Arch Pharm Res Vol 25, No 4, 457-462, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Augmentation of Macrophage Antitumor Activities and Nitric Oxide Production by Oregonin

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(Received March 5, 2002)

Oregonin, a diarylheptanoid derivative from *Alnus hirsuta Turcz, Betulaceae,* was evaluated for its antitumor activity. Oregonin, known to have an antitumor function, and is a novel immunomodulator, which may augment macrophage activity. MTT assays and NO production tests were performed in order to investigate the cytotoxicity of oregonin in tumor cells and to examine its influence on macrophage in detail. In this study, the tumoricidal activity was also evaluated by a MTT assay. The cytotoxicity measurements in the oregonin-treated group both *in vitro* and *in vivo* showed a significant difference from that of the control group. *In vivo*, oregonin significantly increased NO production in a dose-dependent manner, and *in vitro*, the thioglycolate-induced inflammatory macrophages increased NO production in a dose-dependent manner after incubation. These results suggest that oregonin reacts with both the inflammatory and non-inflammatory macrophages in a similar way.

Key words: Oregonin, Macrophage, NO production, Antitumor activity

INTRODUCTION

As commonly understood, there are four therapeutic ways to treat cancer. There are surgical, radio-, immuno- and chemotherapy. Due to the properties of metastasis in cancer, systemic chemotherapy is routinely administered in combination because there are many cases that cannot be completely eliminated with a single treatment at a cancerous site. Generally, an oncologist is faced with great difficulties when treating cancers with existing chemotherapeutic regimes due to the severe side effects. Therefore, it is important to find the most appropriate therapy that can reduce the difficulties associated with chemotherapy. Consequently, there is increasing interest in natural substances, particularly plant extracts. In this study, "oregonin", a diarylheptanoid derivative from *Alnus hirsuta Turcz, Betulaceae,* which is horizontally distributed nationwide excluding Gyung-Buk and Choong-Nam and geographically distributed in Japan, Manturia, Saghalien, Kamchaka and Russia. (Joeng, 1974), was used.

In studies on *Alnus hirsuta Turcz, Betulaceae,* and their derivatives, Karchesy *et al.* first determined the structure of oregonin by tracing the substance that becomes red when cutting the plant from the bark (Karchesy *et al.*, 1974). Suga *et al.* reported its absolute configuration in 1982 (Suga *et al.*, 1982). In addition, Okuyama *et al.* isolated hirsutoside (diarylheptanoid xyloside (oregonin)), a red-colored substance, from *Alnus hirsuta* and *Alnus japonica*. In 1992, Lee *et al.* isolated oregonin from leaves of *Alnus hirsuta var. microphylla*, and in 1993, Yakugaku *et al.* isolated diarylheptanoid from *Acer nikoense* and *Myrica rubra.*

Kiuchi *et al.* elucidated its efficacy through the biosynthesis process of prostaglandin and leukotriene with the diarylheptanoid extracted from *Zingiber officinale* and *Alpinia oficinarum* (Kiuchi *et al.*, 1992), and Doug H. *et al.* found that the diarylheptanoid extracted from *Alpinia blepharocalyx* strongly interfered with the collagen-induced, arachidonic acid-induced and adenosine diphosphate-induced platelet aggregation in human blood (Doug *et al.*, 1998). In addition, YPE-01, a new diarylheptanoid, exhibited an anti-inflammatory effect by the selective inhibition of 5-lipoxygenase in the skin inflammation (Yamazaki *et al.*, 1988), and diarylheptanoid inhibited NO excretion against the inflammatory macrophages induced by LPS (Prasain *et al.*, 1998).

Interestingly, diarylheptanoid derivatives have been understood to have an anti-oxidant effect as well as other antitumor effects and diverse physiological activities (Sheth *et al.*, 1973; Kawai *et al.*, 1990; Saxena *et al.*, 1995).

