Biochemical Pharmacology, Vol. 34, No. 18, pp. 3293–3297, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

INHIBITION OF RAT LIVER HYDROXYMETHYLGLUTARYL-CoA REDUCTASE BY SULFHYDRYL REAGENTS, COENZYME A ESTERS AND SYNTHETIC COMPOUNDS

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(*Received* 26 *November* 1984; *accepted* 9 *April* 1985)

Abstract—The activity of the microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase was assayed with a procedure based on the extraction of the product mevalonolactone in a benzene phase. Diamide is an uncompetitive inhibitor of the reaction, while coenzyme A disulfide and tetraethylthiouram disulfide act as non-competitive inhibitors. Diamide inhibition cooperatively increases with the inhibitor concentration. HMG produces a decrease in enzyme activity that combines with that of coenzyme A disulfide. Both CoASH and coenzyme A esters strongly inhibit the reductase activity. Three new synthetic compounds with either thio-ether or thio-ester groups also show inhibitory effect on the enzyme activity.

3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase)† (EC 1.1.1.34), a key enzyme in cholesterol biosynthesis, is regulated, both *in vivo* and *in vitro*, by factors such as circadian rhythm, hormones, phosphorylation etc. (see reviews [1–3]). Sulfhydryl groups seem essential for its activity, as indicated by *p*-hydroxymercuribenzoate inhibition and the requirement of added thiol compounds for maximal enzyme activity [4]. Recently it has been reported that the enzyme activity is allosterically modulated by thiol concentration [5].

Inactivation of HMG-CoA reductase occurs upon incubation of the enzyme with free coenzyme A or coenzyme A esters [6–8] and it has been shown that the yeast enzyme is drastically inhibited by coenzyme A disulfide (CoAS-SCoA([9]. Gilbert *et al.* [9] also suggested that the inhibition by CoA esters observed by others may be due to either traces of CoA disulfide present in the chemicals, or formation of CoA disulfide from the CoA esters during incubation.

We have studied the inhibition of rat liver microsomal HMG-CoA reductase by different thiol group reagents and free HMG in order to identify some requirements for new synthetic inhibitors with low toxicity. The present study suggests that disulfide compounds are efficient non-competitive inhibitors of the enzyme and also that HMG is an inhibitor whose effects can combine with that of the disulfides.

The inhibitory effects of succinyl-CoA, oleoyl-CoA, CoASH and those obtained with three new compounds, synthesized in order to include either thio-ester or thio-ether groups in chains of different length, are presented and discussed.

MATERIALS AND METHODS

Chemicals. NADP, dithiothreitol, CoASH (lithium salt), CoAS-SCoA, HMG, HMG-CoA, mevalonolactone, tetraethylthiuram disulfide, mersalyl and diamide were purchased from Sigma Chemical Co. (St Louis, MO). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase (yeast) were obtained from Boehringer (Mannheim, F.R.G.), [3-^{14}C]-HMG, [3-^{14}C]-HMG-CoA and [5-^{3}H]-mevalonolactone from New England Nuclear (Dreieichen, F.R.G.). All other reagents were of analytical grade.

Synthetic compounds. Three synthetic compounds with low toxicity and patent pending were tested (a gift of Mr G. Quadro, Medea Res., Milano). They are *N*-3-(2,3,4,5-tetrahydro-tiophen-2-one)-tiophen-2-carboxyamide (MR 869; $C_9H_9NO_2S_2$; mp 127°–129°), 2(tenoyl-thio)-*N*-(3′-tetrahydrothiophenyl-2′-one)-propionyl amide (MR 889; $C_{12}H_{13}NO_3S_2$; mp 115°–120°) and 1,9-bis(2′,3′-dihydroxy-propyl-thio)nonane (MR 764; $C_{35}H_{32}O_4S_2$; mp 72–75°); their structural formulae are reported in Fig. 1.

