9. A. A. Melikian, E. J. LaVoie, S. S. Hecht and D. Hoffmann, *Carcinogenesis* **4**, 843 (1983).
10. S. S. Hecht, A. Rivenson and D. Hoffmann, *Cancer Res.* **40**, 1396 (1980).
11. T. Watabe, T. Ishizuka, T. Aizawa, Y. Hakamata, A. Hiratsuka, K. Ogura and M. Isobe, in *GANN Monograph on Cancer Research No 30, P-450 and Chemical Carcinogenesis* (Eds. Y. Tagashira and T. Omura), pp. 125–139. Japan Scientific Societies Press, Tokyo (1985).
12. T. Watabe, T. Ishizuka, M. Isobe and N. Ozawa, *Science* **215**, 403 (1982).
13. T. Watabe, T. Ishizuka, T. Fujieda, A. Hiratsuka and K. Ogura, *Jpn. J. Cancer Res. (Gann)* **76**, 684 (1985).
14. T. Watabe, T. Fujieda, A. Hiratsuka, T. Ishizuka, Y. Hakamata and K. Ogura, *Biochem. Pharmac.* **34**, 3002 (1985).
15. B. N. Ames, J. McCann and E. Yamasaki, *Mutation Res.* **31**, 347 (1975).

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.

dioxane scintillator according to Bray [12] or with Aquasol 2 (NEN, Dreieich, F.R.G.).

Results

DNA synthesis in normal rat livers after Silibinin treatment. Radioactive thymidine is by far the most commonly used precursor for the analysis of DNA synthesis in experimental animals.

If ^{3}H- or ^{14}C-thymidine is injected into rats to measure liver DNA synthesis, the precursor pool must be precisely measured. A variation in the uptake of thymidine into the cells would result in differences of the specific radioactivity of the newly synthesized DNA. We, therefore, controlled in any case the precursor uptake into liver cells and the other cell lines. Several methods have been used to ascertain equal precursor utilization. In primary hepatocyte cultures from normal and partially hepatectomized rats either treated or untreated with Silibinin the uptake of ^{3}H-thymidine has been measured via acid soluble radioactivity in the cells for the same period as DNA synthesis has been observed (Table 1). From Table 1 it can be seen that the increase in DNA synthesis after partial hepatectomy stimulates thymidine uptake. However, no differences after the treatment with Silibinin can be found.

Second, the *in vivo* precursor concentrations have been measured in liver specimens from Silibinin treated and untreated normal rats and partially hepatectomized rats (Table 2). The ^{3}H-thymidine concentrations in the livers are about 1.5–4-fold the amount incorporated into DNA. After partial hepatectomy the precursor concentrations are higher. The reduced number of hepatocytes per rat injected with the same dose of thymidine of course contributes to this increase. The treatment with Silibinin, however, causes no changes in the precursor pools.

The DNA synthesis in several malign cell lines also will be described. In these experiments again could be shown that Silibinin in the applied dose range does not influence the precursor uptake.

Because of a better solubility in water Silibinin-2',3-dihydrogensuccinate sodium salt was injected instead of Silibinin. This derivative hydrolyses to Silibinin in the cells very quickly [13]. For our experiments with normal livers different amounts of Silibinin were administered to young female rats 20 hr before sacrificing. The controls received a physiological saline solution. One hour before liver resection the DNA was labelled with radioactive thymidine. Then liver nuclei were prepared, the DNA was analysed and the specific radioactivity of the DNA was determined (Table 2a).

No significant differences can be found between the rate of thymidine incorporation into the DNA of rat livers from controls and animals which have been treated with different Silibinin concentrations. A similar result was obtained with ^{14}C-labelled thymidine as a precursor.

