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INHIBITION OF PROSTAGLANDIN BIOSYNTHESIS BY SL-573

YOSHIKAZU YANAGI and TOSHIAKI KOMATSU

Research and Development Center, Pharmaceuticals Division, Sumitomo Chemical Co., Ltd., 4-2-1, Takatsukasa, Takarazuka, Hyogo, 665, Japan

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Abstract—The inhibition of prostaglandin biosynthesis by SL-573 (1-cyclopropylmethyl-4-phenyl-6-methoxy-2-(1H)-quinazolinone), indomethacin, and aspirin was determined by the selective extraction method of prostaglandins, using bovine seminal vesicle microsomes as enzyme sources and arachidonic acid-1-[^{14}C] as a substrate. SL-573 was found to inhibit the prostaglandin biosynthesis. The relative inhibitory potencies of indomethacin, SL-573, and aspirin were 1000, 227, and 1.0, respectively. SL-573, however, differed from other drugs in the nature of the inhibition. The inhibition of SL-573 was reversible, while that of indomethacin and aspirin was irreversible and progressed time-dependently. Furthermore, SL-573 was found to prevent the progressive increase of the irreversible inhibition of indomethacin and aspirin. On the basis of such findings, the lack of the gastrointestinal side-effect of SL-573 was discussed.

The inhibition of prostaglandin biosynthesis has been well established as the mechanism of action for many nonsteroidal anti-inflammatory agents [1–4]. On the other hand, there is some evidence that the same mechanism may be involved in the gastrointestinal side-effect, that is, (i) prostaglandins and prostaglandin synthetase are present in gastrointestinal tract [5, 6], (ii) prostaglandins are related to the normal physiological function of gut [7], (iii) most nonsteroidal anti-inflammatory agents cause gastrointestinal injury [8, 9], (iv) some prostaglandin derivatives possess anti-ulcerogenic activity [10], (v) the intestinal ulcers induced by indomethacin and flufenamic acid are prevented by administration of prostaglandins [11].

SL-573 is a new anti-inflammatory compound of low toxicity, as reported in a previous publication [12]. It was equivalent or superior to mefenamate and phenylbutazone in anti-inflammatory and analgesic activities, but did not cause any gastrointestinal lesions even at a high dose level of 400 mg/kg.

SL-573 inhibited prostaglandin biosynthesis (Tomio Segawa*), so that its lack of adverse effect on the gastrointestinal tract was in conflict with the hypothesis that the inhibition of prostaglandin biosynthesis may result in gastrointestinal injury. The present study, therefore, was carried out to clarify whether SL-573 is different from indomethacin and aspirin as to the nature of prostaglandin biosynthesis inhibition.

MATERIALS AND METHODS

Chemicals. Arachidonic acid-1-[^{14}C] (sp. act. 58 mCi/m-mole, Radiochemical Center, Amersham, England), unlabeled arachidonic acid (Sigma, Grade I: approx. 99%) and aspirin (Sanko Seiyaku Kogyo) were commercially obtained. SL-573 and indomethacin were synthesized in this laboratory. Prostaglandin E_2 and $F_{2\alpha}$ were kindly provided from the Nihon-Upjohn Co., Ltd.

Preparation of enzymes. Bovine seminal vesicle microsomes were prepared by the modified method of Takeguchi *et al.* [13] as follows. Frozen bovine seminal vesicles were thawed, trimmed of fat and other tissues, and cut into small pieces. The seminal vesicles were suspended in 2 vol of 0.1 M potassium phosphate buffer (pH 8.0), and homogenized in a Vertis-45 homogenizer (Vertis Co., Ltd.) for 2 min. The homogenates were centrifuged at 12,000 *g* for 10 min. The supernatant was filtered with a double layer of cheese cloth and centrifuged at 65,000 *g* for 90 min. The precipitated microsomes were suspended in 0.25 M sucrose containing 5 mM phosphate buffer (pH 7.4) to yield the protein concentration of 21.7 mg/ml, and stored at $-70°$ until used. Protein concentration was determined by the method of Lowry *et al.* [14], using bovine serum albumin as a standard.

