and Lys-Val. Lys-Phe, which contains a bulky aromatic amino acid on the carboxy-terminus, was not an effective inhibitor. These observations suggest that the inhibitory capacity of the test compound can be modulated by changing the polarity and the size of the molecule. The differences in the inhibitory capacity of these compounds may also reflect the accommodation of the molecule at the active site of the enzyme, although possible variations in the tissue penetration in this system can not be excluded. Finally, poly-L-lysine in the molecular weight range of 1–4 kD was effective while a similar preparation with a molecular weight range of 30–70 kD was not effective. This difference could also reflect tissue penetration of the compounds.

The exact mechanism of the inhibition of procollagen to collagen conversion by the amines tested here is not clear at present. Previously, Leung *et al.* [9] suggested that arginine might prevent the removal of the C-terminal extension from type I procollagen by interfering with the aggregation of procollagen molecules. We have suggested that the inhibition of removal of the C-terminal extensions from type II procollagen might result from direct inhibition of the procollagen C-proteinase [10]. In support of the latter suggestion was our previous demonstration that the removal of the C-terminal extensions was also inhibited by ε-aminocaproic acid, as well-known proteinase inhibitor with a structural similarity to lysine. Helseth and Veis [14] suggested recently that amines might inhibit the procollagen to collagen conversion through a mechanism which involves elevation of the pH of the lysosomes, thus leading to the inhibition of acidic proteinases which were speculated to participate in the procollagen to collagen conversion. In support of their suggestion was the demonstration that 50 mM Tris was an effective inhibitor of the removal of the carboxy-terminal extension in their system [14]. Under our test conditions, little if any inhibition was noted with 50 mM Tris and ammonia. Thus, our observations further support the hypothesis that critical structural features are necessary for a molecule to be an effective inhibitor of the procollagen to collagen conversion.

Accumulation of collagen is a major pathological feature of various fibrotic diseases affecting the lungs, liver, skin and other animal tissues [5]. Previously, several pharmacologic agents were tested for their potential for limiting collagen deposition in fibrotic conditions [15, 16]. Unfortunately, most of these compounds are not specific for collagen, and their clinical efficacy is frequently compromised by toxicity and long-term side effects. The observations presented in this study suggest that compounds with structural features, which include the presence of a free amino group at the end of an aliphatic carbon chain, appear to inhibit procollagen to collagen conversion in a relatively specific manner. Thus, further development of pharmacologic preparations containing the effective compounds and targeted for tissues exhibiting fibrosis might be helpful in controlling the fibrotic processes.

Acknowledgements—This study was supported by USPHS, NIH Grants AM-28450, GM-28833, AM-35297 and AG-03172, and by a grant from the American Diabetes Association–Southern California Affiliate. During this study, Dr. Oikarinen was a UCLA Silbert International Fellow. The authors thank Dr. Nakos Canellakis, Yale University School of Medicine, for suggesting the use of γ-aminobutyric acid. We sincerely appreciate the excellent secretarial assistance of Ms. C. Aranda in the preparation of this manuscript.

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Biochemical Pharmacology, Vol. 35, No. 3, pp. 535–538, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

A hydroxymethyl sulphate ester as an active metabolite of the carcinogen, 5-hydroxymethylchrysene

(*Received* 17 *June* 1985; *accepted* 23 *August* 1985)

5-Methylchrysene (5-MCR), a well known environmental carcinogen found in tobacco smoke [1] and having as high carcinogenicity to mouse skin as benzo[*a*]pyrene [2], is oxidized in rat liver microsomes to 5-hydroxymethylchrysene (5-HCR) as a major metabolite and to dihydrodiols and phenols [3]. The carcinogenicity of 5-HCR has been reported to be as high as 5-MCR [4], suggesting it to be one of proximate metabolites, while unsubstituted CR is known to have little or only a weak carcinogenic activity [2]. A similar relationship between the introduction of a methyl or hydroxymethyl group(s) to the polynuclear aromatic hydrocarbon and the remarkable increase in its

carcinogenicity is found with a number of methyl- or/and hydroxymethyl-benz[*a*]anthracenes (-BAs), such as 7-methyl-BA, 7-hydroxymethyl-BA (7-HBA), 7,12-dimethyl-BA, 7-hydroxymethyl-12-methyl-BA (7-HMBA), 12-hydroxymethyl-7-methyl-BA (12-HMBA), and 9,10-dimethylanthracene [5–8].

