The Effects of a Newly Developed Nonsteroidal Anti-inflammatory Drug (M-5011) on Arachidonic Acid Metabolism in Rheumatoid Synovial Fibroblasts

Kenji Tobetto1, Yumiko Yamamoto1, Masanori Kataoka1, Takao Ando1, Kenji Sugimoto2 and Michio Himeno2

1Research & Development Laboratories, Maruho Co., Ltd., 1-8-23 Oyodo-naka, Kita-ku, Osaka 531, Japan
2Department of Applied Biochemistry, Faculty of Agriculture, University of Osaka Prefecture, 1 Gakuen-cho, Sakai, Osaka 593, Japan

Received April 11, 1997 Accepted September 16, 1997

ABSTRACT—M-5011 (d-2-[4-(3-methyl-2-thienyl)phenyl]propionic acid) is a newly developed nonsteroidal anti-inflammatory drug (NSAID) that displays potent anti-inflammatory and analgesic properties with low ulcerogenic activities in animal models. In this study, the effects of M-5011 on arachidonic acid (AA) metabolism in synovial fibroblasts from patients with rheumatoid arthritis were evaluated and compared with those of other NSAIDs in vitro. Either M-5011 or ketoprofen potently inhibited prostaglandin (PG) E2 production by cyclooxygenase (COX)-2 from exogenous AA in interleukin-1β (IL-1β)-stimulated cells. The IC50 values of M-5011 and ketoprofen were 4.4 x 10^{-7} and 5.9 x 10^{-7} M, respectively. However, diclofenac and indomethacin were one order less potent. Although the latter two drugs exhibited time-dependent and irreversible inhibition on COX-2 in IL-1β-stimulated cells, the inhibitory effects of M-5011 and ketoprofen were reversible. PGE2 production by COX-1 from exogenous AA in non-stimulated cells was also inhibited by M-5011 with a potency less than that of ketoprofen. In addition, M-5011 inhibited [14C]AA release from prelabeled synovial cells stimulated with bradykinin. However, ketoprofen hardly affected the [14C]AA release. It is likely that the effects of M-5011 on AA metabolism are, in part, responsible for its in vivo efficacy and safety profile.

Keywords: M-5011, NSAID, Synovial cell, Cyclooxygenase, Arachidonic acid metabolism

Cyclooxygenase (COX) converts arachidonic acid (AA) to prostaglandin (PG) H2, which is further metabolized by other enzymes to various PGs and thromboxanes (1). Formation of COX-derived products is dependent on the prior release of AA (2), which is generated from phospholipids by phospholipases (3). COX exists in at least two distinct isoforms, COX-1 and COX-2 (4, 5). The former, expressed constitutively in most cells and tissues (6, 7), is assumed to be responsible for producing PGs involved in maintaining vital functions related to vascular homeostasis, gastric mucosa and kidney (8). COX-2, induced in many cells exposed to pro-inflammatory cytokines such as interleukin-1 (9, 10), is found in synovia of patients with rheumatoid arthritis (RA) (11) and inflammatory tissues of experimental animal models (12, 13).

It is generally accepted that COX is an important target of nonsteroidal anti-inflammatory drugs (NSAIDs) (14). The anti-inflammatory properties of NSAIDs are achieved by the inhibition of COX-2, whereas the common side-effects of NSAIDs, such as gastrointestinal damage and renal dysfunctions, are associated with the inhibition of COX-1 (13, 15). Therefore, it is important to consider the efficacy/side-effect relationship of an NSAID by determining the pharmacological profile of different NSAIDs for COX isoforms.

It has been reported that significant differences exist in the subcellular locations of COX-1 and COX-2; the former functions predominantly in the endoplasmic reticulum, while the latter may be involved in events in both the endoplasmic reticulum and nuclear envelope (16). The differences in site-specific functions may mold the different sensitivities of COX-1 and COX-2 to NSAIDs (17). In addition, intracellular distribution of an NSAID is partly dependent on the pKa and lipid/water partition coefficient of the drug molecule (18). Therefore, enzyme preparations are not ideal for evaluating the pharmacological features of an NSAID for COX activities.