Microsomal preparation. Wistar albino rats (220–250 g) of both sexes, fed *ad libitum* a standard chow diet, were used. The animals were maintained on a reversed light cycle (light from 4 p.m. to 4 a.m., dark from 4 a.m. to 4 p.m.) for four weeks.

At the mid-dark point (10 a.m.) the rats, under ether anaesthesia, were decapitated and their livers were excised and rapidly transferred to an ice-cold medium of 50 mM KCl, 40 mM Tris–HCl pH 7.2, 100 mM sucrose, 30 mM K. EDTA, 10 mM dithiothreitol. The tissue was homogenized with a Teflon pestle and twice centrifuged for 15 min at 12,000 *g*. The supernatant was centrifuged again for 1 hr at

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† Abbreviations: Diamide, azodicarboxylic acid bis-dimethylamide; HMG, 3-hydroxy-3-methylglutarate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MR 869, *N*-3-(2,3,4,5-tetrahydrothiophen-2-one)-2-thiophen-2-carboxymide; MR 889, 2(tenoyl-thio)*N*-(3′-tetrahydrothiophenyl-2′-one)-propionyl amide; MR 764, 1,9 bis (2′,3′-dihydroxy-propyl-thio)nonane.

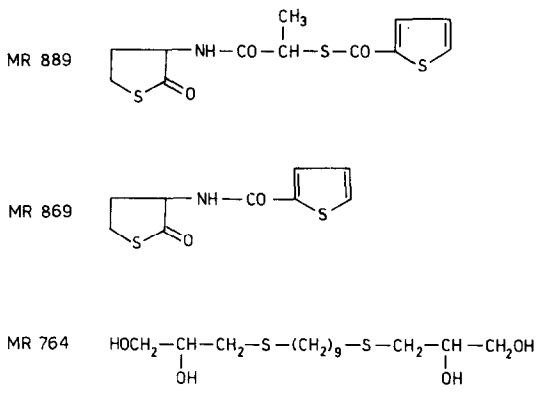

Fig. 1. Structural formulae of new compounds.

100,000 *g* and the pellet was resuspended in the same medium and re-centrifuged for 1 hr at 100,000 *g*. The microsomal suspension contained 60 mg protein/ml.

Determination of HMG-CoA reductase activity. The different partition coefficient of HMG-CoA and mevalonolactone in benzene allows a selective benzene extraction of the mevalonolactone formed in the reaction. The present procedure combines some features of previously described methods [10–13].

A 1.5 mg sample of protein was suspended in 200 μl of 250 mM NaCl, 50 mM potassium phosphate pH 7.2, 10 mM K-EDTA and 10 mM dithiothreitol. The samples were preincubated for 20 min at 37° and the reaction started by addition of 25 μl of 300 mM glucose-6-phosphate, 25 μl of 30 mM NADP, 1 I.U. of glucose-6-phosphate dehydrogenase and 10 μl of 10 mM [3-^{14}C]-HMG-CoA (4.6 μCi/ml). After 20 min incubation at 37° the reaction was stopped with 0.1 ml of 2 N HCl and the samples left 30 min at 37° to allow lactonization of mevalonic acid. They were then cooled in ice and centrifuged for 10 min at 3000 *g*. To 0.2 ml of supernatant 12 μl of 0.5 M mevalonolactone (carrier) and 100 mg of $Na_2S_2O_3$ were added, the final pH of the solution was 6.5. After double extraction with 7 ml of benzene, the radioactivity of aliquots of benzene was measured in Instagel with a Packard counter.

The extraction yield of mevalonolactone in the benzene phase was substantial while that of HMG-CoA and HMG was negligible (Table 1). This was measured by adding, to the incubation medium, known amounts of [5-^{3}H]-mevalonolactone or [3-^{14}C]-HMG-CoA or [3-^{14}C]-HMG. Results, corrected on the basis of the per cent of extraction yields and quenching values, are comparable with those obtained with the method of Shapiro and Rodwell [14] (results not shown).