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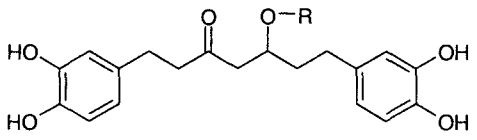

R: Xylose [oregonin]

1.7-bis-(3,4-dihydroxyphenyl)-heptane-3-on-5-O-β-D-xylopyranoside(M.W.=480)

Fig. 1. Chemical Structure of Oregonin

In this study, the antitumor effect of a diarylheptanoid, oregonin (M.W.=480, Fig. 1), was investigated to confirm the above noted functions.

MATERIALS AND METHODS

Materials

Six week-old male ICR mice (20~25 g) were used as the study animals. Oregonin (1.7-bis-(3,4-dihydroxyphenyl)-heptane-3-on-5-O-β-D-xylopyranoside, Fig. 1) was provided by the Department of Herbal Medicine, Chungang University. BSA(Sigma Chem. Co., U.S.A.), DMSO(Sigma Chem. Co., U.S.A.), FBS(Gibco BRL, U.S.A.), Griess Reagent(Sigma Chem. Co., U.S.A.), LPS(*E. coli* serotype 0111 : B4) (Sigma Chem. Co., U.S.A.), MTT(Sigma Chem. Co., U.S.A.), Penicillin-Streptomycin(Gibco BRL, U.S.A.), Recombinant INF-γ (R&D, U.S.A.), RPMI 1640(Sigma Chem. Co., U.S.A.), Sodium nitrite(Sigma Chem. Co., U.S.A.), Thioglycollate broth(Difco, U.S.A), Trypan Blue : Sigma Chem. Co., U.S.A., Trypsin-EDTA(Gibco BRL, U.S.A.) were also used. All other materials used in this study such as buffers and other reagents were prepared in this laboratory. In this study, P-815, a murine mastocytoma cell from the Department of Immunology, school of Pharmacy, SungGyunKwan University, was used.

Methods

Tumor cell culture

The P-815 (murine mastocytotoma cell) cells used in the study were sub-cultured 2~3 times in a week in RPMI-1640 media containing 10% FBS, penicillin 100 U/ml streptomycin 100 μg/ml and were cultured in a CO_2-incubator (37°C, 5% CO_2). The cultured tumor cells that reached the log phases were diluted to a set concentration 20 hours before the study. The FBS used in this procedure was inactivated by heat treatment for 30 minutes at 56°C.

Formulation of cell suspension

In order for the cells to reach the log phase, the tumor cells were cultured until they reached 2~3 × 10^5 cells/ml in 75 cm^2 screw-capped culture flask (Spinner culture) for 24 hours prior to the. In this culture, the number of cells in the culture media generally reached 0.8~1.0 × 10^6 cells/ml after 24 hours. The cell suspension was set at 1~5 × 10^5 cells/ml as the final concentration by diluting it with a fresh medium (Run bottle).

Peritoneal macrophage separation

The 6 week-aged (20~25 g) male ICR mice were sacrificed by a cervical dislocation, and the abdominal skin was pealed off. The serum free RPMI 16~40 medium 6 ml and air 3 ml were then injected into the peritoneum and withdrawn after massaging for 2~3 minutes. After repeating these procedures 3 times, most of the peritoneal cells were obtained. These were centrifuged for 10 minutes at 100 g after a dilution of the appropriate medium. The precipitated cells were suspended in RPMI 1640 medium including 10% FBS to be 1 × 10^6 cell/ml, and plate-adhered macrophages were taken only after a 2 hour culture in a CO_2-incubator, set at 37°C, 5% CO_2, 95% air. The inflammatory macrophages were used to determine the antitumor response against the external stimulation *in vitro* by inoculating thioglycollate (Jennifer *et al.*, 2002). The thioglycollate, 3 ml, was injected into the peritoneal, and the macrophages were taken using the same procedures as reported above. They were then resuspended using trypsin-EDTA and a scraper and finally diluted to the required concentration.

