Pharmacological Studies on 3-Formylamino-7-methylsulfonylamino-6-phenoxy-4*H*-1-benzopyran-4-one (T-614), a Novel Antiinflammatory Agent. 4th Communication: Inhibitory Effect on the Production of Interleukin-1 and Interleukin-6

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In vitro effects of 3-formylamino-7-methylsulfonylamino-6-phenoxy-4*H*-1-benzopyran-4-one (T-614), a novel antiinflammatory compound, on the production of interleukin-1 (IL-1) and/or interleukin-6 (IL-6) by human monocytes and the THP-1 cells of a human monocytic cell line, were examined. T-614 inhibited the release of immunoreactive IL-1 β from these cells stimulated with lipopolysaccharides (LPS) in a dose-dependent manner (0.3—30 μ g/ml). The release of IL-6 from THP-1 cells, as determined by the assays for its hepatocyte-stimulating activities and immunoreactivities, was inhibited by T-614 with the IC₅₀ values of 2.0 and 6.6 μ g/ml, respectively. Northern blotting analysis using LPS-stimulated THP-1 cells indicated that the inhibitory effect of T-614 on IL-1 β production is caused by the suppression of IL-1 β mRNA expression. The inhibition of cytokine production by T-614 may provide an important insight into the additional mechanisms contributing to its antiinflammatory activities.

Keywords — 3-formylamino-7-methylsulfonylamino-6-phenoxy-4*H*-1-benzopyran-4-one (T-614); antiinflammatory agent; interleukin-1; interleukin-6; cytokine production; human monocyte; THP-1 cell

Introduction

Interleukin-1 (IL-1) and interleukin-6 (IL-6) have been detected in the synovial fluids of the patients with rheumatoid arthritis.^{1,2)} Both cytokines are products of activated monocytes/macrophages and other inflammatory cells. In view of the wide range of inflammatory processes which can be induced by IL-1 and/or IL-6, such as fever, acute phase response, cartilage destruction and lymphocyte proliferation, it seems likely that they play an important role in the pathogenesis of rheumatoid arthritis and other chronic inflammatory diseases.^{3,4)} Therefore, a compound which inhibits the production of these cytokines and is able to provide a better symptomatic relief in rheumatoid arthritis would be a desirable therapeutic agent.

3-Formylamino-7-methylsulfonylamino-6-phenoxy-4*H*-1-benzopyran-4-one (T-614) is a member of a novel class of antiinflammatory compounds, which has virtually no gastrointestinal ulcerogenic action.⁵⁾ We have previously demonstrated⁶⁾ that T-614 has unique pharmacological properties such as the inhibitory effects on the granuloma inflammation and on the

joint destruction in adjuvant arthritis. Furthermore, T-614 has been shown to inhibit the production of cyclooxygenase products with an apparently different mode from conventional non-steroidal antiinflammatory drugs (NSAIDs), and additional mechanisms of the antiinflammatory action of T-614 have been suggested.⁷⁾

In this report, we examined the effect of T-614 on the production of IL-1 by human monocytes *in vitro* and the inhibition by this compound was investigated in detail using THP-1 cells, a human monocytic leukemia cell line.

Materials and Methods

Agents Used — T-614 was synthesized in our laboratories. Other test compounds used were indomethacin (Sigma Chemical Co.), ibuprofen (Toyama Chemical Co.) and hydrocortisone acetate (Nacalai Tesque). All test compounds were dissolved in dimethylsulfoxide (DMSO) and added to the reaction medium at the indicated concentration (final concentration of DMSO: 0.5% or less).

Cells — 1) Human Peripheral Monocytes: Human mononuclear cells were isolated from the

peripheral blood of healthy volunteers using differential centrifugation with Ficoll-Paque (Pharmacia LKB). Mononuclear cells suspended in RPMI 1640 medium (Nissui Pharmaceutical Co.) containing 10% heat-inactivated autologous serum were seeded at $3-5 \times 10^6$ cells/ml/well into a 24-well culture plate (CORNING Laboratory Sciences Co.), and cultured at 37 °C for 2 h. Non-adherent cells were removed by rinsing 3 times with serum-free RPMI 1640 medium. The remaining cells were used as the monocyte preparation.

