Review Article

NEW STRATEGY FOR DISCOVERY OF ENZYME INHIBITORS: SCREENING WITH INTACT MAMMALIAN CELLS OR INTACT MICROORGANISMS HAVING SPECIAL FUNCTIONS

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Introduction

Many bioactive compounds including antibiotics have been discovered among the metabolites of microorganisms to date. Researchers have revealed the mechanism of action of some that are practically useful, as well as others that are biologically interesting. Many compounds were found to be specific inhibitors of certain enzymes or enzyme systems. Such enzymes or enzyme systems have been providing targets for selective cytotoxicity or pharmacological intervention. On the basis of this evidence, conventional screening for enzyme inhibitors, such as protease inhibitors[1,2], cholesterol synthesis inhibitors[3], glycosidase inhibitors[4] and so on, has been carried out extensively with assays using target enzymes or enzyme systems. In some cases, purified enzymes, and in other cases partially purified enzyme preparations, such as rat liver microsomal fractions or cell homogenates, were used for the assay of enzyme reactions. Thus, conventional methods for routine assays in the primary screening systems have been established and uncovered many enzyme inhibitors. Among them, acarbose (an α-glucosidase inhibitor)[5], bestatin (an aminopeptidase inhibitor)[6], analogs of compactin and mevinolin (inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase)[7~10] and mutastein (a glucosyltransferase inhibitor)[11] are now under clinical trial or use. However, this screening strategy may suffer from too many false positive inhibitors (*e.g.* those active *in vitro* but inactive *in vivo*) because compounds are easily accessible to enzymes and may nonspecifically affect enzymes or circumstances where enzymes function. Enzymes require

appropriate cellular environments in order to exhibit their activity efficiently. Some enzymes are in the cytosol, some are associated with membranes and others are localized in certain organelles. It is difficult to establish and maintain these environments in converntional screening systems.

In order to resolve these problems, we have attempted to introduce new methods for discovery of enzyme inhibitors into primary screening programs. The fundamental strategy is to establish primary assay systems utilizing intact mammalian cells or microorganisms, in which specific enzyme functions, instead of target enzymes, are relied upon. Utilization of living cells in assays may be much more advantageous as a strategy in searching for enzyme inhibitors for the following reasons. 1) The environment where the enzyme exists *in vivo* is maintained. 2) Responses by false positives, which are always accompanied in direct assays using target enzymes, may be decreased. This addresses the problem of whether an enzyme inhibitor can reach the place where the enzyme exists and functions in cells. This is due to membrane permeability, subcellular distribution, inactivating factors or other factors of an inhibitor. 3) A pro-drug type of inhibitor might be detected. Some inhibitors may exhibit activity only after incorporation into cells and enzymatic modification. Such inhibitors will be overlooked in conventional enzyme assays. Since conventional methods have these defects, there has been a big gap between the efficacy expected from results in an *in vitro* enzyme assay and actual *in vivo* efficacy. Therefore, 4) an inhibitor selected by the method of assaying with living cells will provide a higher possibility for full *in vivo* efficacy. The assay using living cells lies between an *in vivo* and *in vitro* assay and is much closer to the *in vivo* case. Furthermore, it should be noted that a radioactive substrate is often used in an *in vitro* enzyme assay. However, radioactive compounds are not needed in many cases of primary screening assays of the present strategy.

In this review, screening methods for discovery of enzyme inhibitors with assays utilizing intact animal cells or microorganisms which have various fundamental functions and the inhibitors discovered with this strategy will be described.

Screening of Glutamine Synthetase Inhibitors

Glutamine synthetase is the enzyme which synthesizes glutamine by transferring ammonia to glutamate. This enzyme plays an important role in regulating nitrate metabolism. Inhibition of glutamine synthetase results in depletion of glutamine and in build-up of toxic intermediates in nitrate metabolism[12]. In plant cells, excess ammonia is toxic, because ammonia rapidly causes ultrastructural modifications of the chloroplasts resulting in chlorosis[13].

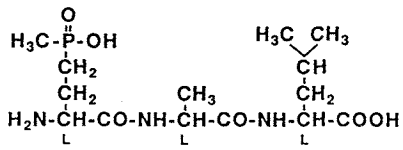
On the basis of these findings, glutamine synthetase was expected to be a target enzyme in search for herbicidal agents. In order to discover inhibitors of glutamine synthetase, we utilized a bacterium *Bacillus subtilis* as a test organism in the primary screening system[14]. This is based on the hypothesis that there exist microbial compounds which inhibit glutamine synthetase, regardless of the source of the enzyme, *e.g.* animals, plants and microoganisms. We screened the culture broths of soil isolates having inhibitory activity against this bacterium grown in DAVIS' minimal medium, which does not contain glutamine, but lacking inhibitory activity against the organism grown in the medium supplemented with glutamine.

Fermented broths of about eight thousand strains of soil actinomycetes were subjected to this screening program. Two novel inhibitors of glutamine synthetase, phosalacine [14,15] and oxetin[16], were discovered and possessed herbicidal activity.

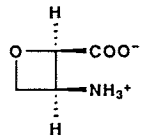
Phosalacine

Phosalacine was produced by a strain of a new species of the genus *Kitasatosporia*, *Kitasatosporia*

Fig. 1. Glutamine synthetase inhibitors, phosalacine and oxetin.



