Biochimica et Biophysica Acta 860 (1986) 91–98
Elsevier

BBA 73216

Characterization of a transporting system in rat hepatocytes. Studies with competitive and non-competitive inhibitors of phalloidin transport

Klaus Münter [a], Doris Mayer [b] and Heinz Faulstich [a,*]

[a] *Abteilung für Physiologie, Max-Planck-Institut für Medizinische Forschung, Jahnstrasse, 29, D-6900 Heidelberg and* [b] *Abteilung für Cytopathologie, Institut für Experimentelle Pathologie, Deutsches Krebsforschungszentrum, Heidelberg (F.R.G.)*

(Received February 12th, 1986)

Key words: Phalloidin transport; Transport inhibitor; Kinetics; (Rat hepatocyte)

Primary cultures of rat hepatocytes were used for assaying several drugs not previously known for inhibiting the transport of phalloidin. In order to have 50% inhibition (IC_{50}) of the entrance of a tritiated phallotoxin derivative ([^{3}H]demethylphalloin, 1 μM) from the medium into the cells the following concentrations (μM) of the various inhibitors were determined: cyclolinopeptide (0.5), Nocloprost® (5.0), Nileprost® (7.0), β-estradiol (42), Verapamil® (70). For comparison, the corresponding IC_{50} values of some known antagonists of phalloidin toxicity were determined by the same method. Moreover, we studied several natural and synthetic phallotoxins and α-amanitin for their ability to displace [^{3}H]demethylphalloin from the transporting system. Lineweaver-Burk plots made it obvious that two groups of inhibitors exist. Competitive inhibitors are, for example, antamanide, β-estradiol, silybin, Nileprost®, taurocholate, and the cyclic somatostatin analog cyclo[Phe-Thr-Lys-Trp-Phe-D-Pro], whereas Verapamil® and monensin inhibit phallotoxin uptake in a non-competitive way. Considering the very different chemical features of the competitive inhibitors, we tentatively conclude that the phallotoxin transport system selects compounds not on the basis of their chemical features, but rather their physical properties. The physical properties of a typical substrate are low molecular mass, lipophilic nature, and, possibly the presence of rigid ring structures. Negative charges accelerate the transport of a substrate, while positive charges have the opposite effect. The phalloidin-transporting system may represent part of a hepatic equipment which clears portal blood from, for example, bile acids, lipophilic hormones, or xenobiotics. By chance, the transporting system incorporates phallotoxins into the hepatocytes leading to the death of these cells.

Introduction

Without exception, the drugs described in the past as antagonists of the mushroom toxin phalloidin (for a review see Ref. 1) are active in affecting the phallotoxin transport system. This was found to apply for drugs inhibiting with high specificity, such as antamanide [2,3], silymarin [4–7], cholate [8], or cyclosporin A [9]. This probably holds true as well for some other compounds representing toxins by themselves, such as rifampicin [10], carbon tetrachloride, or Na-cinchophen [11]; these agents, however, may 'protect' the hepatocytes from phalloidin by damaging, rather than by only inhibiting, the ability of this system to transport phallotoxins.

The transporting system incorporates phallotoxins into hepatocytes with high efficiency. For example, the liver of a mouse accumulates 50% of an i.v. dose of [^{3}H]demethylphalloin within 10 min [2]. In a recirculating, perfused rat liver, more than 80% of a dose of [^{3}H]demethylphalloin was

* To whom correspondence should be addressed.

0005-2736/86/$03.50 © 1986 Elsevier Science Publishers B.V. (Biomedical Division)

found to be taken up within 15 min [1]. Finally, isolated hepatocytes were found to be in equilibrium with a medium containing [^{3}H]demethylphalloin after 30 min at the longest, and probably sooner [12]. Such rapid and preferential uptake into the hepatocytes explains why phalloidin and its homologs act as hepatotoxins.

