. 2B, melanin formation in B16 melanoma was inhibited by NSAIDs treatment.

Tyrosinase activity

We examined the inhibitory effect of NSAIDs (mefenamic acid and diclofenac) on tyrosinase using mushroom tyrosinase. As shown in Fig. 3, mushroom tyrosinase activity was slightly inhibited when treated with either mefenamic acid (approximately 93% at 100 μ M and 87% at 200 μ M or diclofenac (approximately 90% at 200 μ M). Nimesulide also inhibited tyrosinase activity at 100 μ M (79%), but at 200 μ M treatment has no significant inhibition (93%). This inconsistency probably result in yellow color of nimesulide solution, so tyrosinase activity could not measure exactly in our experimental condition.

Effect of NSAIDs on the amount of active form tyrosinase in B16 melanoma cells

To investigate the mechanism of pigmentation inhibition by NSAIDs, we performed DOPA staining on SDS-PAGE gels (Fig. 4.). We can use this to estimate the amount of the active form of tyrosinase generated and removed by de novo synthesis and proteolysis, respectively, because DOPA staining directly detects the tyrosinase activity of protein bands. B16 melanoma cells were treated with either α -MSH alone or α -MSH plus one of the test substances (mefenamic acid or diclofenac at 50 or 100 μ M) for 5 days. Cell lysates were applied to SDS-PAGE and bands that retained tyrosinase activity were detected as described in Materials and Methods. The tyrosinase activity of B16 melanoma cells not stimulated by α -MSH was very low (Fig. 4). In the treatments with α -MSH, tyrosinase activity increased dramatically. Moreover, mefenamic acid, diclofenac and nimesulide inhibited α -MSH enhanced tyrosinase activity in a dose-dependent manner. These results

1 corresponded with the result for the melanin content assays described in
2 Fig. 2.

3 4 5 Western blotting of B16 melanoma proteins treated with NSAIDs

6
7 To investigate whether NSAIDs can influence melanogenic protein
8 expression, Western blotting analysis was carried out using the lysate of
9 B16 melanoma cells treated with NSAIDs and stimulated α -MSH (10
10 nM)(Fig. 5.). When the cells were treated with α -MSH, a significant
11 increase in tyrosinase protein level was observed and TRP-1 expression
12 also increased. All NSAIDs used in this study, inhibited
13 α -MSH-stimulated tyrosinase expression of B16 melanoma cells in a
14 dose-dependent manner. Tyrosinase protein level was decreased by
15 NSAIDs for 3 days treatment (data not shown). TRP-1 expression was
16 slightly inhibited by diclofenac treatment. However, TRP-2 level
17 showed no significant change in the every NSAIDs treatment.

18 19 20 Influence of NSAIDs on melanogenic gene transcription

21
22 To investigate the effects of NSAIDs on melanogenic gene
23 transcription, semi-quantitative RT-PCR analysis was carried out. B16
24 melanoma cells were treated by NSAIDs for 3 days. As shown in Fig. 6,
25 tyrosinase mRNA level was decreased in treatment with mefenamic acid
26 and diclofenac, although nimesulide did not inhibit tyrosinase gene
27 transcription. TRP-1, TRP-2 gene expression showed no change during
28 this experiment. Moreover, the gene expression of transcription factor
29 Mitf also did not change in NSAIDs treatment. The gene expression
30 level of β -actin used as an internal control also showed no change.

Discussion

In the present study, we have demonstrated the inhibitory mechanism of NSAIDs (We used mefenamic acid, diclofenac and nimesulide as NSAIDs in present study.) in B16 melanoma cells. NSAIDs inhibited melanin production that was α -MSH-stimulated melanoma cells. α -MSH acts on cells through the elevation of cAMP concentration resulting in the enhancement of melanin production. α -MSH binds to the melanocortin 1 receptor (MC1R) and activates adenylate cyclase that increases the intracellular cAMP concentration [26]. cAMP activates protein kinase A (PKA), which subsequently phosphorylates cAMP response binding protein (CREB). CREB binds the cAMP response element (CRE) motif of the Mitf promoter and activates Mitf gene transcription [16]. Mitf is a transcription factor that effectively transactivates the expression of tyrosinase and its related genes by binding to their common promoters [27].