Reaction medium. Unless otherwise stated, prostaglandin biosynthesis was carried out in 1 ml of reaction medium consisting of arachidonic acid-1-[^{14}C] (0.082 mM), enzymes (1.1 mg protein/ml), hydroquinone (1.0 mM), ethanol (1.0%) and pH 7.4 potassium phosphate buffer (50 mM). Inhibitors were added as described below. Ethanol was used to dissolved the arachidonic acid and inhibitors. The effect of ethanol on prostaglandin biosynthesis was insignificant.

Thin-layer chromatography. Thin-layer chromatography (t.l.c.) was performed on precoated Silica gel plates (HF_{254}, thickness 0.25 mm, E. Merck A.-G. Darmstadt, Germany). The chromatogram was developed with chloroform–methanol–acetic acid (18:1:1, v/v). R_f values of arachidonic acid, prostaglandin E_2 and $F_{2\alpha}$ were 0.72, 0.35 and 0.20, respectively. The radioactive spots on t.l.c. were located by t.l.c. radioscanner (Aloka, Model 202B) and t.l.c. radioautography.

Selective extraction of prostaglandins. Prostaglandins produced from arachidonic acid-1-[^{14}C] in the above reaction medium at 37° for 60 min were extracted by two different methods as follows.

*Personal communication.

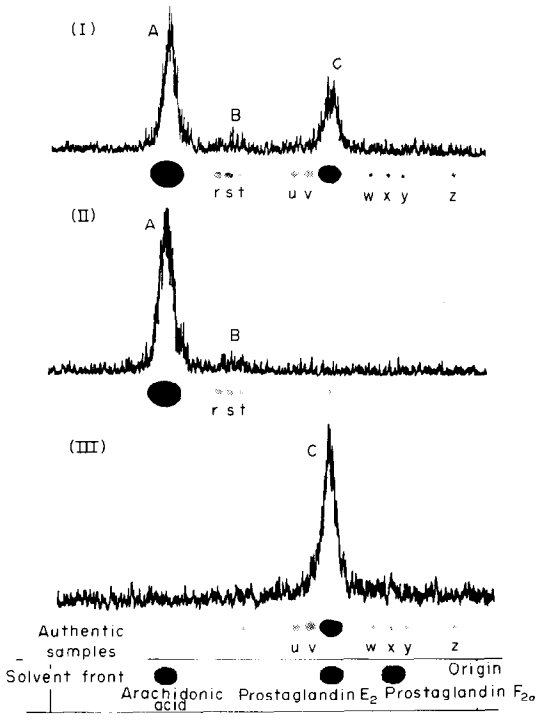

Fig. 1. Selective extraction of prostaglandins from incubation medium. (I) ethyl acetate extract at pH 3.0. (II) *n*-hexane–ethyl acetate (2:1, v/v) extract at pH 7.4. (III) ethyl acetate extract at pH 3.0 after extraction (II). The chromatogram was developed with chloroform–methanol–acetic acid (18:1:1, v/v). The radioactive spots were localized by t.l.c. radioscanner and t.l.c. radioautography. The reaction medium contained arachidonic acid (0.082 mM), hydroquinone (1.0 mM), and enzymes (1.1 mg protein/ml) in 50 mM potassium phosphate buffer (pH 7.4). Incubation was carried out at 37° for 60 min.

First, the reaction was terminated by addition of 0.5 ml of 200 mM citrate buffer (pH 3.0) and immediately 3 ml ethyl acetate were added to the medium. The radioactivity was quantitatively extracted in the ethyl acetate phase. Extraction was repeated three times. The extracts were evaporated *in vacuo*, and submitted to t.l.c. analysis. As shown in Fig. 1–I, three radioactive spots were detected by t.l.c. radioscanner. Spot A and C coincided with arachidonic acid and prostaglandin E_2, respectively, while spot B was unknown. Moreover, besides these spots, various minor unknown products were localized by t.l.c. radioautography. Spot B was found to be made up of three products, (r), (s) and (t). Two products, (u) and (v), were detected between spot B and C, and more than four products, (w), (x), (y), (z), and so on, below spot C.