Influence of Silibinin on the DNA-synthesis in partially hepatectomized rats. If about 68% of the livers are removed

Table 1. Uptake of ^{3}H-thymidine [cpm/5 × 10^{6} cells] into hepatocytes of normal and partially hepatectomized rats with and without pretreatment with Silibinin (27 mg/kg body-weight)

	Time [min]		
	10	20	60
Normal			
+ Silibinin	434 ± 34	444 ± 3	395 ± 16
− Silibinin	431 ± 20	446 ± 20	383 ± 23
Partially hepatectomized			
+ Silibinin	966 ± 45	1041 ± 95	605 ± 54
− Silibinin	961 ± 40	1092 ± 131	608 ± 52

Table 2. DNA-synthesis in livers with and without Silibinin treatment

Silibinin-2',3-dihydrogensuccinate sodium salt (mg/kg body weight)	Number of rats	10^{-3} × dpm/mg DNA (mean values and S.D.)	Precursor pool: Radioactivity 10^{-3} × dpm/g liver tissue (containing about 3 mg DNA)
(a) 0 (controls)	16	105 ± 12	1109 ± 18
17	3	127 ± 26	1017 ± 27
30	10	99 ± 9	1210 ± 9
50	3	118 ± 16	1314 ± 41
(b) 0 (controls)	10	1922 ± 287	10285 ± 1003
	10	1921 ± 338	10339 ± 1636
27	10	2367 ± 326	10261 ± 1846
	10	2306 ± 290	9343 ± 1304

Radioactivities after intraperitoneal injection of each 50 μCi ^{3}H-thymidine 1 hr before sacrificing and 20 hr after i.p. application of different amounts of Silibinin-2',3-dihydrogensuccinate sodiumm salt: (a) in normal rat livers; (b) in hepatectomized (68%) rat livers. 20 hr before sacrificing partial hepatectomy was performed and at the same time Silibinin-2',3-dihydrogensuccinate sodium salt or 0.9% NaCl were injected.

by partial hepatectomy the livers are restored within 2 weeks [9, 14]. During this period the rate of proliferation increases extremely. Afterwards the rate of DNA synthesis drops down to normal values [15, 16]. In the course of this process the DNA content per g liver weight proved to be constant ±5%. In order to look for the influence of Silibinin on DNA synthesis in hepatectomized rat livers the flavonolignane derivative was administered immediately after the operation. Also after treatment with Silibinin the DNA content per g liver remained constant. The values of thymidine incorporation into the liver DNA 20 hr after hepatectomy are listed in Table 2b and the time course is shown in Fig. 1, both in comparison to corresponding data from hepatectomized rats without Silibinin treatment.

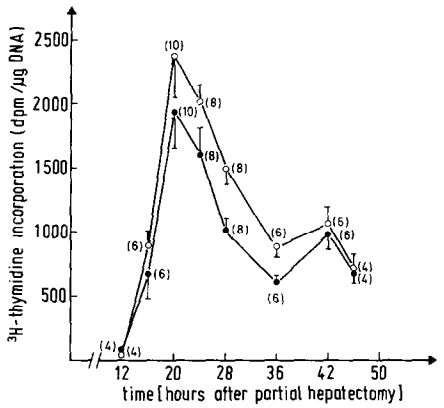

Fig. 1. Time course of DNA synthesis in hepatectomized (68%) rat livers with 27 mg/kg Silibinin (—○—) and without (—●—) controls. Number of experiments in brackets; performance as described in Table 1.

Table 2b shows a clear difference in the thymidine incorporation into the DNA of regenerating rat livers from Silibinin treated and untreated animals. Following the time course of incorporation (Fig. 1), 20–32 hr after partial hepatectomy an increase in DNA synthesis of 23–35% in the presence of Silibinin is observed. In this time interval differences in the mean values result with a significance of $P < 0.005$–0.001. The dose of Silibinin used here was equivalent to that which was found optimal in stimulating RNA synthesis [3].

DNA-synthesis in malign cell lines in the presence of Silibinin. Human hepatoma cells line Alexander [17] and Zajdela acitic hepatoma cells from rat [18], furthermore HeLa cell cultures originating from human portio carcinoma [19], and Raji cells, a lympho-blastoide cell line from Burkitt's lymphoma [20] were used as examples to study the influence of Silibinin on DNA synthesis in fast growing cell lines. In these cell cultures we looked for changes in cell proliferation as well as for the rate of thymidine incorporation.