As for 5-MCR, the bay region diol-epoxides have been strongly suggested to be ultimate forms in bacterial mutagenesis as well as in carcinogenesis since not only the 1,2-dihydro-1,2-diol and 7,8-dihydro-7,8-diol, metabolites in livers of mice [9] and rats [3] both pretreated with polychlorinated biphenyls (PCB), show carcinogenicity towards mouse skin [10], but also the former metabolite shows mutagenicity towards *Salmonella typhimurium* TA100 in the presence of a PCB-pretreated rat liver post-mitochondrial fraction and an NADPH-generating system [3]. The dihydrodiol analogues have also been demonstrated to be metabolites of 5-HCR in PCB-pretreated rat liver [4], but it is equivocal whether they are mutagenic or carcinogenic.

Recently, another biological system has been proposed for the metabolic activation of the hydroxymethyl-BAs, all major metabolites of the corresponding methyl-BAs in untreated rat liver. That is, highly reactive hydroxymethyl sulphate esters have been isolated as metabolites of 7-HBA [11], 7-HMBA [12], and 12-HMBA [13] from the incubation mixture consisting of rat liver cytosol and a 3′-phosphoadenosine 5′-phosphosulphate (PAPS)-generating system. The sulphate esters have potent, intrinsic mutagenicity towards *Salmonella typhimurium* TA strain bacteria [11, 12] and bind covalently to calf thymus DNA in higher ratios than to hepatic cytosolic proteins surrounding it [11, 13, 14].

The present communication deals with (1) isolation and identification of a reactive hydroxymethyl sulphate ester (5-HCR sulphate) as a metabolite of 5-HCR in rat liver cytosol fortified with the PAPS-generating system, (2) intrinsic mutagenicity of 5-HCR sulphate, and (3) covalent binding of 5-HCR sulphate to calf thymus DNA.

5-HCR dissolved in dimethyl sulphoxide (0.1 ml) was incubated at 37° for 20 min in 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 7.4, 0.8 ml) with a dialysed soluble supernatant fraction of a liver homogenate (equivalent to 50 mg of the tissue) from male Wistar rats, weighing 160–180 g, in the presence of PAPS-generating system consisting of ATP (5 μmol), sodium sulphate (5 μmol), magnesium chloride (3 μmol), EDTA (0.1 μmol), and a suspension of *Salmonella typhimurium* TA98 (0.1 ml, 2×10^8 cells) cultured at 37° for 18 hr. According to the method of Ames *et al.* [15], the bacterial suspension was then diluted with soft agar and placed on hard agar plates to count the number of His^+ revertant colonies appearing after 48 hr at 37°. The mutagenicity of 5-HCR towards TA98 was much higher in the presence of the liver cytosol-PAPS system than in the presence of a 9000 *g* supernatant fraction (equivalent to 50 mg of rat liver) fortified with an NADPH-generating system (Fig. 1). The data strongly suggest that sulphate conjugation plays a more important role than monooxygenation in the metabolic activation of 5-HCR in rat liver so far as examined by the bacterial mutation, because microsomal monooxygenation products, such as 5-HCR epoxides, if formed by P-450 in the 9000 *g* supernatant fraction fortified with NADPH, induced the bacterial mutagenesis only to a slight extent. The mutagenicity of 5-HCR, exerted by the cytosol-PAPS system, was not detectable at all either when ATP or sodium sulphate was omitted from the system or when the cytosolic fraction was boiled.

5-HCR (1 mM) was incubated with the hepatic cytosol in the presence of the PAPS-generating system under the aforementioned conditions in order to isolate its active metabolite inducing the mutation of TA98. After removal of the unreacted substrate by extracting three times with ether (10 ml each), the incubation mixture (10 ml) was