Phosalacine
(L-Phosphinothricyl-L-alanyl-L-leucine)



Oxetin

phosalacinea KA-338[17]. The structure of phosalacine is L-phosphinothricyl-L-alanyl-L-leucine (Fig. 1)[15]. Phosalacine inhibited the growth of *B. subtilis* in DAVIS' minimum medium at more than 0.27 μM (0.1 μg/ml) and the inhibition was completely reversed by the addition of L-glutamine at 10 μg/ml. In addition, it showed antimicrobial activity against Gram-positive and Gram-negative bacteria and some fungi in chemically defined minimum media but the inhibition was reversed in media containing glutamine, indicating common inhibitory activity against glutamine synthetases from these sources. On the other hand, phosalacine exhibited very weak inhibition against glutamine synthetase activity when cell-free extracts from *B. subtilis* was used as enzyme source (21% inhibition at 1.37 mM phosalacine when 25 mM glutamic acid was used as substrate). The discrepancy could be explained as follows. After it was incorporated into bacterial or plant cells, phosalacine was considered to be converted to an active inhibitor, phosphinothricin, because 1) phosalacine was easily hydrolyzed to phosphinothricin by cell-free extracts from *B. subtilis* and spinach leaves and 2) phosphinothricin is a potent inhibitor of glutamine synthetase from *B. subtilis* (*Ki*: 81.1 μM, *Km*: 18.2 mM) and spinach leaves (*Ki*: 306 μM, *Km*: 112 mM). Strong herbicidal activity of phosalacine against alfalfa (*Medicago sativa* L.) was observed at 27 μM[14].

This example shows the advantage of our strategy for enzyme inhibitors. If an *in vitro* enzyme assay using isolated glutamine synthetase had been used for the primary screening, phosalacine would not have been discovered, because phosalacine is a very weak inhibitor of the synthetase as describe above. Phosalacine easily enters the cells probably using a transport system for amino acids or peptides, and is hydrolyzed to the active form phosphinothricin which reaches the place where the synthetase reaction takes place. However, phosphinothricin might not enter the bacterial cell, possibly due to the membrane barrier.

L-Phosphinothricyl-L-alanyl-L-alanine, structurally related to phosalacine, was discovered originally as an antibiotic active against fungi by BAYER *et al.* in 1972[18]. Later, this compound named bialaphos[19] was re-discovered and is now in use as a herbicide[20,21].

Oxetin

Oxetin (Fig. 1), produced by *Streptomyces* sp. OM-2317, is the first natural product discovered possessing an oxetan ring[16]. The inhibitory activity of oxetin against *B. subtilis* grown in a minimal medium was reversed by several amino acids such as L-glutamine, L-isoleucine, L-methionine and L-valine. It also exhibited herbicidal activity and inhibited glutamine synthetase activity of spinach leaves.

Oxetin and its three stereoisomers were synthesized[22]. Synthetic oxetin showed the same antimicrobial activity as the natural product but the other three stereoisomers were inactive against *B. subtilis*. However, a subtle difference was observed between the inhibitory effect of the four isomers on glutamine synthetase activity of spinach leaves. The discrepancy might be due to the difference in cell permeability of the isomers.

Screening of Antifolates

Folate metabolites such as tetrahydrofolate is essential in cell metabolism. Tetrahydrofolate functions as an intermediate carrier of 1-carbon (C_1) groups (methyl, methylene, methenyl, formyl and formimino groups) in a number of complex enzymatic reactions. This metabolic pathway has been well defined and, is regarded as one of the most important targets for antibacterial and anticancer chemotherapy[23,24]. Sulfa drugs inhibit the biosynthesis of tetrahydrofolate at the site of dihydropteroate synthase by competition with *p*-aminobenzoic acid[25]. Several synthetic chemotherapeutics such as aminopterin, methotrexate[26], trimethoprim[27] and 5-fluorouracil which inhibit folate metabolism are clinically used (Fig. 2). Nevertheless, compounds originating from microorganisms were virtually unknown.

We developed a new screening method for discovery of antifolates of microbial origin. In this assay *Enterococcus faecium* was used as a test organism[28]. Utilization of this microorganism was expected to have the following advantages. The first is that certain microorganisms including *Enterococcus* spp. and *Lactobacillus* spp. require folate-related compounds for growth and can incorporate them although most common microorganisms cannot. Secondly, the folate metabolic pathway of these microorganisms has been studied extensively (Fig. 2)[24,29,30]. Thirdly, the microorganisms can grow in the folate-free medium supplemented with amino acids such as glycine, serine, histidine, and methionine, and purine and pyrimidine bases such as adenine, guanine and thymine, which are final products after folate and its derivatives function as C_1 donors.

The degree of requirement of folate-related compounds for the growth of *E. faecium* was tested to establish the screening method[28]. The microorganism grew well by adding a very small amount ($1 \sim 10$ ng/ml) of pteroic acid, folic acid, dihydrofolic acid or leucovorin, and by adding a relatively large

Fig. 2. Metabolic pathway of folate in various organisms and inhibition sites of known inhibitors.