Cell propagation was measured over 2–5 days under normal growth conditions but with different concentrations of Silibinin-2',3-dihydrogensuccinate. We did not observe any differences in the growth rates up to 100 µg of the flavonolignane derivative per ml medium.

Time-dependent DNA syntheses in the presence of different Silibinin concentrations were investigated by labelling with ^{3}H-thymidine during the logarithmic growth period (Fig. 2).

Figure 2 shows that the time dependent incorporation of ^{3}H-thymidine into DNA is not influenced by Silibinin in the malign cell line up to 100 µg/ml. Similar curves were obtained with HeLa-cells, Burkitt lymphoma cells line Raji and human hepatoma cells line Alexander.

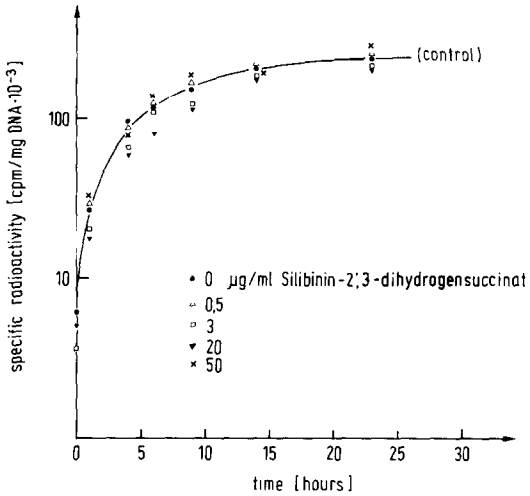

Fig. 2. Time dependent incorporation of ^{3}H-thymidine into the DNA of rat hepatoma cells (Zajdela) (1×10^6 cells/ml with 0.66 µCi ^{3}H-thymidine/ml medium in the presence of different amounts of Silibinin.

Discussion

The uptake kinetics of Silibinin have been studied earlier [21]. Because of its better solubility, the Silibinin-2',3-dihydrogensuccinate sodium salt was injected which is hydrolyzed completely within 30 min by liver esterases [13]. In our experiments with normal livers, no influence of Silibinin on the DNA synthesis can be seen (Table 2a). In undamaged livers the proliferation rate is extremely low, i.e. 1–2 mitosis/10^4 cells [22], and apparently its regulation is strong enough not to be directly driven by changes in RNA- and protein-synthesis induced with Silibinin.

Different results are obtained with hepatectomized rats. As was to be expected, the controls show an enormous increase in DNA synthesis to compensate for the damage. In the presence of Silibinin the rate of thymidine incorporation into DNA is additionally increased from 20 to 32 hr after partial hepatectomy by about 23–35%. The proportion of this increase corresponds to our findings [3] for RNA-synthesis in liver cells, where Silibinin stimulates the ribosomal RNA transcription rate. Apparently, if the regulatory signal for replication initiation is given, the increase in rRNA and protein synthesis [6] also accelerates DNA synthesis.

Our observation that the flavonolignane derivative can stimulate DNA synthesis in cells from hepatectomized rat livers is in good agreement with histological and morphological data from Magliuli *et al.* [23]. These authors found using stathmokinetic indices with colchicine in rats hepatectomized and treated with Silibinin an enhancement of mitosis rate by about 16%. The liver masses show an increase at the second day after partial hepatectomy by 26%. We find a stimulation of DNA synthesis from 23 to 35% at constant DNA amounts per g liver.

To investigate whether proliferation of fast growing cell cultures can also be influenced by Silibinin we used two hepatoma cell cultures and in addition HeLa- and Burkitt lymphoma cells line Raji. Up to relatively high concentrations of Silibinin-2'-3-dihydrogensuccinate in the medium (100 µg/ml) we found no differences between the growth rates of each of these cell lines. The lack of an increase in cell propagation in the presence of Silibinin is confirmed by the data for thymidine incorporation (Fig. 2). Although our findings with these cells cannot be generalized to all malignant tissues, we discuss that in such cell lines the rate of proliferation is already maximal and cannot be further intensified.