Second, the reaction was stopped by addition of 3 ml *n*-hexane–ethyl acetate (2:1, v/v), and the mixture was shaken under neutral pH condition (pH 7.4). T.l.c. demonstrated that spot A and B were extracted into organic solvent phase (Fig. 1–II). The aqueous phase was then acidified with 0.5 ml of 200 mM citrate buffer (pH 3.0) and extracted with 3 ml ethyl acetate. Prostaglandin E_2 was extracted together with minor products, (u), (v), (w), (x), (y), (z), and so on, into the ethyl acetate phase (Fig. 1–III).

Under these reaction conditions, prostaglandin E_2 was a main product which occupied more than 80% of total products. Examining the time-course of formation of minor products, products (r), (s), and (t) did not increase with time, while others did. Accordingly, the data suggest that the former products were intermediates or artifacts produced during extraction procedure, and the latter were by-products, although no detailed analysis of these products was performed.

The above results indicated that arachidonic acid and product (r), (s), and (t) were selectively extracted with *n*-hexane–ethyl acetate (2:1, v/v) under neutral pH condition, while prostaglandin E_2 and by-products remained in the aqueous phase.

Biological estimation of prostaglandin E_2. The rat stomach strip was prepared accordingly to the method of Vane [15] from Sprague–Dawley male rats weighing 200–250 g which had been fasting overnight. Rat stomach strips were suspended with 0.6 g of tension in 10 ml Tyrode's solution at 37°. Contractions were recorded using a isotonic transducer (TD-111S and JD-111S, Nihon Kohden Kogyo, Co., Ltd.) and a recticorder (RJG-3028, Nihon Kohden Kogyo, Co., Ltd.). Prostaglandin E_2 was dissolved in 50% (v/v) polyethylene glycol (av. mol. wt: approx. 400) and added to the tissue bath not to exceed 10 μl. A dose-response curve was obtained by single contractions. After the first contraction, the muscle was overstretched with a 2-g weight for 20 min, the medium was exchanged five times and then the second contraction was performed.

A radioactive spot (spot C in Fig. 1), which coincided with prostaglandin E_2 on t.l.c., was scraped from the plates and extracted with ethyl acetate. The extract was evaporated *in vacuo*, dissolved in 50% polyethylene glycol and added to the tissue bath. The extract contracts the rat stomach strip. The amount of prostaglandin E_2 calculated from a dose-response curve was well consistent with that from the specific radioactivity. These data confirm that spot C in Fig. 1 is prostaglandin E_2.

Assay of prostaglandin biosynthesis activity. The reaction was started by addition of an aliquot of enzymes at 37°. Ten minutes later, the medium was extracted with 3 ml *n*-hexane–ethyl acetate (2:1, v/v) under neutral pH condition. After centrifugation (2500 rpm, 5 min), the aqueous phase was frozen at −25° and the organic solvent phase was discarded. Extraction was repeated twice. The aqueous phase was mixed with 0.5 ml ethanol to minimize protein binding of radioactivity, and centrifuged at 2500 rpm for 5 min. Since prostaglandin E_2 and minor by-products selectively remained in the aqueous phase, the prostaglandin biosynthesis activity was determined by measuring the radioactivity in the aqueous phase with a liquid scintillation spectrometer (Packard Tri-Carb Model 3375). The reaction progressed linearly with time for first 10 min.

Inhibition of prostaglandin biosynthesis. The inhibition of prostaglandin biosynthesis by SL-573, indomethacin and aspirin was investigated under three different incubation conditions as follows.

No pre-incubation: Inhibitor and arachidonic acid were simultaneously added to the medium and immediately the reaction was started by addition of an aliquot of enzymes at 37° for 10 min.