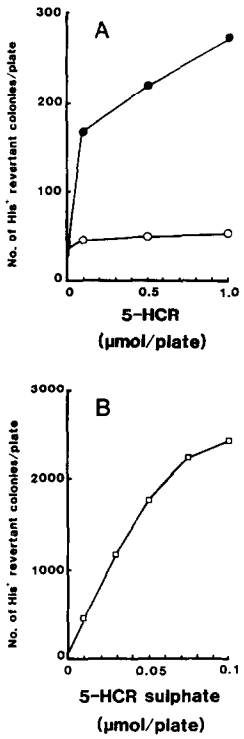

Fig. 1. Mutagenicity towards *Salmonella typhimurium* TA98 exerted by 5-HCR in the presence of a PAPS-generating system or an NADPH-generating system and by 5-HCR sulphate. (A) Closed circles: the incubation mixture contained the following ingredients in a final volume of 1 ml of 0.1 M phosphate buffer, pH 7.4: 5-HCR dissolved in dimethyl sulphoxide (0.1 ml), an overnight culture of bacterial cells (2×10^8), the dialysed rat liver cytosolic fraction (3.5 mg of protein, equivalent to 50 mg of liver, wet weight), Na_2SO_4 (5 mM), ATP (5 mM), $MgCl_2$ (3 mM), and EDTA (0.1 mM); open circles: the incubation mixture (1 ml) was prepared using the 9000 *g* supernatant fraction (equivalent to 50 mg of liver) and the NADPH-generating system by the method of Ames *et al.* [15]. The mixtures containing 5-HCR as the substrate were incubated at 37° for 20 min. (B) 5-HCR sulphate (sodium salt) was dissolved in dimethyl sulphoxide (0.1 ml) and incubated at 37° for 20 min with the bacteria in 0.1 M phosphate buffer, pH 7.4 (0.9 ml).

shaken with ethyl acetate (25 ml) in the presence of tetra-*n*-butylammonium bromide (5 mM) as a counter cation donor for the formation of a hydrophobic ion pair complex with a hydroxymethyl sulphate ester as a possible metabolite of the carcinogen. A residue, obtained on the evaporation of the solvent *in vacuo* at 20° from the ethyl acetate layer separated, was dissolved in methanol and subjected to HPLC carried out on a JASCO model TWINCLE high performance liquid chromatograph equipped with a JASCO model UVIDEC-100 u.v. spectrophotometer and an octadecylsilica column (Nucleosil $7C_{18}$, 7 μm in particle size, 4 × 300 mm) in methanol–water (4:1, 0.8 ml/min) containing tetra-*n*-butylammonium bromide (2 mM). The chromatogram obtained by monitoring at 269 nm showed a single peak at 11.5 min. The peak material that was eluted from the HPLC column showed a single u.v.-absorbing spot at R_f 0.80 on a silica thin-layer plate (Wakogel B-5F) developed with *n*-butanol–acetic acid–water (4:2:1) and u.v.-absorption and fluorescence emission spectra characteristic of 5-alkyl-CR.

The chromatographic and spectral data of the HPLC peak material were identical with those of synthetic sodium 5-HCR sulphate which was obtained by the reaction of 5-HCR with the same molar ratio of redistilled chlorosulphonic acid in dry pyridine, followed by careful neutralization of the reaction mixture with a twice molar ratio of methanolic sodium hydroxide as previously reported [12]. The synthetic sulphate ester, obtained in 64% yield after recrystallization from ethanol–ether, showed the following spectral data: u.v. λ_{max}^{EtOH} nm (log ε); 269 (4.89), 300 (3.84), 312 (3.91), 323 (3.90), fluorescence emission at an excitation wavelength of 271 nm, nm (relative intensity); 373 (90), 392 (100), i.r. ν_{max}^{KBr} cm^{-1}; 1471 and 1433 (ν_{as} SO_2), 1258 and 1201 (ν_s SO_2), n.m.r. δ_{TMS} ppm in d^6-DMSO; 5.50 (s, 2H, $-CH_2-$), 7.66–8.93 (m, 11H, arom. H). For further identification of the water-soluble metabolite isolated by HPLC, it was converted to a lipid-soluble derivative by the treatment with an aqueous sodium ethanethiolate (2 M) solution at room temperature. The reaction mixture was extracted with ethyl acetate. After evaporation of the solvent from the organic phase *in vacuo* at 20°, the residue was dissolved in chloroform and subjected to HPLC. The derivative purified as a single u.v.-absorbing peak appearing at 4.6 min by HPLC on a silica column (Nucleosil 50-7, 7 μm in particle size, 4 × 300 mm) in *n*-hexane-chloroform (19:1, 1 ml/min) showed u.v.-absorption and fluorescence emission spectra characteristic of 5-alkyl-CR and a mass spectrum with a molecular ion peak at m/z 302 (CR—$CH_2SC_2H_5$; $= M^{\dagger}$) and fragment ion peaks at m/z 241 ($M^{\dagger} - SC_2H_5$) and 226 ($M^{\dagger} - CH_2SC_2H_5 - H$). Synthetic 5-HCR sulphate also afforded 5-ethylthiomethyl-CR by the treatment with ethanethiolate under the same conditions, and its chromatographic and spectral data were identical with those of the derivative from the metabolite.

