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PRINCIPAL INVESTIGATOR: Michael B. Sporn, M.D.

CONTRACTING ORGANIZATION: Dartmouth College
Hanover, NH 03755

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Hanover, NH 03755

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13. ABSTRACT *(Maximum 200*

Triterpenoids, natural products related to steroids and retinoids, represent an important class of new structures for drug discovery, with potential applications in many fields of medicine, particularly cancer. This project involves the development of new synthetic triterpenoids for eventual use as agents for chemoprevention or chemotherapy of breast cancer. Although the naturally occurring triterpenoids, ursolic acid (UA) and oleanolic acid (OA), have been shown to have some anti-carcinogenic activity, they are relatively weak agents. During the past year, we have synthesized over 100 new triterpenoids, and many of these have been assayed as inhibitors of de novo formation of inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2), two enzymes highly relevant to the carcinogenesis in the breast. We have also screened these new triterpenoids as inducers of differentiation in HL-60 leukemia cells and as non-cytotoxic suppressors of estrogen-stimulated growth in MCF-7 breast cancer cells. Several new triterpenoids are markedly more active in these assays than their respective parents, UA or OA.

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Michael B. Spera, M.D. 9/26/97

PI - Signature Date

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(5) INTRODUCTION

There is a major need for new drug discovery in the field of breast cancer, and this project is directed toward that goal. There is a particular need for development of new agents that will inhibit progression of premalignant and early malignant lesions to more aggressive, invasive and metastatic stages, since screening techniques are now identifying large numbers of women with such early breast lesions. Furthermore, advances in genetic testing are leading to the identification of many women with a high risk for future development of breast cancer, for whom at present there is no satisfactory preventive modality.

Triterpenoids of an ursane or oleanane structure are very widely distributed in nature, occurring in hundreds of plants all over the world. Although triterpenoids are widely used for medicinal purposes in many Asian countries, this class of molecules, which resemble steroids in their chemical structure, biogenesis, and pleiotropic actions, has not impacted on the practice of Western medicine. Triterpenoids, like the steroids, are formed in nature by the cyclization of squalene, with the retention of all 30 carbon atoms in molecules such as oleanolic acid (OA) and ursolic acid (UA). Although OA and UA are known to have numerous pharmacological activities, including chemoprevention of cancer in experimental animals (Nishino et al., 1988; Huang et al., 1994), the potency of these naturally occurring molecules is relatively weak. Chemical synthesis of new steroid analogs has provided many useful derivatives that are more potent and specific than natural parent structures. With this as a model, and considering the known anti-carcinogenic activities of OA and UA, we have started a new project to synthesize and characterize a new series of synthetic triterpenoid analogs as potential inhibitors of mammary carcinogenesis, using suppression of the formation of nitric oxide and prostaglandins, as well as induction of cadherins/catenins, as assay systems. In addition to these assay systems, we have also performed preliminary assays on new triterpenoids as non-cytotoxic inhibitors of DNA synthesis in human MCF-7 breast cancer cells.

The inducible enzymes that mediate the formation of nitric oxide and prostaglandins (iNOS and COX-2, respectively) are now the focus of major interest in carcinogenesis studies. Elevated activity of both of these enzymes has been particularly implicated in colon carcinogenesis (Takahashi et al., 1997; Prescott and White, 1996), but there is also evidence for their causative involvement in breast cancer (Thomsen et al., 1995; Liu and Rose 1996). Extensive data exist for the role of the cadherin/catenin system in breast cancer (Anzano et al., 1994).

(6) BODY

a) Experimental Methods

1. Studies on Human Breast Cancer Cells

Cell Maintenance:

MCF-7, T47D, or SK-Br-3 cells were maintained in DMEM/F12 with phenol red, 10% fetal bovine serum (Hyclone), Pen/Strep, in a 37°C, 5% CO_2 humidified incubator.

Treatment for Experiment:

Cells were harvested by trypsinization, resuspended in experimental media (RPMI without phenol red, 10% charcoal/dextran-stripped FBS (Hyclone), Pen/Strep), sedimented and washed once with the same media. Cells were then seeded in experimental media at 1200 cells per well in 96-well plates for MTT assay, 6000 cells per well in 24-well plates for ^{3}H-thymidine incorporation, or 10^6 cells per 9-cm dish for RNA extraction.

Addition of reagents:

Equal volume of experimental media containing 17 β-estradiol (final concentration = 10 pM), desired triterpenoid compound dissolved in DMSO, or vehicle alone at final concentration = 0.1% was added to the cells. Unstimulated control wells received vehicle in experimental media without 17 β-estradiol. Cells were incubated in compounds for three days (^{3}H-thymidine incorporation and RNA extraction) or five days (MTT assay).

Assays

1)MTT

1/10 volume of 5mg/ml MTT (Sigma) in experimental media was added to the cells. After 3-4 hours incubation at 37°, the media was aspirated and 100 µl of DMSO was added to each well to solubilize the dye. Absorbance at 570 nm was read using a microtiter plate reader.

2)Thymidine incorporation

5 µCi ^{3}H-thymidine was added to each well. After two hours incorporation time, the media was aspirated, the wells were washed, and the monolayer was fixed with 10% TCA. Nucleic acids were then solubilized with 0.2 N NaOH, 40 µg/ml salmon sperm DNA, and incorporated ^{3}H was measured.

3)Northern blot

Total RNA was extracted using the TRIzol method (Life Technologies) and run on a MOPS-agarose gel with 1.85% formaldehyde. RNA was transferred to a nylon membrane, cross-linked, and hybridized to ^{32}P-labeled probes for two days.

4)Western blot

Total protein was extracted from cells after exposure to compounds for three days. Equal amounts of protein (based on BCA assay) were loaded on

polyacrylamide gels and transferred to nitrocellulose. The membranes were incubated with β-catenin antibodies (Transduction Laboratories). Detection was by chemiluminescence using Amersham ECL reagents, and films were scanned by densitometry.

2. Studies on Macrophages

Full details of methods for cell culture of primary mouse macrophages and the macrophage-like cell line, RAW 264.7, are given in the attached manuscript (submitted for publication), "Novel Triterpenoids Suppress Inducible Nitric Oxide Synthase (iNOS) and Inducible Cyclooxygenase (COX-2) in Mouse Macrophages," by Nanjoo Suh, Tadashi Honda, Heather Finlay, Aaaron Barchowsky, Charlotte Williams, Nicole Benoit, Qiao-wen Xie, Carl Nathan, Gordon W. Gribble, and Michael B. Sporn, which describes the suppression of de novo formation of iNOS and COX-2 by synthetic triterpenoids made with support from this grant. Likewise, methods for assay of mRNA, protein, and enzyme product for bothiNOS and COX-2 are presented in detail in this manuscript.

b) Results and Discussion

1. Synthesis of New Triterpenoids

The synthesis of 17 new triterpenoid derivatives of oleanolic and ursolic acids is described in detail in the attached manuscript, "New Enone Derivatives of Oleanolic Acid and Ursolic Acid as Inhibitors of Nitric Oxide Production in Mouse Macrophages," by Tadashi Honda, Heather Finlay, Gordon Gribble, Nanjoo Suh, and Michael B. Sporn, published in Bioorganic & Medicinal Chemistry Letters 7: 1623-1628, 1997. Structures of other triterpenoids reported here are shown in Figure 1.

2. Results with Human Breast Cancer Cells

Suppression of DNA synthesis in MCF-7 human breast cancer cells by 7 triterpenoids, without evident cytotoxicity, is shown in Figure 2. At this time, there is no apparent set of structure-activity relationships. Studies on modulation of ß-catenin expression have been pursued in SK-Br-3 cells. Figure 3 shows that all-trans-retinoic acid is a potent inducer of ß-catenin expression in these cells, as measured by Western blot analysis after 3 days of treatment. However, we have yet to see a strong inductive effect on ß-catenin with any triterpenoid that we have tested so far. In fact, as shown in Figure 4, 3-keto-oleanolic acid (3-keto-OA) and 3-epi-ursolic acid (3-epi-UA), when tested at 10 micromolar, appear to have an inhibitory effect on ß-catenin expression; furthermore, these two triterpenoids appear to block the stimulatory effect of all-trans-retinoic acid. In contrast, as shown in Figure 4, 3-epi-oleanolic acid (3-epi-OA) has a slight stimulatory activity on ß-catenin expression when tested at 10 micromolar. A large number of new triterpenoids remain to be tested in this assay system. One other significant observation that we have made is shown in Figure 5. TP-82, which was shown in Figure 2 as a potent suppressor of DNA synthesis in MCF-7 cells, is shown in Figure 5 to downregulate the expression of the estrogen

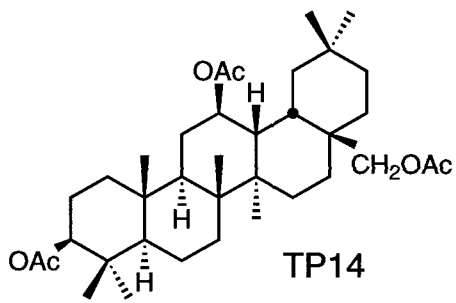
receptor (ER-alpha) in these cells. Since MCF-7 cells are known to be ER-positive, and the growth of these cells is known to be driven by 17-ß-estradiol, this suppression of the estrogen receptor may account, at least in part, for the growth-suppressive activity of TP-82 in the MCF-7 cells.

3. Results with Macrophages

The ability of triterpenoids to suppress de novo formation of two enzymes, inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) is most easily measured in macrophages, using either primary mouse peritoneal macrophages or a mouse macrophage-like cell line (RAW 264.7) as assay systems. The important relevance of iNOS and COX-2 for carcinogenesis (including carcinogenesis in the breast) is discussed in the Introduction. Using either gamma-interferon or lipopolysaccharide (LPS) as inducing agents, we can achieve major inductions of de novo synthesis of both iNOS and COX-2 in the above cells. Two synthetic oleananes, 3,12-dioxoolean-1-en-28-oic acid (TP-69), and 3,11-diooxoolean-1,12-dien-28-oic acid (TP-72) have been shown to be highly active inhibitors of these inductions; the attached manuscript, "Novel Triterpenoids Suppress Inducible Nitric Oxide Synthase (iNOS) and Inducible Cyclooxygenase (COX-2) in Mouse Macrophages," by Nanjoo Suh, Tadashi Honda, Heather Finlay, Aaron Barchowsky, Charlotte Williams, Nicole Benoit, Qiao-wen Xie, Carl Nathan, Gordon Gribble, and Michael Sporn, documents these findings in detail. These data all suggest that further studies on the ability of triterpenoids to suppress iNOS and COX-2 should be pursued, and that we should continue the chemical synthesis and testing program that we have outlined above.

Figure 1

Structures of Growth-Inhibitory Triterpenoids



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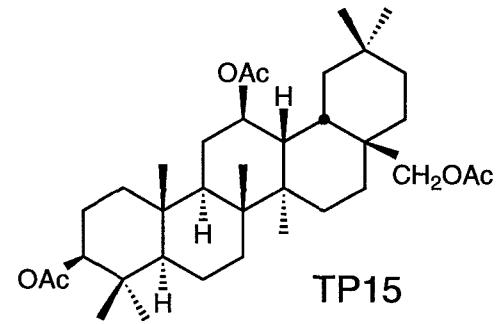

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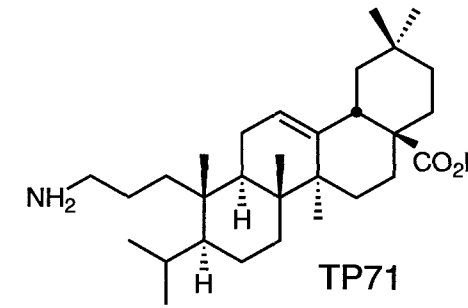

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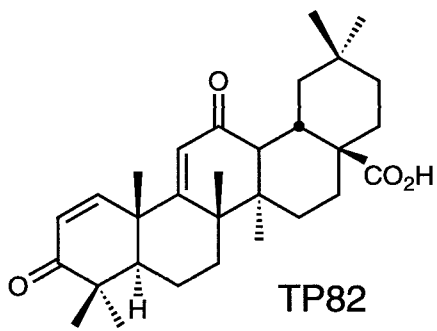

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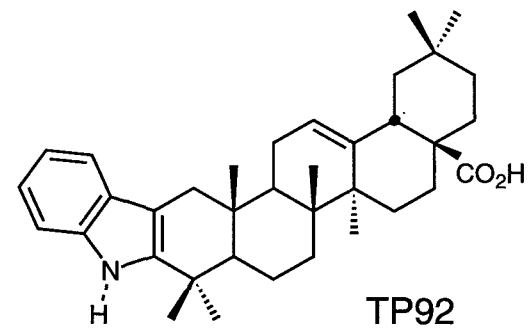

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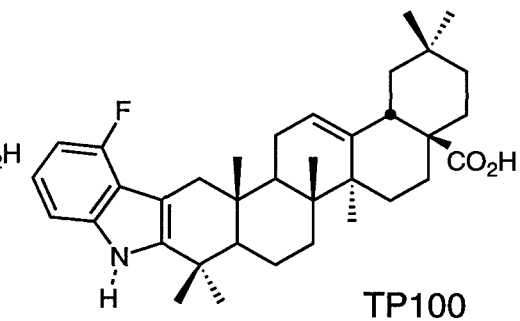