Several pieces of evidence indicate that the phalloidin transporting system is involved in the transport of bile acids as well [8,13]. Recently, two proteins were detected in rat liver plasma membranes (M_r 48000; M_r 54000) which are able to sequester not only affinity-labeled phallotoxins but also affinity-labeled bile acids [14]. For the incorporation of bile acids into hepatocytes it has been proposed [13] that two different transporting systems may exist. So far, however, it is unproved that both systems are involved in phalloidin transport. From the similarity between pictures of rat hepatocytes treated with fluorescent-labeled phallotoxin, and those treated with fluorescent-labeled concanavalin A, it was suggested that a special kind of endocytotic process may be involved in the transport of phalloidin [15].

Materials and Methods

[^{3}H]Demethylphalloin, spec. act. 2 Ci/mmol was our own preparation according to Ref. 16. The cyclo[Phe-Thr-Lys-Trp-Phe-D-Pro] was a donation of H. Kessler and S. Koll, Frankfurt. Nileprost® and Nocloprost® were kindly provided by Schering AG, Berlin and Verapamil® by Knoll AG, Ludwigshafen. Silybin dihemisuccinate, (*O*-carboxymethyl-tyrosine^5) antamanide, and cyclolinopeptide were gifts of Professor G. Vogel, Madaus, Köln and Professor Th. Wieland, Heidelberg, respectively. Somatostatin was received from Diamalt, Munich; monensin from Sigma, Munich; and β-estradiol from Serva, Heidelberg. The mushroom toxins and their derivatives were prepared in our laboratory. Thin-layer chromatography of some of the phallotoxins was performed on silica plates (F_{254}, Merck, Darmstadt) and developed with butanone/acetone/water (30 : 3 : 5, v/v), and detected by ultraviolet light (254 nm), according to Ref. 1.

Isolation and culturing of hepatocytes, as well as the assay procedure, was described recently by us [17]. Penicillin/streptomycin, which is usually added to short-term cultures of hepatocytes, was omitted, because occasionally preparations of these antibiotics interfered with the ability of the hepatocytes to incorporate the phallotoxins. All inhibitors were dissolved in phosphate-buffered saline or in methanol, diluted with phosphate-buffered saline, and then added to the test solution.

Results and Discussion

Short-term cultured rat hepatocytes are useful models for studying transport processes and toxic events occurring in parenchymal liver cells. Since dead cells do not attach to the bottom during incubation and are later washed out, the data obtained is representative for hepatocytes, which remain intact, and is therefore, of a high reproducibility. In studies done with phallotoxins, however, the assay system is limited to a concentration of 5 μM, since at higher concentrations of the toxin, the settling of the hepatocytes is affected.

When incubated with 1 μM [^{3}H]demethylphalloin, a radioactive derivative of the mushroom poison phalloidin, the cells took up 52 ± 16 ng toxin/mg protein within 3 h ($n = 60$). By kinetic studies (see below), we measured K_m and V_{max} of the transport system for [^{3}H]demethylphalloin to be 2.5 μM, and 0.06 nmol $\cdot$ (mg protein)$^{-1} \cdot h^{-1}$, respectively. For comparison, the corresponding values of a probably physiological substrate of this transporting system, taurocholate, were determined to be 43 μM, and 2.72 nmol $\cdot$ (mg protein)$^{-1} \cdot h^{-1}$.