MTT assay

5 mg/ml of the MTT stock solution (Sigma) in pH 7.5 PBS was filtered with a 0.22 μm filter and 10 μlMTT (5 mg/ml) was added to the 100 μl cell suspension or the cell monolayer of the micro titer well and cultured in an incubator containing a humidifier for 3 hours at 37°C. Blue and non-aqueous formazan crystals were mixed with 0.04M HCl 100 μl, dissolved by propan-2-ol till, and the plates were read with an ELISA reader at the test wavelength of 570 nm (reference wavelength 630 nm).

Antitumor activity *in vivo*

There were five 6 week-aged male ICR mice (20~25 g) in each study group. The control mice were injected with the D-PBS buffer and the other 3 groups received 1, 5, 10 mg/kg of oregonin to the active group via the peritoneal in a 0.1 ml volume. The active substances were injected into the mouse peritoneum and the macrophages were isolated after 20 hours of injection. They were then distributed at a concentration of 1 × 10^5cells onto the 96 well plates in order to use them as the effecter cells. The cells were then cultured for 20 hours in a CO_2 incubator to make the ratio of effecter cells (macrophage) to target

cells (P-815) equal to 10:1. The P-815 cells were pre-cultured until they reached the log phase, and the total volume of each well was adjusted to 200 μl. Following these procedures, the MTT assay was used to measure the macrophage antitumor activity.

Antitumor activity *in vitro*

1×10^5 of the inflammatory macrophages isolated from the 6 week-aged male (20~25 g) ICR mice per well were distributed on a 96 well plate and used as the effecter cells. They were then and treated with the D-PBS buffer to make up the control group, 1, 10, 100 μg/ml of oregonin for the active group and IFN-γ 10U/ml & LPS 1 μg/ml for the positive control group for 20 hours. After treatment, the cells were cultured for 20 hours in order for the P-815 to target cell ratio to be 10:1. The total volume was set to 2 μl in each well. Subsequently, the MTT assay was used to measure macrophage antitumor activity *in vitro*.

NO production *in vivo*

In five 6 week-aged male ICR mouse (20~25 g) per group, D-PBS buffer was injected into the control group and 1, 5, 10 mg/kg was injected into the active group via the peritoneum adjusting the volume to 0.1 ml. The mice were inoculated with 2 ml each containing 1×10^5 cells/ml of the macrophages isolated after 20 hours of the injection to mouse peritoneum on the 12 well plate in each well. These were then cultured for 4, 20, 30, 40 hours in a CO_2 incubator and then the 100 μl suspension was then moved to the 96 well plates, and allowed to maintain an equivalent amount of Griess reagent after inoculation. Subsequently, the NO amounts produced were calculated and compared to the standard line of sodium nitrite by measuring the absorbance at 540nm using an ELISA reader. (Y=0.01806 X+0.003; Y:absorbance, X:mole concentration)

NO production *in vitro*

1×10^5 inflammatory macrophages isolated from the 6 week-aged male (20~25 g) ICR mouse per well were distributed on a 96 well plate and cultured in D-PBS buffer for the control group, 1, 10, 100 ml/ of the substance for the active group and IFN-γ 10U/ml & LPS 1 μg/ml for the positive control group for 20 hours in a CO_2 incubator, whilst adjusting the total volume to 2 ml in each well. The cells were then cultured for a further 4, 20, 30, 40 hours in a CO_2 incubator and 100 μl of the suspension was then moved to the 96 well plate, and allowed to maintain an equivalent amount of Griess reagent for 10 minutes after the inoculation. Subsequently, the NO amounts produced were calculated and comparing to the standard line of sodium nitrite by measuring the absorbance at 540nm using an ELISA reader. (Y=0.01806X+0.003; Y: absorbance, X: mole concentration)

Statistical treatment

A Student T-test was used to determine the difference between the control and the active group with the data reported as a mean value ± the standard deviation. A P-value > 0.05 was considered significant.