2) THP-1 Cells (ATCC TIB 202)⁸⁾: The cell line was maintained in RPMI 1640 medium supplemented with 50 μ M 2-mercaptoethanol (2-ME, Wako Pure Chemical Co.) and 10% fetal calf serum (FCS, Whittaker Bioproducts Inc.) and passaged every 6–8 d.

3) HepG2 Cells (ATCC HB 8065),⁹⁾ a Human Hepatoma Cell Line: The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui) with 10% FCS and passaged every 10–14 d. These cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂/95% air.

IL-1 Production by Human Peripheral Monocytes — The cells were cultured with 1 μ g/ml of lipopolysaccharide (LPS, from *E. coli* serotype 0127:B8, Difco Laboratories) in the presence or the absence of a test compound for 18 h in RPMI 1640 with 0.1% human serum albumin (HSA, Sigma Chemical Co.). Then, the culture supernatants were collected and the released levels of IL-1 β were determined by using an enzyme-linked immunosorbent assay (ELISA) kit for IL-1 β (Otsuka Pharmaceutical Co.). To determine the intracellular level of IL-1 β , 0.5 ml of RPMI 1640 was added to each well after removal of the supernatant and the cells were disrupted by sonication.

IL-1 and IL-6 Production by THP-1 Cells — The method of Kovacs *et al.*¹⁰⁾ was used with a slight modification. Briefly, THP-1 cells suspended in RPMI 1640 with 50 μ M 2-ME and 1% FCS were seeded at 1×10^6 cells/ml/well into 24-well plates. Then, LPS was added at 1 μ g/ml into duplicate or triplicate cultures together with or without a test compound. After 48 h culture (unless otherwise noted), the supernatants were

collected for the determination of the levels of IL-1 β and IL-6. The immunoreactive IL-1 β was measured as described above and IL-6 as follows.

Determination of IL-6 — Hepatocyte-stimulating factor, HSF, assay^{11,12)} was employed in order to determine the IL-6 content in the supernatant of cultured THP-1 cells. Briefly, HepG2 cells were seeded at $2-8 \times 10^4$ cells/200 μ l/well into 96-well plates (CORNING) and cultured for 18–48 h. When the cells reached 95% or more confluency, the medium was removed and replaced with 200 μ l of serial three-fold dilutions (1:3–1:2187) of the sample supernatants or the IL-6 standard (human recombinant IL-6, R & D Systems Inc.) in DMEM containing 2% FCS and 1 μ M dexamethasone 21-acetate (Sigma). Following overnight culture, the medium was replaced with fresh DMEM. After an additional 2 h-culture, 50 μ l aliquots of the culture medium were taken and used to quantitate the released levels of fibrinogen induced with HSF activities. The levels of fibrinogen were measured by the biotin-streptavidin sandwich ELISA using a polyclonal goat antibody against human fibrinogen (Cappel Organon Technika Co.), which was developed in our laboratory with reference to the methods of Yamada *et al.*¹³⁾ and Martinez *et al.*¹⁴⁾ The amounts of IL-6 activity, its ability to induce the production of fibrinogen by HepG2 cells in the presence of dexamethasone, were determined by using linear regression analysis of microassay.¹⁵⁾ The results are reported in arbitrary units where one unit is defined as the quantity of IL-6 required to treble the background production of this protein.

An ELISA kit for IL-6 (InterTest-6, Genzyme Co.) was also used for the determination of IL-6 levels in the culture supernatants of THP-1 cells.