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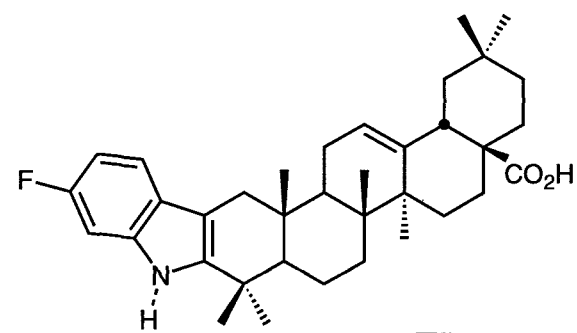

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Figure 2

Suppression of DNA Synthesis in MCF-7 Cells by Triterpenoids

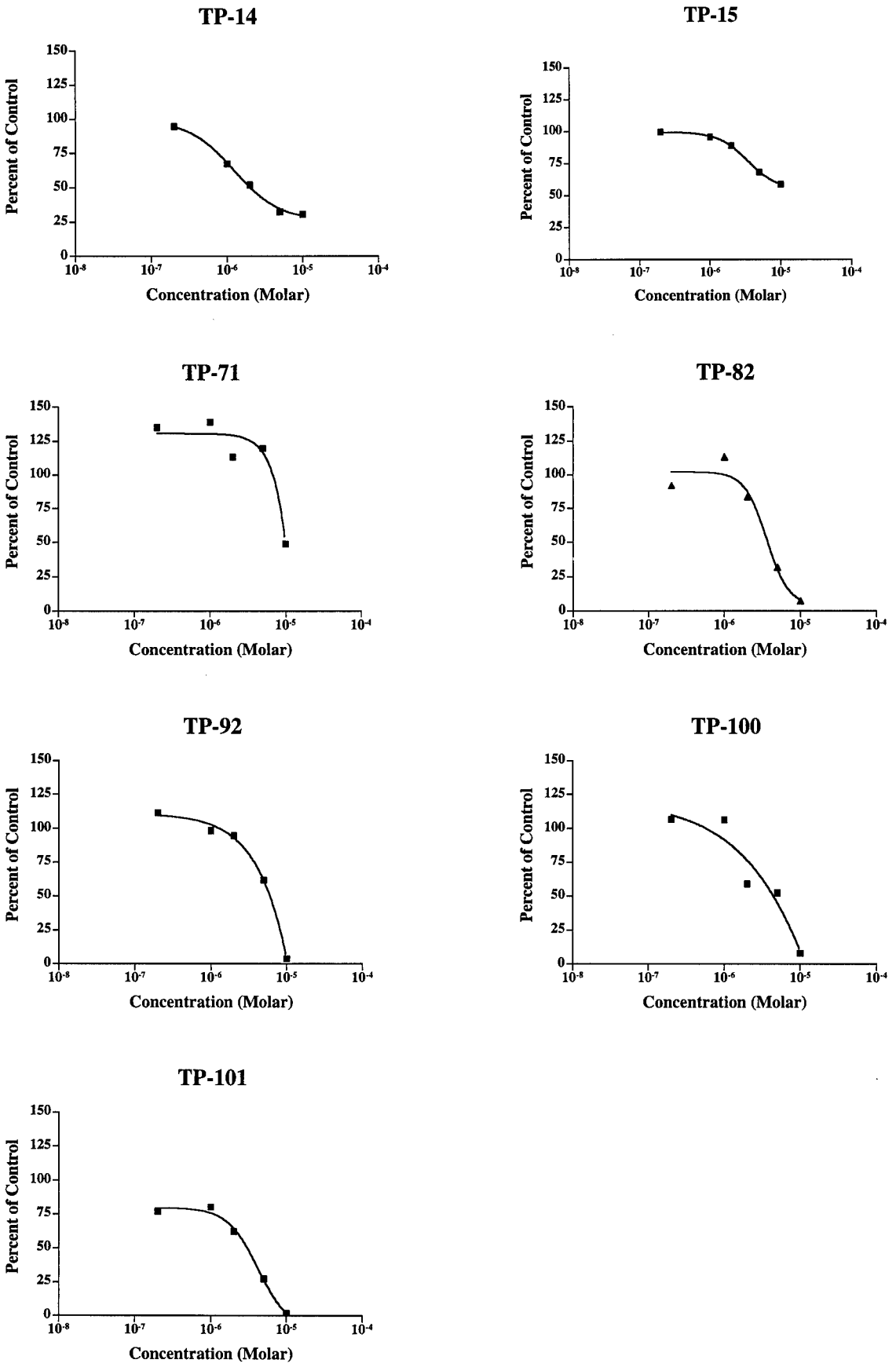


Figure 3

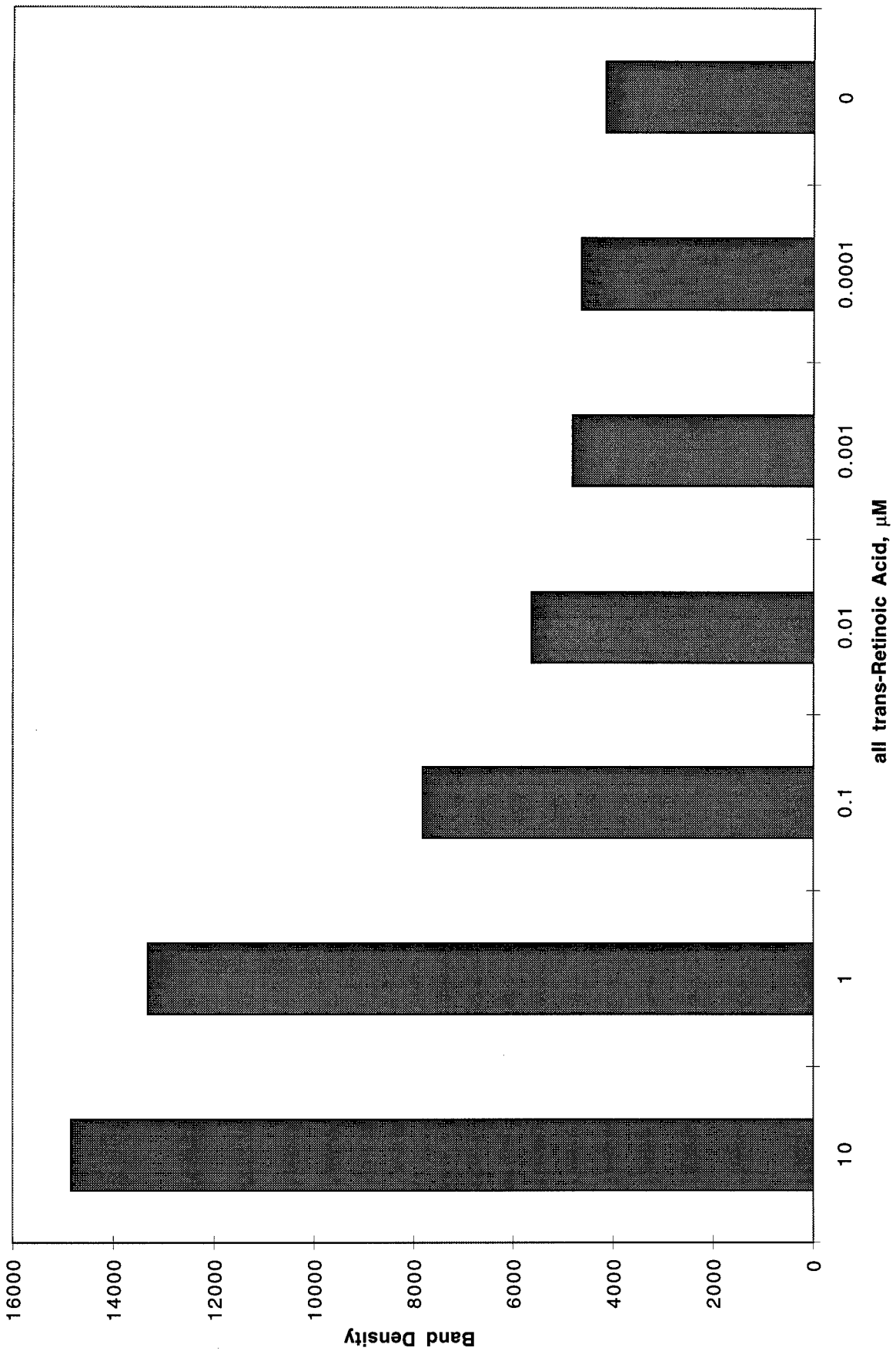

Induction of β-Catenin Expression in SK-Br-3 Cells
Band Density
16000
14000
12000
10000
8000
6000
4000
2000
0
10
1
0.1
0.01
0.001
0.0001
0
all trans-Retinoic Acid, µM

Figure 4

Effects on β-Catenin Expression by Various Triterpenoids and Retinoic Acid

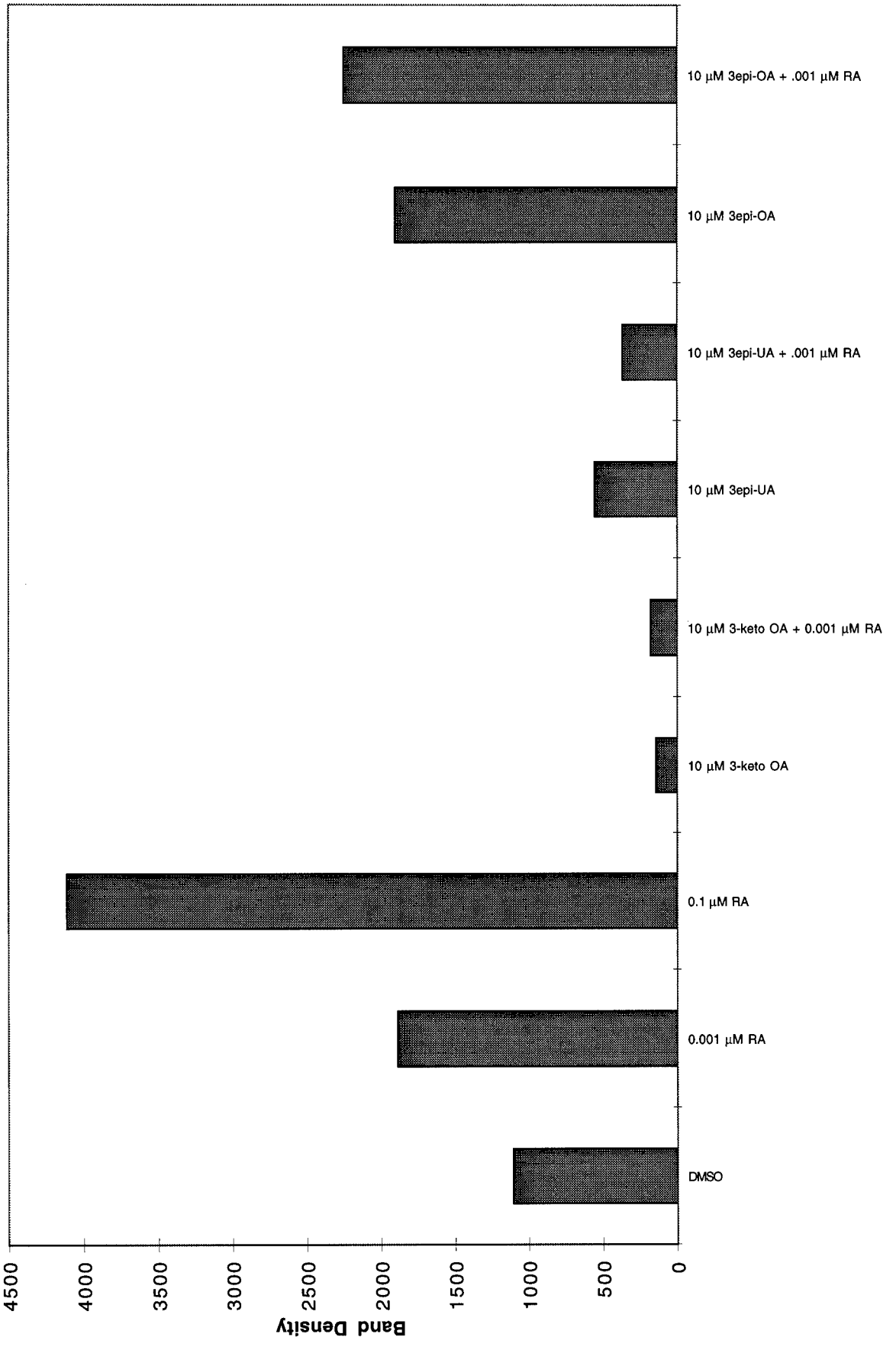
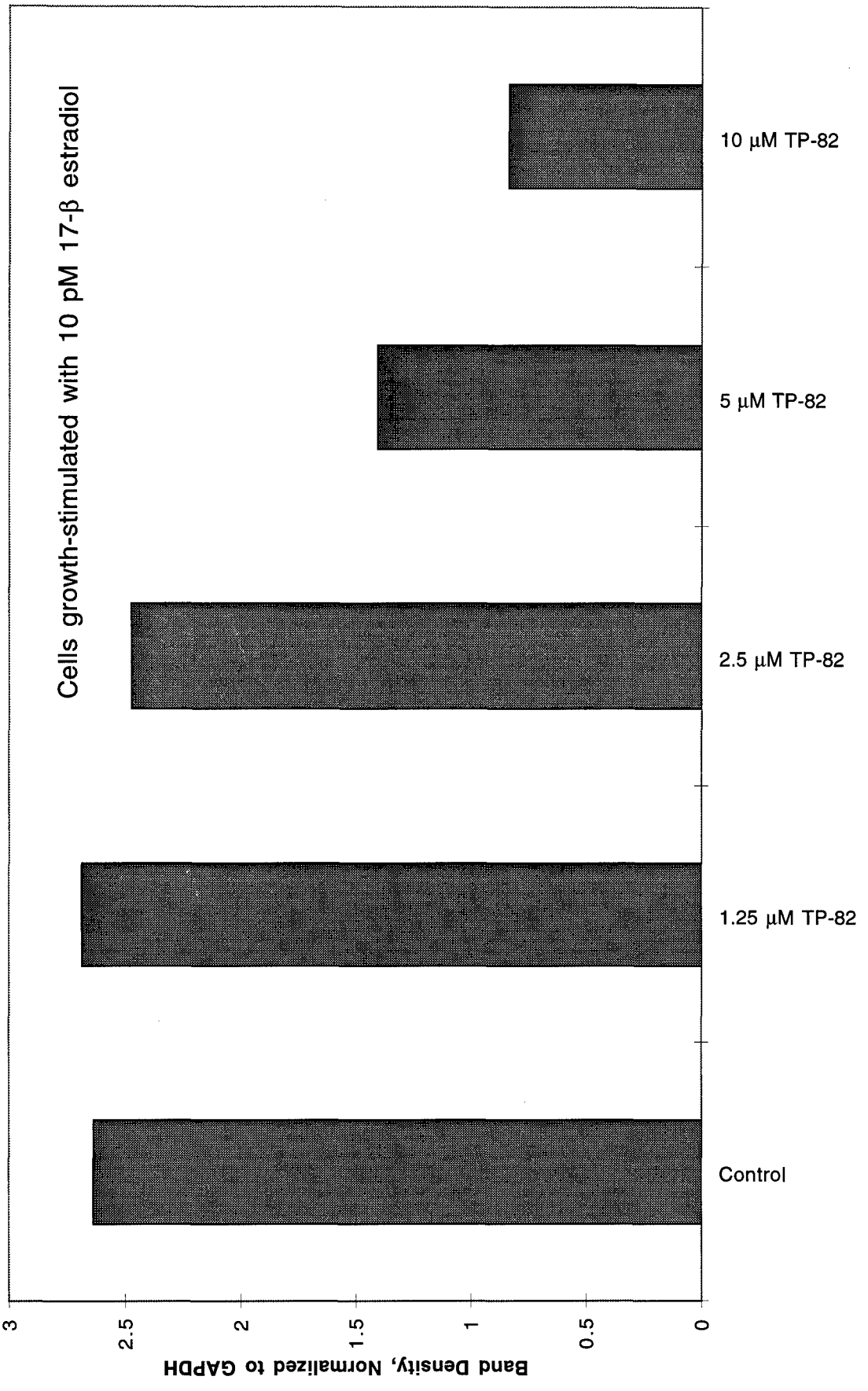