RESULTS

The antitumor activity of macrophage is commonly generated by an anti-microbial mechanism. This anti-microbial mechanism of macrophages occurs via the conversion of active macrophages through activation steps. To represent such activations, the role of cytokines such as IFN is necessary. These produce tumor cells that are easily sensitized. In contrast, Th_1 completely activates the macrophages by secreting INF-γ and providing the CD40 ligand. The membrane-associated TNF-α/TNF-β as well as the LPS used in this study activated the macrophages by playing a similar role to the CD40 ligand. The macrophages increased their phagocytic potency through external stimulations, accelerated the antitumor function and increased the number of cells and various cell secretions. The secreted substances depended upon the types of macrophages passing each activation step. In particular, it is known that macrophages activated by immunomodulators, such as IFN & LPS, secrete toxic substances against tumor cells. The substances secreted by the activated macrophages are TNF-α, IL-1, hydrogen peroxide, nitric oxide (NO), cytolytic protease, all of which are cytotoxic to tumor cells (Hibbs *et al.*, 1988, Jewett *et al.*, 1997)

* The following formula was used to compare the values with the control group.

Numerical value (Value of Y axis) =

| numerical value of the control group - numerical value of the active group | /numerical value of the control group

Antitumor activity *in vivo*

This study investigated whether oregonin directly activates the macrophages *in vivo*. To accomplish this, the macrophages taken from the mice 20 hours after the injection of 1, 5, and 10 mg/kg to the peritoneum, respectively, were measured. As a result, the cytotoxicity of the active group was 23.09%, 53.33%, and 64.23% higher than the control group at 1, 5, 10 mg/kg. (Fig. 2)

Antitumor activity *in vitro*

This study also aimed to determine if oregonin has a direct effect on tumor cells by activating macrophages *in*

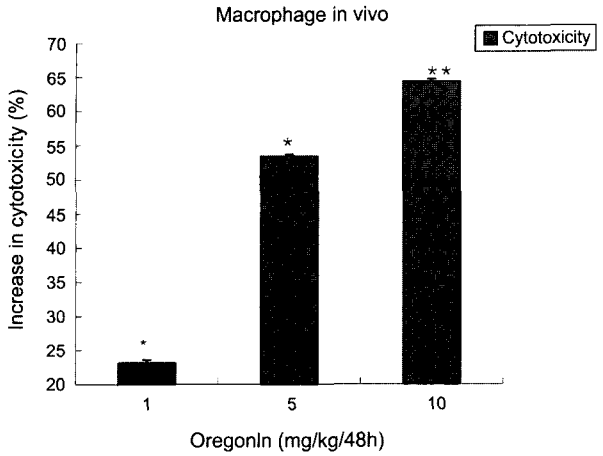

Fig. 2. The effect of oregonin on the antitumor activity of macrophages.
Oregonin was injected i.p. to mice 20 hours prior to the separation of the macrophages. The oregonin -treated macrophages, and effecter cells were co-cultured for 20hrs together with P-815, the target cells (effecter/target ratio=10:1). Tumoricidal activity was evaluated by the cell viability using a MTT-assay. The percentage on the Y-axis shows the cytotoxicity in the comparison with the control group. The cytotoxicity of the oregonin-treated groups were significantly different from that of the control group.(**p < 0.01, *p < 0.05; The percentage of the control group is 0%.)