Pre-incubation: Inhibitors were preliminarily incubated with enzymes (1.1 mg protein/ml) in 50 mM potassium phosphate buffer (pH 7.4) at 37°. After fixed time intervals of preincubation, 0.9 ml of this solution was added to 0.1 ml of 50 mM potassium phosphate buffer (pH 7.4) containing arachidonic acid (0.82 mM) and hydroquinone (10 mM) and the reacreaction was immediately started at 37° for 10 min. Accordingly, the inhibitor concentrations in the reaction medium were diluted to 9/10 of those in the pre-incubated medium. In this paper, the concentration of inhibitors under pre-incubation condition are represented as those in the pre-incubated medium.

Dilution of inhibitors: Inhibitors were preliminarily incubated with enzymes (5.5 mg protein/ml) in 50 mM potassium phosphate buffer (pH 7.4) at 37° for 2 min. The reaction was started by addition of 0.2 ml of this solution to 0.8 ml of 50 mM potassium phosphate buffer (pH 7.4) consisting of arachidonic acid (0.103 mM) and hydroquinone (1.25 mM). Consequently, the concentration of inhibitor was diluted in the reaction medium to 1/5 of that in the pre-incubated medium. In the case of reversible inhibitors, dilution of inhibitors is expected to result in the restoration of the enzyme activity, while in the case of irreversible ones the enzyme activity is not restored.

RESULTS

Inhibition of prostaglandin biosynthesis by SL-573, indomethacin, and aspirin. Under the no pre-incubation condition, SL-573, indomethacin, and aspirin inhibited prostaglandin biosynthesis as illustrated in Fig. 2. The concentrations of inhibitors used were as follows: SL-573: 0.5, 1.0, 2.5, 5.0, and 10 μg/ml, indomethacin: 0.06, 0.1, 0.2, 0.4, 0.6, 1.0, and 2.0 μg/ml, and aspirin: 100, 200, 300, 500, and 800 μg/ml. The ID_{50} values (concentration of inhibitor resulting in 50% inhibition of prostaglandin biosynthesis) were 1.4 μg/ml for SL-573, 0.32 μg/ml for indomethacin, and 320 μg/ml for aspirin, respectively. Accordingly, the relative potencies of indomethacin, SL-573, and aspirin were calculated to be 1000, 227, and 1.0, respectively.

Probably, SL-573 inhibits the early stage of prostaglandin biosynthesis in a manner similar to indomethacin and aspirin, because t.l.c. radioautography

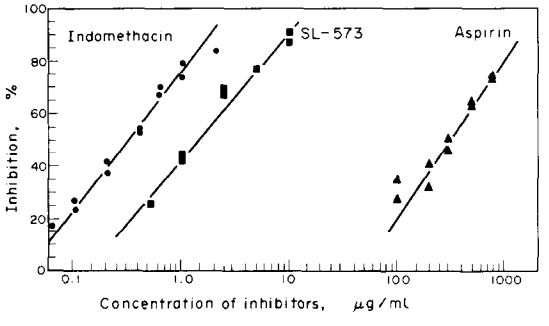

Fig. 2. Inhibition of prostaglandin biosynthesis by indomethacin, SL-573, and aspirin. The reaction medium contained arachidonic acid (0.082 mM), hydroquinone (1.0 mM), various concentrations of inhibitor (indomethacin, SL-573, and aspirin) and 1.1 mg protein bovine seminal vesicle microsomes in 1 ml of 50 mM phosphate buffer (pH 7.4). Incubation was carried out at 37° for 10 min.