Figure 5

Down-Regulation of ER-α mRNA by Triterpenoid in MCF-7 Cells



(7) CONCLUSIONS

We have demonstrated the validity of a new approach to inhibition of carcinogenesis. We have shown that it is possible to synthesize new synthetic triterpenoids that are potent inhibitors of the de novo formation of the enzymes, inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2), that are known to be important etiologic factors in the development of cancer. During the first year of this project, we have published one manuscript on triterpenoid synthesis, which acknowledges support from this grant, and we have submitted a second manuscript (also acknowledging support from this grant) which describes the biological activities of these new triterpenoids. Further studies on the synthesis and testing of new triterpenoids should now be pursued, with the eventual goal being to find a triterpenoid that could be used for chemoprevention of breast cancer in women at high risk for development of this disease.

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(9) APPENDICIES

1. Honda, T., Finlay, H. J., Gribble, G. W., Suh, N., and Sporn, M. B. New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. Bioorg. Med. Chem. Lett., 7: 1623-1628, 1997.

2. Suh, N., Honda, T., Finlay, H.J., Barchowsky, A., Williams, C., Benoit, N. E., Xie, Q., Nathan, C., Gribble, G.W., and Sporn, M.B. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. (Submitted)

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NEW ENONE DERIVATIVES OF OLEANOLIC ACID AND URSOLIC ACID AS INHIBITORS OF NITRIC OXIDE PRODUCTION IN MOUSE MACROPHAGES

Tadashi Honda,[a] Heather J. Finlay,[a] Gordon W. Gribble,*[,a] Nanjoo Suh,[b] and Michael B. Sporn*[,b]

[a]*Department of Chemistry, Dartmouth College, Hanover, NH 03755, U.S.A. and*
[b]*Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, U.S.A.*

Abstract: New derivatives of 3-oxoolean-1-en-28-oic acid and 3-oxours-1-en-28-oic acid were synthesized. Nine of them showed significant inhibitory activity against interferon-γ-induced nitric oxide production in mouse macrophages when assayed at the 1 μM level. 3,12-Dioxoolean-1,9-dien-28-oic acid (**3**) had the highest activity (IC_{50}, 0.9 μM). © 1997 Elsevier Science Ltd.

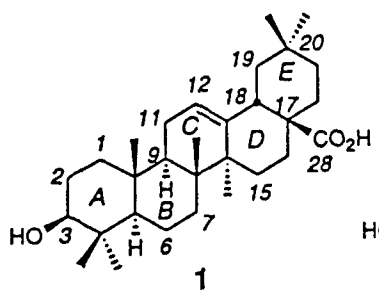
Introduction

Many oleanane and ursane triterpenoids are reported to have interesting biological, pharmacological, or medicinal activities similar to those of retinoids and steroids, such as anti-inflammatory activity, suppression of tumor promotion, suppression of immunoglobulin synthesis, protection of the liver against toxic injury, induction of collagen synthesis, and induction of differentiation in leukemia or teratocarcinoma cells.[1] However, there has never been a systematic study of structure–activity relationships in this set of molecules. Bioassay-directed systematic drug design and synthesis of derivatives of oleanolic acid (**1**) and ursolic acid (**2**), which are commercially available, are of great value in discovering new structures with significant biological activity.

The high output of nitric oxide (NO) produced by inducible nitric oxide synthase (*i*-NOS), which is expressed in activated macrophages, plays an important role in host defense. However, excessive production of NO also can destroy functional normal tissues during acute and chronic inflammation.[2] Thus, inhibitors of NO production in macrophages are potential anti-inflammatory drugs. For this purpose we synthesized oleanolic and ursolic acid derivatives and tested them as inhibitors of NO production. We have found a series of new derivatives of 3-oxoolean-1-en-28-oic acid and 3-oxours-1-en-28-oic acid to have significant inhibitory activity against interferon-γ (IFN-γ)-induced NO production in mouse macrophages.[3] In particular, 3,12-dioxoolean-1,9-dien-28-oic acid (**3**) had the highest activity (IC_{50}, 0.9 μM) in this group of compounds. In this communication, the synthesis, inhibitory activity, and structure–activity relationships are reported for these compounds.

Discovery of Lead Compounds

When we started this project, we had no information about a lead compound. Therefore, about sixty oleanolic and ursolic acid derivatives, e.g., 3-hydroxy-, 3-chloro-, 2-chloro-, C-ring cleaved, and 3-oxo-derivatives (including compounds **4**–**7**), were initially randomly synthesized. In the preliminary screen of these



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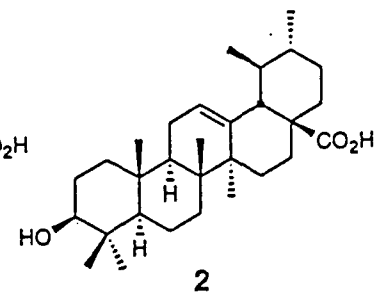

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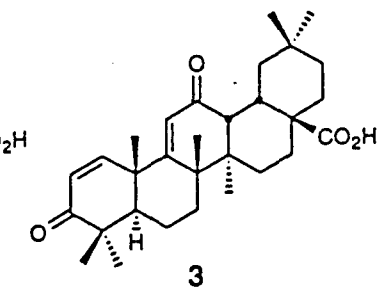

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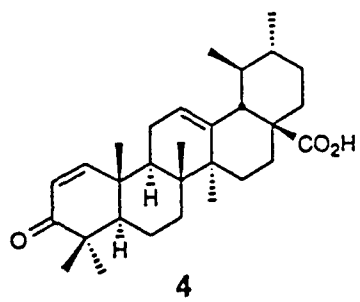

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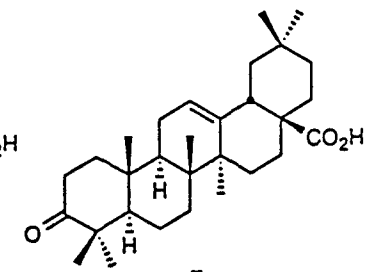

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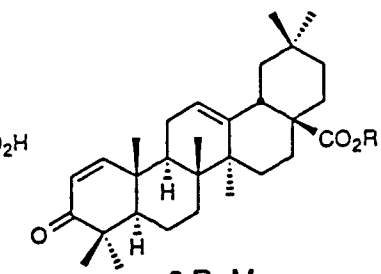
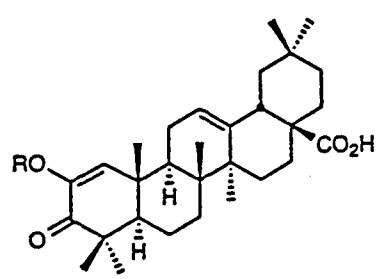
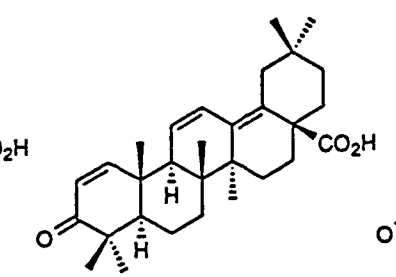


6 R=Me
7 R=H



8 R=Me
9 R=H



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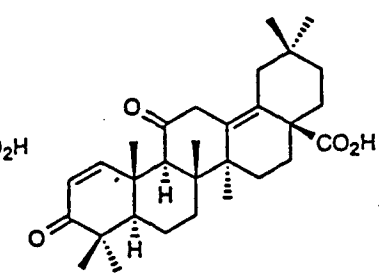

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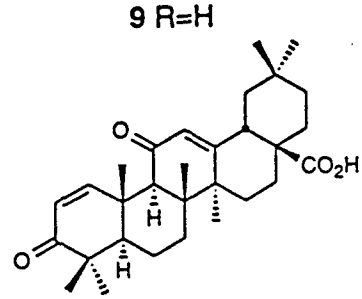

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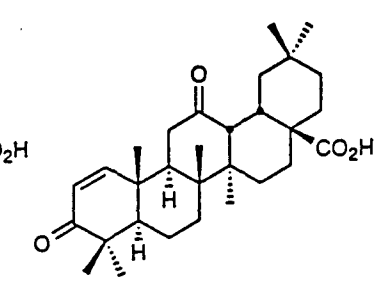

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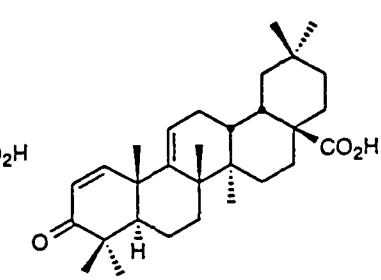

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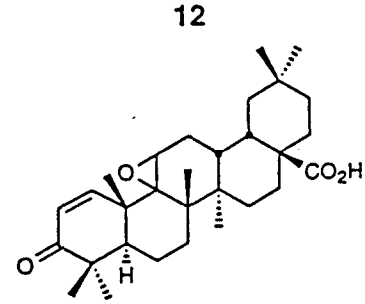