Folate metabolism in various organisms and inhibitors.
(A) Usual bacteria, (B) *Enterococcus faecium*, (C) animal cells.
DHNP: Dihydroneopterin, HMDHP-PP: 6-hydroxymethyl-7,8-dihydropterin pyrophosphate, DHP: dihydropteroate, DHF: dihydrofolate, THF: tetrahydrofolate, Pter.: pteroate, Fol.: folate, LV: leucovorin, TdR: thymidine, TP: trimethoprim, AP: aminopterin, MT: methotrexate, FdUMP: 5-fluorodeoxyuridine monophosphate.

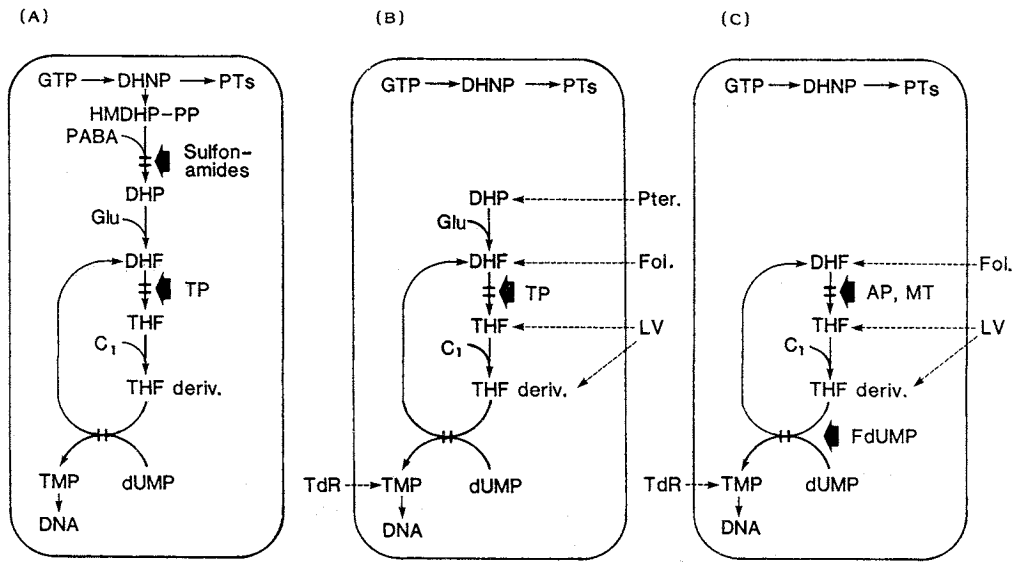


Table 1. Antibacterial activities of antifolates and some antibiotics against *Enterococcus faecium* in media supplemented with folate-related compounds as growth factor.

Site of inhibition	Drugs	Growth inhibition against *E. faecium*				
		Pteroate	Folate	DHF[a]	Leucovorin	TdR (10 μg/ml)
DHP synthase	Sulfa drugs	−	−	−	−	−
DHF reductase	TP, AP, MT	+	+	+	−	−
TMP synthase	5-FU	+	+	+	+	−
Others	Various antibiotics	+	+	+	+	+

[a] For abbreviations, see legend to Fig. 2.
−: No activity, +: positive activity.
Growth factors were added at 1.0 ng/ml, except for TdR added at 10 μg/ml.

amount (1 ~ 10 μg/ml) of thymidine to the medium, Folic Acid Assay Medium "Nissui", which contains the amino acids and purine and pyrimidine bases described above except thymidine. Consequently, folate-related compounds are considered to be necessary only for the biosynthesis of TMP when *E. faecium* grows in this medium.

The possible inhibition sites predicted from the inhibitory patterns of known compounds are shown in Table 1. Trimethoprim, aminopterin and methotrexate which are known to inhibit dihydrofolate reductase showed inhibitory activity against the bacterium in the presence of a small amount (1.0 ng/ml) of pteroate, folate or dihydrofolate, but did not in the presence of leucovorin (1.0 ng/ml) or thymidine (10 ng/ml). 5-Fluorouracil inhibited it even in the presence of leucovorin but did not in the presence of thymidine[28]. As a result, it was ascertained that antifolates and thymidylate synthase inhibitors can be obtained by picking up substances that are active against *E. faecium* in the medium containing a limited amount of pteroate but are inactive in the medium supplemented with thymidine. Thus, the inhibition site (enzyme) of unknown compounds can be easily estimated from the pattern of reversal of the inhibition.

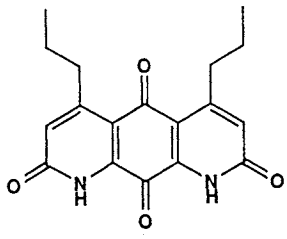
Broth filtrates of about ten thousand strains of soil actinomycetes were subjected to this screening program. Three new antibiotics, diazaquinomycins A and B[31,32], AM-8402[28] and 7-hydro-8-methylpteroylglutamylglutamic acid (HMPGG)[33] were discovered.