Since the synthesis of PGs is limited by the availability of free AA (2), the regulation of AA release may be an
important target for anti-inflammatory drugs in most tissues. Bradykinin (BK) is a potent pain-producing nonapeptide that is formed in damaged tissues (19). This peptide binds to receptors on the cell surface and triggers the activation of phospholipase A2 (20).

It has been reported that a newly developed NSAID, \( d-2-[4-(3\text{-methyl-2-thienyl})\text{phenyl}]\text{propionic acid (M-5011)} \), elicits potent anti-inflammatory and analgesic properties in animal models (21). M-5011 is less potent than some other NSAIDs in inducing stomach and small intestine lesions in mice (21). In this study, the effects of M-5011 on AA metabolism (PGE\(_2\) production and AA release) in human synovial fibroblasts were evaluated and compared with those of other NSAIDs.

**MATERIALS AND METHODS**

**Drugs**

M-5011 \( (d-2-[4-(3\text{-methyl-2-thienyl})\text{phenyl}]\text{propionic acid}) \) was synthesized by Maruho Co., Ltd. (Osaka). Indomethacin, ketoprofen and diclofenac sodium salt were the reference NSAIDs (Sigma Chemical, St. Louis, MO, USA). Stock solutions (100 mM) were prepared by dissolving these drugs in dimethylsulfoxide (Sigma Chemical) and stored at \(-20^\circ\text{C}\) before use.

**Synovial cells**

Human rheumatoid synovium from the knee was obtained from 2 patients (female, age: 46- and 64-year-old) undergoing synovectomy (Hayaishi Hospital, Osaka) with unknown medical history of RA. Adherent synovial fibroblasts, prepared by enzymatic digestion of the synovium (22), were cultured in Dulbecco's modified Eagle's medium (DMEM; ICN Biomedicals, Costa Mesa, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, ICN Biomedicals), 100 U/ml of penicillin (Meiji Seika Co., Ltd., Tokyo) and 100 \( \mu\text{g/ml} \) of streptomycin (Meiji Seika) at \( 37^\circ\text{C} \) in a humidified 5% CO\(_2\) atmosphere. The cells were trypsinized and seeded at a density of \( 2 \times 10^4 \text{cells/cm}^2 \) in 24- and 48-well cell culture plates. Confluent cultures at passages 8-9 were used in all experiments.

**Measurement of PGE\(_2\) production**

"Interleukin-1\( \beta \) (IL-1\( \beta \))-stimulated" cells were synovial cells incubated with human recombinant IL-1\( \beta \) (50 U/ml; Genzyme, Boston, MA, USA) in DMEM containing 10% FBS in 48-well culture plates at \( 37^\circ\text{C} \) for 24 hr prior to washing 3 times with serum-free DMEM. Synovial cells incubated at \( 37^\circ\text{C} \) for 72 hr without changing the medium before rinsing with serum-free DMEM were designated as "non-stimulated" cells.

The effects of drugs on PGE\(_2\) production in synovial cells were evaluated in 2 experiments: i) the instantaneous inhibition of COX activity was determined by exposing IL-1\( \beta \)-stimulated and non-stimulated cells to 0.25 ml/well of DMEM containing 0.1% bovine serum albumin (BSA; Intergen, New York, NY, USA) with AA (10 \( \mu\text{g/ml} \); Serdiary Research Laboratories, London, Canada) in the presence of a drug at the indicated concentrations at \( 37^\circ\text{C} \) for 60 min; and ii) the reversibility of drug inhibition was investigated by incubating IL-1\( \beta \)-stimulated cells with the respective drug (10\(^{-5}\) M) in DMEM containing 10% FBS at \( 37^\circ\text{C} \) for various intervals (up to 60 min) before washing 3 times with serum-free DMEM and exposing to 0.25 ml/well of DMEM containing 0.1% BSA with AA (10 \( \mu\text{g/ml} \) at \( 37^\circ\text{C} \) for 60 min. On completion of the final incubation in all experiments, the medium was collected and stored at \(-20^\circ\text{C}\) before determination of PGE\(_2\) contents by specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA).