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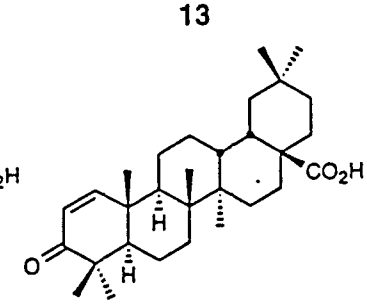
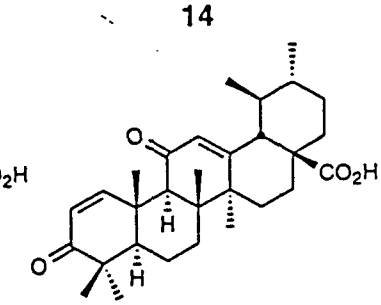
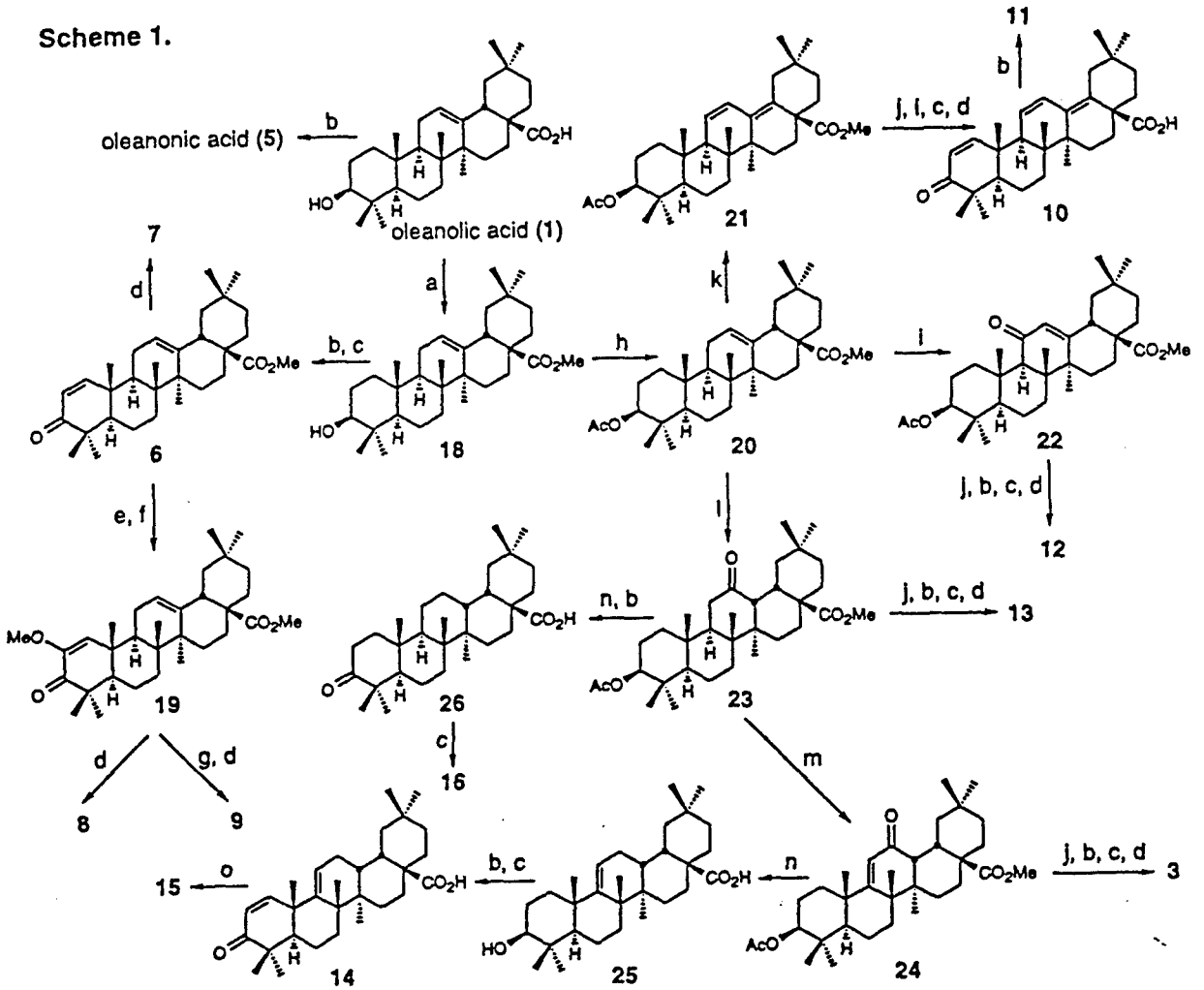


16



17

Scheme 1.


oleanonic acid (5)
oleanolic acid (1)
ursolic acid (2)
a: $CH_2N_2/Et_2O/THF$, b: Jones, c: PhSeCl/AcOEt; 30%H_2O_2/THF, d: LiI/DMF, e: 30%H_2O_2/NaOH/THF,
f: MeONa, g: HCl/AcOH, h: Ac_2O/pyr., i: CrO_3/pyr./CH_2Cl_2, j: KOH/MeOH, k: SeO_2/AcOH,
l: 30%H_2O_2/AcOH, m: Br_2/HBr/AcOH, n: NH_2NH_2/KOH/diethylene glycol, o: *m*-CPBA/CH_2Cl_2

derivatives for inhibition of IFN-γ-induced NO production in mouse macrophages, 3-oxoolean-1,12-dien-28-oic acid (**7**) was found to show significant activity (IC_{50}, 6.0 μM).

Design and Synthesis of New Derivatives

When **7** is compared with the other derivatives (e.g., **1**, **2**, and **4**–**6**), it has the following features: first, it is an oleanane; second, it has a 1-en-3-one structural unit in ring A; third, it has a carboxyl group at C-17. On the basis of these features of **7**, various derivatives with a 1-en-3-one structural unit in ring A and a carboxyl group at C-17 (**3** and **8**–**17**) were designed. The synthesis of these newly designed derivatives and compounds **4**–**7** are illustrated in Schemes 1 and 2.[4] Oleanonic acid (**5**)[5] was prepared in quantitative yield by Jones oxidation of **1**. Enone ester **6** was synthesized by Jones oxidation of methyl oleanolate (**18**)[6] (yield, 90%), followed by introduction of a double bond at C-1 with phenylselenenyl chloride in ethyl acetate and sequential addition of 30% hydrogen peroxide[7] ($PhSeCl-H_2O_2$) (yield, 70%). Enone **7** was synthesized in 88% yield by halogenolysis of **6** with lithium iodide (LiI) in dimethylformamide (DMF).[8] Enone **8** was synthesized in 35% yield by halogenolysis of ester **19** with LiI in DMF, which was prepared by epoxidation of **6** with alkaline hydrogen peroxide (yield, quantitative), followed by sodium methoxide (yield, quantitative).[9] Diosphenol **9** was synthesized by demethylation of the methyl enol ether at C-2 of **19** with hydrochloric acid in acetic acid (yield, 88%), followed by halogenolysis (yield, 18%). Diene **10** was synthesized by alkaline hydrolysis of acetate **21** (yield, quantitative), which was prepared from methyl acetyloleanolate (**20**)[6] according to a known method,[10] sequential Ratcliffe oxidation[11] (yield, 90%), introduction of a double bond at C-1 (yield, 66%), and halogenolysis (yield, 56%). Deconjugated enone **11** was prepared in 28% yield by Jones oxidation of **10**. Bis-enone **12** was synthesized by alkaline hydrolysis of acetate **22** (yield, quantitative), which was prepared from **20** according to our improvement on a known method,[12] sequential Jones oxidation (yield, 91%), introduction of a double bond at C-1 (yield, 97%), and halogenolysis (yield, 43%).[13] Enone **13** was synthesized in 46% yield from C-12 ketone **23**[14] according to the same synthetic route as for **12**. Bis-enone **3** was also synthesized in 26% yield from enone **24**[15] according to the same synthetic route as for **12**. Enone **14** was synthesized by Jones oxidation of acid **25**[16] (yield, 95%), followed by introduction of a double bond at C-1 (yield, 80%). Epoxide **15**[17] was prepared in 46% yield by epoxidation of **14** with *m*-chloroperbenzoic acid in methylene chloride. Enone **16** was prepared in 51% yield by introduction of a double bond at C-1 of acid **26**[18] with $PhSeCl-H_2O_2$. Enone **4** was prepared by introduction of a double bond at C-1 of ketone **28**[19] with $PhSeCl-H_2O_2$ (yield, 66%), followed by halogenolysis (yield, 88%). Bis-enone **17** was synthesized according to the same route as for **12** in 42% yield from enone **29**, which was prepared from **27** according to our improvement on a known method,[16,20]

Biological Results and Discussion

The inhibitory activities [IC_{50} (μM) value] of compounds **1**–**17** and hydrocortisone (a positive control) on IFN-γ-induced NO production in mouse macrophages are shown in the Table. Nine of the new derivatives of 3-oxoolean-1-en-28-oic acid and 3-oxours-1-en-28-oic acid showed significant activity at the 1 μM level. Six of them were superior to the lead compound **7**. Modification of the A and C ring affected activity strongly. In particular, bis-enone type compounds **3** and **12** showed high activity. Surprisingly, ursolic acid (**2**) stimulated NO production although ursolic acid derivatives **4** and **17** showed inhibitory activity. None of the synthesized derivatives were toxic to primary mouse macrophages at 40 μM.

These preliminary results revealed some interesting structure–activity relationships as follows:

(1) In the A ring, a 1-en-3-one structural unit without a substituent is important for significant activity. For example, 1-en-3-one **7** is much more active in comparison with diosphenol **9**, diosphenol methyl ether **8**, C-3 ketone **5**, and C-3 alcohol **1**.

(2) In the C ring: (a) a carbonyl group at C-11 and/or C-12 is important; (b) particularly, an insertion of a double bond at the α position of C-11 and/or C-12 ketone enhances the activity. Bis-enone **3** with 1-en-3-one and 9-en-12-one structural units showed the highest activity. Bis-enone **12**, C-11 ketone **11**, and C-12 ketone **13** also showed high activity, and were more active than **7**. Bis-enone **17** which has an ursane skeleton is also more active than **4**.

(3) At C-17, a carboxyl group (e.g., **7**) gives much more activity than a methoxycarbonyl group (e.g., **6**). Hydrophilic groups seem to be much better than hydrophobic groups.

(4) The oleanane skeleton is more active than the ursane skeleton. **7** and **12** are more active than **4** and **17**, respectively.

On the basis of these structure–activity relationships, further lead optimization is in progress. Studies on the mode of action of these derivatives also are in progress.

Table. IC_{50} (μM) [a] Values for Inhibition of IFN-γ-Induced NO Production in Mouse Macrophages [3]

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
hydrocortisone	0.015	**10**	9.7
3	0.9	**4**	17.6
12	1.8	**9**	26.5
11	2.6	**8**	30.0
13	3.3	**15**	35.5
17	5.1	**5**	37.1
14	5.2	**6**	40.0
7	6.0	oleanolic acid (**1**)	40.0
16	8.5	ursolic acid (**2**)	stimulation [b]

a: All IC_{50} (μM) values were determined over the range of 0.1–40 μM for each compound, except for hydrocortisone, using the computer calculation program Tablecurve® (all were fitted to a log–dose response curve.) Values are an average of two separate experiments.

b: Ursolic acid (**2**) is strongly toxic to primary mouse macrophages (toxic above 5–10 μM).

Acknowledgments

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3. Briefly, the procedure for this assay is as follows: Macrophages were harvested from female mice injected intraperitoneally four days previously with 4% thioglycollate. These cells were seeded in 96-well tissue culture plates and incubated with 4 ng/mL IFN-γ in the presence or absence of inhibitory test compounds. After 48 hours NO production (measured as nitrite by the Griess reaction) was determined. Full details of the assay are given in reference 21.
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Novel Triterpenoids Suppress Inducible Nitric Oxide Synthase (iNOS) and Inducible Cyclooxygenase (COX-2) in Mouse Macrophages

Nanjoo Suh, Tadashi Honda, Heather J. Finlay, Aaron Barchowsky, Charlotte Williams, Nicole E. Benoit, Qiao-wen Xie, Carl Nathan, Gordon W. Gribble, and Michael B. Sporn[1]

Department of Pharmacology and Norris Cotton Cancer Center, Dartmouth Medical School [N. S., A. B., C. W., N. E. B., M. B. S.] and Department of Chemistry, Dartmouth College, Hanover, NH 03755 [T. H., H. J. F., G. W. G.]; Department of Medicine, Cornell University Medical College, New York, NY 10021 [Q.-w. X., C. N.]

Running Title: Novel Triterpenoids

Key Words: Triterpenoids, Nitric oxide synthase, Cyclooxygenase

Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; COX-2, cyclooxygenase-2; COX-1, cyclooxygenase-1; PG, prostaglandin; AA, Arachidonic acid; NMA, *N*-methyl arginine; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; LPS, bacterial endotoxic lipopolysaccharide; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor kappa B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

[1] Michael B. Sporn: To whom correspondence should be addressed.

Department of Pharmacology and Toxicology

Dartmouth Medical School, 7650 Remsen, Hanover, NH 03755

(Tel) (603)-650-6557

(Fax) (603)-650-1129

(e-mail) Michael.Sporn@dartmouth.edu

Abstract

We have synthesized more than 80 novel triterpenoids, all derivatives of oleanolic and ursolic acid, as potential anti-inflammatory and chemopreventive agents. These triterpenoids have been tested for their ability to suppress the *de novo* formation of two enzymes, inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2), using interferon-γ (IFN-γ)-stimulated primary mouse macrophages or lipopolysaccharide (LPS)-activated RAW 264.7 macrophages as assay systems. Two synthetic oleananes, 3,12-dioxoolean-1-en-28-oic acid (TP-69) and 3,11-dioxoolean-1,12-dien-28-oic acid (TP-72), were highly active inhibitors of *de novo* formation of both iNOS and COX-2. Both TP-69 and TP-72 blocked the increase in iNOS or COX-2 mRNA induced by IFN-γ or LPS. In addition, TP-72 suppressed NF-κB activation in macrophages treated with IFN-γ alone or the combination of IFN-γ and tumor necrosis factor. The 3-α(axial)-epimer of ursolic acid suppressed *de novo* formation of COX-2, in contrast to naturally occurring 3-β(equatorial)-ursolic acid. Inhibitory effects of TP-69 or TP-72 on iNOS formation were not blocked by the glucocorticoid receptor antagonist, RU-486, indicating that these triterpenoids do not act through the glucocorticoid receptor, nor does TP-72 act as an iNOS or COX-2 enzyme inhibitor when added to RAW cells in which synthesis of these two enzymes in response to LPS has already been induced. It may be possible to develop triterpenoids as useful agents for chemoprevention of cancer or other chronic diseases with an inflammatory component.