Diazaquinomycins (DQMs)

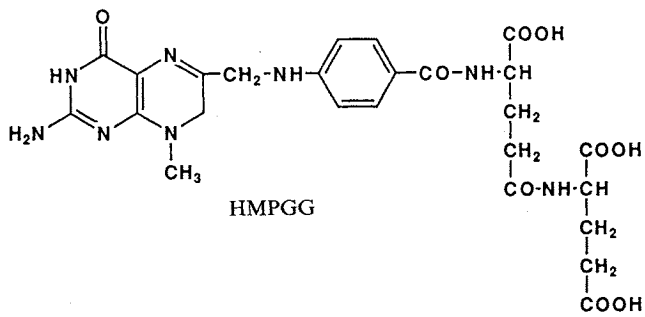
The new antifolate antibiotics, the DQM, are produced by *Streptomyces* sp. OM-704[31]. The structures of DQMs A and B are shown in Fig. 3[32]. DQM show relatively weak antibacterial activity against Gram-positive bacteria and cytotoxic activity against Vero and Raji cells. When one of three compounds (folate, dihydrofolate or leucovorin) was added to the medium at 1.0 ng/ml, inhibitory activity of DQM A against *E. faecium* was observed, but the inhibition was reversed by the addition of thymidine and by the addition of a relatively large amount (1.0 μg/ml) of folate, dihydrofolate or leucovorin[34]. It was shown that DQM A inhibits thymidylate synthase from *E. faecium* and Ehrlich ascites carcinoma cells competitively with 5,10-methylenetetrahydrofolate. The *Km* values were 274 μM and 45 μM for 5,10-methylenetetrahydrofolate and the *Ki* values were 36 μM and 14 μM with the enzymes from *E. faecium* and Ehrlich ascites carcinoma cells, respectively.

DQMs are poorly soluble in various solvents and exhibited no antitumor activity. Therefore, synthesis of diazaquinomycin A analogs was carried out[35]. The 3,7-diethoxy analog was found to show 10-fold more potent activity against thymidylate synthase than DQM A. The 3,7-diacetoxy analog exhibited significant antitumor activity (T/C; 175%) against Meth-A fibrosarcoma in mice.

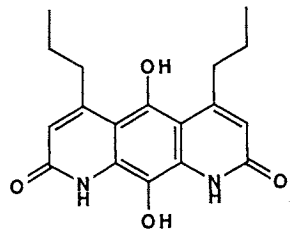
Fig. 3. Antifolates, diazaquinomycins A and B and HMPGG.



Diazaquinomycin A



HMPGG



Diazaquinomycin B

HMPGG and AM-8402

HMPGG (Fig. 3), isolated from the culture broth of a soil actinomycete, did not show any general antimicrobial activity but did inhibit *E. faecium*[33]. The inhibition site of HMPGG was demonstrated to be thymidylate synthase.

AM-8402 is a new antifolate active against Gram-positive bacteria and mycoplasmas[28]. The structure is not well defined but it is related to medermycin. The inhibitory activity against *E. faecium* was reversed by leucovorin and thymidine and partially reversed by dihydrofolate, suggesting that AM-8402 inhibits dihydrofolate reductase. However, AM-8402 exhibited no inhibitory activity against dihydrofolate reductase from rat liver. The inhibition site of AM-8402 in folate biosynthetic pathway is still unclear.

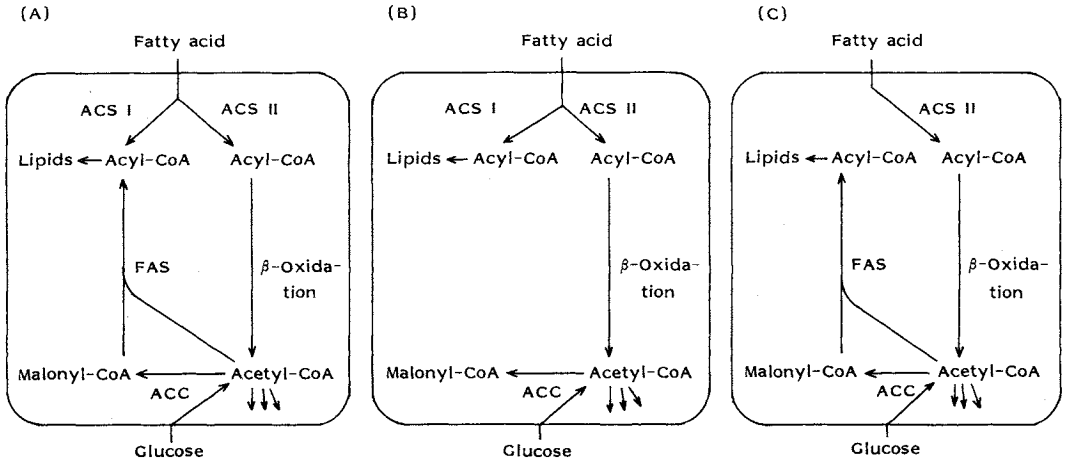
Screening of Lipid Metabolism Inhibitors

Lipid metabolism is balanced elegantly between synthesis and degradation, and closely cooperates with other metabolic activities to maintain homeostasis. However, once the balance of lipid metabolism is lost, a variety of serious diseases develop, including arteriosclerosis (like atherosclerosis), hypertension, obesity, diabetes, functional depression of some organs and so on. Control of lipid metabolism by drugs could lead to the treatment of the diseases described above. Lipid metabolic pathways, such as fatty acid degradation where acyl-coenzyme A (CoA) synthetase and the β-oxidation system are involved, fatty acid synthesis where acetyl-CoA carboxylase and fatty acid synthase are involved, cholesterol synthesis, triacylglycerol synthesis *etc.*, could provide possible target sites for treatment.