NSAIDs significantly inhibited melanin synthesis in α -MSH-stimulated B16 melanoma cells. This result is not explained by the toxicity of NSAIDs because NSAIDs did not show any inhibitory effect on cell proliferation.

There are three well-known enzymes in melanin synthesis, i.e. tyrosinase, TRP-1, TRP-2 [28]. Especially, tyrosinase has a crucial role of melanin synthesis. To clarify the direct inhibitory effect of NSAIDs on mushroom tyrosinase, we performed tyrosinase assay. In tyrosinase assay, NSAIDs slightly inhibited mushroom tyrosinase activity. Diclofenac showed over 40 % inhibition of tyrosinase activity at 2 mM (data not shown). As indicated in Materials and Methods, we applied 50 and 100 μ M at melanin content assay. At 100 μ M, mefenamic acid and diclofenac inhibit approximately 10 %, and nimesulide inhibit 20 %. Therefore, direct inhibitory effect of tyrosinase of these drugs does not

1 have great contribution of anti-melanogenic effect on melanoma cells.
2 For further investigation of the depigmenting mechanism of NSAIDs, a
3 DOPA staining assay was carried out with lysates of α -MSH-stimulated
4 B16 melanoma cells treated with or without chemicals. In this
5 experiment, we could detect intracellular tyrosinase activity in B16
6 melanoma cells. Upon incubation with NSAIDs, activity of intracellular
7 tyrosinase decreased. Therefore, this result shows that NSAIDs might
8 inhibit tyrosinase expression and/or tyrosinase maturation. To
9 investigate the depigmenting mechanism of NSAIDs further, western blot
10 analysis was carried out to show the amount of tyrosinase, TRP-1 and
11 TRP-2. B16 melanoma cells treated with α -MSH showed enhanced
12 tyrosinase expression as compared with unstimulated cells. NSAIDs
13 inhibited tyrosinase expression in a dose-dependent manner. TRP-1
14 protein level also inhibited by diclofenac treatment. On the other hand,
15 mefenamic acid and nimesulide did not decreased protein level either
16 TRP-1 or TRP-2. Diclofenac also did not inhibited TRP-2 expression.

17 For further investigation of the mechanism of down-regulation of
18 tyrosinase protein level by NSAIDs, semi-quantitative RT-PCR was
19 carried out. In this experiment, we assessed tyrosinase, TRP-1, TRP-2
20 and Mitf mRNA levels. Mefenamic acid and diclofenac inhibited
21 tyrosinase mRNA level. However, nimesulide does not inhibited
22 tyrosinase mRNA level. On the other hand, TRP-1 and TRP2, and their
23 transcription factor Mitf did not change by NSAIDs treatment. This
24 result shows that NSAIDs does not influence TRP-1 and TRP-2 mRNA
25 transcription. Also the mRNA level of transcription factor such as Mitf
26 was not inhibited. Mitf plays a crucial role in melanogenesis as the
27 master regulator of tyrosinase, TRP-1 and TRP-2 gene transcription, and
28 subsequently causes hypopigmentation. Therefore, mutation of Mitf can
29 cause pigmentation disorders as observed in Waardenburg Syndrome
30 [29-31], moreover, other transcription factors mutations such as Pax3 and

1 Sox10 cause its disease [32]. Previously, we reported that acetylsalicylic
2 acid (aspirin) inhibit tyrosinase protein level on B16 melanoma cells [22].
3 We also performed RT-PCR assay on aspirin-treated cells. In result of
4 this experiment, aspirin inhibited tyrosinase transcription on 3 days
5 treatment (at 2-3 mM). However, TRP-1, TRP-2 and Mitf mRNA levels
6 were not decreased on same treatment (data not shown). This result is
7 common to mefenamic acid and diclofenac treatment.

