Assay-guided Informatory Screening Method for Antiplatelet Effect of Adenosine Isolated from *Malbranchea filamentosa* IFM 41300: Inhibitory Behaviors of Adenosine in Different Solvents

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A particle-counting aggregometer employing laser-light scattering was used in systematic manners to screen and to detect inhibitor(s) of platelet aggregation from the extract of *Malbranchea filamentosa* (*M. filamentosa*) IFM 41300. The inhibitor was determined as adenosine on the basis of the ^{13}C- and ^{1}H-NMR spectral data. Although adenosine was previously reported as an inhibitor of platelet aggregation, we isolated the compound from the fungus for the first time. Because the method was able to provide us with information on the developmental formation of platelet aggregates in different sizes with incubation time, we further elaborated the inhibitory behaviors of adenosine, as an example, at varied concentrations in different solvents such as dimethyl sulfoxide (DMSO) and saline. We found that DMSO could facilitate to dissolve less water soluble materials from herbs and fungi by using the present assay method.

Key words —— aggregometer, adenosine, *Malbranchea filamentosa*, platelet aggregation, inhibitory effect

INTRODUCTION

It is essential to develop methodologies for the detection of bioactive compounds isolated from natural products such as herbs and fungi in a simple, accurate, sensitive and rapid manner. Such methods may significantly contribute to the preliminary screenings of a wide array of biologically active compounds for potential uses in the prevention and treatment of various diseases.

We directed our interests to evaluate such methods in the study of inhibitory effects of compounds isolated from natural products on platelet aggregation in human blood plasma since platelets play a vital role in the development of thrombi, which are closely associated with lifestyle-related diseases in modern society. For example, platelet microaggregates generated in the early phase of platelet activation are now considered to be able to aggravate thrombus formation which ultimately results in vascular occlusion.[1)]

Platelet aggregation is commonly measured by either the optical density (OD) method[2)] or impedance method.[3)] Although both methods were useful for clinical evaluation of platelet function, they were not able to provide us with information about sizes of platelet aggregates which alter with incubation time. On the contrary, a particle-counting method, which employs laser-light scattering technique, allows us to identify platelet-aggregate sizes over time.[4)]

In our search for new compounds we isolated malfilamentosides A and B[5)] and identified 4-benzyl-3-phenyl-5*H*-furan-2-one as a vasodilator[6)]

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from the extract of the fungus *Malbranchea filamentosa* (*M. filamentosa*) Sigler and Carmichael IFM 41300 found in an Argentine soil sample.[7] Both studies were conducted for the search and isolation of new compounds from the fungus, followed by the examination of the biological activity of these compounds.

In the present study we utilized an aggregometer with the laser-light scattering method and showed the efficiency of this method for a systematic screening of compounds possessing antiplaletet effects with less emphasis on whether the compounds were new or have already been identified. Also, we attempted to demonstrate that the method was simple, rapid, as well as sensitive and accurate for searching antiplatelet compounds as compared to the conventional optical density method because the laser-light scattering method monitored platelet-aggregatory behaviors of isolated compound(s) in different solvents with the development of platelet aggregates in 3 distinct sizes with incubation time.

MATERIALS AND METHODS

Chemicals —— Collagen and adenosine diphosphate (ADP) were purchased from MC Medical, Inc., (Tokyo, Japan). Adenosine and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chem. Ind. Ltd. (Osaka, Japan).

Equipment —— ^{1}H- and ^{13}C-NMR spectra were measured by a Bruker AVANCE-400 (^{1}H: 400 MHz, ^{13}C: 100 MHz) spectrometer (Bruker BioSpin K.K., Ibaraki, Japan). Column chromatography was performed using a Diaion HP-20 (Mitsubishi Chem. Corp., Tokyo, Japan). HPLC was performed with a SSC-3160 pump (flow rate, 2 ml/min, Senshu Scientific Co., Ltd., Tokyo, Japan), equipped with a YRD-883 RI detector (Shimamura Co., Ltd., Tokyo, Japan). HPLC analytical conditions of each fraction were as follows: column, Inertsil ODS-P, 10×250 mm, GL Sciences Inc., Tokyo, Japan; mobile phase, CH_3CN-H_2O (5 : 95); flow rate, 2.0 ml/min; column oven temperature at 40°C; detector, photodiode array (PDA), MD-2010 PLUS, JASCO Ltd., Tokyo, Japan.