5-HCR sulphate showed potent, intrinsic mutagenicity towards TA98 without any activation system (Fig. 1). The sulphate ester was not formed from 5-HCR when incubated with boiled hepatic cytosol under the aforementioned conditions. An HPLC study indicated that the apparent rate of formation of 5-HCR sulphate from 5-HCR by the *in vitro* system was 0.6 nmol/50 mg liver/20 min. When PAPS was added to the incubation mixture instead of its generating system, the rate increased sixfold.

The aforementioned HPLC study also indicated that 5-HCR sulphate decomposed at a half life of 11 hr at 37° in 0.1 M phosphate buffer, pH 7.4, with concomitant formation of 5-HCR; under the chromatographic conditions used the sulphate and 5-HCR were eluted at 11.5 and 25 min, respectively. The decomposition of the sulphate ester was completed within a minute by the addition of an equal volume of 0.5 N sulphuric acid to this solution.

5-HCR sulphate was found to bind to calf thymus DNA (Sigma Type I, 2 mg/ml) as follows. The sulphate ester (0.1 mM) was incubated with the nucleic acid at 37° for 30 min in 0.1 M phosphate buffer, pH 7.4. The DNA was precipitated from the mixture by the addition of ethanol (2 vol.) in the presence of sodium acetate (0.2 M), rinsed successively with ethanol, acetone, and ether. The isolated DNA was re-dissolved in water, re-precipitated and rinsed in the same manner as mentioned above. An aqueous solution of the rinsed DNA showed a fluorescence emission spectrum with peak maxima at 378 and 396 nm at an excitation wavelength of 271 nm. It was estimated from the intensity of the emission spectrum that the sulphate ester bound to the DNA in the ratio 1:1300 base pairs. A fluorospectrophotometric study of the DNA indicated that the binding of the sulphate ester occurred in covalent manner through its 5-methylene carbon with loss of a sulphate anion, because the isolated nucleic acid still showed a strong intensity of the fluorescence emission spectrum, almost superimposable in shape on that of 5-alkyl-CR, after it was treated with 0.5 N sulphuric acid and extensively extracted with ether. 5-HCR incubated with the DNA solution in the absence of PAPS or its generating system was completely removed from the nucleic acid during its precipitation with ethanol in the presence of sodium acetate and subsequent washings with ethanol, acetone, and ether prior to dissolution in water for recording the fluorescence spectrum. Thus, a new mechanism for the metabolic activation of the carcinogen, 5-HCR, related to the bacterial mutation, has been established through the present investigation as illustrated in Fig. 2. A further study, however, would be required to confirm whether the sulphate ester of 5-HCR plays a role as an active metabolite in covalent binding to the biomacromolecules *in vivo*. A study of the mode of the covalent binding of 5-HCR sulphate to DNA bases is now in progress in our laboratory and will be published elsewhere.

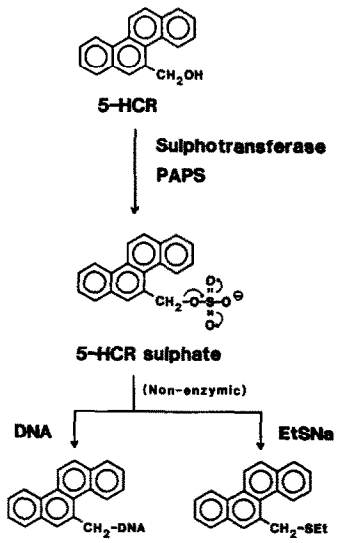

Fig. 2. Sulphotransferase-mediated activation of 5-HCR.

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Biochemical Pharmacology, Vol. 35, No. 3, pp. 538–541, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Stimulatory effect of Silibinin on the DNA synthesis in partially hepatectomized rat livers: non-response in hepatoma and other malign cell lines

(*Received* 4 *December* 1984; *accepted* 13 *August* 1985)

Extracts from the plant *Silybum marianum* are used since many years for a better recovery of patients with liver disease. The flavonolignane derivative Silibinin [1] was detected as the active compound. In many cases this compound is now given as a liver drug over long periods [2]. In preceding publications [3–5] we were able to show a molecular mechanism which explains the stimulating effect of the drug on liver cell metabolism. We demonstrated that Silibinin increases the synthetic rate of the ribosomal RNA species 5.8*S*, 18*S* and 28*S* by about 20% not only in rat livers and hepatocyte cultures, but also in isolated liver nuclei via activation of the DNA-dependent-RNA polymerase I. Subsequently the formation of mature ribosomes is stimulated and as an important consequence the protein biosynthesis in livers is increased as well [6].