8 In this report, we demonstrated the depigmentation effect of
9 NSAIDs. NSAIDs inhibited cAMP-enhanced melanin production in B16
10 melanoma cells. Our results show that mefenamic acid and diclofenac
11 inhibit tyrosinase gene transcription and subsequent protein expression
12 without down-regulation of Mitf. This result implies that these drugs
13 inhibit transcription of tyrosinase. On the other hand, we could not
14 confirm the inhibition of tyrosinase mRNA level by nimesulide treatment.
15 It might be due to suppress of translation of mRNA. The depigmenting
16 mechanism of NSAIDs is not clearly yet. To investigate the further
17 melanogenic mechanism of NSAIDs are very important, and we are
18 studying now underway. This study shows NSAIDs are useful inhibitor
19 of melanogenesis and they might be effective in hyperpigmentation
20 disorders and whitening cosmetics.

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Figure legends

Fig. 1. Effect of NSAIDs (mefenamic acid, diclofenac and nimesulide) on the proliferation of B16 melanoma cells. B16 melanoma cells were cultured for 5 days with or without test substances and harvested as described Materials and Methods. The number of cells was counted. Results are expressed as a percentage of control culture (α -MSH alone treated cells), and data are the means $\pm$ S.D. of at least three separate experiments. $\dagger$ $P < 0.01$ versus media only-treated group, $*P < 0.05$ and $**P < 0.01$ versus α -MSH-treated group.

Fig. 2. (A) Effect of NSAIDs on melanin production. The melanin content of B16 melanoma cells was measured after 5 days of treatment, with or without test substances, as described in Materials and Methods. Results are expressed as a percentage of control (α -MSH treated cells), and data reported are the means $\pm$ S.D. of at least three separate experiments. $\dagger$ $P < 0.01$ versus media only-treated group, $*P < 0.05$ and $**P < 0.01$ versus α -MSH-treated group.

(B) Photograph of B16 melanoma cells after 5 days NSAIDs treatment. B16 melanoma cells were treated with or without NSAIDs (150 μ M). Scale bar: 100 μ m

Fig. 3. Effect of NSAIDs (mefenamic acid or diclofenac) on mushroom tyrosinase activity. Effect of mefenamic acid and kojic acid on L-DOPA oxidation activity of tyrosinase was measured (%) as described in Materials and Methods. Results are expressed as the percentage of the control, and data are means $\pm$ S.D. of at least three separate experiments. $*P < 0.05$ and $**P < 0.01$ versus control

group.

Fig. 4. Effect of NSAIDs (mefenamic acid or diclofenac) on intracellular active tyrosinase amount. B16 melanoma cells were treated with NSAIDs and stimulated with α -MSH. After 5 days treatment, cell lysates were applied to SDS-PAGE gels. The gels were then soaked in DOPA solution as described in Materials and Methods.

Fig. 5. Effect on melanogenic protein expression of NSAIDs. B16 melanoma cells were treated with or without NSAIDs and stimulated with α -MSH (10 nM). After treatment, Western blotting of tyrosinase, TRP-1 and TRP-2 was performed as described in Materials and Methods. The loading control was assessed using anti- β -actin antibody.

Fig. 6. Semi-quantitative RT-PCR analysis of melanogenic gene mRNA levels in NSAIDs-treated B16 melanoma cells. Cells were treated with NSAIDs (200 μ M) for 3 days. Total RNA was extracted and cDNA was prepared. Equivalent amount of cDNA were amplified using primers specific for Tyr, TRP-1, TRP-2 and Mitf. The level of β -actin mRNA was checked as a control to ensure the even loading of target cDNA.

Fig. 1.

A

Fig. 2. A

B

α -MSH 0 nM

α -MSH 10 nM

Mefenamic acid (150 μ M)
+ α -MSH (10 nM)

Diclofenac (150 μ M)
+ α -MSH (10 nM)

Nimesulide (150 μ M)
+ α -MSH (10 nM)

Fig. 2. B

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.