Both incubation time and amount of protein used were within the values yielding a linear formation of mevalonate.

The effect of different compounds on enzyme activity was tested by adding them at the start of preincubation in the absence of dithiothreitol.

Determination of CoAS-SCoA. The hydrolysis of CoA esters and formation of CoAS-SCoA was followed by HPLC with a reversed-phase octasylane column (4.5 m × 25 cm). Incubation was stopped by the addition of 8% (v/v, final concentration) perchloric acid and the mixture centrifuged for 10 min at 3000 *g*. An aliquot of the supernatant was transferred in an ice-bath, neutralized with KOH, and centrifuged again for 10 min at 3000 *g*. The latter supernatant was injected in the chromatographic column at 25°. Elution was carried out by a concentration gradient of methanol 11–13% (v/v) in 0.1 M ammonium phosphate, pH 6.0, and UV absorbance at 254 nm was utilized for detection.

RESULTS AND DISCUSSION

A simple and precise method for measuring microsomal HMG-CoA reductase is described in this paper.

Table 1 shows that the enzymatic product mevalonolactone is extracted with a high yield, while the extracted amounts of the substrate [3-^{14}C]-HMG-CoA and those of the [3-^{14}C]-HMG possibly formed during incubation of [3-^{14}C]-HMG-CoA are quite negligible.

Figure 2 shows the inhibitory effect of mersalyl and diamide, as a function of their concentration, on HMG-CoA reductase. It should be noted that others have shown that mersalyl, diamide and disulfides reversibly react with -SH groups [15]. The inhibition curve we obtained with mersalyl is hyperbolic while that obtained with diamide is sigmoidal. Since it is known that diamide reacts with vicinal SH groups [16] the apparently cooperative inhibition, i.e. the effect of amplification observed, is consistent with the involvement of the two vicinal, interacting SH groups capable of binding such a compound.

Figure 3 shows that the apparent K_m of HMG-CoA increased in the presence of diamide while it did not significantly change in the presence of mersalyl, CoAS-SCoA and tetraethylthiouram. In contrast, all

Table 1. Percent extraction of radioactive mevalonolactone, HMG and HMG-CoA in benzene

Compound	Amount added (nmol)	Amount extracted (nmol)	Extraction yield (%)
[5-^{3}H]-mevalonolactone	20	16 ± 1.7	79.0 ± 4.3
	40	31 ± 1.2	77.5 ± 4.1
[3-^{14}C]-HMG-CoA	100	1.1 ± 0.2	1.1 ± 0.2
[3-^{14}C]-HMG	100	0.3 ± 0.1	0.3 ± 0.1

The specific radioactivity of the three compounds was 0.5 mCi/mmol. Figures are means ± S.D. of four determinations.

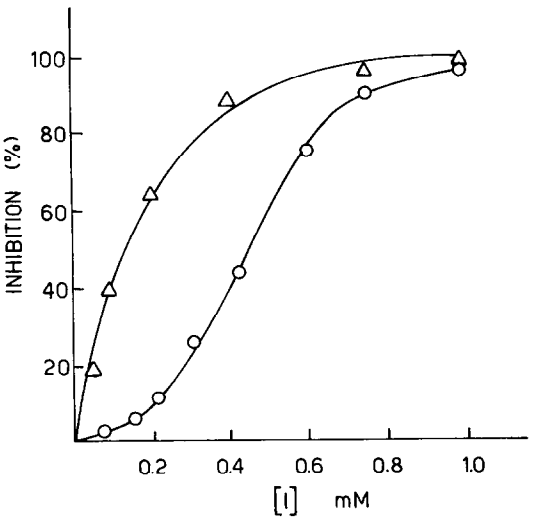

Fig. 2. Inhibition of rat liver HMG-CoA reductase by diamide and mersalyl. Conditions as described in Methods. Mersalyl (△) or diamide (○) were added at the beginning of the preincubation in the absence of dithiothreitol which was subsequently added at the start of the incubation period. The degree of inhibition is calculated by comparison with control experiments carried out without addition of mersalyl or diamide. Values are mean of duplicate experiments carried out with three different microsomal preparations.

