Downregulation of NF-κB activation in human keratinocytes by melanogenic inhibitors

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Key words: Melanogenic inhibitors, ultraviolet light B, transfectant HaCaT cell; NF-κB activity; keratinocyte

Summary

Exposure of skin cells, particularly keratinocytes to various nuclear factor-kappaB (NF-κB) activators [e.g. tumor necrosis factor-α, interleukin-1, lipopolysaccharides, and ultraviolet light] leads to phosphorylation and degradation of the inhibitory protein, IκB. Liberated NF-κB is translocated into the nucleus where it can change or alter expression of target genes, resulting in the secretion of extracellular signaling molecules including melanotrophic factors affecting melanocyte. In order to demonstrate the possible role of NF-κB activation on the synthesis of melanotrophic factors from the keratinocytes, the activities of NF-κB induced by melanogenic inhibitors (MIs) were determined in human HaCaT keratinocytes transfected with pNF-κB-SEAP-NPT plasmid. Transfectant cells released the secretory alkaline phosphatase (SEAP) as a transcription reporter in response to the NF-κB activity and contain the neomycin phosphotransferase (NPT) gene for the dominant selection marker for geneticin resistance. MIs such as niacinamide, kojic acid, hydroquinone, resorcinol, arbutin, and glycolic acid were preincubated with transfectant HaCaT cells for 3 h and then ultraviolet B (UVB) was irradiated. NF-κB activation was measured with the SEAP reporter gene assay using a fluorescence detection method. Of the MIs tested, kojic acid (IC_{50} = 60 μM) was found to be the most potent inhibitor of UVB-upregulating NF-κB activation in transfectant HaCaT cells, which is followed by niacinamide (IC_{50} = 540 μM). Pretreatment of the transfectant HaCaT cells with the MIs, especially kojic acid and niacinamide, effectively lowered NF-κB binding measured by electrophoretic mobility shift assay. Furthermore, these two inhibitors remarkably reduced the secretion level of IL-6, one of melanotrophic factors, triggered by UV-radiation of the HaCaT cells. These observations suggest that MIs working at the *in vivo* level might act partially through the modulation of the synthesis of melanotrophic factors in keratinocyte.

Introduction

Skin is a primary target for stimuli such as UVR and various synthetic chemicals, frequently leading to inflammation. Especially it has been well established that UV-induced melanogenesis is a major physiological stimulus for human skin pigmentation [1, 2]. Keratinocytes and melanocytes form a functional unit called epidermal melanin unit and closely interact to produce and distribute melanin in human skin during the pigmentation process in such a way that while keratinocytes produce paracrine factors that affect melanocytes proliferation, dendricity, and melanin synthesis [3-5], melanocytes control the synthesis of melanin and transfer it to the adjacent keratinocytes [6].

NF-κB is an inducible transcription factor that is activated in response to the variety of environmental stimuli and has a pivotal role in the regulation of genes responsible for inflammatory and immune responses [7-10]. NF-κB, in unstimulated cells, is complexed in the cytoplasm through interactions with the inhibitory protein, IκB. Exposure of skin cells, particularly keratinocytes to various NF-κB activators [e.g., tumor necrosis factor, interleukin-I, lipopolysaccharides, and ultraviolet light] leads to phosphorylation and degradation of the inhibitory protein, IκB [11]. Liberated NF-κB is translocated into the nucleus where it can change or alter expression of target genes, resulting in secretion of extracellular signaling molecules including melanotrophic factors affecting melanocyte [12-15]. The structure, regulation and function of NF-κB have been well studied and reviewed by several investigators [16-22].

Tyrosinase in melanocytes that converts tyrosine to dopaquinone is the rate-limiting enzyme involved in the synthesis of melanin and represents the major regulatory step in melanogenesis [23, 24]. Kojic acid (2-hydroxymethyl-5-hydroxy-γ -pyrone) isolated from *Aspergillus oryzae* is a well-known inhibitor of tyrosinase. This confers kojic acid an indispensable ingredient for downregulation of skin hyperpigmentation [25, 26]. However, kojic acid has been found to confer significant impact on normal keratinocyte physiology such as dramatic suppression of cytokine synthesis and secretion (unpublished observation). Another

longstanding clinical observation is the ability of steroids to suppress skin pigmentation upon its prolonged use without concomitant inhibition of tyrosinase activity or synthesis [27, 28]. These observations leads us to the assumption that many of the compounds currently known as tyrosinase inhibitor might also work as co-modulators, if not primary, of synthesis or secretion of keratinocytes' melanotrophic factors in their *in vivo* applications.