Introduction

Although triterpenoids are widely used for medicinal purposes in many Asian countries, this class of molecules, which resemble steroids in their chemical structure, biogenesis, and pleiotropic actions, has not impacted on the practice of Western medicine. Triterpenoids, like the steroids, are formed in nature by the cyclization of squalene, with the retention of all 30 carbon atoms in molecules such as oleanolic acid (OA) and ursolic acid (UA) (Figure 1). Although OA and UA are known to have numerous pharmacological activities, the potency of these naturally occurring molecules is relatively weak. Chemical synthesis of new steroid analogs has provided many useful derivatives that are more potent and specific than natural parent structures. With this as a model, and considering the known anti-inflammatory and anti-carcinogenic activities of OA and UA (1-4), we have synthesized and characterized a new series of synthetic triterpenoid analogs as potential inhibitors of inflammation and carcinogenesis using suppression of the formation of nitric oxide and prostaglandins as assay systems. We report here the structures and activities of three derivatives of OA and UA which are significantly more potent than their parent structures.

The concept that inflammation and carcinogenesis are related phenomena has been the subject of many studies that have attempted to link these two processes in a mechanistic fashion (5-7). The enzymes which

mediate the constitutive synthesis of NO and prostaglandins from arginine and arachidonate, respectively, have relatively little significance for either inflammation or carcinogenesis. In contrast, inducible nitric oxide synthase (iNOS, EC 1.14.13.39) and inducible cyclooxygenase (COX-2, EC 1.14.99.1) both have critical roles in the response of tissues to injury or infectious agents. These inducible enzymes are essential components of the inflammatory response, the ultimate repair of injury, and carcinogenesis (8-13). While physiologic activity of iNOS and COX-2 may provide a definite benefit to the organism, aberrant or excessive expression of either iNOS or COX-2 has been implicated in the pathogenesis of many disease processes, as diverse as septic shock, cardiomyopathy, acute and chronic neurodegenerative disease, rheumatoid arthritis, and carcinogenesis (14-22).

Immense effort has been devoted to developing new molecules, which are direct inhibitors of the enzymatic activity of either iNOS or COX-2. However, an alternative approach is to find new agents which can prevent expression of the respective genes coding for these enzymes. Glucocorticoids and transforming growth factor-β are such molecules; they both suppress transcription or translation of iNOS and COX-2 genes (23-28). A rationale thus exists to develop more selective agents for suppression of genes that might be over-expressed during the inflammatory or carcinogenic process. In this report we attempt to apply this strategy for the development and evaluation of new triterpenoids.

Materials and Methods

Reagents. Details of the synthesis of TP-69 and TP-72 (see Fig. 1 for structures) have been published (29). TP-52 (3-α-OH ursolic acid) was synthesized by Jones oxidation of ursolic acid, followed by Meerwein-Ponndorf reduction (30). Recombinant mouse IFN-γ (LPS content < 10 pg/ml) was purchased from Genzyme (Cambridge, MA); NF-κB oligonucleotide from Promega (Madison, WI); Goat polyclonal COX-1, COX-2 IgG and anti-goat IgG peroxidase-conjugated secondary antibody from Santa Cruz (Santa Cruz, CA); TGF-β_1 from R&D (Minneapolis, MN); and enzyme immunoassay reagents for PGE_2 assays from Cayman Co. (Ann Arbor, MI). TNF-α was provided by Dr. Jan Vilcek. Lipopolysaccharide (LPS from E.coli 0111:B4 γ-irradiated) and all other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture. RAW 264.7 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum. These cells were treated with LPS (10 ng/ml) for 6-18 h to induce iNOS or COX-2. To obtain primary macrophages, female CD-1 mice, 5-10 weeks old (Charles River Breeding Laboratories, Wilmington, MA), were injected intraperitoneally with 2 ml of 4% thioglycollate broth (Difco Laboratories, Detroit, MI). 4 days after injection, peritoneal macrophages were harvested and processed as described (31). Cells were seeded in 96-well

plates at 2×10^5 cells/well and stimulated with IFN-γ. Triterpenoids were added at the same time.

Measurement of iNOS enzyme activity, protein, and mRNA levels.

NO production in mouse macrophages and RAW 264.7 cell line. Nitrite accumulation was used as an indicator of NO production in the medium and was assayed by the Griess reaction (32). 100 µl Griess reagent was added to 100 µl of each supernatant from LPS, IFN-γ or triterpenoid-treated cells in triplicate. The protein determination was performed by Bradford protein assay. The plates were read at 550 nm against a standard curve of sodium nitrite.

Inhibition of LPS-induced iNOS enzyme activity. RAW 264.7 cells were plated in 100 mm tissue culture dishes (4×10^6 cells) and incubated for 12 h with LPS. The cells were washed twice with PBS. Cells were harvested and plated into a 96-well plate (2×10^5 cells /well) and incubated in the absence or presence of test compounds for 12 h further, with no LPS in the medium. The supernatants were removed, and the Griess assay was performed as above.

SDS-PAGE and Western blot analyses of iNOS protein in mouse macrophages and RAW 264.7 cells. Mouse macrophages or RAW 264.7 cells were plated in 6-well plates (4×10^6 cells per well) and treated, respectively, with IFN-γ or LPS for 18 h. Cells were washed and scraped into

cold PBS, and then centrifuged at 500 *g* for 10 min at 4°C. The cell pellets were resuspended in 50 mM Tris-buffer (pH 7.4), 100 mM NaCl, containing 0.5% NP-40, 5 μg/ml aprotinin, 10 μg/ml leupeptin and 100 μM PMSF, and then centrifuged to obtain whole cell lysates. The proteins (50 μg) were electrophoresed on 7.5% (or 12% in some experiments) reducing SDS-PAGE and transferred in 20% methanol, 25 mM Tris, 192 mM glycine (pH 8.3) to 0.2 micron nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (25 mM Tris, pH 7.5, 150 mM NaCl, 0.02% NaN_3) with 0.2% Tween-20 (Tween-TBS) for 1 h, then incubated with purified rabbit anti-mouse iNOS IgG (33) for 2-3 h, washed and finally incubated for 45 min with a 1:10,000 dilution (iNOS) of secondary antibody conjugated with horseradish peroxidase. The membranes were washed and then developed using a chemiluminescence system (enhanced chemiluminescence detection reagents; Amersham), exposed to film.

Northern blot analyses of iNOS. RNA was isolated from mouse macrophages and RAW cells by a rapid guanidinium isothiocyanate method (34). The total RNA was denatured in formamide (50%)/formaldehyde (6.5%) sample buffer (65°C, 15 min), run on formaldehyde (1.8%)/agarose (0.8%) gels, and transferred to Nytran nylon membranes (Schleicher & Schuell, Keene, NH). After UV-crosslinking, the membranes were prehybridized, hybridized, washed, and exposed to Kodak XAR films. The probe specific for murine iNOS was an 814-bp EcoRI/AccI fragment of iNOS cDNA clone B2 (33), which was random-primer labeled with 50 μCi of [α-^{32}P] dCTP (6,000 Ci/mmol). To control for equal

loading of RNA, the membranes were hybridized with a random-primer radiolabeled mouse glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA.

Measurement of COX-2 enzyme activity, protein, and mRNA levels.

PGE_2 production. RAW 264.7 cells were plated in 6-well plates and incubated with compounds for 6 h, and then the supernatant culture medium was collected to determine the amount of PGE_2 by enzyme immunoassay.

Inhibition of LPS-induced COX-2 enzyme activity. RAW 264.7 cells were plated at 1×10^5 cells per well in a 12-well plate and incubated for 6 h with LPS. The cell supernatants were removed, and cells in each well were washed twice with fresh culture medium and allowed to equilibrate in the absence or presence of test compounds for 30 min. The cells were further incubated with 100 μM arachidonic acid (AA) for 15 min, with no LPS in the medium. The supernatants were removed and assayed for PGE_2 (35).

Western blot analyses of COX-2 in RAW 264.7 cells. RAW 264.7 cells were treated with LPS for 6 h or 18 h. The first steps of the procedure were as described above for Western blots of iNOS. Goat polyclonal COX-1 and COX-2 were used as primary antibodies and the bands were detected with an anti-goat IgG horseradish peroxidase-conjugated secondary antibody (1:2000 dilution), followed by chemiluminescent detection.

Northern blot analysis of COX-2. All procedures were as described above for iNOS. For COX-1 and COX-2 cDNA probes (gifts from Dr. Harvey

Herschman), a 2.76 kb insert of COX-1 in the pBluescript vector was digested with Bsp HI and a 2.3 kb insert of COX-2 in the pGEM7 vector was double digested with Bam HI and Xho I.

Electrophoretic mobility shift assays (EMSA) for NF-κB. NF-κB binding activity in nuclei isolated from uninduced and induced cells was determined by electrophoretic mobility shift assays using previously described methods (36). Nuclear proteins were extracted from macrophages by detergent lysis. Oligonucleotide probes were 5'-end radiolabeled with T4 polynucleotide kinase in the presence of 50 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol) to a specific activity of > 2×10^8 cpm/mg. Five micrograms of nuclear protein were incubated with 10 fmol ^{32}P-labeled double stranded oligonucleotide containing the NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3') binding motif. The specificity of binding was determined by the addition of excess (1.75 pmol) of the same unlabeled oligonucleotide.

Results

Triterpenoids inhibit nitric oxide production in mouse macrophages and RAW 264.7 cells. Active synthetic triterpenoids caused a dose dependent inhibition of NO production both in mouse primary macrophages, induced with IFN-γ and in RAW 264.7 cells, induced with LPS. This inhibition of

NO production was not due to toxicity, as determined by trypan blue exclusion and adherent cell protein determination. Triterpenoids TP-69 and TP-72 (Figure 1) were selected from a primary screening of more than 80 derivatives of OA and UA as the most active ones in suppression of NO production. TP-69 and TP-72 are synthetic enone analogs of OA that have A- and C-ring modifications with one or two enone functional groups. As shown in the dose-response curves in Figure 2A, IC_{50} values for TP-72 are 3.9 μM and 6.7 μM in primary macrophages and RAW 264.7 cells, respectively. The data indicate TP-72 is markedly more active than its parent molecule, oleanolic acid. TP-69 also inhibited NO production in primary macrophages (IC_{50}= 4.2 μM) and RAW 264.7 cells (IC_{50}= 7.8 μM) (curves not shown). While TGF-β_1 is the most potent known inhibitor of inducible nitric oxide formation in primary macrophages (50% inhibition at 200 pg/ml; Refs, 24, 32), it did not suppress NO formation in transformed RAW 264.7 cells (Figure 2B). In contrast, triterpenoids inhibited NO production in both transformed RAW cells and primary macrophages, suggesting that the inhibition by triterpenoids is not mediated by the action of TGF-β.

TP-72 does not inhibit intrinsic iNOS enzyme activity in RAW 264.7 cells. We wished to determine if the inhibitory effect of a triterpenoid on inducible NO production is a direct effect on the intrinsic enzyme activity of iNOS, or whether this inhibition is mediated by some other mechanism.

Dexamethasone is known to inhibit iNOS gene transcription (37, 38). Table 1 shows that addition of either TP-72 or dexamethasone to RAW 264.7 cells, which had been pretreated with LPS to induce NOS, does not affect iNOS enzyme activity in the intact cell. In contrast, N-methyl arginine, an enzyme substrate analog, inhibited this enzyme activity (62% inhibition of NO accumulation at 40 μM). Further confirmation of this lack of direct enzyme inhibition by TP-72 was obtained in experiments in which TP-72 was added to lysates of RAW cells that had been pretreated with LPS to induce NOS. In these experiments, we performed an enzyme assay for iNOS on the lysates, using arginine as added substrate. There was no inhibition of NO formation (measured as nitrite in the Griess reaction) by TP-72 (20 μM) (data not shown). Therefore, TP-72 inhibits NO formation by a mechanism other than direct enzyme inhibition.

Triterpenoids decrease iNOS mRNA and protein levels in mouse macrophages and RAW 264.7 cells. We next investigated whether TP-72 and the related enone, TP-69, might affect levels of iNOS mRNA and the resultant iNOS protein. The data in Figure 3 show that LPS induces the 4.0 kb iNOS mRNA transcript in RAW cells in a dose-dependent manner, over a range from 1-1,000 ng/ml. There is a similar dose-response for the induction of the 130 kDa iNOS protein in these same cells. Both the mRNA and protein responses to LPS are markedly attenuated by TP-72 (10 μM). Densitometer scans of the respective blots show approximately 50% inhibition of iNOS

protein expression at 1 μg/ml of LPS stimulation. As shown in Figure 4A, iNOS protein expression is inhibited by TP-69 or dexamethasone after primary mouse macrophages are stimulated by IFN-γ alone (20 ng/ml) or by IFN-γ in combination with TNF-α (100 ng/ml). In contrast to the strong inhibitory effect of the synthetic triterpenoid, TP-69, the naturally occurring parent molecule, oleanolic acid, was inactive in this assay at an equimolar concentration. Figure 4B shows that IFN-γ is a strong inducer of iNOS mRNA expression in primary mouse macrophages. TP-69 (30 μM) almost totally blocks this induction, with some inhibition of iNOS mRNA levels seen at concentrations as low as 1 μM. In addition, we examined whether these active triterpenoids might suppress constitutive nitric oxide synthase (cNOS) in endothelial cells. In contrast, neither TP-69 nor TP-72 (each at 10 μM) diminished the level of the constitutive NOS in human endothelial cells (data not shown).