Fermentation, Extraction, and Isolation —— *M. filamamentosa* IFM 41300 was cultivated for 3 weeks in five Roux flasks, each containing 150 g of moistened rice. The cultivated rice was extracted with acetone, and the extract was concentrated *in vacuo*. The resulting extract was suspended in ethyl acetate (EtOAc) and extracted with water evaporated *in vacuo* to obtain the aqueous extract. The aqueous extract (about 13 g) was chromatographed using a Diaion HP-20 column, eluted successively with H_2O, 20% MeOH, 40% MeOH, 60% MeOH, 70% MeOH, 80% MeOH, MeOH and acetone. Two liters of each solvent was used to fractionate the extract into 16 fractions (Fr. 1–Fr. 16). Then, 40% MeOH elution fraction or Fr. 5 with a significant inhibitory effect on platelet aggregation was obtained and further separated by HPLC (reverse phase C18 column, 5% MeCN) into 10 fractions (Fr. 5-1–Fr. 5-10). Among the 10 fractions tested for inhibitory effects, Fr. 5-4 demonstrated a significant inhibitory effect, which included compound 1 (about 5 mg).

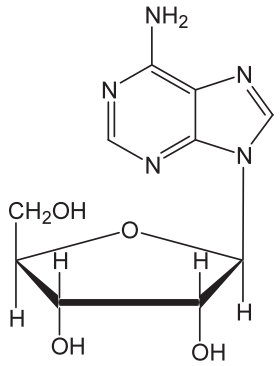

Fig. 1. Structure of Adenosine

Compound 1 (Adenosine) —— Compound 1 was identified as adenosine by comparison with spectral data in the literature.[8]

Preparation of Platelet Suspension —— This protocol was conducted in accordance with the standard of the Declaration of Helsinki with the written approval of the Ethics Committee of the Showa Pharmaceutical University. The procedures were fully explained to all the volunteers in advance. Blood samples from each of the healthy volunteers were collected by venipuncture in a tube containing 3.80% sodium citrate. Centrifugations at about $180 \times g$ (10 min) and $1600 \times g$ (10 min) were performed to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP), respectively. The platelet concentration was then adjusted to $2.5 \pm 0.3 \times 10^5$ platelets/μl by diluting PRP with PPP.

Assay for Platelet-aggregatory Activity —— Platelet aggregation was measured based on the particle counting with laser-light scattering (PA-200, Kowa Company Ltd., Tokyo, Japan) as previously described.[9] Briefly, 300 μl of the platelet suspen-

sion prepared as above was placed in a cuvette and mixed with 24 μl of extract or fraction. Then, the reaction was initiated by stirring at 37°C after calibrating the transmittance (T) or OD using PPP. After reacting for 1 min, 36 μl of collagen at a concentration of 20.0 μg/ml or ADP at 50 μM as a platelet inducer was added to the mixture to monitor platelet activation. DMSO at the final concentration of about 0.5% was used as blank reference. The reaction was recorded for 7 min. The extent of inhibition of platelet aggregation was expressed in following equation:

$$\text{Inhibition}(\%) = (1 - \text{B}/\text{A}) \times 100 \quad (1)$$

where A is T in the presence of collagen or ADP as platelet inducer and B is T in the presence of extract or fraction dissolved in DMSO and collagen or ADP.

Unless otherwise specified, collagen was used as a common platelet inducer for screening inhibitory effects, which occurred during early stage of platelet activation.