Northern Blotting Analysis of IL-1 β mRNA Expression in THP-1 Cells — For determination of mRNA levels, untreated and T-614 treated THP-1 cells (2×10^7 cells) were cultured in the presence or the absence of LPS (1 μ g/ml) for 30 h. Total cellular RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction.¹⁶⁾ Northern blot analysis was carried out by separation of 15 μ g of RNA on 1.2% agarose gels containing formaldehyde, trans-

Fig. 1. Effects of T-614 and Indomethacin on IL-1 β Production by Human Monocytes Stimulated with LPS

Human peripheral monocytes were cultured for 18 h in the presence of LPS (1 μ g/ml) with or without a test compound, and secreted (A, extracellular) and cell-associated (B, intracellular) IL-1 β levels were measured by ELISA. Each column represents the mean with S.E. as the percent of control cultures obtained from 4–5 donors. Extra- and intracellular IL-1 β levels of control cultures were 682 ± 130 pg/ml (mean with S.E., $n = 5$) and 37 ± 29 pg/ml ($n = 4$), respectively.

ferred to nitrocellulose, and hybridized with 32 P-labeled oligonucleotide probe.¹⁷⁾ The probes used in this experiment were 40 mer oligonucleotide for β -actin (Oncogene Science Inc.) and 40 mer for IL-1 β (Oncogene) and were 5'-end-labeled with [γ - 32 P] ATP (Amersham Laboratories, 3000 Ci/mmol) using T4 polynucleotide kinase (Toyobo Co.).¹⁸⁾ The nitrocellulose membranes were exposed for 3 d using intensifying screens.

Results

Effect on IL-1 β Production by Human Monocytes Stimulated with LPS

Figure 1 shows the effects of T-614 and indomethacin on the extra- and intracellular levels of immunoreactive IL-1 β in human peripheral monocytes cultured with LPS (1 μ g/ml). T-614, at 3 and 30 μ g/ml, reduced the extracellular release of IL-1 β , whereas it had no significant effect on the intracellular levels. On the other hand, indomethacin caused a dose-dependent augmentation in the levels of cell-associated

IL-1 β .

Effect on IL-1 β Production by THP-1 Cells Stimulated with LPS

To further elucidate the inhibitory effect of T-614 on cytokine production, THP-1 cells, which has been reported to be stimulated with LPS to produce IL-1,¹⁰⁾ was used instead of peripheral blood monocytes (Fig. 2). The time course of IL-1 β release from control and T-614 (10 μ g/ml) treated THP-1 cells following the stimulation with LPS are shown in Fig. 2A. This cell line released IL-1 β with a longer lag phase of 24 h after the addition of LPS than the peripheral monocytes responding to LPS within several hours.^{19,20)} The inhibition of IL-1 β production by T-614 (10 μ g/ml) was observed from 48 to 72 h after the stimulation with LPS. Figure 2B shows the dose-response curve of each test compound for the inhibition of IL-1 β production by THP-1 cells. T-614 inhibited this response in a dose-dependent manner at a concentration of 1 μ g/ml or more, and the IC_{50} value was calculated to be 3.1 μ g/ml. Indomethacin and ibuprofen did not have any significant

Fig. 2. Inhibitory Effects of T-614 and Other Reference Compounds on IL-1 β Production by THP-1 Cells Stimulated with LPS

A) Time course of IL- β production by control and T-614-treated THP-1 cells. THP-1 cells (1×10^6 cells) were cultured in the presence of LPS (1μ g/ml) with or without T-614 (10μ g/ml) and at certain time intervals secreted IL-1 β levels were measured by ELISA. Each point represents the mean of triplicate cultures. B) Dose-response curves for the inhibition of IL-1 β production. Each test compound was added to the cultures of THP-1 cells in the presence of LPS (1μ g/ml), and after 48 h incubation IL-1 β levels in medium were determined by ELISA. Each point represents the mean with S.E. as the percent of control cultures obtained from 3–6 separate experiments. IL-1 β levels of control cultures were 752 ± 321 pg/ml (mean with S.E., $n = 6$).