Triterpenoids do not act through a glucocorticoid receptor-mediated mechanism. The glucocorticoid antagonist, RU486, was used to determine if the inhibitory effects of triterpenoids on nitric oxide production were mediated through their interaction with the glucocorticoid receptor. Figure 5 shows that, as expected, inhibitory effects of dexamethasone were reversed by the addition of glucocorticoid receptor antagonist RU486 (1 μM). In contrast, the inhibitory activity of TP-69 and TP-72 on nitric oxide production could not be reversed by

RU486. These data strongly suggest that the actions of triterpenoids on the iNOS system are not mediated by their interaction with the glucocorticoid receptor.

Triterpenoids decrease inducible COX-2 mRNA and protein levels, but not constitutive COX-1, in macrophages. LPS induced COX-2 protein (~ 72 kDa) and COX-2 mRNA (4.4 kb) in RAW 264.7 cells in a dose-dependent manner. As shown in Figure 6A, COX-2 protein and mRNA expression induced by LPS (at concentrations ranging from 1-1,000 ng/ml) was markedly decreased by concomitant treatment with TP-72 (10 μM). Figure 6B shows that derivatives of both oleanolic and ursolic acids have inhibitory effects on COX-2 protein expression. In addition to TP-72, the oleanane enone, TP-69, also inhibited COX-2 protein expression. The 3-α-epimer of UA, TP-52, suppresses LPS-induced COX-2 protein expression, while UA itself does not have an inhibitory effect. The amount of the product of the COX-2 enzyme, prostaglandin E_2 (PGE_2), in the supernatants from each treatment of the RAW 264.7 cells was determined and corresponded with the COX-2 protein data (Figure 6B). Thus, LPS markedly increased PGE_2 levels, and oleanolic and ursolic acids did not substantially affect this increase, while TP-69, TP-72, and TP-52 (assayed at 10 μM) all blocked the inductive effect of LPS on production of PGE_2. However, as shown above for suppression of nitric oxide formation, the inhibition of prostaglandin formation by TP-72 is not a result of inhibition of enzyme activity itself. When this triterpenoid was added to RAW cells in which

synthesis of COX-2 had already been induced by LPS, there was no decrease in prostaglandin production, using added arachidonic acid as substrate (Table 2). COX-1 protein levels were not affected by any of the treatments (Figure 6B).

Triterpenoids suppress the activation of NF-κB in nuclear extracts from primary macrophages. Since activation of NF-κB is critical for the induction of both iNOS and COX-2 by LPS or other inflammatory cytokines (39, 40), we determined whether triterpenoids might suppress NF-κB activation in nuclear extracts obtained from primary macrophages induced with IFN-γ, LPS, or TNF-α. As shown in Figure 7A, TP-72 (20 μM) or dexamethasone (1 μM) inhibited the activation of NF-κB in nuclear extracts obtained from macrophages treated with 10 ng/ml IFN-γ. TP-69 also attenuated activation of NF-κB in primary mouse macrophages (data not shown). To confirm the NF-κB binding proteins in the retarded complexes, antibodies specific to either the p65 or p50 subunits of NF-κB were used to demonstrate the retardation of NF-κB. The mobility of bands was further retarded, particularly by antibody to p65. The data in Figure 7B show that TP-72 (20 μM) inhibited the activation of NF-κB in nuclear extracts obtained from macrophages treated with either 10 ng/ml IFN-γ alone (80% inhibition) or by IFN-γ in combination with 10 ng/ml LPS (50% inhibition) or 10 ng/ml TNF-α (70% inhibition).

Discussion

OA and UA have significant, although, relatively weak, anti-inflammatory and anti-carcinogenic actions, particularly *in vivo* (1, 3, 4, 41). However, there has been a paucity of convincing data from cell culture experiments relating to the mechanism of action of OA and UA. It is possible that OA and UA are precursors to more active molecules that are formed by metabolism, as is the case for dietary vitamin A and vitamin D. The experiments reported here now show that synthetic triterpenoids are markedly more active than the parent structures. Among some 80 derivatives we have made, we have found several molecules, namely 3-epi-ursolic acid (TP-52), 3,12-dioxoolean-1-en-28-oic acid (TP-69), and 3,11-dioxoolean-1,12-dien-28-oic acid (TP-72), which are significantly more active than OA and UA in suppression of the formation of either nitric oxide or prostaglandins. We assayed these new synthetic agents as potential suppressors of inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX-2) because of the highly relevant nature of these two enzymes for many disease processes. While numerous agents have been synthesized that are effective inhibitors by acting as substrate analogs for each of these two enzymes, an alternative approach to their control, namely to block *de novo* enzyme formation selectively, has been essentially unexplored.

The anti-inflammatory and anti-carcinogenic activities of the naturally occurring triterpenoids are relatively weak. Much more potent synthetic analogs are needed if this class of compounds is to be of clinical value. The two

synthetic enone derivatives of oleanolic acid, TP-69 and TP-72, represent a first effort in this direction. Both are highly active in suppressing expression of both iNOS and COX-2 mRNA and protein at concentrations at which their parent molecule, oleanolic acid, is inactive. TP-69 and TP-72 exert parallel effects on suppression of the expression of both iNOS and COX-2, suggesting that there may be a common mechanistic basis for this action. Suppression of activation of NF-κB by active triterpenoids may partially account for this, since there are known to be NF-κB response elements on the promoters for both the iNOS and the COX-2 genes (40, 42-46). However, not all genetic regulation of the iNOS or COX-2 systems is transcriptional. It has been shown that both TGF-β and dexamethasone may have potent inhibitory effects on the stability or translatability of iNOS or COX-2 mRNA's (24, 26, 47). Some of the effects of the triterpenoids may be mediated at these levels, rather than by a direct effect on transcription itself.

Although glucocorticoids block the induction of iNOS and COX-2, they are limited in their usefulness for therapy of chronic disease states because of the side effects resulting from activation of the glucocorticoid receptor. In contrast, our data suggest that triterpenoids exert their effects through a receptor system other than the glucocorticoid receptor. The nature of the putative triterpenoid receptor remains to be defined. The steroid-like structure and activity of triterpenoids indicates that such a receptor might have some relationship to the steroid receptor superfamily. The striking difference that we

have shown between ursolic acid (3-β-OH) and its 3-α-hydroxy epimer (TP-52) in their ability to suppress COX-2 synthesis provides particularly strong evidence for receptor-mediated activity, since these two epimers differ only with respect to the conformation of a hydroxyl group (equatorial or axial, respectively) in an essentially planar ring system.

Recently, there have been striking advances which indicate that overexpression of either iNOS and COX-2 may be intimately involved in the pathogenesis of many common debilitating or fatal chronic diseases. These include colon cancer (7, 21, 22, 48), multiple sclerosis (18, 49), Parkinson's disease (19, 50), and Alzheimer's disease (51, 52). There is intense effort to develop enzyme inhibitors that are selective for the inducible forms of these enzymes and do not affect the desirable activity of their respective constitutive isoforms. To the extent that the pathogenesis of the above diseases is promoted by excessive production of nitric oxide or prostaglandins, such selective enzyme inhibitors are promising agents for therapy (53). In addition to these specific inhibitors of the inducible enzymes, the possibility of selective repression of inducible enzyme formation also needs to be considered, and triterpenoids now offer one new approach to this mechanism. The combination of inhibition of inducible enzyme formation, together with selective inhibition of inducible enzyme activity, may provide the most effective therapeutic approach.

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Table 1. The effect of added triterpenoid or dexamethasone after LPS-induction of iNOS enzyme in RAW 264.7 cells.

LPS pretreatment of cells [a]	Addition to LPS-treated RAW cells	NO (nmol/ 2×10^5 cells)[b]
None	DMSO control	0.0 ± 0.0
LPS (10 ng/ml), 12 h	DMSO control	3.9 ± 0.5
	N-methyl arginine (40 μM)	1.4 ± 0.3*
	Dexamethasone (1 μM)	3.6 ± 0.6
	TP-72 (40 μM)	3.9 ± 0.8

[a] RAW 264.7 cells were stimulated for 12 h with LPS (10 ng/ml) in a tissue culture dish, and cells were washed twice with PBS to remove LPS. RAW cells were scraped and placed in a 96-well plate and *N*-methyl arginine (NMA), TP-72 or dexamethasone was then added and incubated for 12 h.

[b] The amount of nitric oxide accumulated in the supernatant was detected by Griess assay as described in Methods section. Data are means ± S.E. of two independent experiments *($P<0.05$, significantly different from LPS alone). In each experiment, triplicate determinations were made for each treatment.

Table 2. The effect of added triterpenoid or dexamethasone after LPS-induction of COX-2 enzyme in RAW 264.7 cells.

LPS pretreatment of cells [a]	Addition to LPS-treated RAW cells	PGE_2 (ng/ml)[b]
None	DMSO control	0.1 ± 0.0
LPS (10 ng/ml), 6 h	DMSO control	3.4 ± 0.1
	Indomethacin (20 μM)	0.2 ± 0.1*
	Dexamethasone (2 μM)	3.4 ± 0.2
	TP-72 (20 μM)	3.5 ± 0.1

[a] RAW 264.7 cells were stimulated for 6 h with LPS (10 ng/ml), and cells were washed twice with fresh medium. Indomethacin, TP-72 or dexamethasone was then added and equilibrated for 30 min. The cells were further incubated with AA (100 μM) for 15 min.

[b] The amount of PGE_2 in the supernatant was assayed as described above. Data are means ± S.E. of two independent experiments *($P<0.001$, significantly different from LPS alone). In each experiment, duplicate determinations were made for each treatment.

Figure legends

Fig. 1. Chemical structures of the natural triterpenoids, oleanolic acid (OA) and ursolic acid (UA), and the synthetic analogs, 3,12-dioxolean-1-en-28-oic acid (TP-69), 3,11-dioxoolean-1,12-dien-28-oic acid (TP-72) and 3-epi-ursolic acid (TP-52).

Fig. 2. Inhibitory effects of OA, TP-72 and TGF-β_1 on nitric oxide production in primary mouse macrophages (closed symbols) and RAW 264.7 cells (open symbols). IFN-γ (4 ng/ml) and LPS (10 ng/ml) were used as inducing agents in primary macrophages and RAW 264.7 cells, respectively. Cells were treated for 18 h (LPS) or 48 h (IFN-γ) with inducers and triterpenoids (or TGF-β_1), and NO was then determined in the supernatants by the Griess reaction. NO production in primary macrophages that received no triterpenoid was 4.3 nmol/2 $\times$ 10^5 cells; in RAW cells in this figure was 3.2 nmol/2 $\times$ 10^5 cells. Data shown are representative of three typical experiments.

Fig. 3. Inhibition of expression of iNOS protein and mRNA by TP-72 in RAW 264.7 cells. LPS dose-dependently induced iNOS protein and mRNA expression at concentrations of 1, 10, 100, and 1,000 ng/ml, and this induction was inhibited by TP-72 (10 μM). LPS and TP-72 were applied simultaneously and cells were harvested 18 h later.

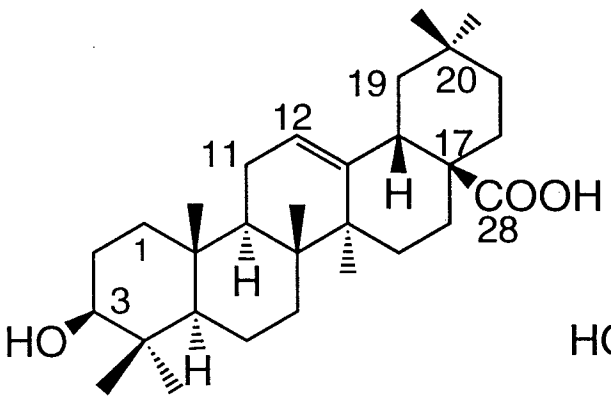
Fig. 4. Inhibition of iNOS protein and mRNA expression by triterpenoids in primary mouse macrophages. (A) Suppression of IFN-γ-induced iNOS protein

expression by TP-69. Primary mouse macrophages were treated with IFN-γ (20 ng/ml) or with IFN-γ plus TNF-α (100 ng/ml) for 18 h, and then harvested to obtain whole cell lysates. TP-69 (10 μM), OA (10 μM) and dexamethasone (1 μM) were added simultaneously with IFN-γ or TNF-α. (B) Inhibition of IFN-γ-induced iNOS mRNA expression by TP-69. RNA samples were obtained from IFN-γ-treated primary macrophages after 18 h incubation.

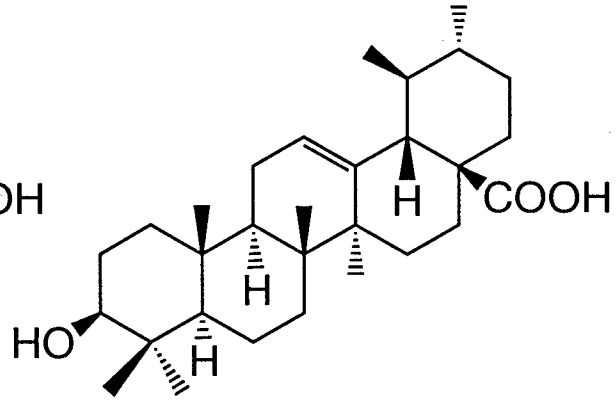
Fig. 5. Blockage by glucocorticoid antagonist RU486 of dexamethasone-inhibited NO production in macrophages but not of triterpenoid-inhibited NO production in macrophages. RAW 264.7 cells were incubated with LPS (10 ng/ml) together with dexamethasone or triterpenoids without RU486 (○); in some cases RU486 (1 μM) was added simultaneously to both dexamethasone- and triterpenoid-treated cell wells (●). Duration of experiment was 18 h. RU486 itself does not interfere with NO production at the concentration tested.