The present study describes several compounds not previously known to act as inhibitors of phalloidin uptake. Included are the prostaglandin analogs Nocloprost® and Nileprost® [18], which strongly inhibit phalloidin transport (Fig. 1, Table I). Their IC_{50} values were determined to be 5 μM and 7 μM, respectively. Since these concentrations are orders of magnitude higher than those needed for hormonal activities of prostaglandins [19] it seems unlikely that Nocloprost® and Nileprost® act as hormones. The steroid hormone β-estradiol was found to inhibit the taking up of phallotoxin at a concentration of 42 μM. Again, this value is much higher than its physiological concentration

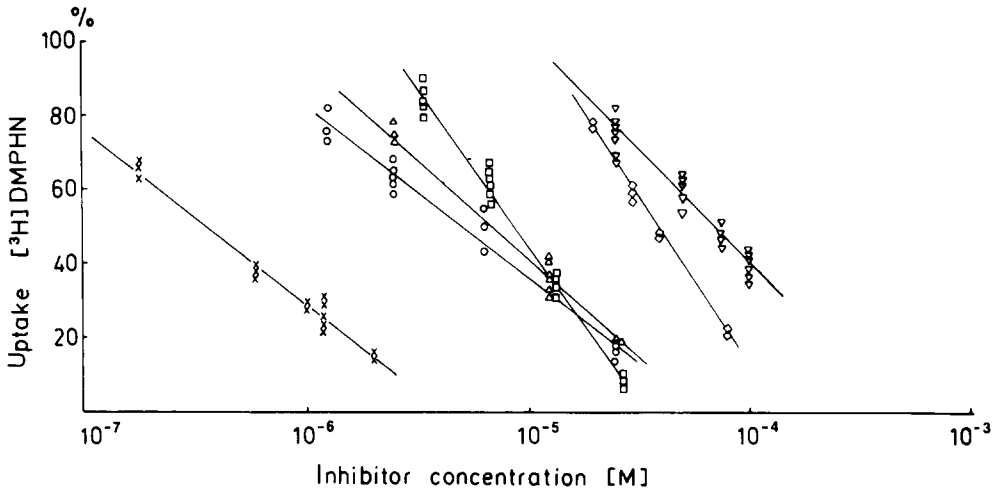

Fig. 1. Inhibition by various drugs of phallotoxin uptake into hepatocytes during 3 h of incubation with 1 μM [^{3}H]demethylphalloin ([^{3}H]DMPHN). 100% uptake corresponds to 52 ± 16 ng toxin per mg of protein. Water-soluble antamanide (×); Nocloprost® (○); Nileprost® (△); silybin dihemisuccinate (□); β-estradiol (◇); Verapamil® (▽).

in plasma, approx. 1 nM [20], and therefore cannot represent a hormonal activity. On the other hand, the fact that a steroid hormone is tolerated as a substrate suggests that one of the physiological functions of the transport system may be the clearance of blood from particular steroid compounds. Certainly, other physiological steroids would merit being investigated by this assay method. Besides prostaglandin analogs and β-estradiol it was detected that the Ca^{2+}-antagonist Verapamil® [21] inhibits phallotoxin incorporation at a concentration of approx. 70 μM. This seems noteworthy, because it shows that even typical therapeutical agents may exert a weak, though significant, effect on this transport system.

In order to obtain further information on the phalloidin transporting system, several monocyclic peptides were assayed, among them the well-known antagonist of phalloidin poisoning, water-soluble antamanide. It was verified that