Statistics —— All results were expressed as mean ± S.E. The statistical evaluation of the data was performed by the Bonferroni-type multiple *t*-test as described (http://chiryo.phar.nagoya-cu.jp/javstat/Bonferro-j.htm). A value of $p < 0.05$ or $p < 0.01$ was considered statistically significant.

RESULTS AND DISCUSSION

We first detected an inhibitory effect of the aqueous extract of *M. filamamentosa* IFM 41300 and found its inhibitory effect on platelet aggregation induced by collagen at the final concentration of about 330 μg/ml. The extract was successively separated into fractions, in which Fr. 5 and Fr. 5-4 showed significant inhibitory effects at the final concentrations of about 70 μg/ml and 130 μg/ml, respectively. The aqueous extract and the fractions exhibited about 68% inhibition, with increased inhibitory levels found in bioactive compound isolated from *M. filamamentosa* IFM 41300. The other fractions tested did not demonstrate significantly higher inhibitory effects on platelet aggregation; therefore, we eliminated these fractions for further screenings.

The finding of a higher inhibitory effect of Fr. 5 even at a lower concentration than Fr. 5-4 indicated that Fr. 5 contained compound(s) which might have acted as inhibitor(s) at varied degrees and therefore

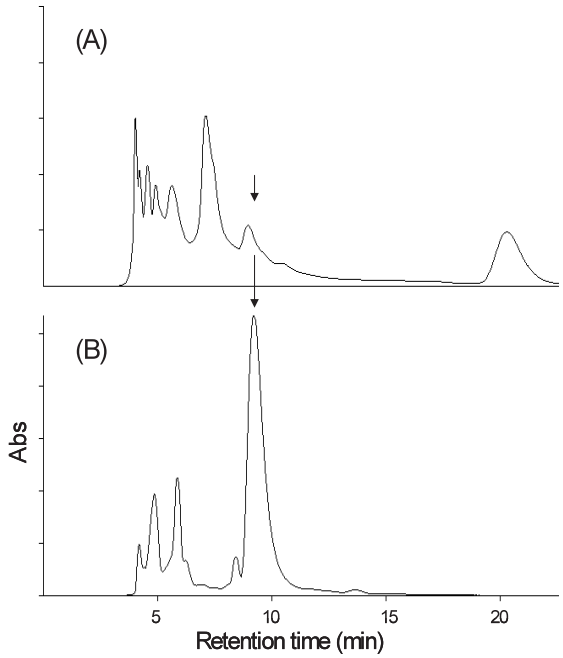

Fig. 2. HPLC Chromatograms of Fractions which Exhibited Significant Inhibitory Effects on Platelet Aggregation

(A) HPLC chromatogram of Fr. 5. (B) HPLC chromatogram of Fr. 5-4. Both arrows show peaks corresponding to a same compound with inhibitory effect. Absorbance was at 210 nm.

synergistically exerted the enhanced inhibitory effect. In other words, Fr. 5 which might have contained other inhibitor(s) differed from the major inhibitory compound in Fr. 5-4 as shown by arrow (Fig. 2) and such inhibitor(s) could be separated into other fractions during the course of isolating the major inhibitors in Fr. 5-4. In fact, the major inhibitor isolated from Fr. 5-4 was highly potent, and thus the inhibitor was thought to achieve its maximal inhibition even at very low concentrations with our present assay method; furthermore, the inhibitory behavior of the suspected compound was elaborated to understand such phenomenon as below.

It is interesting to note that a major peak designated by an arrow on the chromatogram of Fr. 5-4 was also present on the chromatogram of Fr. 5 (Fig. 2). This observation suggested that the compound which corresponded to the peak might be a platelet-aggregatory inhibitor. In addition, we did not detect any potent inhibitory effects on the fraction containing the peaks which were eluted before the major peak in Fr. 5-4 (unpublished data).