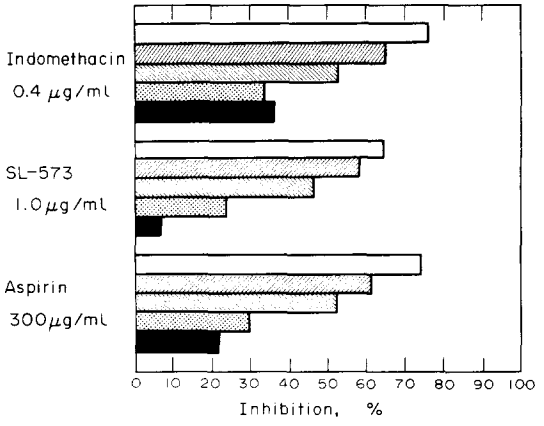

Fig. 3. Effect of substrate concentration on prostaglandin biosynthesis inhibition by indomethacin, SL-573, and aspirin. Substrate concentrations: □, 0.033 mM; ▨, 0.049 mM; ▧, 0.082 mM; ▨, 0.164 mM; ■, 0.328 mM. The reaction medium contained various concentrations of arachidonic acid, hydroquinone (1.0 mM), inhibitor (indomethacin, 0.4 μg/ml; SL-573, 1.0 μg/ml; aspirin, 300 μg/ml) and 1.1 mg protein of bovine seminal vesicle microsomes in 1 ml of 50 mM phosphate buffer (pH 7.4). Incubation was carried out at 37° for 10 min.

indicated that these inhibitors suppressed the formation of all products of arachidonic acid to the same extent.

Effect of substrate concentration on inhibition of prostaglandin biosynthesis by SL-573, indomethacin, and aspirin. Different concentrations of arachidonic acid (0.033, 0.049, 0.082, 0.164, and 0.328 mM) were added to the reaction medium simultaneously with the inhibitors, and the reaction was started by addition of an aliquot of enzymes. Inhibitors were used at a fixed concentration (SL-573: 0.5 μg/ml, indomethacin: 0.4 μg/ml, and aspirin: 100 μg/ml). As illustrated in Fig. 3, the inhibition of prostaglandin biosynthesis decreased concomitantly with an increase of substrate concentration. At the lowest concentration of arachidonic acid (0.033 mM), the inhibition was 64.5% in SL-573, 76.0% in indomethacin, and 74.2% in aspirin, while at the highest concentration (0.328 mM) the inhibition was 6.4% in SL-573, 36.0% in indomethacin, and 21.2% in aspirin. From these data, the inhibition is assumed to be competitive.

Kinetic confirmation of such an assumption was not performed in the present study because Lineweaver–Burk's plot indicated no linear relationship in the presence of inhibitors. In the absence of inhibitors, Lineweaver–Burk's plot was observed to be linear, so that K_m- and V_{max}-values of this enzyme were determined to be 0.106 mM and 2.0×10^{-9} moles/min/mg protein, respectively.

Effect of pre-incubation of inhibitors with enzymes on prostaglandin biosynthesis. The inhibition of prostaglandin biosynthesis by SL-573, indomethacin, and aspirin was compared under both conditions of pre-incubation and no pre-incubation. Inhibitors were used at the following concentration: SL-573: 0·5 μg/ml, indomethacin: 0.1 μg/ml and aspirin: 100 μg/ml.

The inhibitory activities of indomethacin and aspirin were remarkably potentiated by their pre-incubation with the enzymes as illustrated in Fig. 4.

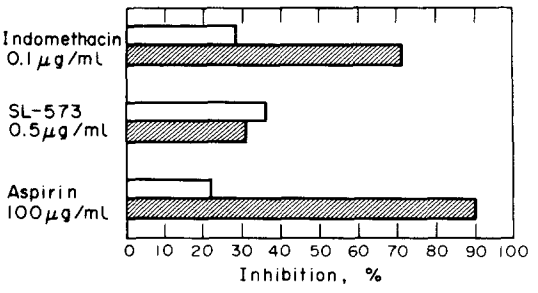

Fig. 4. Effect of pre-incubation of inhibitors with enzyme on prostaglandin biosynthesis. □, no pre-incubation (substrate and inhibitor were simultaneously added to incubation medium). ▨, pre-incubation (inhibitor was pre-incubated with enzyme at 37° and 2 min later substrate was added). The reaction medium contained arachidonic acid (0.082 mM), hydroquinone (1.0 mM), inhibitor (indomethacin, 0.1 μg/ml; SL-573, 0.5 μg/ml; aspirin, 100 μg/ml) and 1.1 mg protein of 50 mM phosphate buffer (pH 7.4). Incubation was carried out at 37° for 10 min.