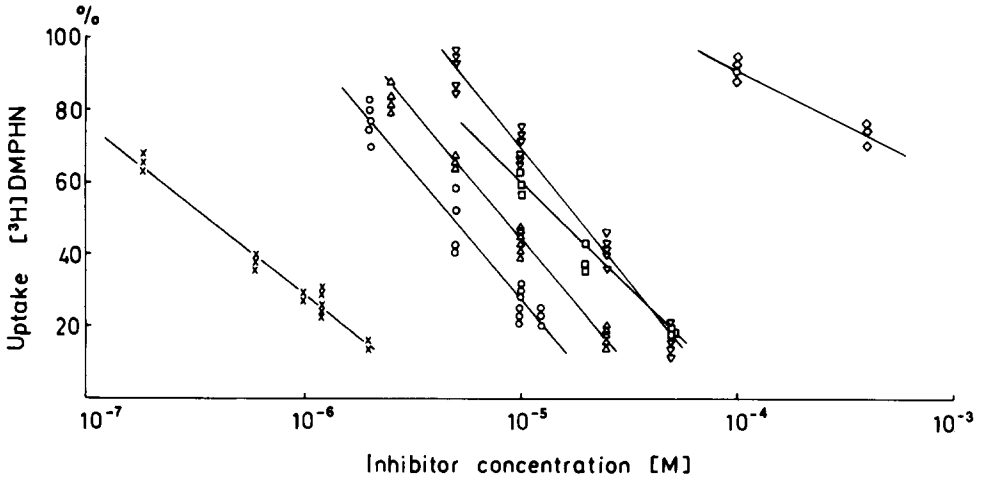

Fig. 2. As Fig. 1. Water-soluble antamanide (×); monensin (○); somatostatin analog cyclo[Phe-Thr-Lys-Trp-Phe-D-Pro] (△); taurocholate (□); cholate (▽); somatostatin (for comparison) (◇).

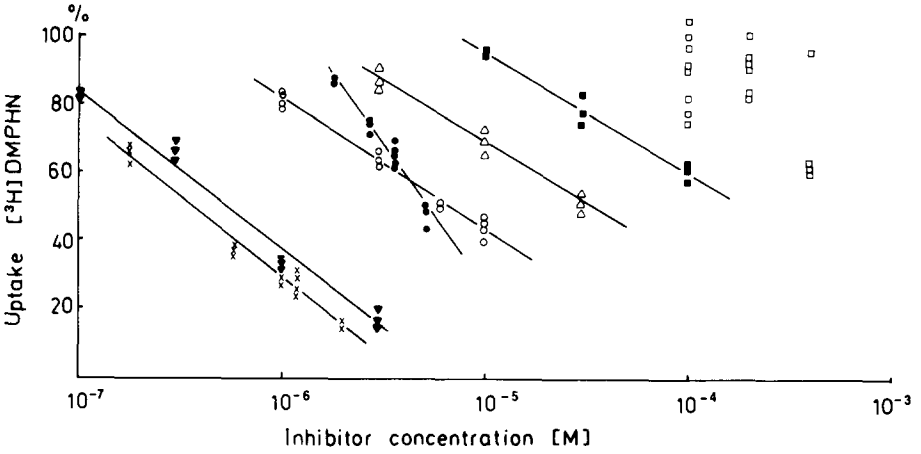

Fig. 3. As Fig. 1. Water-soluble antamanide (×); cyclolinopeptide (▼); phallacidin (○); fluoresceine-labeled phalloidin (●); aminophalloidin (△); secophalloin (■); α-amanitin (□).

antamanide is the most effective inhibitor of [^{3}H] demethylphalloin uptake into short-term cultured hepatocytes, as it is in other systems (Fig. 2). Surprisingly, we detected that cyclolinopeptide [22,23], which lacks any antagonistic activity in phalloidin poisoning (Wieland, Th., personal communication), does inhibit the transport of phallotoxins, being nearly as effective (0.5 μM) as water-soluble antamanide (0.3 μM) (Fig. 3). Two possible explanations may account for this: either, cyclolinopeptide is sequestered in vivo by other tissues and, therefore, cannot interfere with phalloidin toxicity; or antamanide has, in addition to its ability to inhibit the transport of phalloidin, an antitoxic activity within the hepatocytes which is not known as of yet. More than one order of magnitude less effective on phalloidin transport in cultured hepatocytes than antamanide and cyclolinopeptide is the cyclic somatostatin analog cyclo[Phe-Thr-Lys-Trp-Phe-D-Pro] (8.5 μM). The inhibitory activity of this compound was not unexpected, because the somatostatin analog also inhibits the transport of bile acids into isolated rat hepatocytes [24]. Native somatostatin was tested for comparison; by extrapolation, we estimated that its inhibitory capacity is nearly 270-times less than that of its cyclic analog.