Inhibitory patterns of the fractions are shown, for example, in Fig. 3. Typical recorded outputs of Fr. 5 and Fr. 5-4 clearly demonstrated that there were less large-sized platelet aggregates in Fig. 2B and 2D against Fig. 2A and 2C as negative controls,

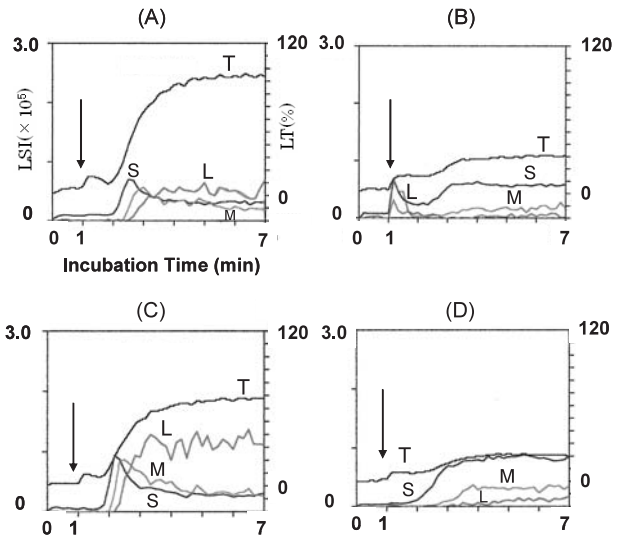

Fig. 3. Collagen-induced Platelet Aggregation by Changes in Light Scattering Intensity (LSI) with Incubation Time

Incubating PRP and DMSO (about 0.5%) as a blank with collagen (2.0 μg/ml) added after 1 min. (B) Incubating PRP with Fr. 5 (70 μg/ml) dissolved in DMSO (about 0.5%) with collagen (2.0 μg/ml) added after 1 min. (C) Incubating PRP and DMSO (about 0.5%) as a blank with collagen (2.0 μg/ml) added after 1 min. (D) Incubating PRP with Fr. 5-4 (130 μg/ml) dissolved in DMSO (about 0.5%) with collagen (2.0 μg/ml) added after 1 min. The arrows indicate the time of collagen addition. All concentrations were expressed as final concentrations. (A) as negative control for (B) and (C) as negative control for (D) were performed on separate experimental days against the same volunteer. Symbols: S, small-sized platelet aggregates (9–25 μm); M, medium-sized platelet aggregates (25–50 μm); L, large-sized platelet aggregates (50–70 μm); T, transmittance.

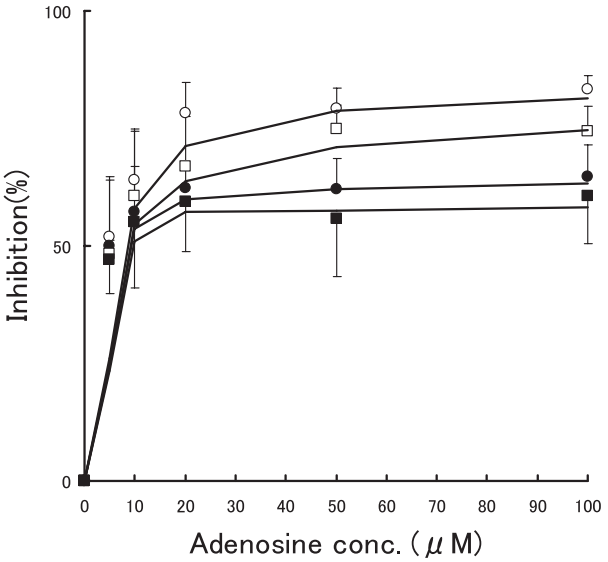

Fig. 4. Percentage Platelet-aggregatory Inhibition by Various Concentrations of Adenosine Dissolved in DMSO or Saline as Induced by Collagen (at Final Concentration 2.0 μg/ml) and ADP (at Final Concentration 5.0 μM)

Final concentrations of DMSO in reaction mixtures are about 0.5%. Symbols: DMSO, collagen-induction (□) and ADP-induction (■); Saline, collagen-induction and (○) and ADP-induction, (●).

respectively.