We have been interested in compounds from microbial sources to control lipid metabolism[36]. Based on our strategy of utilizing intact animal cells or microorganisms with special functions, screens for discovery of inhibitors of fatty acid metabolism and mevalonate biosynthesis were constructed. Eventually, two kinds of interesting inhibitors, triacsins[37] and 1233A[38], were discovered.

Fig. 4. Metabolic pathway of fatty acids in *Candida lipolytica* and the deletion site of mutant strains, A-1 and L-7.

(A) Wild type, (B) mutant A-1, (C) mutant L-7.
ACS: Acyl-CoA synthetase, ACC: acetyl-CoA carboxylase, FAS: fatty acid synthase.



1. Fatty Acid Metabolism

Fatty acid metabolism in *Candida lipolytica* has been studied extensively by NUMA and his co-workers[39~43] and they have isolated a number of mutant strains. The scheme of lipid metabolism in *C. lipolytica* is shown in Fig. 4. This yeast possesses two distinct acyl-CoA synthetases which activate a free long chain fatty acid to produce the corresponding acyl-CoA. Acyl-CoA synthetase I is responsible for the synthesis of cellular lipids whereas acyl-CoA synthetase II provides an acyl-CoA that is exclusively degraded *via* β-oxidation to yield acetyl-CoA[40,41]. Acyl-CoA synthetase I is distributed among different subcellular fractions including microsomes and mitochondria. On the other hand, acyl-CoA synthetase II is localized in peroxisomes where the acyl-CoA-oxidizing system is located[42]. To be utilized for cellular lipid synthesis, acyl-CoA is also provided *via* fatty acid synthase. Target sites of inhibitors of fatty acid metabolism were defined as acyl-CoA synthetases, β-oxidation system and fatty acid synthase. For the screening of fatty acid metabolism inhibitors, two mutant strains of *C. lipolytica*, L-7 and A-1, were utilized as test organisms. Mutant strain L-7 is defective in acyl-CoA synthetase I[41] and mutant strain A-1 lacks fatty acid synthase activity[44]. To isolate mutant L-7, cerulenin (an inhibitor of fatty acid synthase[36] discovered in 1963 by our group) was used[41]. These mutant strains were grown on two different media, one containing fatty acid as sole carbon source and the other containing glucose and a small amount of fatty acid (0.01%, w/v). The target of inhibition by unknown inhibitors from microbial sources can be determined by evaluating their pattern of inhibitory activity against the two mutant strains grown in the two different media. The possible inhibition site predicted from the inhibitory pattern is shown in Table 2.

Triacsins

During the course of this screening program, broth of *Streptomyces* sp. SK-1894 showed inhibitory activity against strain A-1 grown in the two media but no effect of strain L-7 in the both media, indicating that the broth contains acyl-CoA synthetase I inhibitors. Four active principles named triacsins A, B, C and D (Fig. 5) were isolated[37]. Triacsins C and D are identical to WS-1228 A and B, respectively, which were originally isolated as vasodilators[45,46]. Triacsins have an eleven-carbon

Table 2. Possible inhibition site in fatty acid metabolism predicted from inhibitory patterns against *Candida lipolytica* mutant strains L-7 and A-1 in a medium containing only fatty acid or glucose as carbon source.

Inhibitory pattern				Possible inhibition site
Mutant L-7		Mutant A-1		
Fatty acid	Glucose[a]	Fatty acid	Glucose[a]	
−	−	+	+	Acyl-CoA synthetase I
+	−	+	−	Acyl-CoA synthetase II or β-oxidation
+	+	−	−	Fatty acid synthase

[a] A small amount of fatty acid (0.01%) was supplemented.
+: Growth inhibition, −: growth.

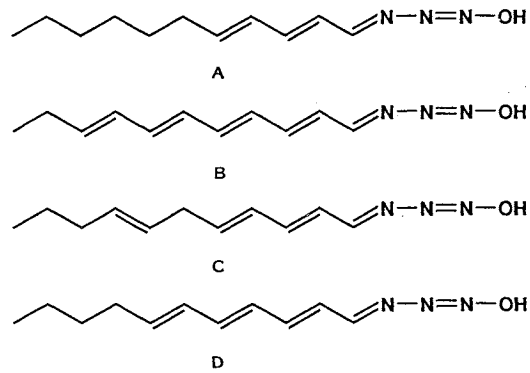
Table 3. Effects of triacsins on acyl-CoA synthetases and acetyl-CoA synthetase.

Enzyme	IC_{50} (μM)				
	Triacsin A	Triacsin B	Triacsin C	Triacsin D	*E,E,E*-2,4,7-Undecatrienal
Acyl-CoA synthetase					
Pseudomonas sp.	17	>200	3.6	>200	—
Rat liver	18	>200	8.7	>200	—
Raji cells	5.3	>100	3.2	>100	—
Acetyl-CoA synthetase					
Saccharomyces cerevisiae	—	ND	—	ND	ND

—: No inhibition at 200 μM.
ND: Not determined.
>100: Inhibited by 40~45% at 100 μM of triacsins.
>200: Inhibited by 40~45% at 200 μM of triacsins.