Normally the mitotic activity of a cell is regulated in conjunction with RNA and protein synthesis by proliferation active factors [7, 8]. With respect to pharmacological aspects it seemed therefore important to investigate any influence of the drug Silibinin on DNA replication. We here describe the influence of Silibinin on DNA synthesis in normal rat livers and in livers from partially hepatectomized rats. As examples for fast growing cells we used rat and human hepatoma cells and HeLa- and Burkitt lymphoma cell cultures. In hepatectomized livers a remarkable increase in DNA synthesis caused by the flavonolignane derivative can be observed. No effect was found in the case of normal livers or with the malign cell lines.

Materials and methods

Specific pathogen-free female Wistar rats (Gesellschaft für Strahlen- und Umweltforschung. Neuherberg, F.R.G.) weighing 95–105 g were used in all experiments. Animals were maintained at a room temperature of 22° and humidity of 50 ± 5%, and were fed food (Altromin 1324) and water *ad libitum*. Room lighting was controlled on a 12 hr dark : 12 hr light regime. Partial hepatectomy (68%) was performed under ether anaesthesia according to the method of Higgins and Anderson [9]. All animals were killed between 9.00 a.m. and 10.00 a.m. and the time of partial hepatectomy and injection was calculated accordingly.

Silibinin (Madaus & Co, Köln, F.R.G.) was applied as Silibinin-2',3-dihydrogensuccinate sodium salt* dissolved in physiological NaCl-solution. As a precursor for DNA we used ^{3}H-thymidine (specific radioactivity 23.9 Ci/mmol) or ^{14}C-thymidine (specific radioactivity 61 mCi/mmol). The precursor and the flavonolignane were both injected intraperitoneally at separate sites in the abdomen. In order to control the precursor supply in the livers 100 mg portions of each liver tissue were dissolved overnight at 55° in 1 ml Protosol (NEN, Dreieich, F.R.G.) and by subsequent addition of 0.5 ml H_2O_2. After adding 15 ml Aquasol-2 (NEN) the radioactivity was determined. In order to determine the precursor uptake into liver cells 20 hr after i.p. application of Silibinin hepatocytes were isolated [4]. After 30 min preincubation 1.67 μCi/ml ^{3}H-thymidine were added to 5×10^{6} cells/ml. 250-μl samples were taken, centrifuged at 0° for 1 min/50 × *g*, the cells washed with 250 μl medium. After 4 min in total 10% TCA was added, and the radioactivity measured in the supernatants after centrifugation at 1500 *g*.

Zajdela ascitic hepatoma cells and human hepatoma cells line Alexander, furthermore HeLa cells and the Burkitt lymphoma cell line Raji were cultivated in medium RPMJ 1640 (Biochrom, Berlin, F.R.G.) with 10% foetal calf serum (Biochrom), 100 U/ml penicilline and 100 μg/ml streptomycine in Falcon flasks (Becton Dickinson GmbH, Heidelberg, F.R.G.) at 37° without shaking. The combined trypsin-versen dispersion method according to Bonin [10] was used for harvesting HeLa cells and Alexander cells. For labelling the DNA ^{3}H-thymidine was added during the logarithmic growth periods. After incubation the cells were isolated by centrifugation for 10 min at 1500 *g*, then washed twice by resuspension in cold 0.32 M sucrose and centrifuged again.

For DNA isolation the rat livers were homogenized in 9 vol. 0.32 M sucrose + 3 mM $MgCl_2$ + 10 mM Tris buffer, pH 7.5. The nuclei were separated by centrifugation for 10 min at 1200 *g* and, the nuclear pellet resuspended in 60% sucrose and again centrifuged 5 min at 9000 *g*. In order to isolate the nuclei from the cell cultures the cells were resuspended in 0.32 M sucrose + 3 mM $MgCl_2$ + 10 mM Tris buffer, pH 7.5 and softly homogenized. After a first centrifugation for 10 min at 1200 *g* the nuclei were purified as above in 60% sucrose.

Aliquots of the nuclear pellets were suspended in 3 ml 0.25 M perchloric acid and stored at 4° for 30 min. After centrifugation each pellet was treated with 0.5 ml 0.5 M NaOH for 30 min at room temperature. Then 4.5 ml 0.5 M perchloric acid were added and after 30 min at 4° the material was again centrifuged. The pellets were then boiled for 20 min in 2.5 ml 0.5 M perchloric acid, centrifuged and the supernatant kept. After repeating the heat extraction both supernatants were combined and used for analysis of the DNA.

DNA was determined according to Burton [11]. The radioactivity of the DNA samples was measured with a

* Formerly Silybindihemisuccinate.