CHEMICAL & PHARMACEUTICAL BULLETIN

Vol. 28, No. 9 September 1980

Regular Articles

[Chem. Pharm. Bull. 28(9)2553—2564(1980)]

Biochemically Active Substances from Microorganisms. IV.[1] Establishment of Several *in Vitro* Anti-inflammatory Probes Applicable to Microbial Broths and the Effects of Non-steroidal Anti-inflammatory Drugs and Antibiotics on Them

YASUYUKI T. NINOMIYA, YOOSUKE YAMADA, MITSUKO ONITSUKA (née ONO),[2a] YUSHIRO TANAKA, TSURUMI MAEDA,[2b] and HIROMI B. MARUYAMA[2a,c]

Department of Microbiology and Chemotherapy[2a] *and Department of Pharmacology*,[2b] *Nippon Roche Research Center*

(Received October 18, 1979)

Four assay systems, *i.e.* membrane stabilization, inhibition of protein denaturation, and inhibition of collagen- and ADP-induced platelet aggregation, were examined for possible use as *in vitro* anti-inflammatory screening probes applicable to microbial metabolites. All these systems were simple and reasonably selective for known platelet anti-aggregants or non-steroidal anti-inflammatory agents. As regards sensitivity, detection should be possible at levels of several to several hundred μg/ml for an indomethacin class substance existing in the broth; such concentrations are attainable in the broth in the case of useful antibiotic production. Each system was hardly disturbed by medium components in the broth and the procedures were successfully applied to actinomycetes cultures. Upon examination of typical non-steroidal anti-inflammatory drugs to determine their effects on these systems, it was found that they could be classified into four distinct types in terms of their mode of action. The correlation of these effects with results in several *in vivo* models is discussed. Furthermore, the effects of various antibiotics including several types of ionophores on these probes were thoroughly studied.

Keywords——biochemical screening probe; platelet aggregation; heat hemolysis; protein heat denaturation; anti-inflammatories; membrane stabilizer

We believe that microbial metabolites include many potentially useful compounds other than antibiotics, but suitable screening procedures are required to identify them.[3] When one attempts to find pharmacologically active compounds of microbial origin, however, it is practically impossible in most cases to use established animal models for screening the minute amounts of the active principle present in the culture broth or for monitoring the purification process. For instance, although various *in vivo* evaluation systems have been established for anti-inflammatory compounds, those cannot be directly used for microbial screening due to the complicated procedures and high cost.

1) Part III: H. Azuma, Y. Kotoh, Y. Suhara, and H.B. Maruyama, *Antimicrob. Ag. Chemother.*, 7, 377 (1975).

2) *a*, *b*) Location: *200 Kajiwara, Kamakura-shi 247, Japan*; *c*) To whom all correspondence should be addressed.

3) H.B. Maruyama and H. Azuma, *Protein, Nucleic Acid and Enzymes*, 20, 1132 (1975).

The purpose of the present paper was to evaluate several *in vitro* biochemical phenomena that are known to be at least partly related to the inflammation process, and to determine whether they could be used, singly or collectively, as simplified screening probes for microbial broths which might contain anti-inflammatory substances. We have chosen 4 systems as candidates and evaluated their simplicity, sensitivity, applicability to microbial broths and selectivity. These were the inhibition of bovine platelet aggregation induced by ADP (APA), the inhibition of bovine platelet aggregation induced by collagen (CPA), the inhibition of heat hemolysis of rat erythrocytes (HH), and the inhibition of protein denaturation (PD). This paper describes the establishment of these systems as screening probes. A comparison of the effects of various known anti-inflammatory compounds and antibiotics has led to a classification of these compounds on the basis of mechanism of action.

Experimental

Inhibition of Bovine Platelet Aggregation (APA and CPA Assays)——Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by differential centrifugation essentially following Itoh *et al.*,[4] from freshly slaughtered bovine blood. The aggregation assay was done essentially by Born's method:[5] in a reaction mixture containing 0.9 ml of PPP, 4.0 ml of Tris-ACD[6] and 0.1 ml of PRP, with 50 μl of drug, the final platelet concentration was adjusted to 3 to 5×10^8 platelets/ml. The reaction was started by the addition of ADP (1 to 3 μM ADP and 15 mM $CaCl_2$, final) for ADP-induced aggregation (APA) or soluble collagen (30 μg/ml and 15 mM $CaCl_2$, final) for collagen-induced aggregation (CPA), and the turbidity at 600 nm was monitored.

The aggregation activity was calculated from the difference (ΔOD_{max}) between OD_{max} and OD_{min} during aggregation for APA and from the maximal rate of decrease of OD for CPA. The activities of drugs were indicated as the concentration causing 50% inhibition of the platelet aggregation.[7]

Heat Hemolysis of Rat Erythrocytes (HH Assay)——The inhibitory activity towards rat erythrocytes hemolysis was assayed by a modification of the method of Nakanishi *et al.*[8] Fresh blood from male Wistar rats (200—250 g) was centrifuged and the pellet was suspended in 0.15 M phosphate buffer, pH 7.4, to give a final concentration of 1% (w/v). The reaction mixture contained 3 ml of the rat erythrocytes suspension and 50 μl of a broth or a solution of a drug to be tested. After preincubation for 15 min at 37°, the mixture was incubated at 54° for 25 min. After spinning down the precipitate, the A_{540} of the supernatant was determined. The inhibition activity of the drug was indicated as the concentration causing 50% inhibition of the heat hemolysis determined from A_{540}.[9]

Inhibition of Protein Denaturation (PD Assay)——Heat denaturation of 0.2% (w/v) bovine serum albumin (Fraction V; Sigma) in 0.2 M phosphate buffer, pH 5.3, was assayed by the method of Mizushima[10] in which the solution was heated at 67° for 4.5 min in the presence or absence of the test drug (50 μl). Turbidity at 650 nm was determined and the inhibition (percent) was calculated as in the HH assay.

