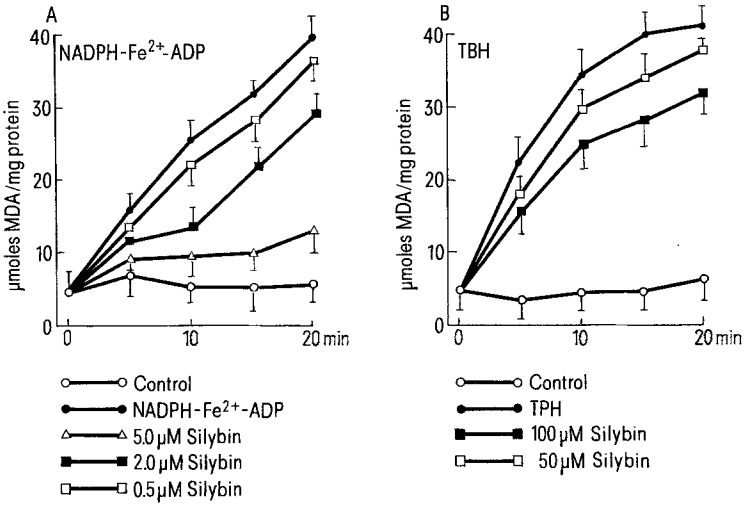


Figure 2. Effect of silybin on the malonaldehyde (MDA) formation induced by NADPH-Fe^{2+}-ADP (A) and TBH (B) on rat liver microsomes. The assay system contained 3.5 mg/ml of microsomal protein in a final volume of 10 ml. Silybin was added 2 min before the addition of the catalyst (time = 0) to final concentrations indicated in the figure. Aliquots of 0.5 ml were taken every 5 min for MDA determination. Each point represents the promedium of five experiments ± SEM.

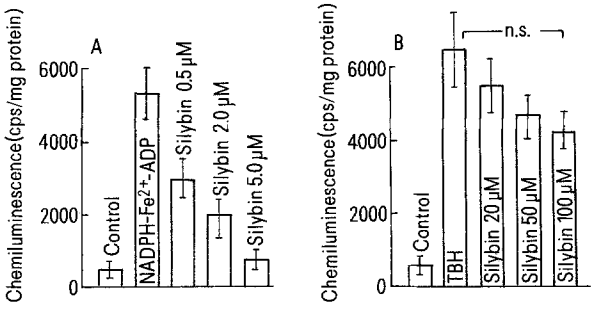


Figure 3. Effect of silybin on the low level chemiluminescence induced by NADPH-Fe^{2+}-ADP (s) and TBH (B) on rat liver microsomes. The assay system contained 3.5 mg/ml of microsomal protein in a final volume of 3 ml. Silybin was added 2 min before the addition of the catalyst (time = 0) to final concentrations indicated in the figure. After 10 min of incubation at 37 °C, 2 ml of a 1:100 dilution was counted in a glass standard vial. Results represent the promedium of six experiments ± SEM. Differences with $p > 0.05$ were considered to be without significance (N.S.) and are indicated in the figure.

is the excited molecule which is most important in the NFeA-induced chemiluminescence, and is only secondary in the TBH-induced mechanism, the differential action of silybin could be explained assuming that this flavonoid acts by trapping preferentially the $^1O_2$ species.

Our results support the free radical scavenger action ascribed to silyb and to silymarin. It is possible that the flavonoid preferentially traps certain structures such as the ·OH free radical and/or the $^1O_2$ excited species. More complex free radical structures such as the t-butoxy radical would not be scavenged by the flavonoid. The membrane stabilizing action ascribed to silymarin and to silyb, as the basis of many of its experimental and therapeutical effects, may well be a consequence of its antioxidant properties and their scavenging actions as proposed here.