the tested compounds caused a decrease of the V_{max} of HMG-CoA reductase; it seems, therefore, that the inhibition shown by diamide has the features of an uncompetitive inhibition, while those of CoAS-SCoA and tetraethylthiuram disulfide seem non-competitive with the substrate HMG-CoA. The use of different concentrations of inhibitors (results not shown) confirm these deductions.

Different authors have reported that CoASH and its esters are inhibitors of HMG-CoA reductase [4, 6, 8, 17, 18] and recently it has been proposed that, at least for the yeast enzyme, this inhibition is actually due to the CoAS-SCoA, present as a contaminant or formed during incubation [9].

In the present study, we found that the enzyme prepared from rat liver is also inhibited by CoAS-SCoA. The results reported in Table 2, however, indicate that the inhibition caused by CoASH, succinyl-CoA and oleolyl-CoA is not to be ascribed to the CoAS-SCoA present as a contaminant in the chemicals or formed during the incubation. In fact, the amount of CoAS-SCoA present in the medium either at the beginning or at the end of incubation with CoASH or CoA-esters was undetectable. In addition, it should be noted that incubations were carried out in the presence of 10 mM dithiothreitol which would cause the reduction of any coenzyme A disulfide present. In fact the amount of externally added CoAS-SCoA decreased during the incubations. From these results it can be concluded that although the inhibitory efficiency of CoAS-SCoA is higher than that of other CoA-esters, the enzymatic inhibition found in the presence of the latter compounds is due to the compounds themselves rather than to the coenzyme disulfide produced by them. The influence of species difference or that due to purification of the enzyme might explain the discrepancy between our conclusion and that of others who claim that inhibition exerted by

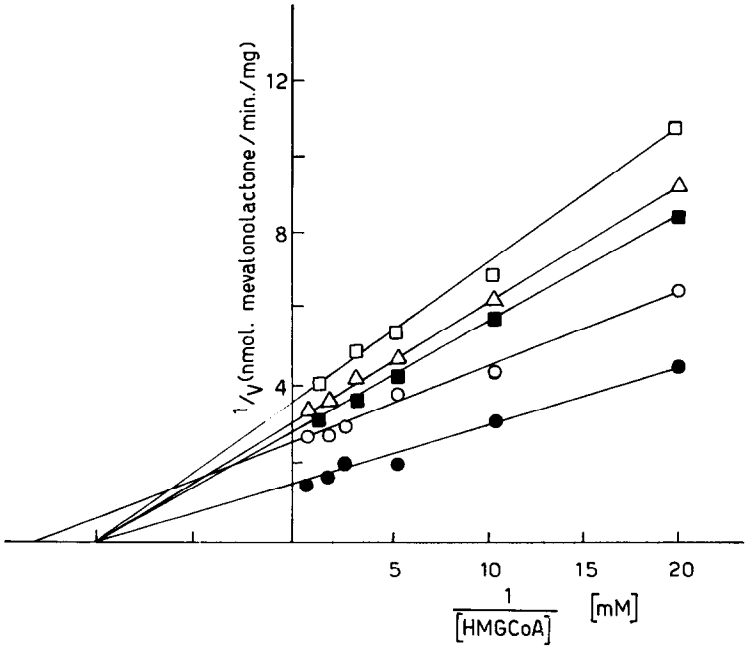

Fig. 3. Inhibitory effect of different thiol group reagents on rat liver HMG-CoA reductase. Control (●), 0.15 mM mersalyl (△); 0.3 mM diamide (○); 0.3 mM CoAS-SCoA (□); 0.3 mM tetraethylthiuram disulfide (□). Other experimental details as in Fig. 2.