In order to directly compare the inhibitory capacities of the newly detected compounds with those of some already well-known inhibitors, some of the latter were assayed in short-term cultured hepatocytes. Silybin dihemisuccinate, taurocholate, and cholate were found to be 30-, 45- and 60-times, respectively, less inhibitory than the most effective inhibitor, water-soluble antamanide. For mutual comparison, the IC_{50} values of all inhibitors investigated in this study are compiled in Table I.

Inhibitors of phallotoxin transport differ widely in structure. In order to establish some sort of classification the kinetics of phalloidin uptake as a function of inhibitor concentration was studied. From plots according to Lineweaver and Burk (Fig. 4), it is evident that some of the inhibitors act in a competitive manner, while others behave in a non-competitive way. The latter group is comprised of monensin and Verapamil®. Nileprost®, β-estradiol, water-soluble antamanide, taurocholate, silybin dihemisuccinate and the cyclic somatostatin analog were found to be competitive inhibitors.

Regarding the structures of the competitive inhibitors, one is unable to detect any chemical features common to all of them which could eventually be recognized by the phalloidin-transporting system. For example, competitive inhibitors may be as different in structure as peptides, prostaglandins, steroids and flavonoids. Therefore, we speculate that the hepatic transport system recognizes phallotoxins and their competitive inhibitors not by their chemical features, but rather by distinct physical properties.

TABLE I

IC_{50} VALUES FOR SEVERAL DRUGS ASSAYED FOR THEIR INHIBITORY CAPACITY OF [^{3}H]DEMETHYLPHALLOIN UPTAKE INTO RAT HEPATOCYTES

Name	Class of compound	Mass (Da)	IC_{50} (μM)	Relative inhibitory capacities (phalloidin = 1)
(*O*-Carboxymethyl-tyrosine^5)antamanide	cyclic peptide	1221	0.3	11.11
Cyclolinopeptide	cyclic peptide	1041	0.5	7.14
Demethylphalloin (for comparison)	cyclic peptide	759	2.1 [a]	1.67
Phalloidin	cyclic peptide	789	3.5	1.00
Fluorescein-labeled phalloidin	cyclic peptide	1251	5	0.71
Monensin	antibiotic	671	5	0.71
Nocloprost®	prostaglandin analog	402	5	0.71
Phallisin	cyclic peptide	805	6	0.59
Nileprost®	prostaglandin analog	403	7	0.50
Phallacidin	cyclic peptide	847	7	0.50
Somatostatin analog cyclo[Phe-Thr-Lys-Trp-Phe-D-Pro]	cyclic peptide	806	8.5	0.42
Silybin dihemisuccinate	flavonoid	728	10	0.33
Taurocholate	steroid	516	14	0.25
Cholate	steroid	409	18	0.20
Aminophalloidin	cyclic peptide	862	32	0.11
β-Estradiol	steroid	272	42	0.08
Verapamil®	Ca-antagonist	455	70	0.05
Phalloidinsulfoxide (*R*)	cyclic peptide	805	100	0.034
Secophalloin	cyclic peptide	772	200	0.018
Phalloidinsulfoxide (*S*)	cyclic peptide	805	1000	0.003
α-Amanitin	cyclic peptide	918	> 1000	< 0.003
Somatostatin	peptide	1638	> 1000	< 0.003

[a] Greater than 1, because the transport system was assayed below its K_m.