Adjuvant Arthritis in Rats——Male Sprague Dawley rats weighing 170—210 g received an intradermal injection of *Mycobacterium butyricum* cell powder (Difco) (0.5 mg in 0.1 ml liquid paraffin (Wako)) in the right hindpaw. The drug suspended in 1% carboxymethyl cellulose solution was administered once daily from Day 21 to Day 27 after the adjuvant injection to test for healing effect.

Carrageenin-induced Edema in Rat Hindpaw——Male Sprague Dawley rats received a subcutaneous injection of 0.1 ml of 5% carrageenin solution[11] in the right hindpaw. Drugs were orally administered immediately after carrageenin injection. The anti-inflammatory effects were determined using Randoll-Sellito's instrument[11] at 2, 3, and 4 hr after dosing.

Reagents, Chemicals and Preparation of Actinomycetes Broths——All drugs were dissolved in and diluted with distilled water, ethanol or dimethylsulfoxide (DMSO), depending upon the solubility, and the solutions were stored at −20° until use.

The test drugs used as representatives of neurotropic drugs, vasotropic drugs or steroidal and non-steroidal anti-inflammatory drugs were: imipramine (Geigy: Tofranil®), chlorpromazine (Ro 01-8079), di-

4) M. Itoh, Y. Nishimura, O. Takenaka, and Y. Inada, *Thrombos. Diathes. Hemorrh.* (Stugg), **31**, 452 (1974).
5) G.V.R. Born, *Nature* (London), **194**, 927 (1962).
6) Tris-ACD: 1 part of ACD (65 mM citric acid, 85 mM sodium citrate, 2% dextrose) and 5 parts of 115 mM NaCl, 15 mM KCl, 5 mM dextrose, 25 mM Tris, pH 7.35.
7) Inhibition% = (1 − aggregation activity with drug/aggregation activity with vehicle) × 100
8) M. Nakanishi, H. Imamura, and K. Gotoh, *Yakugaku Zasshi*, **90**, 548 (1970).
9) Inhibition% = $(1 - (A_{540}$ sample $- A_{540}$ blank$)/(A_{540}$ control $- A_{540}$ blank$)) \times 100$
10) Y. Mizushima and S. Sakai, *J. Pharm. Pharmacol.*, **21**, 327 (1969).
11) M. Maeda, Y. Tanaka, T. Suzuki, and K. Nakamura, *Folia Pharmacol. Jpn.*, **73**, 757 (1977).

pyridamole (Boehringer: Persantin®, extracted with $CHCl_3$), theophylline (Wako Pure Chem. Co. Ltd.), papaverine (Ro 01-6939), hydrocortisone (Wako Pure Chem. Co. Ltd.), and aspirin (Asp) (Nakakita Yakuhin Co. Ltd.). Other non-steroidal anti-inflammatory compounds were obtained from F. Hoffmann-La Roche AG, Basle, Switzerland. These included chloroquine (CQ), phenylbutazone (PB), aldrithiol-2 (AT2), MK830, ketoprofen (KP), oxyphenbutazone (OHPB), flufenamic acid (FA), indomethacin (IM), frusemide (FS), ibuprofen (IP), proquazone, diclofenac-Na (DCF), piroxicam (PRX), Ro 20-5720 (carprofen), Ro 20-8011 (*l*-carprofen), Ro 20-8012 (*d*-carprofen), naproxen (NPX), sudoxicam (SDX), Ro 21-2056 (+,−), Ro 21-5521 (+), Ro 21-5522 (−), and aminopyrine (AMP).

The ionophore antibiotics used were given by Dr. Westley, Hoffmann-La Roche Inc., Nutley, U.S.A., and included nigericin, dianemycin, lysocelline, A-23187, nonactin, and enniatin B. Azathioprine and other purine derivatives were given by Roche Products Ltd., Welwyn, England. The following antibiotics were obtained from the indicated companies: penicillin G, streptomycin and tetracycline (Meiji Seika), ampicillin (Toyo Jozo), carbenicillin, cefazolin and trichomycin (Fujisawa), cefamandole (Lilly), kanamycin and valinomycin (Sigma), gentamicin (Shionogi), spiramycin and mitomycin (Kyowa Hakko Kogyo), nystatin (Sankyo), lincomycin (Upjohn), polymyxin B (Pfizer), bleomycin (Nippon Kayaku), aminopterin (Rare and Fine Chem.), puromycin (Nutritional Biochemicals), kasugamycin and erythromycin (gift from the Institure of Microbial Chemistry), chloramphenicol, novobiosin, and anthramycin (Hoffmann La Roche Inc.). Fatty acid derivatives were provided by Prof. Kabara, Univ. of Michigan, U.S.A., and included lauric acid, 1-monolaurin, sucrose laurate, dodecylamine, M-1, M-3, M-20, M-31,[12] and N,N-dimethyllaurylamide.

The acid-soluble collagen was prepared from bovine Achilles tendon collagen (Sigma, Insoluble Type I) by the method of Cazenave[13] with 1 M acetic acid. After repeated precipitation to remove acetic acid, the final preparation was suspended in 1 μM $CaCl_2$, stored at 4° and used within a week.

Microbial broths were prepared by our routine methods, mainly from soil actinomycetes.[14]

Results

Effects of Known Platelet Anti-aggregants on the APA, CPA, HH and PD Systems

First of all, the effects of several types of drugs known to inhibit platelet aggregation in other systems[15–20] were examined using the present assays. The results, summarized in Fig. 1 a and b, indicate that the present systems have sufficient selectivity and show the expected inhibitory activity for these platelet aggregation inhibitors.[15–20] The effects of typical drugs are as follows:

i) Aspirin and indomethacin, anti-inflammatory drugs which are well known as inhibitors of prostaglandin biosynthesis,[21] were strong inhibitors on CPA, but had no effect on APA. Over 30 anti-inflammatory agents were assayed with both APA and CPA, and none of them showed inhibitory activity in APA.

ii) Theophylline, papaverine and dipyridamole, known to be cyclic AMP enhancers through the inhibition of phosphodiesterase, gave similar IC_{50} values on both APA and CPA. Their inhibitory activities were in the order: theophylline<dipyridamole<papaverine.

iii) Imipramine and chlorpromazine, reported to be membrane stabilizers,[22] showed moderate inhibitory activity on both APA and CPA.