chain and a common *N*-hydroxytriazene moiety at the terminus. Effect of triacsins on acyl-CoA synthetase was studied[47] using acyl-CoA synthetases from *Pseudomonas aeruginosa*, rat liver and Raji cells as enzyme sources. The IC_{50} values are summarized in Table 3. Triacsins inhibited acyl-CoA synthetase activity from all the sources tested. Triacsin C is the most potent with IC_{50} values of 3.2~8.7 μM followed by triacsin A with IC_{50} values of 5.3~18 μM. Triacsins B and D are much less potent. The *N*-hydroxytriazene moiety is essential for inhibitory activity, because the hydrolytic products (*E,E*-2,4-undecadienal and *E,E,E*-2,4,7-undecatrienal) have no inhibitory activity. Furthermore, triacsins A and C have a common structural feature of a conjugated dienylidine *N*-hydroxytriazene moiety, while triacsins B and D have a longer conjugated polyene in their structures. This suggests that the common structural feature of triacsins A and C is responsible for potent inhibitory activity against acyl-CoA synthetase. On the other hand, acetyl-CoA synthetase from *Saccharomyces cerevisiae* (short chain acyl-CoA synthetase) is not inhibited by triacsins. It will be interesting to see the effect of triacsins on medium-chain acyl-CoA synthetase.

Fig. 5. Acyl-CoA synthetase inhibitors, triacsins A, B, C and D.



The effect of triacsins on the two synthetases from *C. lipolytica* was also investigated (H. TOMODA *et al.*, unpublished data). Triacsins inhibited acyl-CoA synthetase I and their inhibitory potency was within the same levels as those shown in Table 3. On the other hand, the inhibitory effect of triacsins on synthetase II is much weaker than that on synthetase I. These findings coincide with the result of the growth inhibition of triacsins against the two mutant strains L-7 (inactive) and A-1 (active), which were employed as indicator strains for screening. The different effect of triacsins on acyl-CoA synthetases I and II may be also explained by the different characteristics of the two synthetases[42].

One of the interesting biological characteristics of triacsins is that tiracsins show no antimicrobial activity but exhibit potent growth inhibition against animal cells such as Vero, HeLa and Raji. In animal cells, the same hierarchy of triacsin inhibitory potency against acyl-CoA synthetase, lipid synthesis and cell growth was demonsrated; triacsin C > triacsin A ≫ triacsin D ≧ triacsin B. This suggests that the inhibition of acyl-CoA synthetase by triacsins causes the inhibition of lipid synthesis and eventually cell growth[48]. Triacsins do not show any antimicrobial activity[37]. For example, no effect was observed on the growth of *P. aeruginosa* though the long chain acyl-CoA synthetase purified from *P. aeruginosa* is inhibited by triacsins[37,47]. It might be that acyl-CoA can be synthesized by at least two pathways in microorganisms, that is, acyl-CoA synthetase and fatty acid synthase. Fatty acid synthase in microorganisms is classified into two types I and II[49] which produce acyl-CoA and acyl-ACP, respectively. Furthermore, acyl-ACP plays the same role as acyl-CoA in lipid biosynthesis in microorganisms containing type II fatty acid synthase. Further experiments would be needed to elucidate this hypothesis. Triacsins, unique inhibitors of acyl-CoA synthetase, discovered from our screening program are now being applied to the field of lipid research as biochemical tools[50,51].

2. Mevalonate Biosynthesis

Much attention has been paid to inhibitors of cholesterol biosynthesis as potential hypocholesterolemic agents. The fungal metabolites compactin (ML-236B) (Fig. 6)[52,53] and mevinolin (monacolin K)[54,55] were discovered as inhibitors of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Recently, their analogs pravastatin (CS-514)[7,9] and simvastatin (synvinolin)[8,10] have been developed and marketed as hypocholesterolemic agents.

The enzymatic product, mevalonate, is a key intermediate in the cholesterol pathway. It is produced from acetyl-CoA by three enzymes, namely, acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase (Fig. 6). These enzymes provide promising targets for pharmacological intervention by hypocholesterolemic agent.

KANEKO *et al.*[56] reported that the growth of cultured animal cells was inhibited by compactin and that the inhibition was overcome by the addition of mevalonate to the medium. By contrast, it is believed that most microorganisms cannot incorporate mevalonate into the cell. On the basis of these findings, cultured animal cells were expected to be an ideal test organism to find inhibitors of mevalonate biosynthesis. Eventually, Vero cells (an established cell line from kidney cells of the African Green monkey) were selected for primary screening. Vero cells are sensitive to compactin (morphological changes at 0.25 to 1.0 μM and complete growth inhibition occurs at more than 1.0 μM) and both morphological changes and growth inhibition are reversed by the addition of 1 mM mevalonate. The candidate microbial cultures chosen were those which inhibited the growth of Vero cells in the EAGLE's minimum essential medium supplemented with 2% calf serum (2% CS-MEM) but lacked inhibitory activity against the growth in 2% CS-MEM supplmented with 1 mM mevalonate[57].

Fig. 6. Biosynthetic pathway of mevalonate and the inhibition site of compactin.