The findings prompted us to isolate the compound which was shown in the arrowed peak for platelet-aggregatory inhibition (Fig. 2). We isolated the compound and identified it to be adenosine by employing ^{1}H- and ^{13}C-NMR spectrometer. As a result, adenosine was isolated from *M. filamamentosa* IFM 41300 and reported for the first time based on the result of our literature search.

The presence of adenosine was reported from a wide range of natural products such as safflower (*Carthamus tinctorius*),[8] *Auricularia auricula*,[10] *Ganoderma lucidum*,[11] and tomato,[12] revealing inhibitory effects of platelet aggregation.

Factors such as the type of *in vitro* assays, the choice of solvents used to dissolve the extracts, fractions or compounds obtained from various natural products all play a crucial role in the successful use of aggregometer which is commonly utilized in aqueous experimental conditions. Consequently, we compared DMSO with saline solution (saline) as solvents for adenosine as a model to facilitate the selection of appropriate solvents for future experiments using the aggregometer with the laser-light scattering method. Although DMSO was reported as a platelet-aggregatory inhibitor when used at high concentrations,[13] concentrations of DMSO at less than 0.5% showed little or no effect on platelet aggregation.[14] Moreover, the inhibitory effects of adenosine on different solvents such as DMSO and saline at various concentrations were further examined by monitoring the percent distribution of platelet aggregates according to size and changes in T with the laser-light scattering method (Fig. 4, Tables 1 and 2).

The inhibition of platelet aggregation induced either by collagen or ADP plateaued at concentrations ranging from 20 to 100 μM under the *in vitro* experimental conditions using human platelets (Fig. 4). Furthermore, when saline was used to replace DMSO as a blank, it showed similar inhibitory manners with slightly higher percent in platelet-aggregatory inhibition as induced by collagen then by ADP. In spite of the above results, the use of DMSO in the assay system could contribute to accurate screenings and detections of platelet-aggregatory inhibitors in extracts and fractions attributed to the enhancement of solubility of target compounds as performed in the present study.

Tables 1 and 2 exhibit the extent of platelet ag-

Table 1. Distribution of Collagen-induced Platelet Aggregates by Size

Test compound	Conc. (μM)	Platelet aggregates by size (%)					
		Large (50–70 μm)		Medium (25–50 μm)		Small (9–25 μm)	
		DMSO	saline	DMSO	saline	DMSO	saline
Collagen	2.0[a)]	51.7 ± 5.5	55.0 ± 2.0	19.3 ± 0.7	18.3 ± 0.7	29.0 ± 6.1	26.7 ± 2.4
Adenosine	5.0	21.7 ± 7.3**	18.0 ± 8.2**	20.3 ± 2.2	21.7 ± 3.3	58.7 ± 9.0	60.3 ± 11.5*
	10.0	14.3 ± 6.1**	8.3 ± 3.8**	18.7 ± 6.0	18.0 ± 5.5	67.0 ± 12.1*	73.3 ± 9.0**
	20.0	4.7 ± 1.3**	2.7 ± 0.9**	15.0 ± 5.1	14.0 ± 4.2	80.3 ± 6.4**	83.7 ± 5.4**
	50.0	3.7 ± 0.3**	1.3 ± 0.3**	12.3 ± 4.7	9.3 ± 4.4	84.3 ± 5.3**	89.3 ± 4.7**
	100.0	4.7 ± 2.0**	2.7 ± 1.5**	13.0 ± 5.5	5.7 ± 3.3	82.3 ± 7.0**	91.3 ± 3.8**

a) μg/ml. Values are mean ± S.E. of 3 volunteers. *,**: Statistical significance of differences from collagen in DMSO or saline as control at $p < 0.05$ and $p < 0.01$, respectively. The Bonferroni-type multiple *t*-test was performed. All concentrations were expressed as final concentrations.