The pre-incubation of indomethacin altered its inhibitory activity from 28.5% in no pre-incubation to 71.6%, and in the case of aspirin from 22.5% to 90.4%. In addition, the inhibition by indomethacin and aspirin was found not to be restored by their dilution. These data show that indomethacin and aspirin inhibit the enzymes in an irreversible manner. SL-573, on the other hand, produced almost identical inhibition under both conditions, namely 31.6% in pre-incubation and 36.9% in no pre-incubation. Dilution of SL-573 from 0.5 μg/ml in the pre-incubated medium to 0.1 μg/ml in the reaction medium resulted in full restoration of original activity, so that the inhibition of SL-573 appears to be reversible.

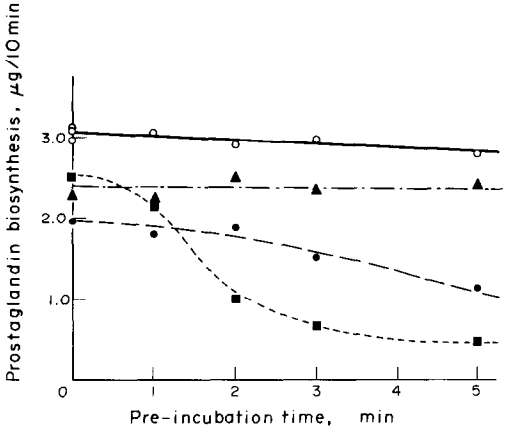

Fig. 5. Effect of pre-incubation time on prostaglandin biosynthesis inhibition by SL-573, indomethacin, and their combined use. —○—○—, control; -·▲-·▲-·-, SL-573 (0.5 μg/ml); - ■ ■ -, indomethacin (0.1 μg/ml); —●—●—, SL-573 (0.5 μg/ml) + indomethacin (0.1 μg/ml). Inhibitors were pre-incubated with enzymes at 37° for 0, 1, 2, 3, and 5 min and the reaction was started by addition of substrate. The reaction medium contained arachidonic acid (0.082 mM), hydroquinone (1.0 mM), inhibitor and enzymes (1.0 mg protein/ml) in 50 mM phosphate buffer (pH 7.4). Incubation was carried out at 37° for 10 min.

Various concentrations of inhibitors were preliminarily incubated with the enzymes at 37° for 2 min, and the ID_{50} values were determined to be 2.2 μg/ml for SL-573, 0.032 μg/ml for indomethacin, and 16 μg/ml for aspirin, respectively. As described above, the ID_{50} values under no pre-incubation condition were 1.4 μg/ml for SL-573, 0.32 μg/ml for indomethacin, and 320 μg/ml for aspirin. The potentiation of inhibitory activities by 2-minute pre-incubation, therefore, is calculated to be ten times for indomethacin and twenty times for aspirin. In the case of SL-573, the ID_{50} value under pre-incubation conditions was slightly lower than the no pre-incubation one. Probably, this is due to the fact that SL-573 is a reversible inhibitor and its concentrations in the reaction medium under pre-incubation conditions were 9/10 of those under no pre-incubation one (see Materials and Methods).

Effect of SL-573 on time-dependent progressive inhibition of indomethacin. Inhibitors were pre-incubated with enzymes at 37° for 0, 1, 2, 3, and 5 min, and the reaction was started by addition of 0.9 ml of the pre-incubated medium to 0.1 ml of 50 mM potassium phosphate buffer (pH 7.4) consisting of arachidonic acid (0.82 mM) and hydroquinone (10 mM). As illustrated in Fig. 5, the inhibition of prostaglandin biosynthesis by indomethacin (0.1 μg/ml) progressed time-dependently, while that of SL-573 (0.5 μg/ml) did not. In the combined use of indomethacin and SL-573, the enzyme activity also decreased time-dependently, but more slowly than in indomethacin alone. In addition, the effect of SL-573 was found to be synergistic to the instant inhibition of indomethacin, and antagonistic to the time-dependent increase of inhibition. These data suggest that there are at least two processes involved in the inhibition of prostaglandin synthetase by indomethacin; the same observation was made with aspirin.