Fig. 6. Inhibition of COX-2 protein and mRNA expression by triterpenoids in RAW 264.7 cells. (A) LPS dose-dependently induced COX-2 protein and mRNA expression over the ranges of 1, 10, 100, and 1,000 ng/ml, and this was suppressed by TP-72 (10 μM). RNA samples were obtained from LPS-treated RAW 264.7 cells after 18 h treatment. (B) RAW 264.7 cells were treated with compounds and LPS (10 ng/ml) for 6 h. All of the triterpenoids were tested at 10 μM and dexamethasone was at 1 μM. The amount of PGE_2 from the supernatant was determined by PGE_2 kit.

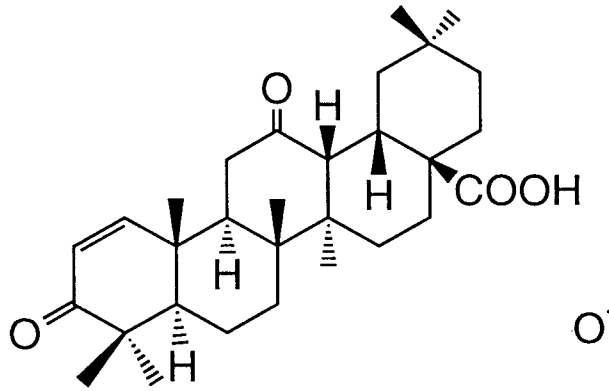
Fig. 7. Suppression of NF-κB by triterpenoids in nuclear extracts from mouse macrophages. Electromobility shift assay using a 5'-end-labeled consensus oligonucleotide was performed with nuclear extracts prepared from primary mouse macrophages. (A) Macrophages were pretreated with compounds for 1 h, and then IFN-γ (10 ng/ml) was added for 1 h. Antibodies to either p65 (anti-p65) or p50 (anti-50) were added to the incubation mixtures for an additional hour. 1: DMSO; 2: IFN-γ (10 ng/ml); 3: IFN-γ + dexamethasone (1 μM); 4: IFN-γ + TP-72 (20 μM). (B) Macrophages were pretreated with compounds for 1 h (dexamethasone, 1 μM; TP-72, 20 μM), and then IFN-γ (10 ng/ml), TNF-α (10 ng/ml) and LPS (10 ng/ml) were added for 1 h further before making nuclear extracts.



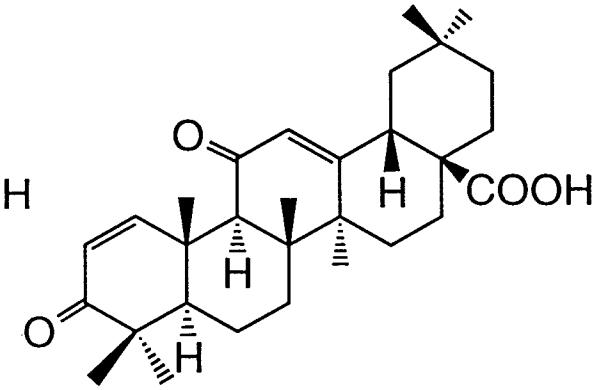
Oleanolic acid (OA)



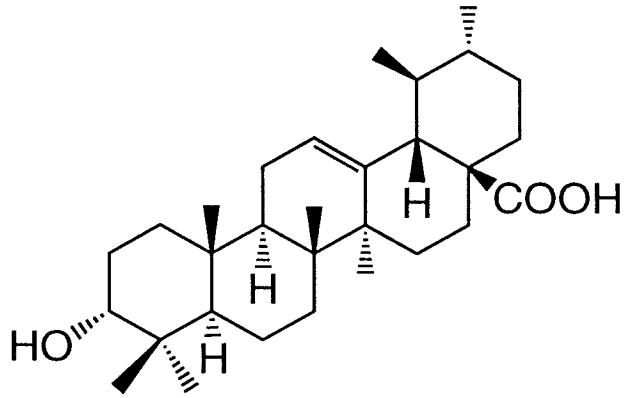
Ursolic acid (UA)



3,12-Dioxoolean-1-en -28-oic acid (TP-69)



3,11-Dioxoolean-1,12-dien -28-oic acid (TP-72)



3-epi-Ursolic acid (TP-52)

Figure 1

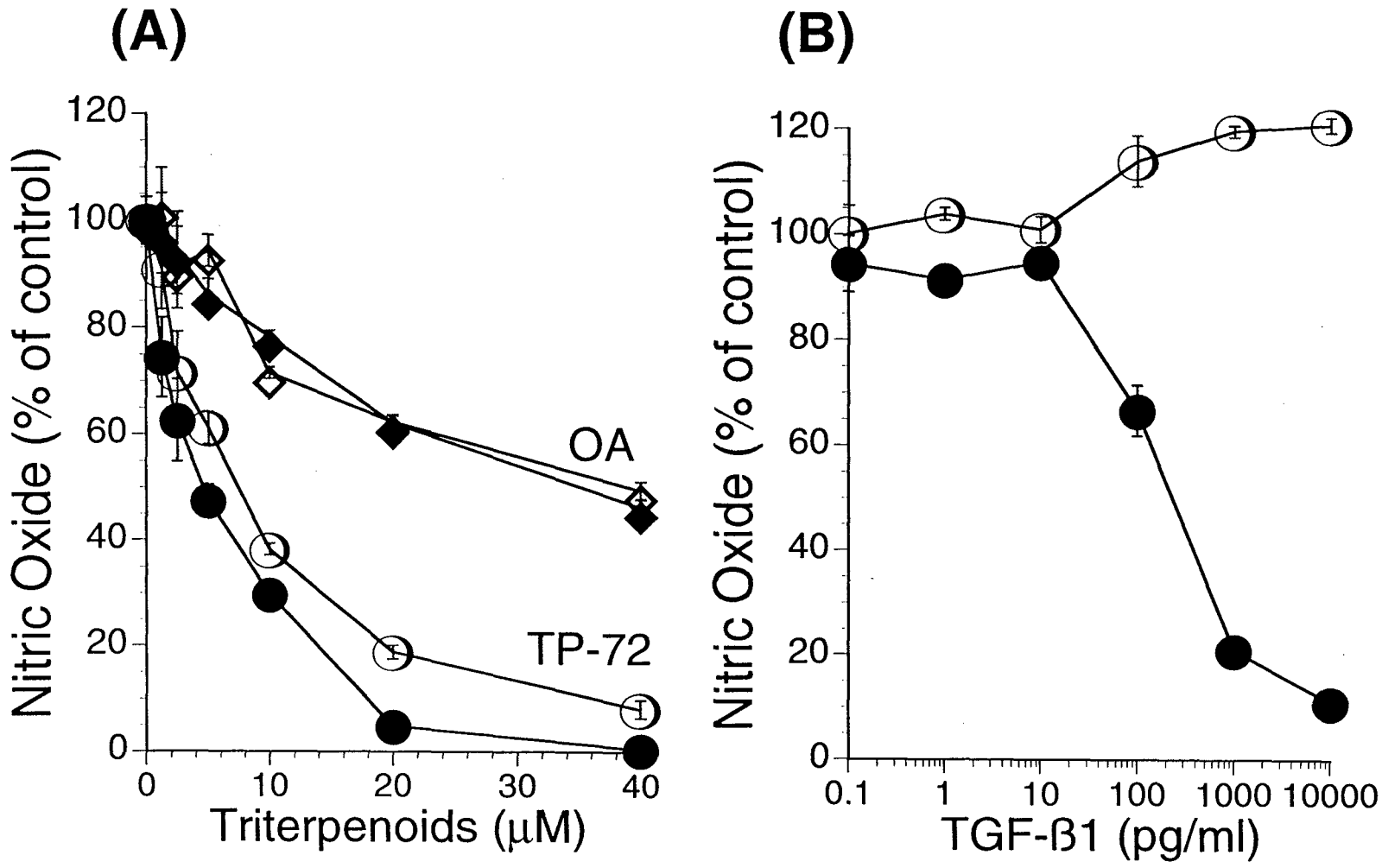

(A)
Nitric Oxide (% of control)
120
100
80
60
40
20
0
0
10
20
30
40
Triterpenoids (μM)
OA
TP-72
(B)
Nitric Oxide (% of control)
120
100
80
60
40
20
0
0.1
1
10
100
1000
10000
TGF-ß1 (pg/ml)

Figure 2

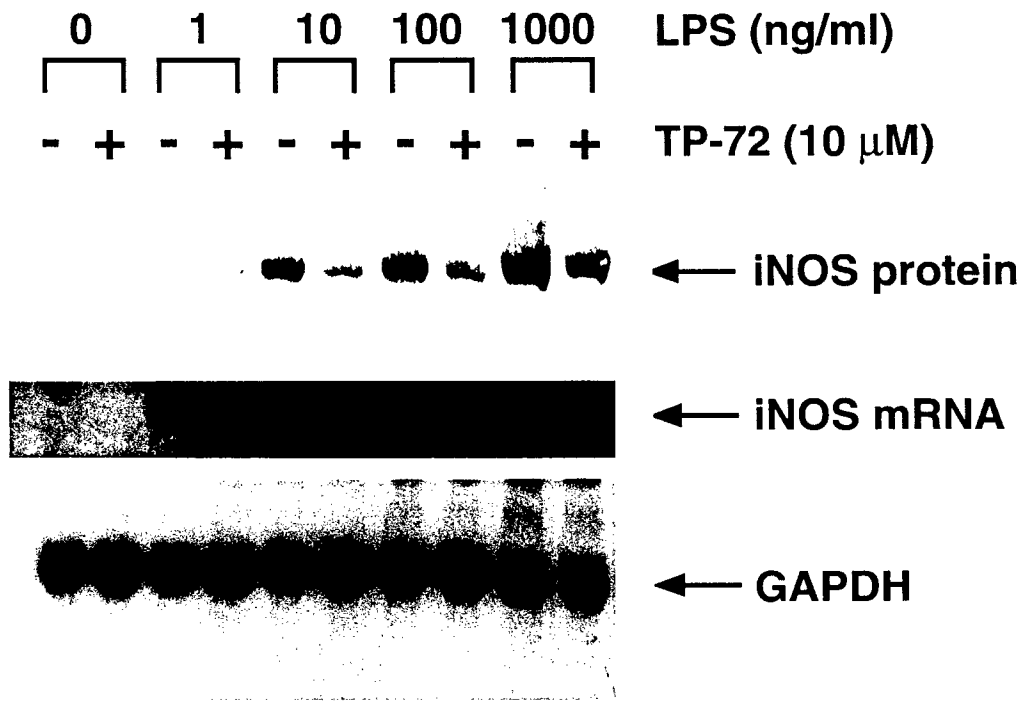

0 1 10 100 1000
LPS (ng/ml)
- + - + - + - + - +
TP-72 (10 μM)
iNOS protein
iNOS mRNA
GAPDH

Figure 3

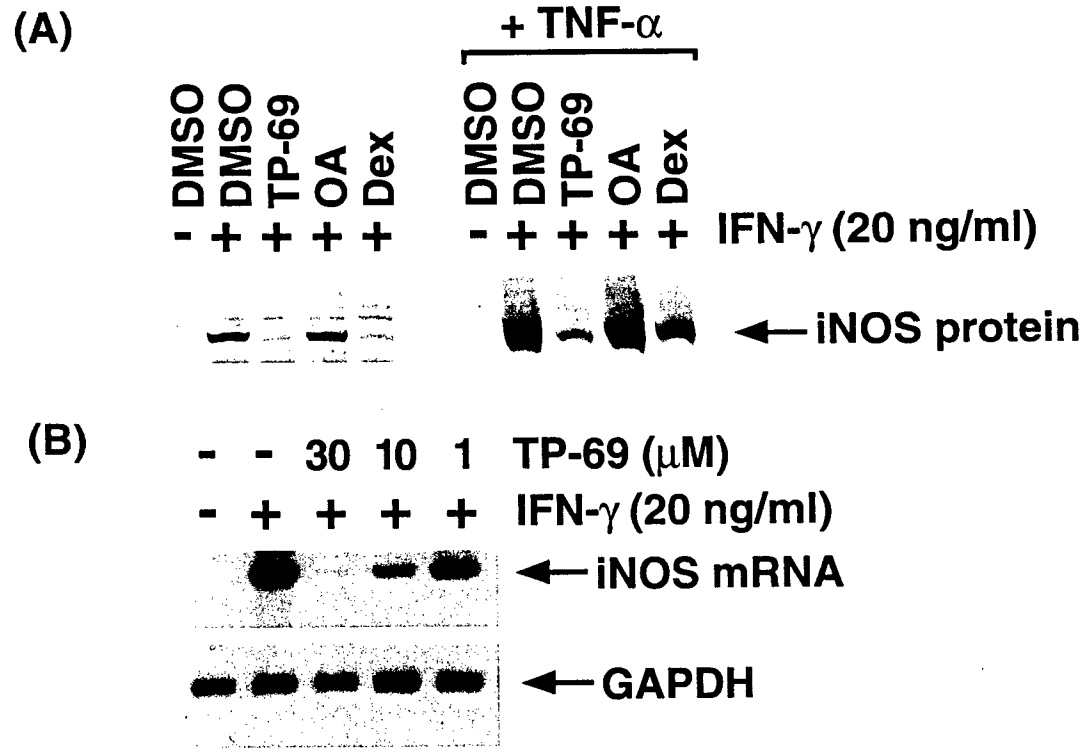

(A)
+ TNF-α
- DMSO
+ DMSO
+ TP-69
+ OA
+ Dex
- DMSO
+ DMSO
+ TP-69
+ OA
+ Dex
IFN-γ (20 ng/ml)
iNOS protein
(B)
- - 30 10 1 TP-69 (μM)
- + + + + IFN-γ (20 ng/ml)
iNOS mRNA
GAPDH