12) M-1: 1,1-dimethyl-1-(2-hydroxypropyl)amine dodecanoimide; M-3: 1,1-dimethyl-1-(2-hydroxypropyl)-amine hexadecanoimide; M-20: dimethyl-2-hydroxytetradecylamine methacrylimide; M-31: monobenzyl quaternary acetimide.
13) J. Cazenave, M.A. Packham, and J.F. Mustard, *J. Lab. Clin. Med.*, **82**, 978 (1973).
14) H.B. Maruyama, Y. Suhara, J. Suzuki-Watanabe, Y. Maeshima, N. Shimizu, M. Ogura-Hamada, H. Fujimoto, and K. Takano, *J. Antibiot.* (Tokyo), **28**, 636 (1975).
15) F. Markwardt, W. Barthel, E. Glusa, and A. Hoffmann, *Experientia*, **22**, 578 (1966).
16) P.R. Emmons, M.J.G. Harrison, A.J. Honour, and J.R.A. Mitchell, *Lancet*, **2**, 604 (1965).
17) L.C. Best, T.J. Martin, M.B. McGuire, E.F. Preston, R.G.G. Russel, and D.S. Segal, *Lancet*, **2**, 846 (1978).
18) M.B. Zucker and J. Peterson, *J. Lab. Clin. Med.*, **76**, 66 (1970).
19) L. Caprino, "Platelet Aggregation and Drugs," ed. by L. Caprino and E.C. Rossi, Academic Press, New York, 1974, p. 143.
20) D.C.B. Mills and G.C.K. Roberts, *Nature* (London), **213**, 35 (1967).
21) J.B. Smith and A.L. Wills, *Nature* (London) New Biology, **231**, 235 (1971).
22) P.M. Seeman, *Int. Rev. Neurobiol.*, **9**, 145 (1966).

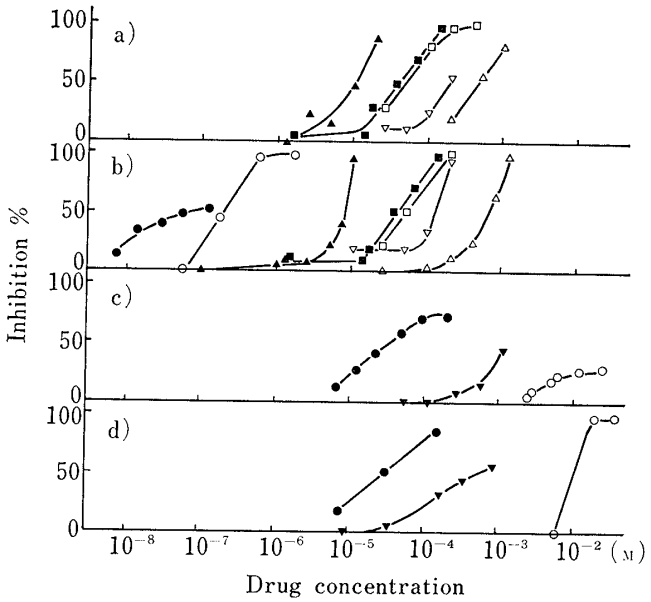

Fig. 1. Dose–Response Curve of 8 Typical Drugs on a) APA, b) CPA, c) HH, and d) PD Systems

—○—: aspirin; —●—: indomethacin; —△—: theophylline; —▲—: papaverine; —▽—: dipyridamole; —□—: imipramine; —■—: chlorpromazine; —▼—: phenylbutazone.

It should be noted that none of these typical platelet anti-aggregants exhibited selective APA inhibition without any effect on CPA.

The effects of these typical platelet anti-aggregants on the other two systems, *i.e.* HH and PD, were then examined at various concentrations (Fig. 1c and d). Among them, indomethacin exhibited a significant inhibition of hemolysis at a concentration of 10^{-6} to 10^{-4} M in a biphasic manner, as reported by Inglot and Wolna.[23] The IC_{50} of indomethacin was 3×10^{-5} M. Indomethacin, phenylbutazone and aspirin showed an inhibitory effect on the PD system too. The order of inhibitory activity of these non-steroidal anti-inflammatory drugs was the same as in the HH and PD systems: indomethacin > phenylbutazone ≫ aspirin. This order seems in some respects to reflect their anti-carrageenin edema activity. Correlation of these *in vitro* assays with *in vivo* anti-inflammatory action may be expected to a considerable extent. On the other hand, steroidal anti-inflammatory drugs such as hydrocortisone inhibited neither HH nor PD at all. Theophylline and papaverine also had no effect on either system.

Comparative Effects of Non-steroidal Anti-inflammatory Drugs on 4 *in Vitro* Probes[24]

Table I shows the inhibitory effects of 23 known anti-phlogistics and related compounds of various structures on collagen-induced platelet aggregation (CPA), inhibition of erythrocytes hemolysis (HH) and protein denaturation (PD). None of them showed any inhibition of the primary aggregation of platelets by ADP(APA). An overall summary of the results in comparison with the anti-inflammatory activities *in vivo* is shown in Table II. Some of the features are summarized as follows:

Arylpropionic Acid Group (a) in Table I)——Among 9 compounds tested, naproxen showed moderate inhibition in all three systems. Ibuprofen showed strong inhibition in PD, but moderate inhibition in CPA and HH. On the other hand, carprofen,[25] its O-analog and their enantiomers (Ro 20-8011, -8012, 21-5521, -5522) strongly inhibited HH and PD, but showed a weak inhibition in CPA. Among the enatiomers, no marked difference was observed in HH and PD, but in CPA, the *d*-form exibited 2- to 4-fold higher inhibition than the *l*-form, corresponding to their anti-inflammatory activities *in vivo*. No difference was found between carprofen and its O-analog in the three assay systems.