It seemed to us that the most promising approach to finding out such physical parameters was to study the inhibitory capacities of compounds closely related to [^{3}H]demethylphalloin, such as natural and synthetic derivatives of phalloidin. We found that the phallotoxin derivatives were selected according to their lipophilicity. The most efficient derivative was the unlabeled demethylphalloin, which among the phallotoxins assayed, is the most lipophilic ($R_F = 0.66$, like the natural toxin phalloin). It was followed in efficiency by phalloidin ($R_F = 0.45$) and phallisin ($R_F = 0.4$). Although much less lipophilic in nature ($R_F = 0.15$), the acidic phallacidin is nearly as effective as phallisin. We conclude that negative charges of a substrate accelerate the transport process. On the other hand, positive charges are obviously repelled by the transporting system. This was shown to be true for an amino derivative of phalloidin as well as for the zwitterion secophalloin (Table I). This observation is in agreement with recent studies [25,26] reporting that bile acids with cationic or zwitterionic charges show a diminished uptake into rat hepatocytes or into perfused rat liver.

Transportability is decreased not only by cations but also by strong dipoles like sulfoxides. For example, both epimers of phalloidinsulfoxide [1] are only weak inhibitors of [^{3}H]demethylphalloin transport. The *R*-configurated compound has an IC_{50} value of 100 μM; the *S*-configurated sulfoxide is nearly inactive ($IC_{50} = 1000$ μM). Presence of the polar sulfoxide moiety may also explain why amatoxins, for example α-amanitin, only inhibit the phallotoxin transporting system at very high concentrations (> 1000 μM), if at all.