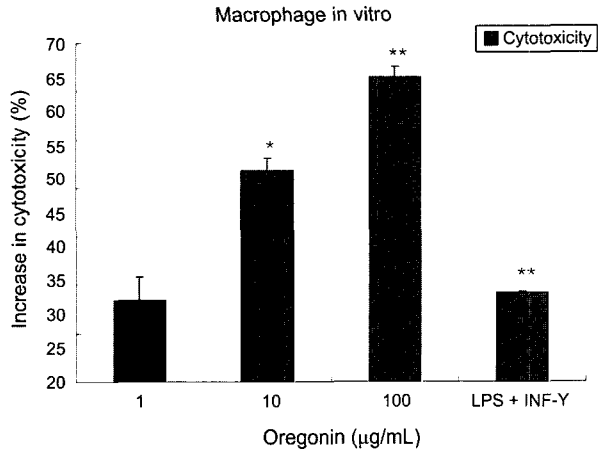

Fig. 3. *In vitro* effect of oregonin on macrophage antitumor activity.
Thioglycolate-elicited macrophages from the mice were collected by a peritoneum lavage. The peritoneal inflammatory macrophages, the effecter cells, were pretreated with oregonin for 20hours and co-cultured for 20hours with P-815, the target cells(effecter/target ratio= 10 : 1). The tumoricidal activity was evaluated by cell viability using a MTT-assay. The percentage on the Y-axis shows the cytotoxicity in the comparison with the control group. (** p 0.01, * p 0.05; The percentage of the control group is 0%.)

vitro. In line with this, the inflammatory macrophages taken from the test mouse were examined. The cytotoxicity of the macrophages increased in a dose-dependent manner up to 43.61%, and 62.91% higher than that of the control group, particularly at 10 and 100/.

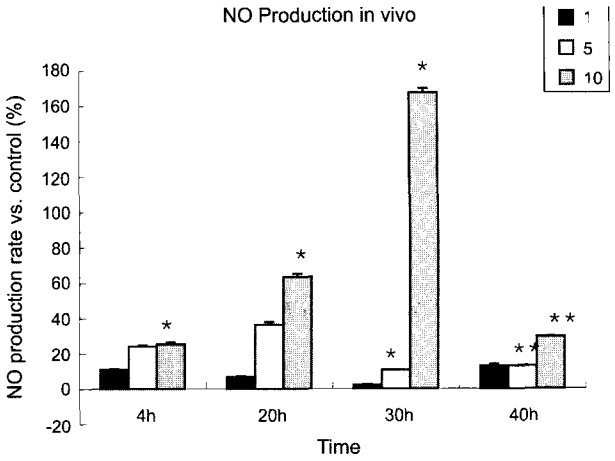

Fig. 4. The effect of oregonin on nitric oxide (NO) production of macrophages *in vivo*.
Oregonin was injected i.p. into the mice 20 hours prior to the separation of the macrophages. Oregonin-treated macrophages were incubated in the medium for the time indicated. The supernatant in the medium was collected and used for the NO assay. The NO production rate of the oregonin-treated groups were significantly different from that of the control group. (**p < 0.01, *p < 0.05; The percentage of the control group is 0%.)

Those results show that the active group is more effective than that of positive control group in terms of LPS & INF-γ (Fig. 3)

NO production *in vivo*

The amount of NO secretion after 4, 20, 30, 40 hours, respectively, was measured by isolating the macrophages from the mice 20 hours after the injection to the peritoneum at 1, 5, 10 mg/kg. As a result, the peak at 1 mg/kg was reached within 4 hours, within 20 hours at 5 mg/kg and within 30 hours at 10 mg/kg, respectively. This may be due to the half-life of the drug. In the case of 10 mg/kg, the rate of NO secretion increased by up to 167.38% compared to the control group after 30 hours. In addition, the amount of NO secretion tended to increase in a dose-dependent manner.(Fig. 4)

NO production *in vitro*

In order to confirm the influence of oregonin, which may give a specific impact to macrophages, the nitric oxide (NO) levels, one of the substances secreted when macrophages are activated, was measured. By isolating the inflammatory macrophages from the mice, the amount of NO secreted was confirmed by culturing by a further 4, 20, 30, 40 hours in fresh media after treating with oregonin for 20 hours at 1, 10, 100 μg/ml. As a result, the positive control group was found to show a continued increase of NO secretion when compared to the control