We have previously investigated for the inhibitory effect of kojic acid on the activation of NF-κB in transfectant HaCaT and SCC-13 keratinocytes and proposed that antimelanogenicity of kojic acid might be mediated by the modulation of NF-κB activation in keratinocytes [29]. We extend this study to the various well-known MIs (i.e., niacinamide [30, 31], hydroquinone [32-34], arbutin [35, 36], resorcinol [37], glycolic acid [38], and kojic acid [27, 28]) (Fig. 1) to obtain the proof of concept. First, we examined the NF- B activities in UV-radiated transfectant HaCaT cells with or without pretreatment of MIs using our cell-based reporter gene expression assay, which was then confirmed by electromobility shift assay. Further, we investigated the NF- B-associated cytokines or melanotrophic factors released from the HaCaT cells the level of which showed the similar pattern of NF-kB activity upon the treatment of MIs [39, 40].

We now report the effects of known MIs on NF-κB activation in human transfectant HaCaT cells and their possible roles as the modulators of secretion level of NF-κB-associated keratinocyte's paracrine mediators in downregulation of melanogenesis.

Material and methods

Materials

Great EscAPe Fluorescence detection kit was obtained from Clontech Laboratories, Inc., Palo Alto, CA. Geneticin (antibiotic G-418) and cell culture media from Gibco BRL, Grand Island, NY. Kojic acid, arbutin, glycolic acid, resorcinol, niacinamide, hydroquinone and dimethyl sulfoxide (DMSO) from Sigma Chemical Co., St. Louis, MO and other chemicals and

solvents from Aldrich Chemical Co., Inc., Milwaukee, WI. Arbutin was first dissolved in DMSO and then made to a final concentration in culture media of 0.1 %, and other chemicals directly dissolved in the culture media. Human HaCaT cells were originally obtained from Dr. Norbert E. Fusenig (German Cancer Research Center, Heidelberg, Germany). The pNF-κB-SEAP-NPT plasmid (Fig. 2) that permits expression of the secretory alkaline phosphatase (SEAP) reporter gene in response to the NF-κB activity and contains neomycin phosphotransferase (NPT) gene for the geneticin resistance in host cells was constructed and transfected into HaCaT cells as previously described [41]. ELISA kits for the measurement of cytokines were purchased from R & D System (Minneapolis, MN, U. S. A.)

Cell culture

Transfectant human HaCaT cells were cultured with 500-600 μg/mL of geneticin (100 mg/mL) for selection and maintenance of stable transformants. These cells were maintained at subconfluence in a 95% air, 5% CO_2 humidified atmosphere at 37°C. The medium used for routine passage was Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were counted with a hemocytometer and the number of viable cells was determined by trypan blue dye exclusion.

Ultraviolet radiation of cells

Transfectant human HaCaT cells (3×10^6) were UV-radiated with a peak emission at a wavelength of 312 nm in the tissue culture dishes [1, 5]. The source of ultraviolet was a Spectrolinker XL-1000 UV (Spectronics Co., USA) stimulator equipped with a UVB radiation source of microprocessor-controlled energy (mJ/cm^2). In brief, single cell-derived stable transfectant HaCaT (3×10^6) cells were plated on a tissue culture dish with 5 ml medium, which was decanted after 24 h of incubation. Then the cells were washed twice with phosphate-buffered saline (PBS) and added to fresh media. MIs were directly added to the culture medium at 0 h and incubated for 3 h. UVB was radiated to tissue culture dish directly at 60

mJ/cm^2. The aliquots (25 μl) from both the control and MIs-treated cultures were taken at 0, 3, 6, 9, and 12 h, heated at 65 °C for 5 min to eliminate the endogeneous alkaline phosphatase activity, and used immediately or stored at –20 °C until assayed.

Reporter (SEAP) gene assay

Reporter enzyme activity (SEAP) was measured as previously described [41]. In brief, MIs were directly added to the culture medium after 24 h incubations. Incubation mixtures, containing dilution buffer (25 μl), assay buffer (97 μl), culture media (enzyme source, 25 μl), and 4-methylumbellifery phosphate (MUP, 1 mM, 3 μl) in 96 well plate were left for 60 min in the dark at room temperature. Fluorescence from the product of the SEAP/MUP was measured using a 96 well plate fluorometer (Molecular Devices, Gemini XS) by excitation at 360 nm and measuring light emission at 449 nm.