1 This research was supported in part by grant B-2019-8412 from Dirección de Investigación y Bibliotecas, Universidad de Chile.
2 To whom correspondence should be addressed.
3 Vogel, G., Arzneimittel-Forsch. *18* (1968) 1063.
4 Greimel, A., and Koch, H., Experientia *33* (1977) 1417.
5 Valenzuela, A., Guerra, R., and Videla, L.A., submitted to Lipids (1985).
6 Bindoli, A., Cavallini, L., and Siliprandi, N., Biochem. Pharmac. *26* (1977) 2405.
7 Auclair, C., and Lecompte, M.C., Biochem. biophys. Res. Commun. *85* (1978) 946.
8 Hochstein, P., and Ernster, L., Biochem. biophys. Res. Commun. *12* (1963) 388.
9 Lowry, D.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
10 Ottolenghi, A., Archs Biochem. Biophys. *79* (1959) 355.
11 Videla, L.A., Experientia *39* (1983) 500.
12 Lai, C.S., and Piette, L.H., Archs Biochem. Biophys. *190* (1978) 27.
13 Walling, C., in: Oxidases and related redox systems, p. 85. Eds T.E. King, H.S. Mason and M. Morrison. Pergamon Press, Oxford 1982.
14 Encina, M.V., Rivera, M., and Lissi, E.D., J. Polym. Sci. *16* (1978) 1709.
15 Boveris, A., Cadenas, A., and Chance, B., Fedn Proc. *40* (1981) 195.

0014-4754/86/020139-03$1.50 + 0.20/0

Influences of the chemical structure on the activity of new inhibitory compounds of the angiotensin-converting enzyme

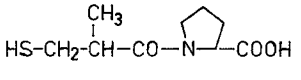
W.-E. Siems*, G. Heder*, K.D. Jentzsch*, U. Burkhardt**, S. Johne**, H. Kühmstedt, K. Kottke, I. Wunderlich and P. Oehme*

**Institute of Drug Research, Academy of Sciences of the GDR, DDR-1136 Berlin (German Democratic Republic), **Institute of Biochemistry of Plants, Academy of Sciences of the GDR, DDR-4010 Halle (Saale) (German Democratic Republic), and Section of Pharmacy, Ernst-Moritz-Arndt-University Greifswald, DDR-2200 Greifswald (German Democratic Republic), 10 December 1984*

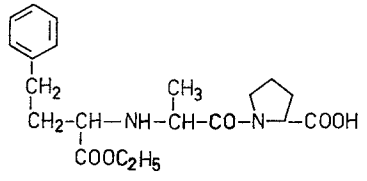
Summary. The ACE inhibitory activity of some perimidines, chinazolinones and amidinohydrazones is described. Relations were found between the chemical structure and the inhibitory activity on the ACE.
Key words. Angiotensin-converting enzyme (ACE); new ACE inhibitory compounds; perimidines; chinazolinones; amidinohydrazones; structure-activity-relations.

Experientia 42 (1986), Birkhäuser Verlag, CH-4010 Basel/Switzerland

Since the late 1970s, a new method has been used in the treatment of hypertension; the inhibition of angiotensin-converting enzyme (ACE)[1-4]. Inhibitors of ACE cause a decrease of angiotensin II formation and a potentiation of bradykinin action. The basis for the development of ACE inhibitors was the knowledge about the ACE inhibitory and antihypertensive actions of some 'bradykinin-potentiating-peptides' (BPP) from snake venom fractions[5,6]. A hypothetical model of the active center of the ACE was developed based on the C-terminal sequences of the BPP and on the presumed analogy to the well characterized zinc-containing carboxypeptidase A (CPA). On the basis of this model many oral ACE inhibitors were developed[3,7]. Most of them are interpretable as analogues of BPP and the action of these compounds can be well understood on the basis of the active site model developed by Cushman[1] and Petrillo[3]. Modern ACE inhibitors are oral antihypertensives with a wide therapeutic application[7-9]. Furthermore, ACE inhibitors have been used with good success in the treatment of congestive heart failure (reduction of peripheral resistance)[10]. The two most important types of ACE inhibitors have been derived from captopril (SQ 14.225) (for example YS-980; CL-242815; RHC-3659) and enalapril (MK-421) (for example HOE-498; SCH 31846; CGS 13945; MK-521).