Figure 4

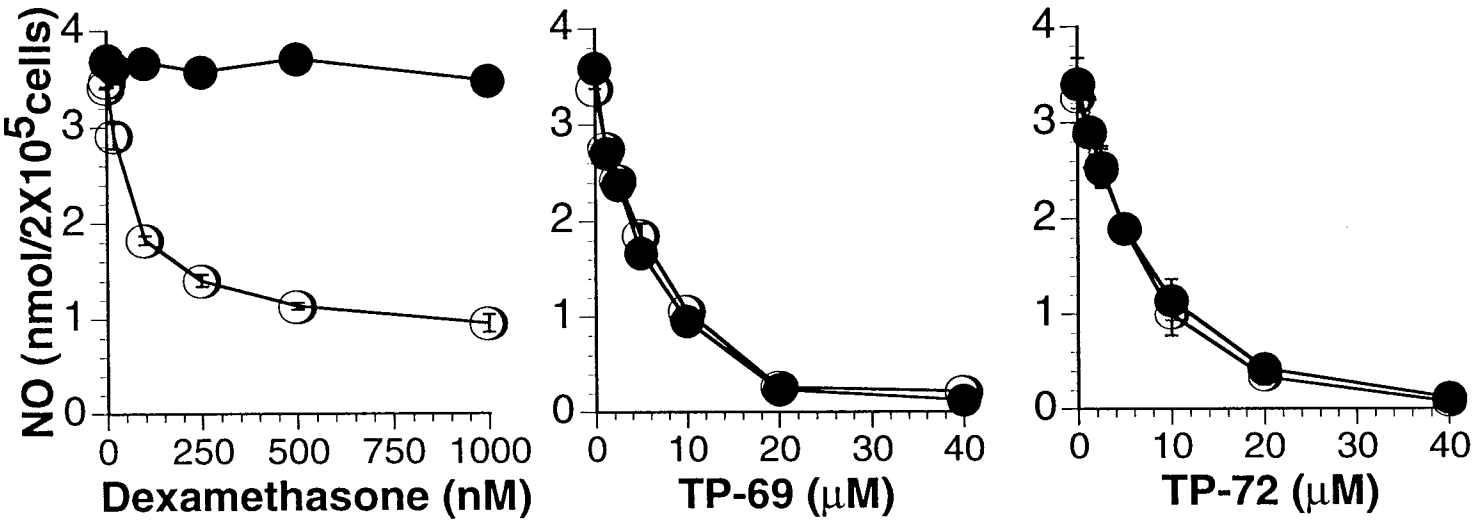

NO (nmol/2X10^5cells)
0
1
2
3
4
0 250 500 750 1000
Dexamethasone (nM)
0 10 20 30 40
TP-69 (μM)
0 10 20 30 40
TP-72 (μM)

Figure 5

(A)

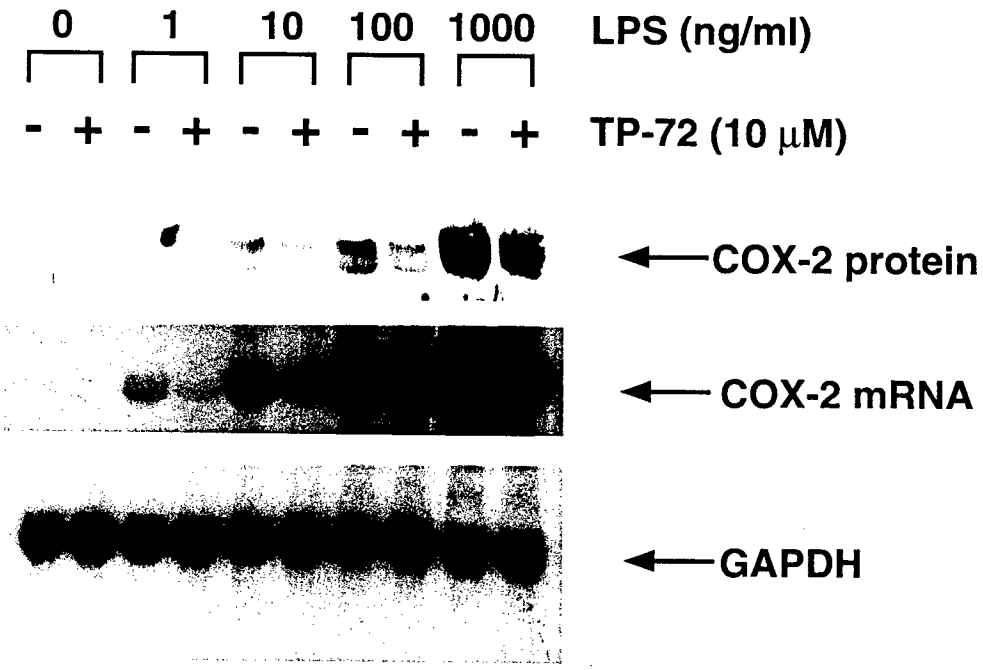
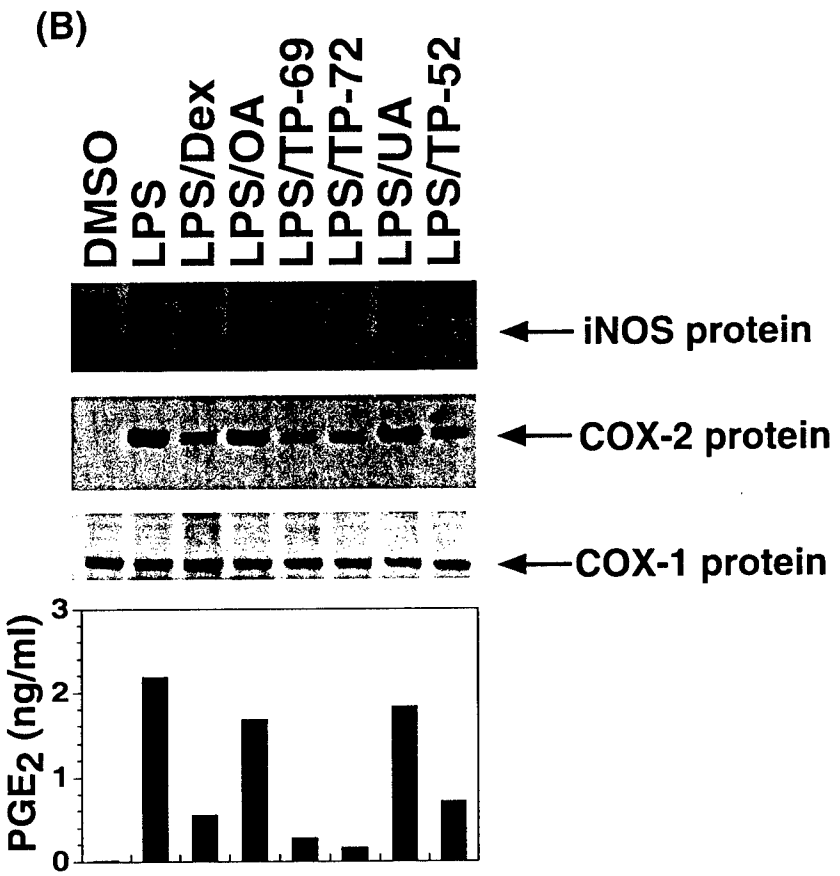

0
1
10
100
1000
LPS (ng/ml)
- + - + - + - + - +
TP-72 (10 μM)
COX-2 protein
COX-2 mRNA
GAPDH

Figure 6A

Figure 6B


(B)
DMSO
LPS
LPS/Dex
LPS/OA
LPS/TP-69
LPS/TP-72
LPS/UA
LPS/TP-52
iNOS protein
COX-2 protein
COX-1 protein
PGE2 (ng/ml)
3
2
1
0

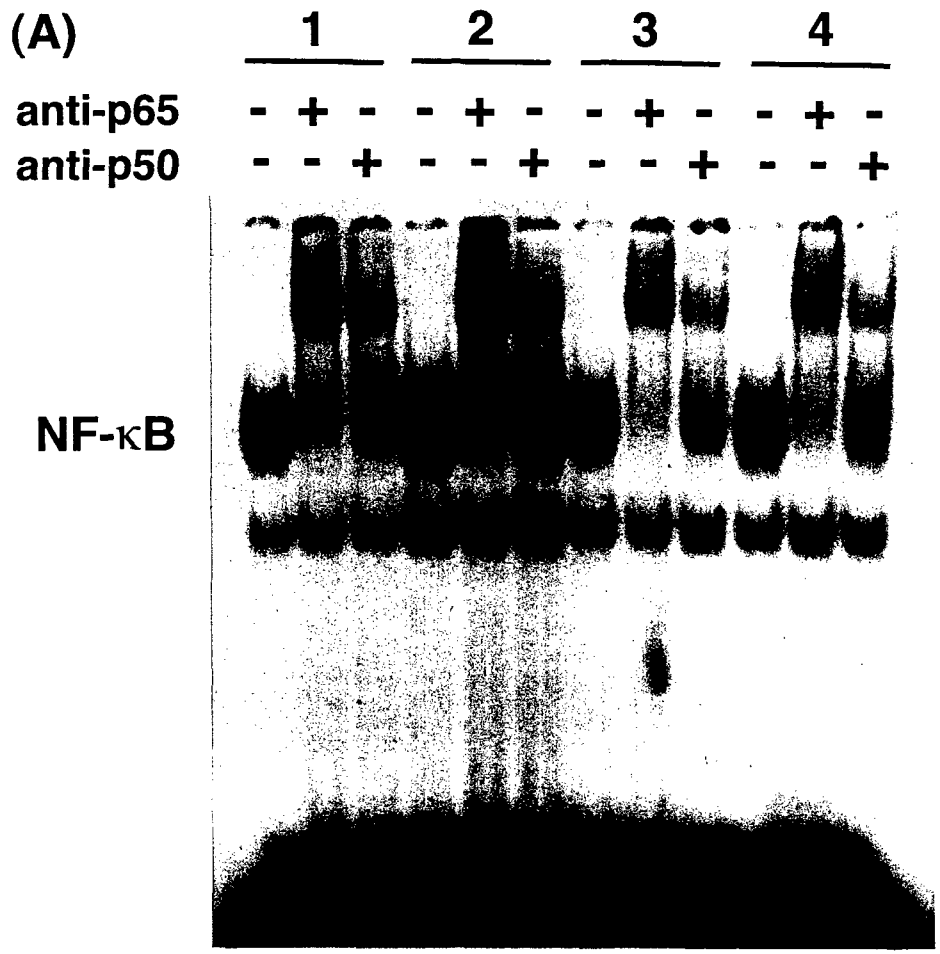


Figure 7A

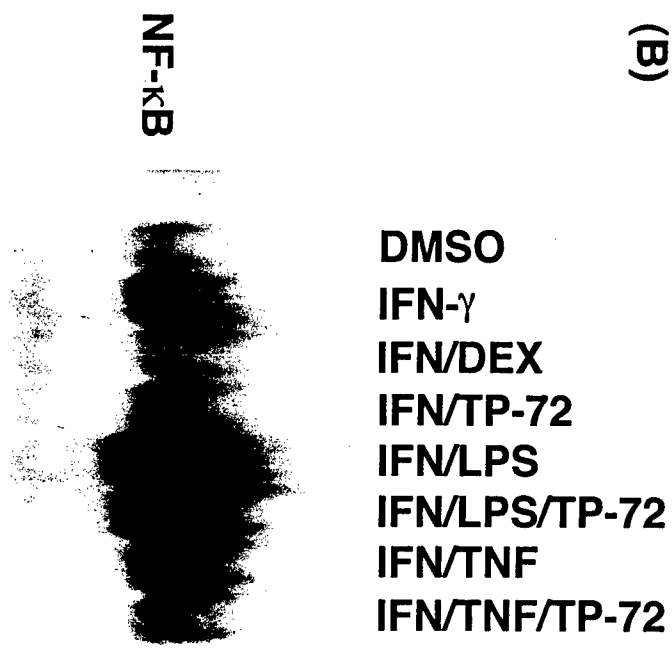

(B)
NF-κB
DMSO
IFN-γ
IFN/DEX
IFN/TP-72
IFN/LPS
IFN/LPS/TP-72
IFN/TNF
IFN/TNF/TP-72

Figure 7B