Preparation of nuclear extracts

Nuclear proteins were extracted by using a modification of Andrew's method [42]. Normal HaCaT cells were plated at the density of 10^5 cells/cm^2 on six-well dishes. Twenty hours later, cells were treated as described previously [29, 41]. Cells were incubated for 3h after UVB radiation (60 mJ/cm^2). Scrapped and pelleted cells were resuspended in 1 ml of ice-cold lysis buffer (10 mM Tris-Cl, pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 1% NP-40) and incubated for 15 min on ice with an occasional vortexing. After centrifugation at 900 g for 10 min at 4°C and washing of the nuclei pellet, 1 ml of Buffer A (20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 ml KCl 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) was added. After centrifugation at 900 g for 10 min at 4°C and washing of the nuclei pellet, 30~50 μl of ice-cold hypertonic buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DDT, 0.5 mM PMSF, and protease inhibitors cocktails) was added and the pellet was incubated at 4°C for 40 min with a constant shaking. Nuclear extracts were isolated by centrifigation at 14,000 g for 30 min at 4°C and the protein content in aliquots was determined

by Bradford assay [43]. Nuclear extracts were stored at -70°C until use.

Electrophoretic mobility shift assays (EMSA)

The oligonucleotide probe used for EMSA contained the NF- B consensus sequence. Double-stranded NF-κB consensus oligonucleotide (AGT TGA GGG GAC TTT CCC AGG C) and mutant one (AGT TGA GGC GAC TTT CCC AGG C) were purchased from Promega. The probes were 5'-end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (Promega Corp., Madison, WI) and separated from the unincorporated label by mini column chromatography. Binding reaction was performed at room temperature for 30 min with 10 μg of nuclear protein in 20 μl of binding buffer (100 mM Tris-Cl, pH 7.9, 250 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 0.5% NP-40, 2.5% BSA, 50% glycerol) containing 1 μg of Poly (dI-dC) and 2 μl of 5'-labeled probe (15,000 to 30,000 cpm). In the competition experiments, a 40-fold molar excess unlabeled probe or unlabeled mutated probe (sc-2511, Santa Cruz) was added before the labeled probe. DNA-protein complex was separated from the unbound probe on 6% polyacrylamide gel in 0.25X TBE running buffer at 100 V. After electrophoresis, the gels were dried and visualized by autoradiography.

Measurement of cytokines in HaCaT cells

After pretreatment of MIs, the culture media from non-exposed and UV-exposed HaCaT cells were collected, sterile-filterd, and stored at -70℃ until assayed. The concentration of IL-6 was determined by the commercial ELISA kit according to the manufacturer's instructions. Results are expressed as the mean ± SD for triplicate determination.

Cytotoxicity of melanogenic inhibitors

Cytotoxicity of MIs was evaluated by MTT assay [44]. In brief, transfectant HaCaT ($3x10^4$) cells were plated on 96 wells of ELISA plate, incubated for 48 h, and washed twice with 100 l of PBS. They were incubated with MIs for 3 h and then exposed to UV (60 mJ/cm^2)

directly. After 9 h incubation, the extension of incubation for another 3 h at 37°C was made with 200 l of MTT in PBS. Then the solution was removed and formazan dissolved in 200 l dimethyl sulfoxide was added. The absorbance of the solution was measured at 540 nm using an ELISA plate reader. The control was made of medium without MIs and UV radiation.

Data analysis

Data were summarized as mean ± SD. For the statistical analysis, we perfomed a one-way analysis of variance (ANOVA) for repeated measurements of the same variable. Then, Duncan's multiple range t-test was used to determine which means were significantly different from the mean of the control. We considered significant difference at $p<0.05$ and $p<0.01$.

Results

Suppression of UV-upregulating NF-κB activity in transfectant HaCaT cells by MIs

UV radiation (60mJ/cm^2) increased the level of NF-κB activities in transfectant HaCaT cells in a time-dependent manner upto 3-fold at 9h incubation compared to the unradiated control (data not shown). Treatment of the transfectant HaCaT cells with MIs such as niacinamide (5 mM), hydroquinone (50 μM), arbutin (10 mM), glycolic acid (10 mM), resorcinol (10 mM) and kojic acid (3 mM) reduced the level of UV-upregulating NF-κB activity by 95, 47, 74, 70, 63 and 90%, respectively with respect to the unradiated control (Fig. 3). Based on the further elaboration of this experiment, the 50% inhibitory concentrations (IC_{50}) of MIs were determined and summarized in Table 1. Kojic acid was the most potent suppressor of UV-upregulating NF-κB activity among the MIs tested, most of which behaved in the concentration-dependent manner except hydroquinone showing cellular toxicity at higher than 50 μM. This suggests that modulation of the level of NF-κB activity in keratinocytes appeared to be associated with the potency of melanogenic inhibition.