TABLE I. Effects of T-614 and Other Reference Compounds on IL-6 Production by THP-1 Cells Stimulated with LPS

Compounds	Concn. (μ g/ml)	HSF activity ^{a)}		ELISA ^{b)}	
		Units/ml	(% of control)	pg/ml	(% of control)
Control	—	23.6 ± 5.4		2484	
T-614	0.3	21.4 ± 5.2	(84)	2039	(82)
	1	15.0 ± 3.7	(63)	1802	(73)
	3	9.5 ± 3.7	(37)	1545	(62)
	10	5.7 ± 2.6	(22)	991	(40)
	30	3.2 ± 0.7	(15)	851	(34)
Indomethacin	10	37.5 ± 20.9	(154)	5754	(232)
Ibuprofen	10	49.5 ± 19.5	(204)	3245	(131)
Hydrocortisone acetate	0.01	NT ^{c)}		1197	(48)
	0.1	NT		<313	(<13)

Each test compound was added to the cultures of THP-1 cells in the presence of LPS (1μ g/ml), and after 48 h incubation IL-6 levels in medium were determined by the assay for its hepatocyte-stimulating factor (HSF) activities and by ELISA. a) Each value represents the mean with S.E. obtained from 4–5 separate experiments. b) Each value represents the mean of triplicate samples. c) not tested.

Fig. 3. Effects of T-614 and Indomethacin (IDM) on Expression of IL-1 β and β -Actin mRNA in THP-1 Cells Stimulated with LPS

THP-1 cells (2×10^7 cells) were cultured in the presence of LPS (1μ g/ml) with or without a test compound. After 30 h cultures, cellular RNA was extracted and analyzed by northern blot hybridization using radiolabeled oligonucleotide probes of human IL-1 β (A) and β -actin(B).

effect on IL-1 β release even at a high concentration of 10μ g/ml. Hydrocortisone acetate was more effective than T-614 with an IC_{50} value of 0.015μ g/ml.

Effect on IL-6 Production by THP-1 Cells Stimulated with LPS

Subsequently, we measured the release of IL-6 to medium from THP-1 cells stimulated with LPS. Determination of IL-6 levels was performed by both assays for its activities as HSF and the immunoreactivities. THP-1 cells responded to LPS (1μ g/ml) by producing substantial amounts of IL-6 with the similar time course to that of IL-1 β (data not shown). Table I summarized the effects of T-614 and other test compounds on the IL-6 production by THP-1 cells cultured with LPS for 48 h. T-614 exhibited a dose-dependent inhibitory effect on its production and the IC_{50} values were calculated to be 2.0μ g/ml for the HSF assay and 6.6μ g/ml for the ELISA. In contrast to the case of IL-1 β production, indomethacin and ibuprofen increased the IL-6 levels at a concentration of 10μ g/ml in either assay. Because of the presence of dexamethasone, the effect of hydrocortisone was not assessed in the HSF assay system, however, this drug also showed a potent inhibition of the release of immunoreactive IL-6.

Effect on Expression of IL-1 β mRNA in THP-1 Cells

For better understanding of the inhibitory

mechanism of IL-1 production by T-614, we studied the effect of this compound on IL-1 β mRNA levels. Northern blot analysis of mRNA in THP-1 cells stimulated with LPS for 30 h is shown in Fig. 3. In contrast to non-treated cells, the LPS-stimulated cells had a considerable amount of IL-1 β mRNA and the addition of T-614 (10μ g/ml) to the culture of LPS-stimulated cells resulted in an obvious reduction of the levels. Densitometric scans of the films revealed that the decrease in this expression by T-614 was reached by about 30% as compared with control cells. On the other hand, indomethacin did not affect the amounts of IL-1 β mRNA in THP-1 cells. The levels of β -actin mRNA were unchanged by either treatment with LPS or T-614.