Table 2. Distribution of ADP-induced Platelet Aggregates by Size

Test compound	Con. (μM)	Platelet aggregates by size (%)					
		Large (50–70 μm)		Medium (25–50 μm)		Small (9–25 μm)	
		DMSO	saline	DMSO	saline	DMSO	saline
ADP	5.0	38.7 ± 3.7	48.0 ± 1.0	26.7 ± 2.3	24.0 ± 1.5	34.0 ± 1.0	28.0 ± 0.6
adenosine	5.0	8.0 ± 7.0**	7.0 ± 6.0**	19.0 ± 7.0	20.3 ± 6.9	72.0 ± 14.0	73.0 ± 13.1
	10.0	8.7 ± 7.7**	7.0 ± 4.6**	13.0 ± 11.0	17.3 ± 9.8	78.3 ± 18.7	75.7 ± 14.3*
	20.0	3.3 ± 3.0**	1.0 ± 0.6**	11.7 ± 11.7	8.7 ± 5.0	84.7 ± 14.3	90.3 ± 5.4**
	50.0	5.0 ± 5.0**	4.3 ± 2.4**	12.0 ± 12.0	12.7 ± 11.2	82.7 ± 16.3	83.0 ± 13.6*
	100.0	2.3 ± 0.3**	1.7 ± 0.7**	10.7 ± 9.7	11.0 ± 7.8	86.3 ± 9.7	87.7 ± 8.5**

Values are mean ± S.E. of 3 volunteers. *,**: Statistical significance of differences from ADP in DMSO or saline as control at $p < 0.05$ and $p < 0.01$, respectively. The Bonferroni-type multiple *t*-test was performed. All concentrations were expressed as final concentrations.

gregation on the basis of percentage distribution of platelet aggregates by size. The effect and potency of platelet-aggregatory inhibition were assessed by values of percent distributions of small- (9–25 μm), medium- (25–50 μm) and large-sized (50–70 μm) aggregates as determined by the Area Under the Curve (AUC). For example, high percent distribution of small-sized aggregates or low percent distribution of large-sized aggregates indicates high inhibitory rates, suppressing the developmental formations of large-sized aggregates from small-sized aggregates (Fig. 3).

As shown in Tables 1 and 2, inhibitory effects were noted at all the concentrations of adenosine tested on platelet aggregations induced by collagen or ADP as indicated by the small percent distribution of large-sized platelet aggregates as compared with the platelet inducers in DMSO or saline as controls. Additionally, the collagen-induction demonstrated significant differences in the percent distribution of small- and large-sized platelet aggregates at concentrations of adenosine at 5.0 μM or above particularly in saline, while ADP-induction only showed significant differences in the percent distribution of large-sized platelet aggregates particularly in DMSO. Similar inhibitory effects were detected in both DMSO and saline groups. Although the difference in the inhibitory behavior of adenosine dissolved in DMSO or saline on aggregation induced by collagen or ADP is apparent using the present aggregometer, such difference in inhibitory effects cannot be observed when a conventional T or OD method is used because neither method is capable of obtaining information on the developmental formation of platelet aggregates by size with incubation time.

In conclusion, we demonstrated that the platelet aggregometer with a laser-light scattering or laser-scattering technology was simple, rapid, accurate and sensitive to screen and detect compounds in extracts or fractions of natural products for antiplatelet effects since additional information on the development of platelet aggregates at varied sizes with time could help further elaboration on the extent of platelet-aggregatory inhibition, as well as platelet aggregating behaviors using different inhibitors such as adenosine which was found in the extract of *M. filamentosa*. We also studied the effect of solvents such as DMSO and saline on the inhibitory effects and behaviors of adenosine as a

platelet-aggregatory inhibitor. It became possible that increase in solubility of testing extracts, fractions, or compounds could facilitate the screening and assessment of platelet-aggregatory effects of testing materials, thus enhancing the detection level of less water-soluble inhibitors from natural products.

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