To confirm the co-linearity of the level of NF-κB activity in terms of reporter gene

product with the relative amount of NF-κB, electromobility shift assay was performed by incubating the nuclear extracts from transfectant HaCaT cells which had been pretreated with MIs for 2h and then UVB-radiated (60 mJ/cm^2) with the labeled oligonucleotides for 3h. The strong banding of NF-κB in response to UVB radiation was dramatically weakened by pretreatment of most MIs tested except glycolic acid and resorcinol (Fig. 4A). After band intensities were converted into bars, it was easy to find kojic acid as the most potent suppressor of UV-upregulating NF-κB activity (Fig. 4B). Banding specificity was verified by the competition experiment; radioactivities were not detected with excess amount of unlabeled κB olionucleotides, but detected either with labeled mutant oligonucleotides or in the absence of unlabeled oliognucleotides (Fig. 4C). These results indicated that the binding protein fractions of nuclear extracts is specific for NF-κB. Even though each of MIs showed difference in the level of suppression of UV-upregulating NF-κB amount, it was evident that MIs could protect the cells from UV damage and the band intensities shown by electromobility shift assay was remarkably similar to the pattern of the cell-based reporter gene assay system in Fig. 3.

Suppression of IL-6 secretion from the UV-radiated transfectant HaCaT cells by MIs

Among the representative cytokines responsive to UV radiation, the level of IL-6 in the supernatants was detected as a significant biomarker in response to the MIs added in the culture medium of UV-radiated transfectant HaCaT cells (Fig. 5). The concentration of IL-6 secreted in the culture medium increased dramatically after UV radiation to the level of 600 pg/ml compared to the less than 200 pg/ml in the unradiated control. This elevation of IL-6 was effectively suppressed by pre-treatment of MIs except hydroquinone and glycolic acid. Again, the suppression pattern of IL-6 secretion was remarkably similar to the suppression of NF-κB activity in the previous set of experiments with niacinamide, arbutin, resorcinol and kojic acid (Fig. 4).

Cell viability not affected by the UV dose used and the concentration of MIs tested

The UV dose and the concentration of MIs tested throughout the experiments in this

study were not harmful to affect the cell viability. No significant difference in the cell viability was found between UV-radiated and unradiated cells, and in the 9h pretreatment of MIs at the selected concentration.

Discussion

While the chemical basis of melanogenesis inside melanosome in melanocytes has been well documented, the cellular and molecular basis of some well-known MIs' action mechanism in cytoplasm and cell membrane of melanocytes has not been fully explored. In human skin, each of melanocytes is surrounded by keratinocytes at the ratio of 1:35 forming an epidermal melanin unit, which serves a basis for the importance of two types of skin cells in skin pigmentation. This is exemplified by the cell-cell interaction of those two types of cells; UV activates keratinocytes to produce extracellular signaling molecules including melanotrophic factors such as ET-1 [45], GM-CSF [46], and MSH which render signals to nearby melanocytes to promote melanogenesis [47]. Since these extracellular signaling molecules or melanotrophic factors are normally outcome of NF-κB activation in response to UV-radiation on keratinocytes and any topical agents should work first at the level of keratinocytes *in vivo*, we assumed that well-known MIs might regulate melanogenesis through the modulation of NF-kB activation in the cells at the uppermost layer of skin, i.e, keratinocytes.

Using HaCaT cells, spontaneously immortalized human keratinocytes capable of undergoing normal differentiation mimicking normal human keratinocytes [48, 49], we have shown that UV radiation elevates NF-κB activity[23]. To obtain the early evidence of MIs as NF-κB modulators, we tested well-known antimelanogenic compounds such as niacinamide, hydroquinone, arbutin, resorcinol, glycolic acid, and kojic acid on the HaCaT cells (Fig. 1). These MIs, especially kojic acid and niacinamide, were found to remarkably suppress the NF-κB activity which otherwise had been significantly elevated by UVB radiation. These results strongly suggested that MIs could also act as suppressors of NF-κB activation in the keratinocytes, possibly reducing the secretion level of melanotrophic factors such as ET-1 [39].

In fact, it has been generally accepted that UV-induced ROIs (reactive oxygen intermediates) activate NF-κB in the keratinocytes, allowing secretion of melanotrophic cytokines or hormones [50].