**Determination of COX mRNA levels by the reverse transcriptase-polymerase chain reaction (RT-PCR)**

Non-stimulated synovial cells were incubated in DMEM containing 10% FBS with and without IL-1\( \beta \) (50 \( \text{U/ml} \)) at \( 37^\circ\text{C} \) for various intervals (up to 24 hr). The cells were rinsed with phosphate-buffered saline (PBS) and harvested for total RNA preparation by the guanidinium thiocyanate procedure. Reverse transcription was carried out by use of the first-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden). According to the kit manual, total RNA (5 \( \mu\text{g} \)) dissolved in 20 \( \mu\text{l} \) of RNase-free water was incubated for 1 hr at \( 37^\circ\text{C} \) with 13 \( \mu\text{l} \) of first-strand cDNA reaction mixture containing Moloney murine leukemia virus reverse transcriptase, RNAGuard, RNase/DNase-free BSA, dNTPs and 0.2 \( \mu\text{g} \) of random hexamers. The samples were then heated at 95\(^\circ\text{C}\) for 5 min to terminate the reverse transcription. The resulting cDNA was mixed with 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (final volume of 100 \( \mu\text{l} \)). Aliquots (1 \( \mu\text{l} \)) were used for PCR amplification with either COX- or glyceraldehyde-3 phosphate dehydrogenase (GAPDH)-specific primers. To assess if the amplification was achieved in an exponential range with a fixed number of cycles (23), cDNA samples diluted in the same buffer to 1 : 2 and 1 : 8 were also amplified. A 1.1-kb cDNA coding for human GAPDH (50 pg; Clontech Laboratories, Palo Alto, CA, USA) was amplified and used as a standard reference. PCR was performed in a reaction buffer (50 \( \mu\text{l} \)) containing 10 mM Tris-HCl (pH 8.9), 1.5 mM MgCl\(_2\), 80 mM KCl, 0.5 mg/ml BSA, 0.1% sodium cholate, 0.1% Triton X-100, 0.2 mM dNTPs and 26 pmol of each sense and antisense primer with 2 units of Tth DNA polymerase (Toyobo Co., Ltd., Osaka). The reaction (30 cycles) was performed as follows: 94\(^\circ\text{C}\) (1 min) to 55\(^\circ\text{C}\) (1 min) fol-
lowed by 72°C (2 min) for each cycle. For human COX-1, primers were 5'-GCT GGG AGT TCT CTA ACG TGA G-3' (a 25-mer sense oligonucleotide at position 417) and 5'-GGC AAT GCG GTT GCG GTA TTG GAA CT-3' (a 26-mer antisense oligonucleotide at position 1138). For human COX-2, primers were 5'-TAC TAG AGC CCT TCC TCC TGT GCC T-3' (a 25-mer sense oligonucleotide at position 402) and 5'-TGC TCA GGG ACT TGA GGA GGG TAG A-3' (a 25-mer antisense oligonucleotide at position 803). For GAPDH, primers were 5'-TCC ACC CAT GGC AAA TTC CAT GGC A-3' (a 25-mer sense oligonucleotide at position 211) and 5'-TTT CTA GAC GGC AGG TCA GGT CCA C-3' (a 25-mer antisense oligonucleotide at position 811). Aliquots of PCR products were electrophoresed in 2% agarose gels with Tris-borate/EDTA buffer (pH 8.0) before staining with ethidium bromide.