Captopril



Enalapril

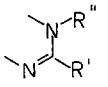
The aim of the present work was the search for new classes of ACE inhibitory compounds with structures completely different from the well know ACE inhibitors. This could be the starting point for the development of a new family of ACE inhibitors in the future.

Material and methods. The ACE used in our experiments was polyacrylamide-gel-electrophoretically pure with a specific activity of 80 µmoles His-Leu/min/mg protein. The isolation of the enzyme from pig lungs includes the following procedures: Homogenization, centrifugations, DEAE ion-exchange chromatography, separation on hydroxylapatite and gel filtration on Sephadex G-200.

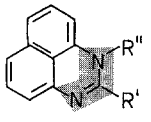
ACE activity was measured by a slight modification[11] of the fluorimetric method first described by Friedland and Silverstein[12]. The ACE inhibitory activities are expressed in IC_{50}-values. These are the molar concentrations required for 50% inhibition of the enzyme under standard conditions. The substrate Hip-His-Leu was synthesized in the Department of Drug Synthesis of the Institute of Drug Research of the Academy of Sciences of the GDR. The His-Leu used for the standardization of the assay was produced by Serva (Heidelberg/FRG).

The specifity of the ACE inhibition was characterized by comparison with the inhibition of some other selected zinc-containing enzymes. Most substances were tested for their action on CPA with Hip-Phe as substrate and on carboxypeptidase B (CPB) with Hip-Lys as substrate. The most active substances were also tested on leucine aminopeptidase (LAP) with leucinehydrazide as substrate.

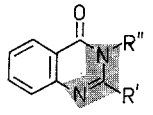
Results. In the result of a broad screening of substances with structures relatively far from those of captopril, enalapril, and other known ACE inhibitors, compounds with the following structural segment were found to be potential ACE inhibitors.



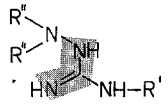
This effect was established with the following three types of compounds. The dependence of the ACE inhibitory activity of these groups of compounds on the substituents is summarized in the tables 1–3.



Perimidines



Chinazolinones



Amidinohydrazones

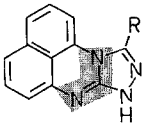
Discussion. All three types of compounds presented here contain the same structural feature, as illustrated by the segments with dark background in the structures of perimidines, chinazolinones, and amidinohydrazones in the tables 1–3. We suppose that the ACE inhibitory activity of these compounds is due to this structural segment incorporated in a mesomeric system. Our assumption is supported by some analogously substituted chinoxaline and pyrimidine compounds in which the structural feature is changed. These compounds have very low or no inhibitory activities on ACE.

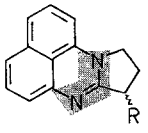
In all three cases the IC_{50}-value of the ACE inhibition depends on the kind of substitution. Substituents with free electron pairs and +I or +M effects at the position R′ (see general formula) support the mesomeric system and cause an increase of the ACE inhibition (amines, thioalkohols, thioethers, hydrazines, semicarbazides, etc.). In the case of perimidines 1a-1g the substituent R′(hydrazone) is incorporated in a triazolring system. The action of substituents at the position R′ is very clearly shown by comparing the perimidine compounds 1a-1g with 1h, 1i and by comparing the thioethers of chinazolinones 2a and 2b with the sulfone 2c (R′ = R_1).

Larger hydrophobic groups at the position R″ lead to stronger ACE inhibition. Unambiguous examples for it are the increasing alkyl substituents (R) connected over the triazole ring with the perimidines 1a-1g and the increasing O-alkyl in the chinazoline compounds 2d-2g (R″ = $-C_6H_4-O-R$).

For the amidinohydrazone compounds it is important that substances with three aromatic substituents (3a, 3b) are more potent inhibitors in relation to not completely substituted compounds (3c-3e). An olefinic linkage of the aromatic residues to the amidinohydrazone center results in a relatively good inhibitory activity of the compounds 3f, 3g. It may be important that the greater distance between the hydrophobic groups (aromats) and the amidinohydrazone basic structure is more suitable for the interaction with the active center of the ACE molecule. All amidinohydrazones presented here are nitrates or iodides.