Arylacetic Acid Group (b) in Table I)——Diclofenac was found to inhibit CPA strongly, and

23) A.D. Inglot and E. Wolna, *Biochem. Pharmacol.*, **17**, 269 (1968); Using rat erythrocytes (1% w/v) suspended in 0.01 M phosphate buffer, pH 7.4, containing 75 mM NaCl.

24) In all cases, the inhibitory activity *in vitro* was expressed by IC_{50} (concentration required to produce 50% inhibition compared with the control, in mole (M) or μg/ml). When IC_{50} could not be obtained, IC_{30} is given or the term "weak inhibition" is used. Stimulating activity is indicated by "S". "NE" means no effect. Some *in vivo* data are cited from M. Maeda, Y. Tanaka, T. Suzuki, and K. Nakamura, *Folia Pharmacol. Jpn.*, **73**, 757 (1977).

25) Carprofen: 6-chloro-α-methylcarbazole-2-acetic acid.

Table I. Effects of Drugs on Anti-inflammatory Screening Systems

	Drug	IC_{50} (μM)				ED_{20} (mg/kg, *p.o.*)	ED_{30} (mg/kg, *p.o.*)
		HH	PD	CPA	APA	AA	CE
a)	Carprofen (*d*-form)	17	40	110	NE		
	Carprofen (*d,l*-form)	18	43	240	NE	2.5	12.0
	Carprofen (*l*-form)	19	47	400	NE		
	Ro 21—2056 (+, −)[a]	37	38	150	NE	ND	7.0
	Ro 21—5522 (−)	36	39	220	NE	ND	>16.0
	Ro 21—5521 (+)	42	36	150	NE	ND	4.0
	Naproxen	290	140	22	NE	8.4	4.8
	Ibuprofen	630	28	17	NE		
	Ketoprofen	NE	Weak	NE	NE		
b)	MK 830	50	11	<40	NE		
	Diclofenac-Na	210	11	0.23	NE	4.9	7.1
c)	Indomethacin	32	25	0.11	NE	0.5	3.4
	Ro 03—7080[b]	35	>73(S)	≃29	NE		
d)	Flufenamic acid	35	8.9	25	NE	41.4	33.4
	Frusemide	250	Weak	Weak	NE		
	Aspirin	Weak	Weak	0.19	NE	361.6	162
e)	Sudoxicam	260	Weak	0.45	NE	3.7	2.0
	Piroxicam	190	Weak	0.91	NE	1.2	3.1
	Aldrithiol-2	130	>230(S)	NE	NE		
f)	Phenylbutazone	1200	310	89	NE	57.4	25.6
	Oxyphenbutazone	Weak	Weak	180	NE		
	Aminopyrine	NE	Weak	130	NE		
g)	Chloroquine	NE	>100(S)	Weak	NE		

Weak: weak inhibition, NE: no effect, S: stimulation, ND: not done.
a) 8-Chloro-α-methyldibenzofurane-3-acetic acid.
b) 1-(*p*-Chlorobenzyl)-5-methoxy-2-methylindole-3-acetic acid.

showed strong PD inhibition and moderate inhibition of HH. MK830 showed strong inhibition of PD and HH, but moderate inhibition of CPA.

Indole Acetic Acid-containing Group (c) in Table I)——Indomethacin showed strong inhibition in all systems. Among others, it elicited the strongest inhibition on CPA among the drugs tested (IC_{50}=0.11 μM).

Substituted Benzoic Acid Group (d) in Table I)——Flufenamic acid elicited strong inhibition in PD and in HH but only moderate inhibition in CPA. On the other hand, the inhibition by frusemide was moderate in HH but weak in PD and CPA. The effect of aspirin is quite characteristic: it showed very strong inhibition in CPA, while its inhibition in HH and PD was very weak.

Thienothiazine and Thiol-containing Group (e) in Table I)——Sudoxicam and piroxicam elicited strong inhibition of CPA, although they showed moderate membrane stabilization in HH, and they were unique in showing only marginal inhibition in PD.

Aldrithiol-2 exhibited moderate inhibition of HH, but caused no inhibitory effect in the other two systems. A high concentration (more than 200 μM) of aldrithiol-2 elicited an apparent stimulation of PD denaturation.

Phenylbutazone and Related Compounds (f) in Table I)——Phenylbutazone showed weak to moderate activity in all three systems; CPA, HH and PD. The introduction of a hydroxyl moiety into the phenyl group (oxyphenbutazone, a metabolite of phenylbutazone) reduced the inhibitory activities by a factor of 2 or more.

Aminopyrine elicited weak but distinct effect in CPA and PD systems.

Chloroquine (g) in Table I)——Chloroquine did not show any activity in the PD and HH systems, while possessing weak activity in CPA.

Table II summarizes the above results in comparison with the *in vivo* activities. As far

Table II. Effects of Drugs on Anti-inflammatory Screening Systems

	In vitro				
	IC_{50}	PD	HH	IC_{50}	CPA
I	<100 μM	Indomethacin Flufenamic acid Carprofen Ibuprofen Diclofenac	Indomethacin Flufenamic acid Carprofen	<10 μM	Indomethacin Aspirin Sudoxicam Piroxicam Diclofenac
II	<1 mM	Phenylbutazone Naproxen	Diclofenac Ibuprofen Naproxen Sudoxicam Piroxicam	<100 μM	Flufenamic acid Ibuprofen Naproxen Phenylbutazone
III	<10 mM	Oxyphenbutazone	Phenylbutazone Oxyphenbutazone	<1 mM	Oxyphenbutazone Carprofen
IV	Weak	Aspirin Sudoxicam Piroxicam Ketoprofen	Aspirin		Frusemide
—	NE	Chloroquine D-Penicillamine	Chloquine D-Penicillamine Ketoprofen		Chloroquine D-Penicillamine