**Western blot analysis**

Non-stimulated synovial cells in 150-cm² culture flasks were incubated in DMEM containing 10% FBS with and without IL-1β (50 U/ml) at 37°C for various intervals (up to 24 hr). On decantation of the medium, the cells were rinsed 3 times with ice-cold PBS and scraped into 1.5-ml samples of 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 10 mM EDTA, 1 mM diethyldithiocarbamate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 50 μM pepstatin A and 1% Triton X-100. COX polypeptides were solubilized by sonication of cell samples for 5 sec, and the sonicate was centrifuged at 15,000 x g for 30 min to remove insoluble contaminants. Protein contents in the supernatant were determined by DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as a standard. Solubilized proteins, precipitated by adding 3 vol. of cold acetone (−20°C), were dissolved in 80 mM Tris-HCl (pH 8.0) containing 0.3 mM diethyldithiocarbamate, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 50 μM pepstatin A and 1% Triton X-100. COX polypeptides were solubilized by sonication of cell samples for 5 sec, and the sonicate was centrifuged at 15,000 x g for 30 min to remove insoluble contaminants. Protein contents in the supernatant were determined by DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as a standard. Solubilized proteins, precipitated by adding 3 vol. of cold acetone (−20°C), were dissolved in 80 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 10 mM EDTA, 1 mM diethyldithiocarbamate, and 0.1% Tween 20 to afford a 20 mg/ml solution. Dissolved proteins were then heated at 100°C for 1 min with gel-loading buffer (50 mM Tris-HCl (pH 6.8) containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 2 mg/ml bromphenol blue) in a ratio of 1 : 1 (v/v). Sample proteins (160 and 20 μg/lane for COX-1 and COX-2 analyses, respectively) were separated by SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide gel and transferred electrophoretically (2 mA/cm², 1 hr) to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) with a transfer buffer containing 25 mM Tris and 192 mM glycine. After blocking the membranes with 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (TBS) and 10% non-fat dry milk, the membrane was incubated overnight at 4°C with either 1 : 100 rabbit anti-COX-1 antibody (Cat. No. PG16; Oxford Biomedical Research, Oxford, MI, USA) or 1 : 500 rabbit anti-COX-2 antibody (Cat. No. 160107, Cayman Chemical) in TBS containing 0.05% Tween 20 (TBST) and 3% BSA. The membranes, washed with TBST and incubated for 30 min at room temperature with 1 : 20000 goat anti-rabbit IgG-alkaline phosphatase (Jackson Immunoresearch Laboratories, West Grove, PA, USA), were then incubated with CDP-Star (Tropix, Bedford, MA, USA), an enzyme substrate, before exposing them to X-ray film for chemiluminescence. Purified COX-1 (100 ng/lane) and COX-2 (80 ng/lane) polypeptides, derived respectively from ram seminal vesicles and sheep placenta (Cayman Chemical), were used as positive controls.

**Release of AA from synovial cells**

Synovial cells in 24-well cell culture plates were incubated with [1-14C]AA (1.9 kBq/0.5 ml/well, 2.0 GBq/mmol; Du Pont, Wilmington, DE, USA) in DMEM containing 10% FBS for 24 hr at 37°C to label membrane phospholipids with radioactive AA (24). After washing 3 times with serum-free DMEM, the labeled cells were exposed to 0.5 ml/well of DMEM containing 10% FBS with BK (20 μM; Bachem, Torrance, CA, USA) at 37°C for 3 hr in the presence of a drug at the indicated concentrations. At the end of incubation, the medium was collected and centrifuged at 2000 x g for 15 min at room temperature to remove any cells. The supernatant was isolated and its radioactivity was measured by a liquid scintillation counter. The free [14C]AA content in the medium was analyzed by thin layer chromatography as previously described (24).

**Incorporation of AA in synovial cells**

Synovial cells in 24-well cell culture plates were incubated with [1-14C]AA (0.6 kBq/0.5 ml/well) in DMEM containing 10% FBS with or without M-5011 (10−6 M) at 37°C for various intervals. At the end of incubation, the residual radioactivity in the medium was measured.