We further investigated for the existence of any relationship between NF-κB activation and IL-6, a melanotrophic cytokine in keratinocytes. NF-κB activation was found to be closely associated with upregulation of IL-6 in keratinocytes by UV radiation. Imokawa *et al.* also reported that the UV-induced increase in IL-1α in epidermis triggers the release of endothelin-1 from keratinocytes, which in turn stimulates melanin production by melanocytes [39]. Since there is a report that UVB-enhanced IL-6 production and mRNA expression might be mediated by IL-1α [40], it would be possible that NF-κB activation is associated with the synthesis and secretion of IL-6 as well as endothelin-1 via IL-1α within keratinocytes, eventually stimulating melanogenesis [51]. Suppression of UV-induced NF-κB activation was not due to the harmful effect of the MIs, especially kojic acid and niacinamide, because any visible signs adverse to the cell viability such as cytotoxicity and morphological change due to MIs were not detected. We also examined the change in NF-κB activity induced by MIs under the condition of UV-unradiated transfectant HaCaT cells and found that MIs did not give any detectable suppression of NF-κB activation (data not shown). These results suggest that MIs are capable of suppressing the UV-induced NF-κB activation beyond the basal level in the keratinocytes.

Most MIs currently used as skin lightening agents have been selected out by their warrant of tyrosinase inhibition *in vitro*. However, when these compounds were applied onto the skin, they might not work by passing through the several layers of keratinocyte directly to the melanocytes in the basal layer of epidermis, but rather work on the keratinocytes at the upper-most layer. Therefore it is possible that even the chemicals with high potency of specific tyrosinase inhibition in melanocyte may or may not good anti-melanogenic topical agents in practical sense.

In this study we provided evidence that the MIs frequently used in practice indeed were found to affect the cell physiology of keratinocytes by suppressing the NF-κB activity

otherwise elevated by UV radiation, and thereby reduce one of the melanotrophic cytokines, IL-6. At the same time, we propose that our cell-based assay system using transfectant HaCaT cells would be a useful tool for the selection of chemicals suppressing the NF-κB activation triggered by external physicochemical stimuli, which could be exploited for the clinically applicable anti-melanogenic agents with much ease.

Acknowledgements

This work was supported by the grant from the Korea Health 21 R & D Project, Ministry of Health & Welfare, Republic of Korea (01-PJ2-PG6-01NA01-002)..

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Table. 1. Determination of IC_{50} of MIs on human HaCaT cells

Melanogenic Inhibitors	IC_{50} (mM)
Niacinamide	0.54
Hydroquinone	*N.E.
Arbutin	3.62
Glycolic acid	6.79
Resorcinol	9.87
Kojic acid	0.06

* N.E. : no effect

Figure legends

Fig 1. Chemical structures of melanogenic inhibitors

Fig 2. Diagram of pNF-κB-SEAP-NPT plasmid

Fig. 3. Suppression of UV-upregulating NF-κB activity in transfectant HaCaT cells by melanogenic inhibitors

1. UV-radiated; **2.** UV-unradiated control; (**3-8**) MI-treated before UV-radiation; **3.** 5 mM niacinamide; **4.** 50 μM hydroquinone; **5.** 10 mM arbutin; **6.** 10 mM glycolic acid; **7.** 10 mM resorcinol; **8.** 3 mM kojic acid. Data are expressed as mean ±SD (n=3). Significant differences in NF-κB activities between UV-radiated and MI-treated before UV radiation are indicated by *p <0.05 and **p<0.01. Significant difference in NF-kB activities between UV-radiated and UV-unradiated control is indicated by $^{\#\#}$p<0.01.

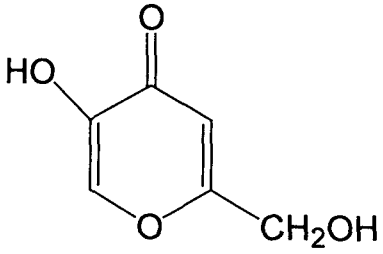
Fig. 4. Confirmation of the co-linearity by electromobility shift assay of the change in the level of NF-κB in transfectant HaCaT cells by melanogenic inhibitors

Nuclear protein extracts (10 μg) from transfectant HaCaT cells were incubated with ^{32}P-labeled κB oligonucleotides, and the resultant DNA-protein complexes resolved by polyacrylamide gel electrophoresis (A). Corresponding band intensities were shown in (B). Competition experiment using unlabeled κB oligonucleotides (cold) or labeled mutated κB oligonucleotide (mut) confirmed the binding specificity of the κB oligonucleotides (C). **1.** UV-radiated; **2.** UV-unradiated; (**3-8**) MI-treated before UV-radiation; **3.** 5 mM niacinamide; **4.** 50 μM hydroquinone; **5.** 10 mM arbutin; **6.** 10 mM glycolic acid; **7.** 10 mM resorcinol; **8.** 3 mM kojic acid.