	In vivo			
	ED_{30} (mg/kg *p.o.*)	Carrageenin edema	ED_{20} (mg/kg *p.o.*)	Adjuvant arthritis
I	<10 mg	Indomethacin Naproxen Sudoxicam Piroxicam Diclofenac		Indomethacin Naproxen Sudoxicam Piroxicam Diclofenac Flufenamic acid Carprofen
II	<100 mg	Flufenamic acid Ibuprofen Phenylbutazone Carprofen		Phenylbutazone
III	<1000 mg	Aspirin		Aspirin

Weak: weak inhibition, NE: no effect.

as the effect of known anti-inflammatory drugs is concerned, the inhibition in CPA seems to show significant correspondence with the *in vivo* activity, except in the aspirin and carprofen groups. The HH system also appears to correlate with *in vivo* activities, except for the thienothiazine group, which showed moderate activity in membrane stabilization and very weak activity in preventing protein denaturation.

The HH system has a high correlation with PD when the activities of known drugs in both systems are compared (Fig. 2 a). A similar correlation can be seen with HH and CPA to some extent, in spite of the presence of some compounds which have a much stronger effect on one of the systems (Fig. 2 b). A similar situation exists with the PD and CPA systems (Fig. 2 c). This suggests a need for the use of more than one system. Figure 3 a, b and c show the correlation of theses systems with two *in vivo* animal model systems. The apparent positive correlation is encouraging, but should be further examined with various animal models and test compounds.

Effects of Compounds Other Than Anti-inflammatory Drugs

To evaluate the selectivity of these screening systems, it is necessary to show how physiologically active compounds which lack anti-inflammatory activity affect them, in addition to confirming positive effects of known anti-inflammatory drugs, For this purpose, we examined the effect of azathioprine analogs (which may have immunosuppressive activity), 9 fatty acid derivatives provided by Prof. A. Kabara, Univ. of Michigan, U.S.A.,[26] and typical antibiotics of microbial origin. None of the azathioprine analogs showed marked membrane

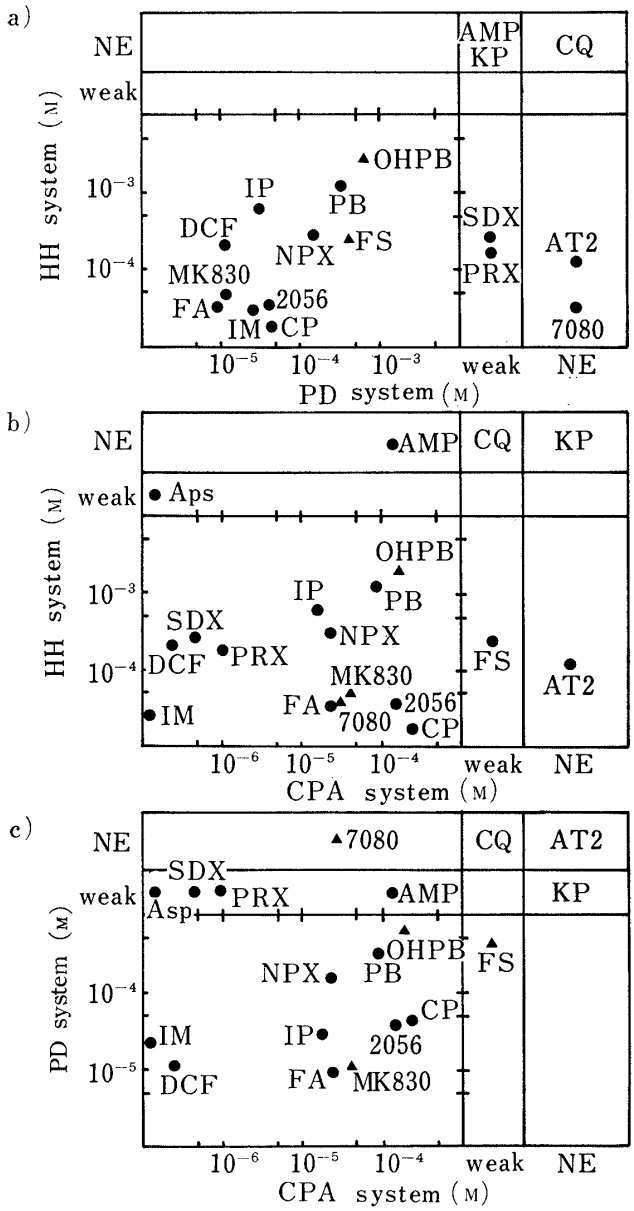

Fig. 2. Correlations among the Effects of Known Non-steroidal Anti-inflammatory Drugs on *in Vitro* Systems

●, IC_{50} in both systems were estimated.
▲, IC_{50} in one system and IC_{30} in the other system.
weak: weak inhibition; NE: no effect.