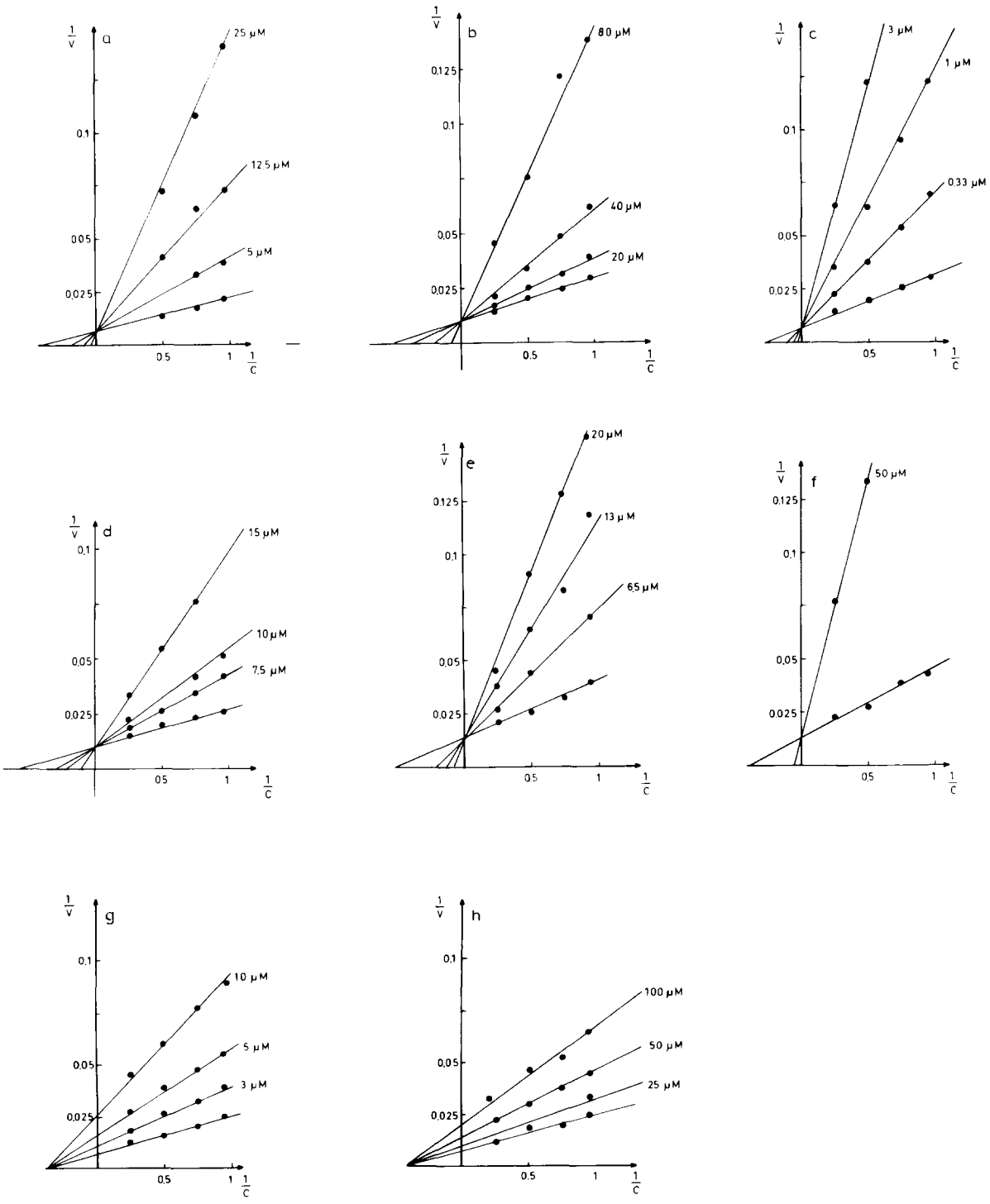

Fig. 4. Lineweaver-Burk plots of some competitive (a–f) and non-competitive inhibitors (g, h) of [^{3}H]demethylphalloin incorporation into rat hepatocytes. Nileprost® (a); β-estradiol (b); water-soluble antamanide (c); silybin dihemisuccinate (d); somatostatin analog cyclo[Phe-Thr-Lys-Trp-Phe-D-Pro] (e); taurocholate (f). Non-competitive: monensin (g) and Verapamil® (h).

Besides lipophilic nature and negative charges, the presence of rigid ring structures may facilitate the transport of substrates. This was concluded on the one hand from the structures of silybin and β-estradiol, and on the other, from the cyclic peptides, phalloidin, antamanide, cyclolinopeptide, and somatostatin analog. Ring structures which possibly accelerate the transport of a substrate could be represented by the flavonoid or the steroid backbone, or also by the side chains of aromatic amino acids, particularly when the side chains are restricted in their conformational freedom by the backbone of cyclic peptides. It was documented by NMR measurements [27] that the α-substituted indole nucleus of the phallotoxins is exposed on the surface and fixed in this position by the backbone of the bicyclic peptide. Similar to the contribution of tryptophan in phallotoxins or in the somatostatin analog, the hepatic transport of antamanide or cyclolinopeptide may be facilitated by the side chains of phenylalanine or tyrosine.

In summary, one can say that rat hepatocytes possess a transporting system which works with an unusually low specificity. It is postulated that the system recognizes substrates not by their chemical features but by their physical properties. A typical substrate could be described as follows: (1) lipophilic in nature, i.e. of low solubility in water; (2) low molecular mass, usually 400–1200 Da; (3) presence of negative charges, while cationic groups are at the same time absent; and, possibly, (4) presence of rigid ring structures. The physiological function of this transport system is probably to remove compounds of the described physical features from portal blood. This is particularly important for bile acids, which are resorbed in the gut and not intended to be distributed by blood circulation. Another function of the transport system may be to extract compounds, which are designed for metabolization and subsequent excretion from the serum into bile, such as steroid hormones and xenobiotics. By chance, one of the xenobiotics incorporated into hepatocytes by the transporting system is the hepatotoxin phalloidin, which, by interference either with cell actin [1] or mitochondria [17], deteriorates the structure and function of these cells.

Acknowledgements

The authors gratefully acknowledge the provision of various compounds by Th. Wieland, Heidelberg (cyclolinopeptide, water-soluble antamanide), H. Kessler, Frankfurt (cyclo[Phe-Thr-Lys-Trp-Phe-D-Pro]), W. Seifert, Schering, Berlin, (Nocloprost®, Nileprost®), G. Vogel, Madaus, Köln (Silybin dihemisuccinate) and Knoll A.G., Ludwigshafen (Verapamil®).