**Statistical analyses**

IC50 values were calculated by the least squares method. Statistical significance of data was evaluated by the unpaired Student’s t-test.

**RESULTS**

**Expression and activities of COX-1 and COX-2 in cells**

When the COX mRNA levels in non-stimulated cells were assessed with the RT-PCR method, which is more sensitive than the Northern blot procedure, COX-1 mRNA but not COX-2 mRNA was observed (Fig. 1a). Dilution of cDNA samples followed by amplification demonstrated that PCR was performed in a semiquanti-
Incubation of non-stimulated cells with IL-1β resulted in marked induction of COX-2 mRNA, which was detectable at 1 hr and attained a high content at 6 hr post-incubation. The COX-2 mRNA expression persisted for at least 24 hr (Fig. 1b). In contrast, the COX-1 mRNA contents were slightly affected by IL-1β. Furthermore, 2-hr incubation with 10% FBS, but not 0.1% BSA (data not shown), induced slight COX-2 mRNA expression.

Western blotting manifested induction of COX-2 polypeptide at a position of 70 kDa in response to IL-1β. The COX-2 accumulation, detectable at 1 hr, displayed the maximum content at 6 hr post-incubation, and it remained elevated thereafter for up to 24 hr (Fig. 2). COX-2 polypeptide was undetectable in non-stimulated cells. Parallel expression kinetics of COX-2 mRNA and protein induced with IL-1β were observed. A weak COX-1 polypeptide band (70 kDa), unaffected by IL-1β for 24 hr, was observed in non-stimulated cells at a position similar to that of authentic COX-1 (Fig. 2).

**Fig. 1.** RT-PCR analyses of COX-1 and -2 mRNA expression in synovial cells. Non-stimulated cells were incubated with (+) and without (−) interleukin-1β (IL-1β, 50 U/ml) at 37°C for the indicated periods. Total RNA isolated from non-stimulated (a) and IL-1β-stimulated (b) cells was used in the RT-PCR assay for COX transcript. GAPDH mRNA was used to assess the integrity and sample equivalence. A sample of 50 pg of authentic GAPDH cDNA (Std) was amplified by PCR as the standard. PCR products were electrophoresed in an agarose gel, visualized by adding ethidium bromide and photographed under u.v. lighting. A 100-base-pair (bp) ladder was used as the molecular wt. marker.

**Fig. 2.** Western blot analyses of COX-1 and COX-2 polypeptide levels in synovial cells. Non-stimulated cells were incubated with (+) and without (−) interleukin-1β (IL-1β, 50 U/ml) at 37°C for the indicated periods and lysed. The contents of COX-1 and COX-2 proteins were determined by Western blots using polyclonal antibodies for COX-1 and COX-2. Purified COX-1 polypeptide (100 ng/lane) from ram seminal vesicles and COX-2 polypeptide (80 ng/lane) from sheep placentae were used as the positive controls (PC).
COX activities of IL-1β- and non-stimulated cells were measured with 10 μg/ml AA as the substrate in DMEM containing 0.1% BSA. The kinetics of PGE2 production in cells were examined for 6 hr (Fig. 3). PGE2 production in IL-1β-stimulated cells exceeded that in non-stimulated cells by 10.1-fold at 60-min incubation.

**Instantaneous inhibition of COX activities**

The instantaneous effect of a drug on COX activities in synovial cells was evaluated by adding the drug and AA (10 μg/ml) simultaneously to the cells. All tested NSAIDs inhibited COX activities in IL-1β-stimulated cells dose-dependently (Fig. 4). The IC50 values of M-5011 and ketoprofen were 4.4 × 10^{-7} and 5.9 × 10^{-7} M, respectively, whereas those of diclofenac (2.0 × 10^{-6} M) and indomethacin (>5.0 × 10^{-6} M) were one order higher. Within the range of 10^{-8}–5 × 10^{-6} M, although M-5011 was less potent than ketoprofen, the inhibition plateaus elicited by M-5011 and ketoprofen (propionic acid derivatives) were approximately 30–40% of the COX activities in non-stimulated cells (Table 1).