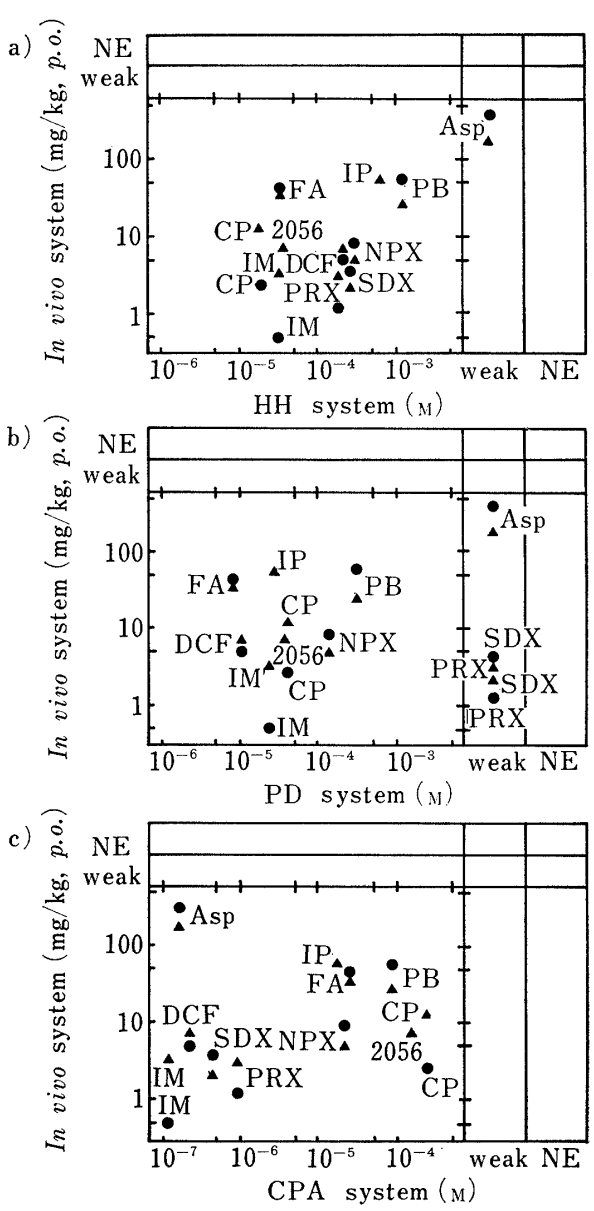

Fig. 3. Correlations among the Effects of Known Non-steroidal Anti-inflammatory Drugs on *in Vivo* Animal Models and *in Vitro* Systems

●, adjuvant arthritis (ED_{20}) (therapeutic effect).
▲, Carrageenin edema (ED_{30}).
weak: weak inhibition; NE: no effect.

26) Some fatty acid derivatives of bacterial origin were reported to have anti-inflammatory activity (*e.g.* Tsutomu Mimura, Noboru Onishi, Snigeru Toyoda, Norio Muto and Shigeru Aonuma, *Yakugaku Zasshi*, **97**, 1240 (1977)).

stabilization (HH). Azathioprine exhibited inhibition in PD and CPA, though it was weak. Among the 9 fatty acid derivatives tested, M-1, M-3, M-20, M-31 and dodecylamine showed moderate inhibition in CPA. These compounds also exhibited inhibition in the APA system, in contrast to known anti-inflammatory drugs. In the HH system, all of them showed a labilizing tendency except for dodecylamine, which showed moderate inhibition. Their anti-protein denaturation activity in the PD system was rather remarkable, with the exception of M-31. They had no detectable *in vivo* effects on carrageenin edema and scalded edema.

Effects of Various Antibiotics on the Four *in Vitro* Probes

Since many antibiotics have been found in microbial broths, it is important to know how these antibiotics affect the present anti-inflammatory probes. The effects of various groups of antibiotics available were thus investigated. Among 24 commonly used antibiotics tested (Table III), anthramycin, novobiosin, and valinomycin showed positive inhibition of HH. Novobiosin also showed inhibition in PD. In the CPA system, nystatin, valinomycin, and anthramycin showed weak inhibition. In view of the effect of valinomycin, the 5 groups of ionophore antibiotics in Westley's classification[27] were studied.

Table III. Effects of Antibiotics on *in Vitro* Anti-inflammatory Screening Probes

Group	Drug	IC_{50} (μM)			
		PD	HH	CPA	APA
β-Lactam	Penicillin G–Na	—	—	—	—
	Ampicillin–Na	—	—	—	—
	Carbenicillin	—	—	—	—
	Cefazolin	—	—	—	—
	Cefamandole	—	—	—	—
Aminoglycoside	Kanamycin	—	—	—	—
	Streptomycin	Weak	—	—	—
	Gentamicin	—	—	—	—
	Kasugamycin	—	—	—	—
Macrolide	Erythromycin	—	—	—	—
	Spiramycin	Weak	—	—	—
Polyene	Nystatin	Weak	—	Weak	Weak.
	Trichomycin	—	—(S>0.83 μg/ml)	Lysis	Lysis
Others	Chloramphenicol	—	—	—	—
	Tetracycline	—	—	—	110
	Lincomycin	—	—	—	—
	Novobiosin	46	54	—	—
	Polymyxin B	—	—	—	—
	Mitomycin C	—	—	—	—
	Bleomycin	—	—	—	—
	Aminopterin	—(S>50 μg/ml)	—	—	—
	Puromycin	—	—	—	—
	Valinomycin	—(S>12.5 μg/ml)	0.05	Weak	—
	Anthramycin	—	0.26	290	Weak

Weak: weak inhibition, —: no inhibition, S: stimulation, Lysis: Cell lysis occurred.

As shown in Table IV, all types of ionophores except for monovalent polyethers exhibited strong inhibition of HH. The strong activity of lysocelline ($IC_{50}=6\times10^{-10}$ M) is noteworthy. When the effect on the osmotic hemolysis (OH)[23] of rat erythrocytes was examined in parallel, it was found that the inhibitory activity in OH did not necessarily correspond to that in HH. In CPA, all polyether-type ionophores tested, except for lysocelline, showed strong inhibition

27) J.W. Westley, *J. Antibiot.* (Tokyo), **29**, 584 (1976).