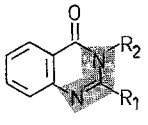
Table 1. Inhibitory activities of perimidine derivates[13,14] against ACE, CPA, CPB and LAP

	Comp.	R	IC_{50} (10^{-6} moles/l) ACE	CPA	CPB	LAP
	1a	H	28	> 100	> 200	n.t.
	1b	CH_3	16	> 200	> 200	n.t.
	1c	C_6H_5	35	n.t.	n.t.	n.t.
	1d	$C_{11}H_{23}$(n)	1.3	23	20	> 200
	1e	$C_{13}H_{27}$(n)	0.65	13	n.t.	> 400
	1f	$C_{15}H_{31}$(n)	0.75	27	17	> 400
	1g	$C_{17}H_{35}$(n)	0.55	15	34	n.t.

	Comp.	R	IC_{50} (10^{-6} moles/l) ACE
	1h	OH	> 100
	1i	H	> 100

n.t. = not tested.

Table 2. Inhibitory activities of chinazolinone derivates[15,16] against ACE, CPA, CPB and LAP

	Comp.	R_1	R_2	IC_{50} (10^{-6} moles/l) ACE	CPA	CPB	LAP
	2a	S-C_3H_7	$C_6H_3(CH_3)_2$[3,4]	7.5	> 100	52	n.t.
	2b	S-C_4H_9	$C_6H_3(CH_3)_2$[3,4]	4.7	> 200	30	> 200
	2c	SO_2-C_2H_5	C_6H_4Cl[4]	> 100	n.t.	n.t.	n.t.

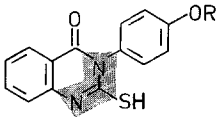
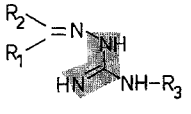
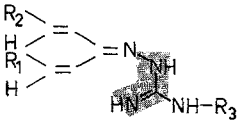
	Comp.	R	IC_{50} (10^{-6} moles/l) ACE	CPA	CPB	LAP
	2d	CH_3	80	150	83	n.t.
	2e	C_2H_5	45	58	> 200	n.t.
	2f	C_4H_9(n)	7.0	12.5	210	n.t.
	2g	C_5H_{11}(n)	4.7	17	142	> 200

Table 3. Inhibitory activities of amidinohydrazone derivates[17,18] against ACE, CPA, CPB and LAP

	Comp.	R_1	R_2	R_3	IC_{50} (10^{-6} moles/l) ACE	CPA	CPB	LAP
	3a	C_6H_5	C_6H_5	C_6H_5	4	100	> 200	> 400
	3b	C_6H_5	C_6H_5	C_6H_4Br(p)	1	17	25	400
	3c	C_6H_5	C_2H_5	C_6H_5	15	20	> 200	> 400
	3d	C_9H_6N(Chinoline)	H	H	100	n.t.	n.t.	n.t.
	3e	C_9H_6N(Chinoline)	H	C_6H_5	17	n.t.	n.t.	n.t.
	3f	C_6H_5	C_6H_5	H	0.75	> 200	> 100	> 400
	3g	C_6H_5	C_6H_5	C_6H_5	0.60	61	150	> 400

We suppose that the common structural feature in all three classes of compounds is responsible for a blockade of the zinc ion in the active center of the ACE. This effect can be supported by suitable substituents in the position R'. On the other hand the positive influence of the larger hydrophobic residues on the ACE inhibition is presumably the consequence of an interaction of these groups with the hydrophobic pockets in the neighborhood of the central zinc ion of the enzyme. The blockade of the zinc ion of the ACE could lead to an inactivation of the normal function of the cation on peptide hydrolysis, i.e. the activation of the carbonylfunction of the peptide bond between the amino acids two and three of the substrates. Our assumption is supported by the fact that other zinc-containing peptidases such as CPA and CPB are also inhibited by these three classes of compounds, but in most cases the value of enzyme inhibition is much lower in comparison with ACE. Our compounds show no or very low inhibitory activities on the aminopeptidase LAP. These different results regarding the specifity of our compounds can be explained by the fact that the carboxypeptidases CPA and CPB are more closely related to ACE than the aminopeptidase LAP. Our experiments on the specifity of ACE inhibition, however, point to a relatively good selective action of some of our com-