**Time-dependent inhibition of COX-2**

The mode of M-5011 action on COX-2 was examined with IL-1β-stimulated cells. Cells incubated with a drug were subjected to increasing time intervals, washed and exposed to AA per se. Indomethacin and diclofenac revealed irreversible inhibitions on the COX-2 activity, which was dependent on the time of incubation with these inhibitors (Fig. 5). Especially with respect to diclofenac, a rapid decay in COX-2 activity was observed. However, M-5011 and ketoprofen, which did not induce time-dependent inhibition of COX-2 activities, behaved as reversible inhibitors.

**Inhibition of [14C]AA release**

Prebabeled synovial cells were incubated for 3 hr with or without BK (20 μM) in the presence of either M-5011 (10^{-6} M) or ketoprofen (10^{-6} M). The amount of radiolabel released after the incubation in BK-stimulated cells was twofold that of the unstimulated cells (Table 2). When free [14C]AA contents against the total releases in media incubated with BK, BK plus M-5011, BK plus ketoprofen or with the medium alone were expressed as a

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Concentration (M)</th>
<th>PGE2 level (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>—</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>4.79 ± 0.34</td>
</tr>
<tr>
<td>M-5011</td>
<td>10^{-4}</td>
<td>3.75 ± 0.11**</td>
</tr>
<tr>
<td></td>
<td>5 × 10^{-4}</td>
<td>3.49 ± 0.56*</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>3.32 ± 0.27**</td>
</tr>
<tr>
<td></td>
<td>2 × 10^{-3}</td>
<td>3.33 ± 0.05**</td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>3.72 ± 0.34*</td>
</tr>
<tr>
<td></td>
<td>5 × 10^{-6}</td>
<td>3.09 ± 0.28*</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>10^{-4}</td>
<td>2.09 ± 0.23**</td>
</tr>
<tr>
<td></td>
<td>5 × 10^{-4}</td>
<td>2.90 ± 0.25**</td>
</tr>
<tr>
<td></td>
<td>2 × 10^{-5}</td>
<td>2.70 ± 0.31**</td>
</tr>
<tr>
<td></td>
<td>10^{-6}</td>
<td>2.95 ± 0.08***</td>
</tr>
<tr>
<td></td>
<td>5 × 10^{-6}</td>
<td>2.77 ± 0.03**</td>
</tr>
</tbody>
</table>

Non-stimulated synovial cells were incubated with arachidonic acid (10 μg/ml) at 37°C for 60 min in the absence (control) or presence of either M-5011 or ketoprofen at the indicated concentrations before determinations of prostaglandin (PG) E2 contents in the medium. Each value represents the mean ± S.D. of 3 separate experiments. Significant differences of P<0.05 (*), P<0.01 (**) and P<0.001 (***) were values compared to the control.
percentage, the values were 85.0%, 81.8%, 84.6% or 82.5%, respectively. Therefore, the counts in the medium were mainly attributed to free [14C]AA. Prelabeled synovial cells were incubated for 3 hr with BK in the presence of either M-5011 or ketoprofen at various concentrations. M-5011 dose-dependently inhibited the release of [14C]AA from synovial cells within the concentration range of $10^{-8}$ to $10^{-6}$ M (Table 2). However, ketoprofen hardly affected the [14C]AA release.

Fig. 4. Concentration-inhibition curves of the drugs against COX activities in interleukin-1β (IL-1β)-stimulated synovial cells. Synovial cells pretreated with IL-1β (50 U/ml) for 24 hr were incubated with arachidonic acid (10 μg/ml) at 37°C for 60 min in the absence (○, control) or presence (●) of a drug at the indicated concentrations before determinations of prostaglandin (PG) E2 contents in the medium. Each point represents the mean ± S.D. (unless S.D. is smaller than the size of the symbol) of 3 separate experiments. Significant differences of $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) were values compared to the control.