Table IV. Effects of Ionophore Antibiotics on *in Vitro* Anti-inflammatory Probes

Class	Name	IC_{50} (M)				
		PD	HH	OH	CPA	APA
Polyether:						
Monovalent	Nigericin	— (S>6.3 μg/ml>NE)	— (S>0.001 μg/ml>NE)	6.9×10^{-8}	1.0×10^{-6}	Labilize.
Monovalent Monoglycosides	Dianemycin	— (S>6.3 μg/ml>NE)	— (S>0.00042 μg/ml>NE)	— (S>0.0042 μg/ml>NE)	3.6×10^{-6}	Labilyze.
Divalent	Lysocellin	— (S>3.1 μg/ml>NE)	6.4×10^{-10}	— (S>0.0084 μg/ml>NE)	4.8×10^{-6}	Inhibit.
Divalent pyrrole-ether	A-23187	— (S>3.1 μg/ml>NE)	7.5×10^{-7}	5.7×10^{-7}	6.5×10^{-5}	Labilyze.
Non-polyether:						
	Nonactin	— (S>12.5 μg/ml>NE)	9.5×10^{-7}	2.7×10^{-7}	—	Stimulate
	Enniatin B	— (S>6.3 μg/ml>NE)	1.0×10^{-5}	1.0×10^{-4}	Weak	—

S: stimulation, NE: no effect, —: no inhibition.

with cell lysis. The primary aggregation in APA could not be measured in these cases due to the cell lysis. The relationships among the structures, ionophoric properties and these inhibitory activities remain to be clarified. On the other hand, all these ionophore antibiotics showed no inhibition in the PD system and even exhibited stimulation at levels over 10 μg/ml.

Classification of Active Compounds in Terms of Differential Effects on Each *in Vitro* Probe

The present study has shown that strong anti-inflammatory drugs generally exhibit inhibition in the CPA, HH and PD systems, but not in the APA system, suggesting a possible application of these systems as simple *in vitro* anti-inflammatory probes in combination. Nevertheless, the relative activity patterns of the drugs varied considerably, and there appeared to be several distinct activity patterns.

When the relative activity of each drug on the CPA, PD, HH and APA systems is plotted quadrilaterally, it was found that known anti-phlogistic agents could be classified into four distinct patterns as shown in Fig. 4. Most of them gave a regular triangle, like indomethacin (Type I). Type II represents the pattern with a weaker CPA activity, which includes carprofen and Ro 21-2056. Type III includes thienothiazine which have a relatively low activity in protein denaturation inhibition. Type IV is that of aspirin, which exhibits an extraordinarily high CPA activity in relation to other activities.

On the other hand, some antibiotics gave a quite different shape due to higher activity in APA. For example, pyrrothine antibiotics have CPA, APA and HH activities but no activity in PD (Type V; details will be presented in the subsequent paper.[28]) Anthramycin is another example which exhibited a relatively high HH activity (Fig. 4; Type VI) compared with aspirin. It is interesting that each ionophore shows a unique pattern (Fig. 4; Type VI, lysocellin). Although it is not yet known how these patterns correlate with *in vivo* efficacy and what the classification really means in terms of the mechanism of action, it may be useful

28) Y.T. Ninomiya, Y. Yamada, H. Shirai, M. Ono-Onitsuka, Y. Suhara, and H.B. Maruyama, "Biochemically Active Substances from Microorganisms. V. Pyrrothines, Potent Platelet Aggregation Inhibitors of Microbial Origin," *Chem. Pharm. Bull.*, in press.

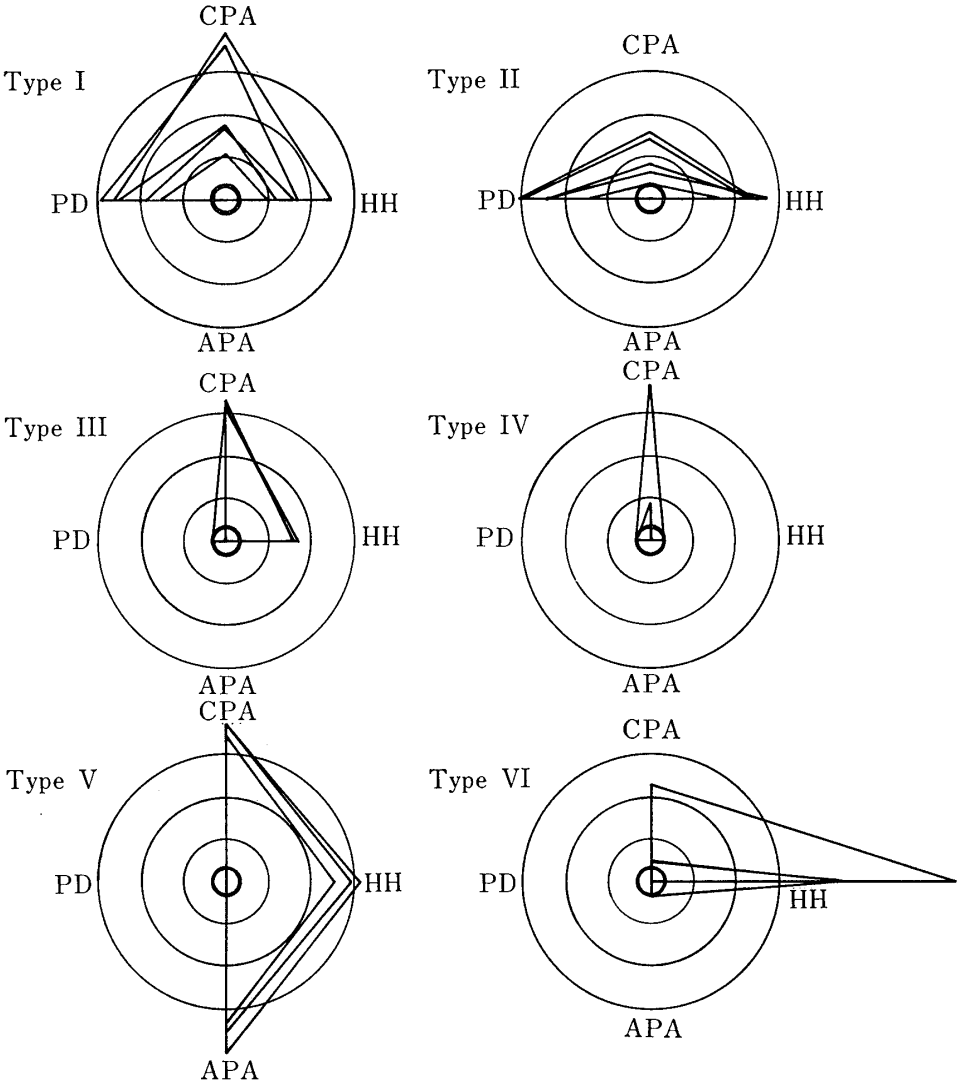

Fig. 4. Quadrilateral Profiles of the Relative Activities on 4 *in Vitro* Probes of Non-steroidal Anti-inflammatories (Type I: IM, DCF, NPX, PB, *etc.*, Type II: carbofen, Ro 21—2056, MK830, FA, *etc.*, Type III: PRX, SDX, Ro 03—7080, *etc.*, Type IV: Asp, *etc.*) and Antibiotics (Type V: thiolutin, aureothericin, *etc.*, Type VI: anthramycin, lysocellin)