pounds. This mainly applies to the amidinohydrazones 3f and 3g. But also for the less specific chinazolinones, perimidines and the remaining amidinohydrazones the IC_{50}-values for ACE are always clearly smaller in relation to those of CPA and CPB.

We have presented here new types of ACE inhibiting compounds. These substances differ very clearly from all well-known ACE inhibitors. The ACE inhibition of the presented compounds has to be evaluated in relation to the IC_{50} of captopril and other well-known ACE inhibitors measured under the same conditions (see table 4). Until now these new classes of ACE inhibitory compounds are of course not able to complete or replace the most active ACE inhibitors. We expect, however, a further increase in the activity of these compounds by a systematic modification of the side chains of our compounds in accordance with the conceptions of the active ACE center as proposed by Cushman[1] and Petrillo[3].

Table 4. ACE inhibitory activities of captopril, enalapril and HOE-498

Comp.	IC_{50} (10^{-6} moles/l)
Captopril	0.03
Enalapril (maleat)	4.0 (ester; prodrug)
HOE-498	1.7 (ester; prodrug)
HOE-498	0.0012 (free acid)

Immediately after the submission of the manuscript, K.-C. Liu and L.-Y. Hsu published an article about 'Synthesis and antihypertensive activity of some quinazolinone derivates' (Archs Pharm. *318* (1985) 502). The mechanism of the hypotensive action is not described, but all these antihypertensive compounds are structurally closely related to our substances. These studies, consequently, support the aim of our work.

1 Cushman, D.W., Cheung, H.S., Sabo, E.F., and Ondetti, M.A., Biochemistry *16* (1977) 5484.
2 Antonaccio, M.J., A. Rev. Pharmac. Toxic. *22* (1982) 57.
3 Petrillo, E.W., and Ondetti, M.A., Med. Res. Rev. *2* (1982) 1.
4 Patchett, A.A., Harris, E., Tristam, E.W., Wyvratt, M.J., Wu, M.T., Taub, D., Peterson, E.R., Ikeler, T.J., ten Broeke, J., Payne, L.G., Ondeyka, D.L., Thorsett, E.D., Greenlee, W.J., Lohr, N.S., Hoffsommer, R.D., Joshua, H., Ruyle, W.V., Rothrock, J.W., Aster, S.D., Maycock, A.L., Robinson, F.M., Hirschmann, R., Sweet, C.S., Ulm, E.H., Gross, D.M., Vassil, R.C., and Stone, C.A., Nature *288* (1980) 280.
5 Ferreira, S.H., Bartelt, D.C., and Greene, L.J., Biochemistry *9* (1970) 2583.
6 Ondetti, M.A., Williams, N.J., Sabo, E.F., Pluščeč, J., Weaver, E.R., and Kocy, O., Biochemistry *10* (1971) 4033.
7 Sweet, C.S., and Blaine, E.H., in: Cardiovascular Pharmacology, p. 119. Ed. M.J. Antonaccio. Raven Press, New York (1984).
8 Ram, C.V.S., Archs intern. Med. *142* (1982) 914.
9 Overlack, A., Higuchi, M., Kolloch, R., and Stumpe, K.O., Klin. Wochenschr. *61* (1983) 377.
10 Romankiewicz, J.A., Brogden, R.N., Heel, R.C., Speight, T.M., and Avery, G.S., Drugs *25* (1983) 6.
11 Siems, W.-E., Heder, G., and Komissarowa, N.W., Z. med. Labor-Diagn. *26* (1985) 232.
12 Friedland, J., and Silverstein, E., Am. I. clin. Pathol. *66* (1976) 416.
13 Burkhardt, U., and Johne, S., J. prakt. Chem., in press 1985.
14 Burkhardt, U., and Johne, S., DD-Application WP C 07 D/250 459 6 from 2.5.1983.
15 Kottke, K., Dissertation-B, Universität Greifswald 1983.
16 Kottke, K., Friedrich, F., Knoke, D., and Kühmstedt, H., Pharmazie *32* (1977) 540.
17 Dau, K., Dissertation-A, Universität Greifswald 1977.
18 American Cyanamid Co., Fr. Demande 2.422.646 (Cl. C 07 D 239/18), 9.11.79, Appl. 77/27 372, 9.9.1977.