Effect of M-5011 on [14C]AA incorporation
To determine if M-5011 had reduced the free [14C]AA level in the medium through an enhancement in [14C]AA uptake, the effect of M-5011 on [14C]AA incorporation in synovial cells was examined. Cells were incubated with [14C]AA in the presence or absence of M-5011 at $10^{-6}$ M (where the peak inhibition of BK-induced [14C]AA release was observed). M-5011 revealed slight inhibitions but did not enhance the [14C]AA uptake by cells for up to 6 hr (Table 3).
Table 3. Effects of M-5011 on incorporation of labeled arachidonic acid (AA) in synovial cells

<table>
<thead>
<tr>
<th>Time after incubation (hr)</th>
<th>Residual radioactivity in medium (dpm x 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (without M-5011)</td>
</tr>
<tr>
<td>0</td>
<td>3.46±0.02</td>
</tr>
<tr>
<td>1</td>
<td>3.20±0.03</td>
</tr>
<tr>
<td>3</td>
<td>2.81±0.02</td>
</tr>
<tr>
<td>6</td>
<td>2.44±0.00</td>
</tr>
</tbody>
</table>

Synovial cells were incubated with [1^4C]AA (3.46×10^4 dpm) in the presence (10^-6 M) or absence (control) of M-5011. At the end of incubation, the residual radioactivity in medium was measured. Each value represents the mean±S.D. of 3 separate experiments.

DISCUSSION

Although mRNA and polypeptide of COX-2 were not observed, both these cell components of COX-1 were located in the non-stimulated synovial cells (Figs. 1a and 2). Incubation of non-stimulated cells with IL-1β resulted in a parallel induction of COX-2 mRNA and COX-2 polypeptide 1 hr after initiation, and the expression persisted for at least 24 hr (Figs. 1b and 2). In contrast, COX-1 expression only showed a minor variation under the same condition. These results suggest that PGE2 production by IL-1β-stimulated and non-stimulated cells from exogenous AA is mainly attributed to the actions of COX-2 and COX-1, respectively.

M-5011 and ketoprofen were more potent instantaneous inhibitors of COX-2 activities in IL-1β-stimulated cells than diclofenac and indomethacin (Fig. 4). Both M-5011 and ketoprofen were reversible inhibitors of COX-2 (Fig. 5). In contrast, indomethacin and diclofenac, which elicited time-dependent irreversible inhibition, manifested respective peak suppressions at 10 and 2 min post-incubation. This is in agreement with a report that indomethacin exhibits time-dependent inhibition of COX-2 activities in vitro (25). It has been reported that COX-2 activity is responsible for PGE2 production in the fluid exudate in rat subcutaneous air-pouch inflammation induced by carrageenan (26). In this model of inflammation, we have confirmed that orally administered M-5011 revealed an effective inhibitory activity (ID50) on PGE2 production in the pouch exudate at 0.28 mg/kg, a dose that exhibited a potency higher than indomethacin (1.00 mg/kg), diclofenac (0.48 mg/kg) and ketoprofen (0.87 mg/kg) (H. Kido et al., unpublished data). Although the mode of action of M-5011 and ketoprofen on COX-2 in vitro was partly different from that of indomethacin and diclofenac, these drugs may have similar in vivo anti-inflammatory effects.

PGE2 production in IL-1β-stimulated cells elevated
markedly for the first 60 min followed by gradual increases up to 6 hr after AA addition (Fig. 3). Therefore, if the irreversible inactivations of COX-2 by indomethacin and diclofenac occurred within a few minutes in the presence of AA, the instantaneous inhibitions of these drugs would be more potent than those of M-5011 and ketoprofen 60 min after incubation with IL-1β-stimulated cells. However, as the potencies of instantaneous inhibitions on COX-2 activities by indomethacin and diclofenac were not as predicted, the irreversible inactivations of COX-2 by these drugs could have been attenuated or abolished in the presence of AA.