Discussion

The data reported in this paper indicate that T-614 is an inhibitor of IL-1 β production, especially the extracellular release of this cytokine, by human monocytes *in vitro*. This inhibitory effect seems unlikely to be due to its inhibitory activity on the prostaglandin production, because endogenous prostaglandins have been shown to modulate suppressively the production of IL-1 and cyclooxygenase inhibitors are reported to cause an augmentation in the LPS-induced IL-1 production.²¹⁾ In fact, the addition of indomethacin resulted in a dose-dependent increase

of intracellular levels of IL-1 β in our experiments. The inhibitory effect of T-614 on IL-1 production has been observed in monocytes stimulated by other phagocytic stimuli including zymosan and silica particles (data not shown), suggesting that the action site of T-614 is unrelated to the initial interaction of the stimulatory agent and its putative receptor.

Since the effect of T-614 on IL-1 production was found to be apparently different from the conventional cyclooxygenase inhibitors, we further investigated its inhibitory effects on cytokine production using THP-1 cells, which has been shown to be stimulated with LPS to produce IL-1.¹⁰⁾ The kinetic studies of the production of IL-1 β and IL-6 by the LPS-stimulated THP-1 cells indicated that the latency of about 24 h post-stimulation was required for the cytokine response. Cochran and Finch-Arietta²²⁾ have reported that the "priming" step mediates between the transition of THP-1 cells from resting monocytes to fully activated macrophages capable of cytokine production upon exposure to LPS. However THP-1 cells own a peculiar cytokine response, we have thought that THP-1 cells would provide a useful model to study the effects of various compounds on cytokine production, as was reported by Reisman *et al.*,²³⁾ because of the easiness of cell preparation and the reproducibility.

T-614 suppressed dose-dependently both cytokines productions by the LPS-stimulated THP-1 cells, whereas indomethacin and ibuprofen had no effect on IL-1 β production and the significantly augmentative effects on IL-6 production. The precise mechanism of the different effects of these cyclooxygenase inhibitors on IL-1 β and IL-6 remains obscure. Hydrocortisone also exhibited a potent inhibitory effect on both productions in this cell line, as has been shown in the peripheral monocytes.¹⁹⁾ Northern blotting analysis of LPS-stimulated THP-1 cells demonstrates that the inhibitory effect of T-614 on IL-1 β production is based on the suppression of IL-1 β mRNA expression, suggesting that T-614 might act mainly at the pretranscriptional level. T-614 had no inhibitory effect on either cell viability or total RNA level and β -actin mRNA. This indicates that the inhibition of cytokine

production is not due to a non-specific inhibition of metabolic functions or to cellular toxicity.

T-614 has been tested in the chronic inflammation models, in which IL-1 and/or other cytokines should be involved.²⁴⁻²⁶⁾ Oral administration of this compound leads to the significant improvement in not only the hind paw swelling but the joint destruction in adjuvant arthritis, and inhibits the granuloma formation in carrageenin-induced air-pouch type inflammation in rats.⁶⁾ These *in vivo* experiments suggest that T-614 has apparently dissimilar action to the classical NSAIDs and its inhibitory effect on cytokine production may partly account for the favorable *in vivo* actions. Further studies will be required to investigate its possible relevance *in vivo*. We attempt to examine whether T-614 might reduce the production of IL-1 and IL-6 in the inflammatory sites of several animal models or not. If so, T-614 may provide an important insight into the treatment of disease states such as rheumatoid arthritis, where aberrant cytokine expression is implicated. By virtue of its effective inhibition of the production of the inflammatory cytokines, T-614 represents a new class of steroid replacing antiinflammatory drugs (SRADs) which may serve as a new therapy for the treatment of chronic inflammatory diseases.

In summary, the data presented here demonstrate that T-614 has a considerable suppressive effect on the production of IL-1 and IL-6 in human monocytic cells *in vitro*. The inhibition of IL-1 β production seems to be caused by the suppression at the step up to the transcription of IL-1 β mRNA in THP-1 cells. Our findings also suggest that THP-1 cells may provide a convenient model to study the effects of various test compounds on the monocytic functions.

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