DISCUSSION

The inhibition of prostaglandin biosynthesis by SL-573, indomethacin, and aspirin was determined by the method of selective extraction of prostaglandins. The assay of prostaglandin biosynthesis activity has been carried out by various methods, namely spectrophotometric [13, 16, 17], radiometric [18–20], polarographic [21], immunochemical [22, 23], g.l.c.-mass spectrometric [24, 25], g.l.c. techniques [26], and biological assay methods [27–29]. The advantage of the present method is to be rapid, accurate, and simple in comparison with other methods. Its disadvantage is the lack of specificity, because several unknown by-products were measured together with prostaglandin E_2.

Flower *et al.* [20] have reported that indomethacin and aspirin inhibited the formation of the cyclic endoperoxide, an early stage of prostaglandin biosynthesis, while phenylbutazone suppressed the conversion of the cyclic endoperoxide into prostaglandin E_2 and $F_{2\alpha}$ and did not inhibit the formation of by-products such as prostaglandin D_2 and hydroxy acid. Since SL-573 was found to inhibit the formation of all products from arachidonic acid to the same degree, it is

considered to inhibit the formation of the cyclic endoperoxide as well as indomethacin and aspirin. Recently, the prostaglandin synthetase system was solubilized and separated into two enzymes, namely the oxygenase which catalyzed the formation of the cyclic endoperoxide and the isomerase which converted the cyclic endoperoxide to the E-type prostaglandin, by Miyamoto *et al.* [30]. They have reported that indomethacin and aspirin inhibited the oxygenase. The present assay method, therefore, appears to be valid for the inhibitors of the oxygenase, while unsuitable for the inhibitors of the isomerase.

The inhibitory activities of indomethacin and aspirin were potentiated by their pre-incubation with the enzymes, and their inhibition was not restored by dilution. These results are in agreement with those of other workers [19, 21, 31, 32], suggesting that indomethacin and aspirin inhibit prostaglandin synthetase in a time-dependent and irreversible manner. SL-573, on the other hand, was found to be a reversible inhibitor. The *in vivo* effect of reversible inhibitors such as SL-573 is considered to be a short-lasting inhibition parallel with tissue concentration, while that of the irreversible ones is a long-lasting inhibition. The low adverse effect of SL-573 in gastrointestinal tract, therefore, may be accounted for by the short-lasting inhibition of prostaglandin synthetase in the gut.

The combined use of SL-573 and indomethacin revealed that there are at least two different processes involved in the inhibition of prostaglandin synthetase by indomethacin. Its first process is the instant inhibition, to which the effect of SL-573 is synergistic, and the second one is the progressive irreversible inhibition, to which the effect of SL-573 is antagonistic. Such effects of SL-573 are very similar to those of *o*-phenanthroline as shown by Smith *et al.* [21], although SL-573 was much more potent (approx. one thousand times) than *o*-phenanthroline.

The findings that SL-573 prevented the time-dependent progressive inhibition of indomethacin suggest that SL-573 may modify the *in vivo* effect of indomethacin. In fact, Hiroshi Awata *et al.** have shown that SL-573 blocked the gastrointestinal damage induced by indomethacin without concomitant decrease of their anti-inflammatory and analgesic activities in rats. Accordingly, these data led us to the assumption that the irreversible and time-dependent progressive inhibition of prostaglandin synthetase by indomethacin type drugs may be correlated to their gastrointestinal complication. Further investigation is being undertaken to clarify this assumption.

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*Personal communication (the data will be published in the near future).

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