Although both M-5011 and ketoprofen are propionic acid-derived NSAIDs, Murakami et al. (21) have reported that M-5011 displays lower ulcerogenicity than ketoprofen in orally administered mice; effective ulcerogenic doses (UD50) in the stomach and small intestines were 88.23 and 46.09 mg/kg for M-5011 and 20.04 and 10.75 mg/kg for ketoprofen, respectively. Since the NSAID-associated ulcerogenicity is attributed to inhibition of COX-1 (8), the effect of M-5011 on COX-1 activities in non-stimulated cells was compared with that of ketoprofen. Both M-5011 and ketoprofen failed to manifest apparent dose-dependent inhibitions on COX-1 activities at all concentrations used (Table 1). Although M-5011 was less potent than ketoprofen on COX-1 activities, the low ulcerogenicity of M-5011 could not be sufficiently explained by its lower inhibitory activity on COX-1. Formation of COX-derived products is dependent on the prior release of AA from membrane phospholipids (2, 3). Therefore, the effect of M-5011 on AA release from synovial fibroblasts stimulated with BK, an important inflammatory mediator (19), was further compared with that of ketoprofen. M-5011 inhibited [14C]AA release from synovial cells incubated with BK in a dose-dependent manner, whereas ketoprofen hardly affected the response (Table 2). It has been reported that intracellular AA levels are regulated by the equilibrium between AA release from and uptake in phospholipids (27). Thus, two possible mechanisms underlying the decrease in AA in the medium by M-5011 are conceivable. Firstly, M-5011 may actually inhibit AA release from synovial cells. Secondly, the agent may enhance AA uptake into synovial cells. To evaluate these possibilities, the effects of M-5011 on [14C]AA uptake in synovial cells were examined in this study. It was found that M-5011 at 10^-6 M did not enhance the [14C]AA uptake into the cells and revealed, in fact, a slight suppressive effect (Table 3). This result suggests that the decreased rate of [14C]AA release by M-5011 is more likely due to the inhibition of [14C]AA release. Preliminary studies performed with human osteoarthritic synovial cells showed that M-5011 but not ketoprofen at 10^-10 - 10^-5 M was also effective in inhibiting [14C]AA release from prelabeled cells stimulated with BK (data not shown). These results may explain, in part, the lower ulcerogenicity of M-5011 compared with ketoprofen in an animal model (21).

While a plausible mechanism for the inhibition of [14C]AA release by M-5011 is not known at present, decreased AA levels by M-5011 in the extracellular space may result in diminished production of PGs, since AA is taken up by neighboring cells before being catabolized to PGs (28). In this study, effects of M-5011 on COX-1 and COX-2 activities were evaluated with the use of exogenous AA as the substrate. Further investigations to evaluate the effect of M-5011 on PGE2 production from endogenous AA at a cellular level are warranted.

In summary, the present study demonstrated that M-5011 elicited an instantaneously potent inhibition against COX-2 and manifested a reversible inhibition on COX-2 activity in intact synovial cells. In addition, the cellular COX-1 activity was inhibited by M-5011, although to a lower extent (Table 1). Furthermore, M-5011 inhibited [14C]AA release from prelabeled synovial cells. It is likely that the effects of M-5011 on AA metabolism are, in part, responsible for its in vivo efficacy and safety profile.

REFERENCES

10 Ristimäki A, Garfinkel S, Wessendorf J, Maciag T and Hla T:


15 Seibert K and Masferrer JL: Role of inducible cyclooxygenase (COX-2) in inflammation. Receptor 4, 17-23 (1994)


22 Dayer JM, Krane SM, Russell RGG and Robinson DR: Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. Proc Natl Acad Sci USA 73, 945–949 (1976)


27 Irvine RF: How is the level of free arachidonic acid controlled in mammalian cells. Biochem J 204, 3–16 (1982)