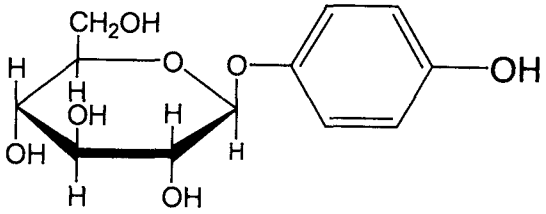
Fig. 5 . Pattern of suppression of IL-6 secretion in the UV-radiated transfectant HaCaT cells by melanogenic inhibitors

Confluent transfectant HaCaT cells were preincubated with MIs and then UV-radiated (60 mJ/cm^2). The culture media were collected at 9h of post-UV radiation and the level of IL-6 determined by ELISA. **1.** UV-radiated; **2.** UV-unradiated control; (**3-8**) MI-treated before UV-radiation; **3.** 5 mM niacinamide; **4.** 50 μM hydroquinone; **5.** 10 mM arbutin; **6.** 10 mM glycolic acid; **7.** 10 mM resorcinol; **8.** 3 mM kojic acid. Significant differences in NF-κB activities between UV-radiated and MI-treated before UV radiation are indicated by *p <0.05 and **p <0.01. Significant difference in NF-kB activities between UV-radiated and UV-unradiated control is indicated by $^{\#\#}$p <0.01. ■stands for IL-6 secretion unaffected by corresponding MI.

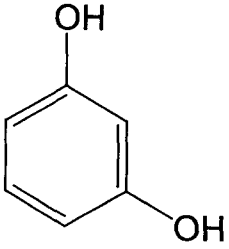
Fig. 1



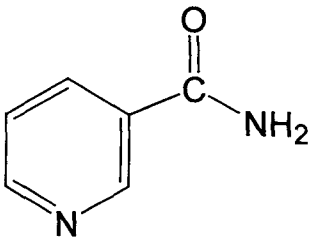
Kojic acid



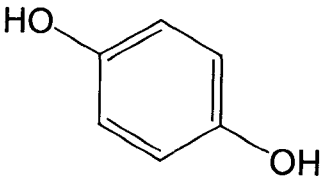
Arbutin



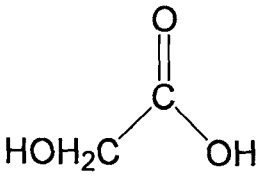
Resorcinol



Niacinamide



Hydroquinone



Glycolic acid

Fig. 2

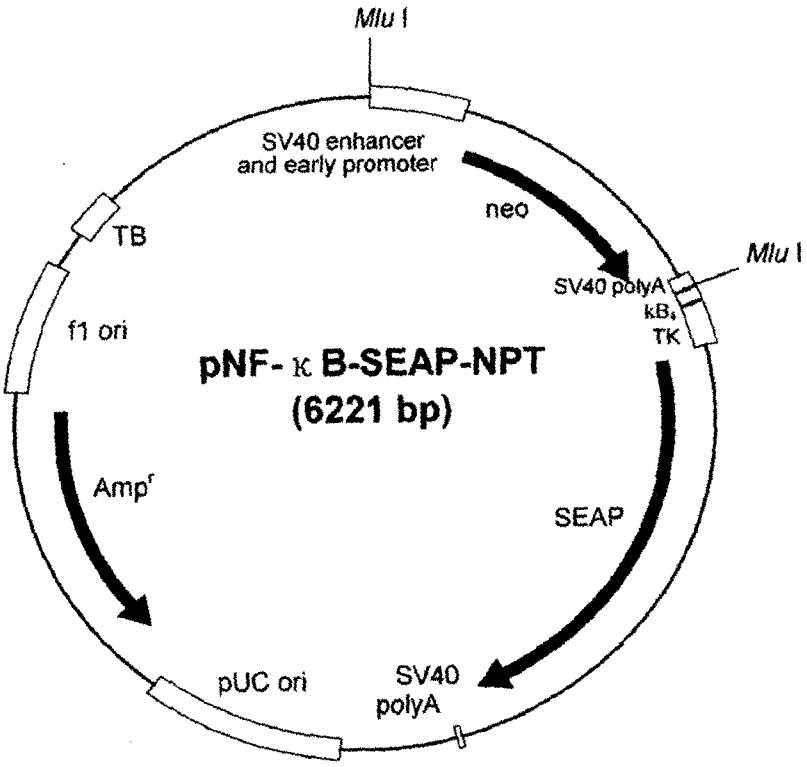

Mlu I
SV40 enhancer
and early promoter
neo
TB
Mlu I
SV40 polyA
kB4
TK
f1 ori
pNF-κB-SEAP-NPT
(6221 bp)
Amp^r
SEAP
pUC ori
SV40
polyA