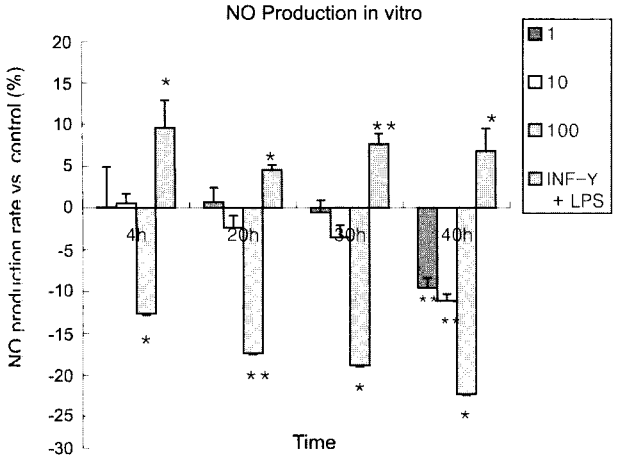

Fig. 5. *In vitro* effect of oregonin on macrophage NO production. Thioglycolate-elicited macrophages from the mice were collected by a peritoneum lavage. The peritoneal inflammatory macrophages were incubated with oregonin for 20 hrs. The supernatant in the medium was then collected and used for the NO assay 4, 20, 30, 40hours after incubation, respectively. The NO production rate decreased with increasing oregonin dose. It is believed that oregonin can reduce the NO production rate of the inflammatory macrophages. (**p < 0.01, *p < 0.05; The percentage of the control group is 0%.)

group, whereas the active group decreased in dose-dependent manner. These results correspond to the results reported by Prasain JK *et al.*. From such *in vivo*, and *in vitro* results, it is believed that oregonin may accelerate NO production in macrophages, whereas the macrophages already activated by thioglycolate or LPS decreased NO production. When investigating those results, it is believed that oregonin may represent an immunomodulatory function through an action both in the immuno-active and immuno-suppressive case. (Fig. 5)

DISCUSSION

The main aim of this study was to determine the antitumor effect of oregonin. Various types of excretion were observed when the macrophages were stimulated by a second transmitter generated from the binding sites such as the N-formulated peptide and Fc portion. Of those secretions, NO exhibits its cytotoxicity by inactivating the cell enzymes including Fe (iron) by forming an iron-nitrosyl complex and enzymes (Lancaster et al., 1990). Bredt and Snyder reported that macrophages kill tumor cells or play a role in killing microorganisms because NO inhibits the enzymes of the target cell (Bredt et al., 1994). Some researchers have reported that NO inhibits mitochondrial respiration, aconitase of the TCA cycle and the ribonucleotide reductase of tumor cells. In addition, others have reported increased NO production in macrophages induced from thioglycolate (Chen et al., 1996)

With regard to the antitumor activity and NO production test for the macrophages *in vivo* and *in vitro*, tests were carried out using various concentrations and the MTT assay was used to measure the antitumor function. The cytotoxicity at 10, 100 μg/ml *in vitro* was higher than that of the positive control administered as a combination of IFN-γ and LPS. Therefore, oregonin enhances the antitumor activity of macrophages.

In order to investigate the NO production of macrophage, the mutual action of oregonin in sensitized and non-sensitized macrophages was observed. The amount of NO secreted from the macrophages *in vivo* increased in a dose-dependent manner with maximum values at 1 mg/kg, 5 mg/kg, 10 mg/kg after 4, 20, 30 hours, respectively. These were attributed to the half-life of the drug. Although the positive control group increased NO secretion for up to 30 hours *in vitro*, the active group showed a higher inhibitory effect on NO secretion with increasing dose. This means that oregonin inhibits the macrophages induced by thioglycolate. Therefore, oregonin enhances the antitumor activity against non-activated macrophages and inhibits the inflammatory macrophages. This suggests that oregonin functions as an immunomodulator both in activated and inhibited cases of immunity.

Overall, oregonin, one of diarylheptanoids extracted from the leaves of *A. hirsute,* can show an activated response to immune cells. This is due to its enhancement function of immunity rather than its cytotoxicity both *in vivo* and *in vitro.*

In conclusion, more studies on the efficacy and function of oregonon as an antitumor as well as an anti-inflammatory are required to obtain more precise information are recommended.

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