0014-4754/86/020141-04$1.50 + 0.20/0

Ultrastructural localization of catalase and D-amino acid oxidase in 'normal' fetal mouse liver

A.S. Dabholkar

Department of Biological Sciences, Wright State University, Dayton (Ohio 45435, USA), 9 January 1985

Summary. In the hepatocytes of 'normal' fetal mice from mothers which were carriers of muscular dysgenesis, catalase and D-amino acid oxidase (DAAO) positive as well as negative peroxisomes were observed. DAAO reaction product was occasionally localized in patches around cell membranes and DAAO-positive peroxisomes were frequently observed near mitochondria.
Key words. Ultrastructure; catalase; D-amino acid oxidase; fetal mouse liver; hepatocytes; peroxisomes; muscular dysgenesis.

Peroxisomes (microbodies) are now recognized as respiratory cellular organelles. Enzymes present in peroxisomes (peroxisomal enzymes) are not synthesized in peroxisomes but in the cytosol. Peroxisomal proteins in rat liver are synthesized on membrane bound polysomes[2] or on free polysomes[3], released into the cytosol and transported to peroxisomes[2,3]. Cytosolic catalase has been localized in liver parenchyma[4] but its absence from the Golgi apparatus and rough endoplasmic reticulum suggests that the channeling of newly synthesized catalase from the rough endoplasmic reticulum to the peroxisome is unlikely[5]. No connections were demonstrated between peroxisomes and endoplasmic reticulum by electron microscopic cytochemistry[6]. Biogenesis of peroxisomes is still not well understood.

Peroxisomes contain the marker enzyme catalase and one or more oxidases. Some of these enzymes have been demonstrated by cytochemical techniques in peroxisomes but not at the sites of their synthesis or in their pathways to the peroxisomes. Since peroxisomes are formed during tissue development and differentiation, as has been shown by various studies[7], an embryonic stage may show peroxisomal enzymes at the sites of synthesis or en route to peroxisomes. With this assumption, the present study of only two of the cytochemically demonstrable enzymes, catalase and DAAO, was undertaken in 'normal' fetal mouse liver. Another objective was to learn whether or not heterogeneous populations of hepatic peroxisomes with respect to catalase and DAAO do exist, at least during development. Catalase plays a protective role against peroxide toxicity by degrading H_2O_2 while oxidases generate H_2O_2[8]. Thus, H_2O_2 generation and degradation occur in the peroxisomes. DAAO catalyzes the oxidation of D-d-amino acids[9] and its role in metabolizing substrates such as biogenic amine and glyoxylate is beginning to unfold[10].

Material and methods. The liver from a 'normal' mouse embryo (fetus of 19 days gestation obtained from the mother carrier of muscular dysgenesis) was dissected out under anesthesia, cut into pieces and fixed in cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. For catalase cytochemistry, the liver tissue was fixed for 2 h, washed in 0.1 M cacodylate buffer and incubated for 20–60 min at 37 °C in Novikoff's medium[11] slightly modified and used at pH 9.7. The incubation medium contained 0.020 g 3,3'-diaminobenzidine (DAB) tetrahydrochloride (Sigma Chemical Co.), 9.8 ml 0.05 M 1,2-propanediol buffer at pH 9.0, 0.2 ml 1% hydrogen peroxide. The tissue was subse-