References

1 Wieland, Th. and Faulstich, H. (1978) Crit. Rev. Biochem. 5, 185–260
2 Wieland, Th., Faulstich, H., Jahn, W., Govindan, M.V., Puchinger, H., Kopitar, Z., Schmaus, H. and Schmitz, A. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1337–1345
3 Wieland, Th., Lüben, G., Ottenheym, H. and Schiefer, H. (1969) Liebigs Ann. Chem. 722, 173–178
4 Vogel, G., Trost, W., Braatz, R., Odenthal, K.P., Brüsewitz, G., Antweiler, H. and Seeger, R. (1975) Arzneim. Forsch. 25, 82–89
5 Frimmer, M. and Kroker, R. (1975) Arzneim. Forsch. 25, 394–396
6 Wagner, H., Diesel, P. and Seitz, M. (1974) Arzneim. Forsch. 24, 466–471
7 Weil, G. and Frimmer, M. (1970) Arzneim. Forsch. 20, 862–863
8 Petzinger, E. and Frimmer, M. (1980) Arch. Toxicol. 44, 127–135
9 Ziegler, K. and Frimmer, M. (1984) Biochim. Biophys. Acta 805, 174–180
10 Floersheim, G.L. (1971) Agents Actions 2, 142–149
11 Floersheim, G.L. (1966) Biochem. Pharmacol. 15, 1589–1593
12 Faulstich, H., Wieland, Th., Schimassek, H., Walli, A.K. and Ehler, N. (1977) in Membrane Alterations as Basis of Liver Injury (Popper, H., Bianchi, L. and Reutter, W., eds.), pp. 301–310, MTP Press, Lancaster
13 Frimmer, M., Petzinger, E. and Ziegler, K. (1980) Naunyn-Schmiedeberg's Arch. Pharmacol. 313, 85–89
14 Wieland, Th., Nassal, M., Kramer, W., Fricker, G., Bickel, U. and Kurz, G. (1984) Proc. Natl. Acad. Sci. USA 81, 5232–5236
15 Faulstich, H., Trischmann, H. and Mayer, D. (1983) Exptl. Cell Res. 144, 73–82
16 Puchinger, H. and Wieland, Th. (1969) Liebig's Ann. Chem. 725, 238–240
17 Faulstich, H., Münter, K. and Mayer, D. (1984) FEBS Lett. 167, 241–244
18 Research products of Schering AG, Berlin
19 Allen, G.E. and Rasmussen, H. (1972) in Prostaglandins in Cellular Biology (Ramwell, P.W. and Phariss, B.B., eds.), pp. 27–60, Plenum Press, New York

20 Roy, E., Harkness, R.A. and Kerr, M.G. (1965) J. Endocrinol. 31, 177–178
21 Fleckenstein, A., Tritthart, H., Fleckenstein, B., Herbst, A. and Grün, G. (1969) Pflügers Archiv. 307, R25
22 Kaufmann, H.P. and Tobschirbel, A. (1959) Chem. Ber. 92, 2805–2809
23 Balasubramanian, D., Chopra, P. and Ardeshir, F. (1976) FEBS Lett. 65, 69–72
24 Ziegler, K., Frimmer, M., Kessler, H., Damm, I., Eiermann, V., Koll, S. and Zarbock, I. (1985) Biochim. Biophys. Acta 845, 86–93
25 Hardison, W.G.M., Bellentani, S., Heasley, V. and Shelhamer, D. (1984) Am. J. Physiol. 246, G477–G483
26 Anwar, S.M., O'Maille, E.R.L., Hoffmann, A.F., DiPietro, R.A. and Michelotti, A. (1985) Am. J. Physiol. 249, G479–G488
27 Patel, D.G., Tonelli, A.E., Pfaender, P.A., Faulstich, H. and Wieland, Th. (1973) J. Mol. Biol. 79, 185–196