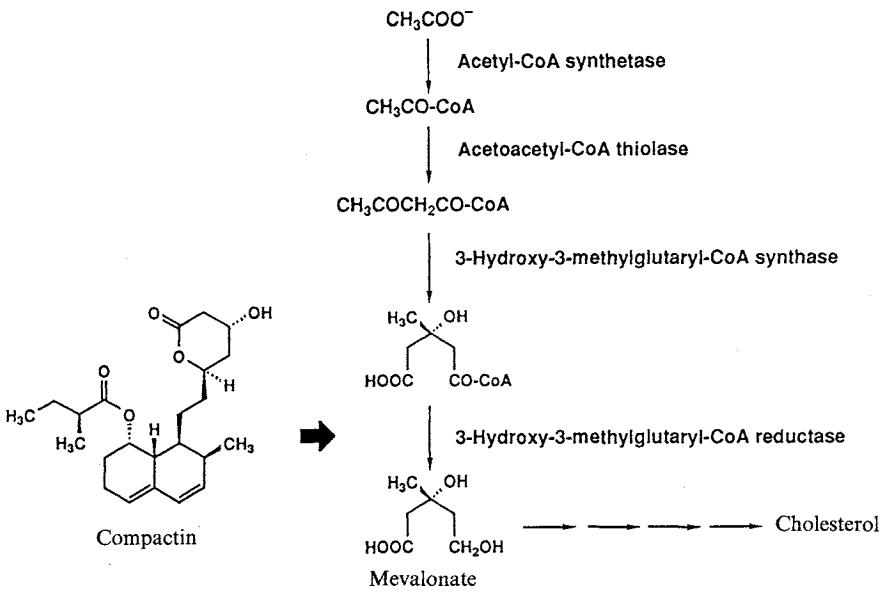


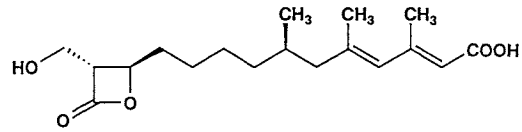
Table 4. Summary of IC_{50} values of 1233A and compactin for various enzymatic reactions involved in mevalonate biosynthesis in a rat liver enzyme system.

Inhibitor	IC_{50} (μM)						
	[^{14}C]Precursor incorporation			Partial enzyme reaction			
	Acetate	Acetyl-CoA	Mevalonate	Acetoacetyl-CoA thiolase	HMG-CoA synthase	HMG-CoA reductase	Thiolase~Synthase[a]
1233A	1.8	1.8	>150	>150	0.20	>150	0.68
Compactin	0.0062	NT	>130	NT	>130	0.046	NT

[a] Sequential reaction of acetoacetyl-CoA thiolase and HMG-CoA synthase.
NT: Not tested.

1233A

Fig. 7. HMG-CoA synthase inhibitor, 1233A.



Broth filtrates of about ten thousand soil isolates were subjected to this screening program. A cultured broth of a fungal stain, *Scopuraliopsis* sp. F-244, showed the above described activity with Vero cells as an inhibitor[57,58]. The active principle F-244 was isolated[38] and identified as 1233A (Fig. 7), originally isolated from *Cephalosporium* sp. as an antibiotic[59,60]. 1233A shows weak antimicrobial activity[58].

The inhibition site of 1233A in mevalonate biosynthesis was studied[61,62]. The incorporation experiments with ^{14}C-labeled precursors in a rat liver enzyme system (Table 4) showed that the inhibition sites of 1233A lies within the steps between acetyl-CoA and mevalonate. This evidence demonstrates the rationality of the screening method using Vero cells. Further studies on the inhibition site revealed that 1233A is a potent inhibitor of HMG-CoA synthase (Table 4). Under the same conditions, compactin inhibited HMG-CoA reductase specifically at almost the same level as reported[3].

Study of the effect of 1233A analogs and ebelactones, esterase inhibitors[63] structurally related to 1233A, on HMG-CoA synthase indicated that both β-lactone and hydroxymethyl moieties of 1233A are responsible for inhibitory activity against the synthase[61]. CHIANG *et al.*[64] reported the absolute configuration of 1233A (L-659,699) to be that shown in Fig. 7. Recently, the absolute configuration of the β-lactone ring moiety was found to be important for specific HMG-CoA synthase inhibition (ŌMURA *et al.*; unpublished data). In order to obtain more potent inhibitors synthetic studies of 1233A analogs are now in progress.

Thus, the first naturally occurring inhibitor of HMG-CoA synthase, 1233A, was discovered in the screening system of Vero cells.

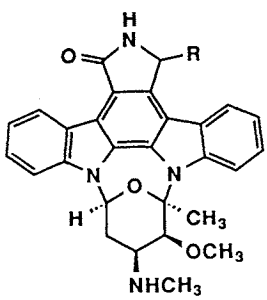
Screening of Protein Kinase C Inhibitors

The functional role of Ca^{2+} is well defined as a second messenger for regulating a variety of cell functions such as secretion, contraction, phototransduction, cell division and differentiation[65]. It has been demonstrated unambiguously that the Ca^{2+}-messenger system involves protein kinase C (a phospholipid-sensitive Ca^{2+}-dependent protein kinase) as a mediator of Ca^{2+} action[66] in addition to calmodulin. In living cells, diacylglycerol, formed from inositol phospholipid turnover stimulated by extracellular informational signals, activates protein kinase C[67]. The kinase is also activated directly by tumor promoters such as phorbol esters, suggesting that protein kinase C plays key roles in both signal transduction and cellular proliferation. Moreover, the kinase activity was proposed to be closely correlated with secretion, platelet aggregation and smooth muscle contraction.