Table 2. Inhibition of microsomal rat liver HMG-CoA reductase activity by CoA-esters and HMG

Addition	[CoAS-SCoA] (μM)	HMG-CoA reductase activity (nmole/min/mg protein)	Inhibition (%)
None	n.d.	0.72 ± 0.13	—
SuccinylCoA (0.15 mM)	n.d.	0.51 ± 0.05	28
SuccinylCoA (0.30 mM)	n.d.	0.40 ± 0.05	44
OleoylCoA (0.15 mM)	n.d.	0.55 ± 0.06	23
OleoylCoA (0.30 mM)	n.d.	0.45 ± 0.06	37
CoASH (0.15 mM)	n.d.	0.57 ± 0.04	20
CoASH (0.30 mM)	n.d.	0.50 ± 0.04	30
CoAS-SCoA (0.05 mM)	21	0.54 ± 0.05	25
CoAS-SCoA (0.30 mM)	185	0.21 ± 0.03	71
HMG (1 mM)	—	0.65 ± 0.07	10
HMG (5 mM)	—	0.55 ± 0.06	24
HMG (1 mM) + CoAS-SCoA (0.05 mM)		0.44 ± 0.05	38
HMG (5 mM) + CoAS-SCoA (0.30 mM)		0.11 ± 0.03	84

The CoAS-SCoA concentration in the medium was measured both at the start and at the end of incubations. Oleoyl-CoA was dissolved in 12% (v/v) dimethylsulfoxide that caused 22% activity inhibition by itself, this inhibition was subtracted from that shown by oleoylCoA solutions. Results are means ± S.D. of four experiments with different liver preparations. Protein was determined according to Lowry *et al.* [22].
n.d.: non detectable; i.e. less than 1 μM.

Table 3. Microsomal rat liver reductase activity assayed in the presence of new synthetic thiophene derivatives

Addition	Concentration	Reductase activity (nmole/min/mg protein)	Inhibition (%)
None	—	0.72 ± 0.13	—
MR 869	0.5 mM	0.68 ± 0.12	5
MR 869	5.0 mM	0.55 ± 0.12	23
MR 889	0.5 mM	0.67 ± 0.15	7
MR 889	5.0 mM	0.52 ± 0.10	28
MR 764	0.5 mM	0.65 ± 0.11	10
MR 764	5.0 mM	0.39 ± 0.13	45

The new compounds MR 869, MR 889 and MR 764 were dissolved either in propyleneglycol or in 12% (v/v) dimethysulfoxide. The values, corrected for the inhibition caused by the solvent (14% inhibition by propyleneglycol and 22% by dimethysulfoxide), are means ± S.D. of duplicate determinations on four different liver preparations.

CoA esters on yeast HMG-CoA reductase is almost completely due to contaminations of CoA disulfide [9].

It has been shown by other workers that HMG is a non-competitive inhibitor of cholesterol biosynthesis acting at the level of HMG-CoA reductase [16, 18–20]. Table 2 suggests that the inhibition due to HMG can combine with that of CoAS-SCoA.

The inhibitory effect of different compounds on the activity of the enzyme can give useful clues to the planning of new synthetic inhibitors, ideally containing in the same molecule an HMG moiety and a disulfide portion.

As a first approach three new compounds with low toxicity and potentialities as chemical precursor of such an "ideal" inhibitor were synthesized and Table 3 shows their inhibitory effects on HMG-CoA reductase. The compounds indeed cause an inhibition of the reductase activity and this result was confirmed by parallel experiments with HMG-CoA reductase preparations purified and assayed according to Alberts [21] (results not shown). The more effective among the inhibitors seems that with thioether groups and a long chain while those with shorter chains and thio-ester cyclic groups are less effective. The difference in activity, however, is not substantial.

Acknowledgements—The help of Rex Parker, Department of Biochemistry, I.U.M.C. Indianapolis, in some experiments and the financial support of Medea Researches, Milano, are gratefully acknowledged.

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