Fig. 3

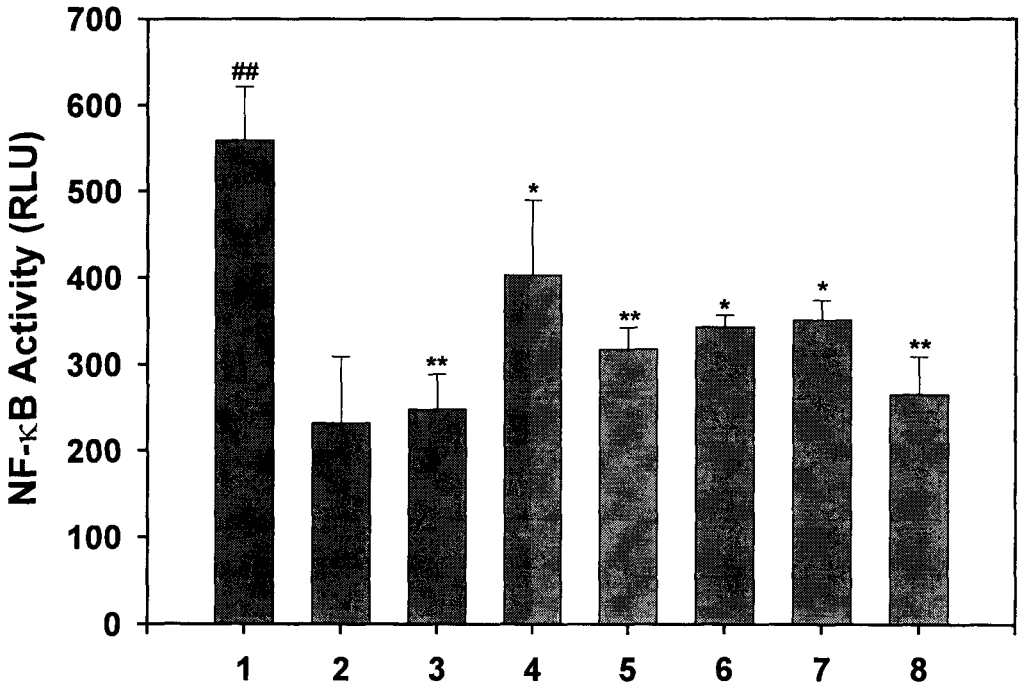


Fig. 4

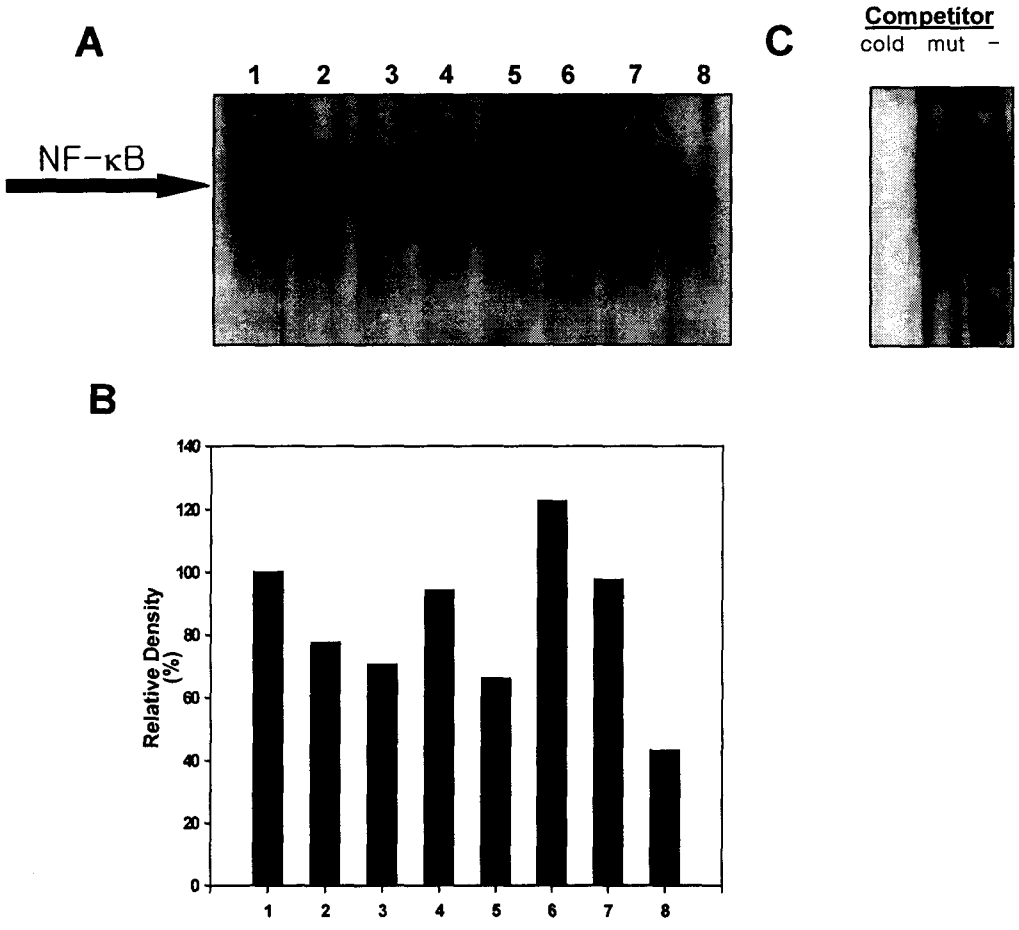
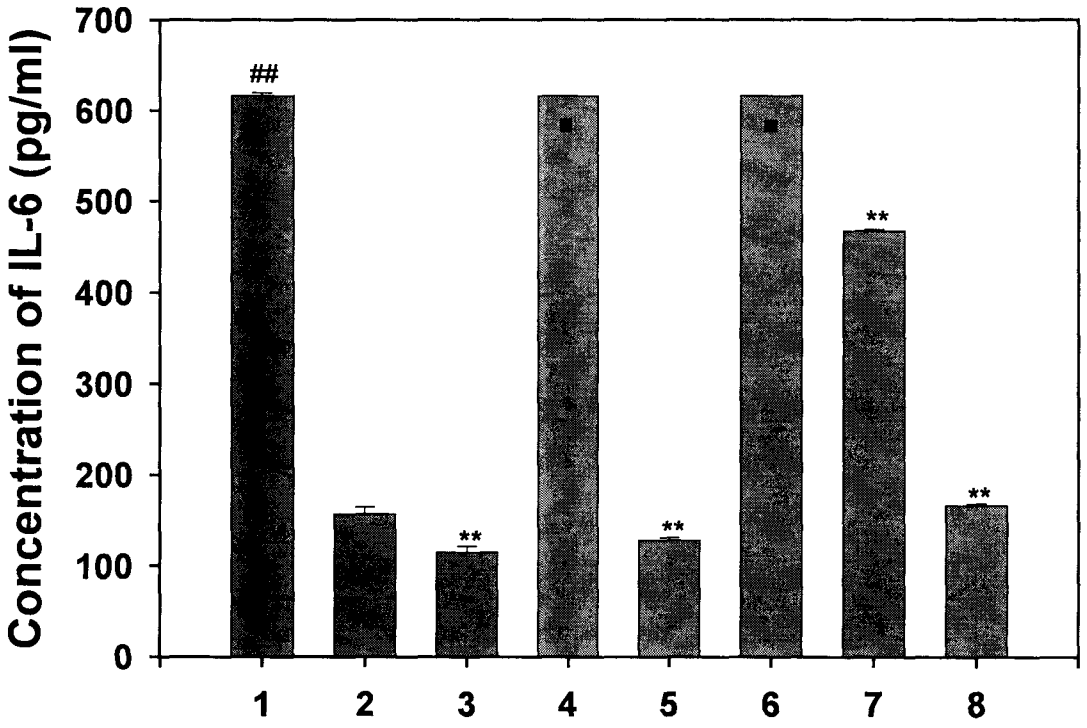

A
1 2 3 4 5 6 7 8
NF-κB
C
Competitor
cold mut -
B
Relative Density (%)
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8

Fig. 5


Concentration of IL-6 (pg/ml)
700
600
500
400
300
200
100
0
##
**
**
**
**
1
2
3
4
5
6
7
8