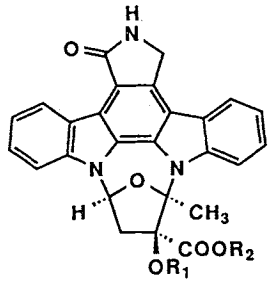
Many researchers who have been interested in inhibitors of protein kinase C carried out screening studies mostly with *in vitro* enzyme assays[68]. Staurosporine and its related compounds (Fig. 8) obtained from actinomycetes were discovered as inhibitors of protein kinase C by several groups[69~71]. Thus far, staurosporine, originally isolated as a microbial alkaloid by ŌMURA *et al.*[72], is the most potent among the known inhibitors; it has a *Ki* value of 0.7 nM[73].

OSADA *et al.* have established a new screening method utilizing K562, a human chronic myeloid leukemia cell[74]. When K562 was treated with phorbol ester or teleocidin which are activators of protein kinase C, many blebs appeared on the cell surface of K562 within 10 minutes. This bleb formation is inhibited by inhibitors of protein kinase C such as staurosporine and H7. The unique bleb formation was

Fig. 8. Protein kinase C inhibitors, staurosporine and related compounds.



Staurosporine R=H
UCN-01 R=OH



K-252a R_1=H R_2=CH_3
K-252b R_1=R_2=H
KT5720 R_1=H R_2=$(CH_2)_5CH_3$
KT5822 R_1=R_2=CH_3

observed only in K562 among the related cell lines tested. The correlation between the bleb formation and the kinase activity is still unknown. About one thousand strains of soil actinomycetes were screened using the bleb formation assay. Staurosporine, isoflavones and sangivamycin were identified as inhibitors and teleocidins and tautomycin as activators[75], demonstrating the rationality of this screening system.

Concluding Remarks

A novel strategy of using intact mammalian cells or microorganisms having various functions for the discovery of new enzyme inhibitors is presented in this article with examples of screening methods and inhibitors discovered by the methods. It has been noticed that success in new drug discovery is based on the following factors; 1) isolation of microorganisms, 2) effective screening systems, 3) fermentation conditions and 4) isolation of desired compounds[76]. The strategy presented in this review provides a novel idea concerning category 2), which is likely to lead to the discovery of new enzyme inhibitors.

As clearly shown in this review, screening systems should be established on the basis of biochemical and related scientific achievements. In most cases, it is neccessary to know the metabolic net work in which the target enzyme is involved. The metabolic pathways to folate in various organisms and of lipid in *C. lipolytica* had been studied in detail. Selection of a suitable test organism for screening is also important. In case of the screening of antifolates, a bacterium, *E. faecium* was used as test organism because folate-related compounds can enter the cells and are utilized for growth. In the screening of mevalonate biosynthesis inhibitors, Vero cells were chosen among several animal cell lines because of the high sensitivity to compactin in addition to mevalonate permeability through animal plasma membrane. For protein kinase C inhibitors, K562 was selected because only this cell line showed the peculiar phenomenon of bleb formation.

As expected, some advantages of this strategy have been demonstrated. First, a pro-drug type of inhibitor was discovered. Phosalacine itself is a very weak inhibitor of glutamine synthetase, but in cells it is easily converted to an active inhibitor, phosphinothricin. Secondly, false positives could be reduced. It was reported that long chain fatty acids, arachidonate and linoleate, inhibit HMG-CoA synthase in an *in vitro* enzyme assay[77]. These fatty acids are hardly expected to show *in vivo* efficacy. In the screening for mevalonate inhibitors, in fact, the inhibition of Vero cell growth by arachidonate was not reversed by the addition of mevalonate, and linoleate showed no effect on Vero cell growth[57], indicating the false positive activity caused by the fatty acids could be eliminated. Thirdly, all the primary screens described here were carried out without radioactive substrates. However, it must be emphasized that it is often troublesome to keep the desirable characteristics of animal cells conserved during subculture. In addition, costs for animal cell cultures are rather high for routine screening work. It may take more time to evaluate the inhibitory potency in assays with living cells. Therefore, unstable inhibitors might be overlooked.

Mammalian cells and peculiar microorganisms have been used in various screening studies. However, most of these studies were not directed toward enzyme inhibitors. For example, screening for anticancer drugs have been carried out by observing cytotoxicity to various cell lines derived from various cancers such as HeLa, KB, L1210 cells *etc.* Later studies revealed that some of anticancer drugs are enzyme inhibitors. In a screening of immunomodulating agents, KINO *et al.*[78,79] discovered FK-506, a very potent immunosuppressor, with a mixed lymphocyte reaction assay. Very recently, FK-506 and cyclosporin A[80,81] have been found to be potent inhibitors of peptidyl-prolyl *cis-trans* isomerase (N. TAKAHASHI *et al.*; personal communication).

Hopefully, studies on the mechanism of action of drugs will be fed back to develop new screening systems depending on the strategy presented in this article. Furthermore, research in the field of cell biology is now advancing remarkably. On the basis of achievements in cell biology, unique screening systems will be established and provide many effective inhibitors. We believe that this strategy could be extended for discovery of many types of enzyme inhibitors in the field of screening research and development.

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