The outermost, the second and the third circles represent IC_{50} values of 10^{-5}, 10^{-4}, and 10^{-3} M in HH and PD, and 10^{-6}, 10^{-5}, 10^{-4} M in CPA and APA, respectively. The innermost circle represents weak inhibition in all systems.

to know at an early stage of purification what types of known compounds the unknown microbial products in the broth resemble. A broth showing categories other than Type I might be promising as a source of new drugs. Activity in APA could be unfavorable as regards potential anti-inflammatory drugs, but this possibility requires further study.

Applicability of Four Biochemical Screening Systems to Microbial Broths

The four probes presently proposed are simple enough for routine screening. The sensitivity of these systems can be estimated as follows: if indomethacin or its equivalents exist in a broth, detection would be possible at a level of a few μg/ml by CPA, whereas in HH and PD, 650 μg/ml and 350 μg/ml in the broth are needed to give 50% inhibition, respectively (Table II).

Our past experiences suggest that if more than 20% of broths show activity, the inhibitory principle originated most likely from the medium components or something of this sort. The

effects of actinomycetes metabolites on these four systems were examined with several hundred broths newly isolated from soil. The results gave a normal distribution of their effects around 100% to control (no effect) having a mean ($\bar{X}$) of 114% (APA), 115% (CPA), 104% (HH), and 86% (PD), while approximately 1% of the broths tested contained "specific" inhibitory substances (less than 40% to control): these facts indicate enough selectivity and sensitivity of these systems as the screening probes applicable to microbial culture broths. As an example of an inhibitor found with the aid of these screening systems, details of pyrrothines will be presented in the subsequent paper.[28]

Discussion

Various compounds are known to cause inhibition of platelet aggregation through several different mechanisms.[29] This phenomenon could thus be useful as a biochemical screening probe to detect potentially interesting, particularly anti-inflammatory, metabolites in microbial broths, for which many established animal models are inappropriate. The mechanisms of action of several known anti-inflammatory drugs are thought to involve stabilization of the membrane and inhibition of protein denaturation[10]: for instance, some non-steroidal anti-inflammatory drugs stabilized the erythrocyte membrane under hypotonic conditions[23,30] or on heat hemolysis[8] and inhibited the heat denaturation of certain serum proteins. Thus, simultaneous examination of the inhibition of HH and PD could provide a more positive differentiation for possible anti-inflammatory compounds.[8]

The present four assay systems, APA (bovine platelet primary aggregation by ADP), CPA (bovine platelet secondary aggregation by collagen), HH (heat hemolysis of rat erythrocytes) and protein denaturation, were found to be simple as regards handling, reasonably sensitive and selective for either anti-platelet aggregants or non-steroidal anti-inflammatory agents. Trials with several hundred actinomycetes culture broths tested with these systems indicated that medium components did not disturb the assays.

In the present paper, we sought to determine how non-steroidal anti-phlogistics and other agnets (such as antibiotics) affect these *in vitro* systems and how their effects correlate with those on *in vivo* inflammatory models. These studies have led us to classify the drugs as shown in Fig. 4: by considering the relative effects of unkown compounds on various systems through a patternal comparison, it should be possible to predict a similarity to certain types of known anti-phlogistics or to identify a potentially unique anti-inflammatory agent differing in mode of action from typical known drugs. For this purpose, the following three points were considered: (A) the effects of known anti-inflammatory drugs on the *in vitro* probes and the correlation with *in vivo* efficacy, (B) the effects of non-anti-inflammatory compounds including antibiotics, and (C) patternal grouping of these compounds on the basis of differential effects on each *in vitro* system. In other words, once a substance is screened and isolated through the present assay systems, one should evaluate at the earliest stage as possible what type of pharmacological effects can be expected from it. Though well-established *in vivo* animal models can be used when the substance is available in reasonably large amounts, the combined use of various *in vitro* probes to investigate the mode of action may still be useful for secondary evaluation in a relatively simple way using small amounts of samples.

It should be noted that inhibitors of APA or CPA may have various modes of action, because of the complexity of the aggregation phenomenon. Such an inhibitor could be a membrane stabilizer (imipramine), a microtubule inhibitor (colchicine), a regulator of cellular

29) J.L. Gordon (ed.), "Platelets in Biology and Pathology," Elsevier/North-Holland Biomedical Press, Amsterdam, 1977; K. Yasunaga, "Thrombosis," ed. by M. Murakami, Nippon Medical Center, Tokyo, 1977, p. 199.

30) E.M. Glenn, B.J. Bowman, and J.C. Koslowske, *Biochem. Pharmacol.*, Supplement, **1968**, 27.

cyclic AMP level or of Ca^{2+} level, an inhibitor of intracellular release of arachidonic acid, or an inhibitor of prostaglandin biosynthesis. The latter two kinds of inhibitors are speicific to secondary aggregation, as would be most non-steroidal anti-inflammatory agents. This again indicates the potential usefulness of the present simple assay systems as screening probes for pharmacologically active substances of various types in microbial metabolites.

Acknowledgement We thank Dr. K. Nakamura, Department of Pharmacology, Nippon Roche Research Center, for facilities, data on *in vivo* models and critical discussions.