In conclusion and in addition to former results [3, 5] on the stimulatory effect of Silibinin on RNA and protein synthesis we show here that the drug increases DNA replication by about 23–35% in hepatectomized rat livers. This again gives proof for the liver cell regenerating capacity of the flavonolignane derivative supporting the clinical reports [2]. Our experiments with some malign cell lines cells should be seen as a first attempt to investigate any influence of Silibinin on malignant tissues and to ascertain that there are no stimulatory effects which should be a precondition for a clinical use.

Acknowledgements—We are gratefully indebted to Dr R. Madaus and his colleagues, Köln, for giving us pure charges of Silibinin. We thank Prof Dr. H. P. Hofschneider and Mrs I. Dick (Max-Planck-Institut für Biochemie) for the Alexander and HeLa cells and Prof. Dr H. Wolf and Mrs H. Schmidt (Max-v.-Pettenkofer-Institut, Univ. München) for Burkitt lymphoma cells line Raji.

Abt. Membranbiochemie JOHANN SONNENBICHLER
**Abt. Experimentalle Medizine* MICHEL GOLDBERG†
Max-Planck-Institut für Biochemie LYDIA HANE
Martinsried bei München IFEANYI MADUBUNYI
†Inst. für Physiol. Chemie SIEGFRIED VOGL*
und Ernährungsphysiol, ISOLDE ZETL
Universität
München, Federal Republic of Germany

REFERENCES

1. H. Wagner, L. Hörhammer, and R. Münster, *Naturwissenschaften* **52**, 305 (1965).
2. V. Fintelmann and A. Albert, *Therapiewoche* **30**, 5589 (1980).
3. J. Sonnenbichler and I. Zetl, *Hoppe Seyler's Z. physiol. Chem.* **365**, 555 (1984).
4. J. Sonnenbichler, J. Mattersberger and H. Rosen, *Hoppe Seyler's Z. physiol. Chem.* **357**, 1171 (1976).
5. F. Machicao and J. Sonnenbichler, *Hoppe Seyler's Z. physiol. Chem.* **358**, 141 (1977).
6. J. Sonnenbichler, I. Zetl and I. Madubunyi, *Hoppe Seyler's Z. physiol. Chem.* in preparation.
7. N. L. R. Bucher, J. A. McGowan, W. E. Russel, in *Nerve, Organ, and Tissue Regeneration* (Ed. . J. Seil), pp. 455–469. Academic Press, New York (1983).
8. M. Goldberg, *J. Cell. Biochem.* **27**, 291 (1985).
9. G. M. Higgins and R. M. Anderson, *Archs Path.* **12**, 186 (1931).
10. O. Bonin, in *Quantitative virologische Methodik*, p. 94. Georg Thieme Verlag, Stuttgart (1973).
11. K. Burton, *Biochem. J.* **62**, 315 (1956).
12. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
13. H. Koch and J. Tscherny, *Archs Pharm.* **316**, 426 (1983).
14. N. L. R. Bucher and M. N. Swaffield, *Cancer Res.* **24**, 1611 (1964).
15. R. G. Gerdes, K. G. Rientis and J. F. Williams, *Cytobios* **4**, 193 (1964).
16. D. M. Hays, *Surg. Gynec. Obstct.* **139**, 609 (1974).
17. J. J. Alexander, E. M. Bey, E. W. Geddes and G. Lecatsas, *S. Afr. med. J.* **50**, 2124 (1976).
18. F. Zajdela, *Quelques Problemes Poses par la Cellule Cancereuse*, p. 914. Gauthiers – Villars, Paris (1964).
19. W. F. Scherer, J. T. Syverton and G. O. Gey, *J. exp. Med.* **97**, 695 (1953).
20. R. J. V. Pulvertaft, *Lancet* 238 (1964).
21. J. Sonnenbichler, J. Mattersberger and G. Hanser, *Hoppe Seyler's Z. physiol. Chem.* **361**, 1751 (1980).
22. R. Sheimin, J. Humbert and R. E. Pearlman, *A. Rev. Biochem.* **41**, 277 (1978).
23. E. Magliulo, P. G. Carosi, L. Minoli and S. Gorini, *Arzneimittelforschung* **23**, 161 (1973).

Biochemical Pharmacology, Vol. 35, No. 3, pp. 541–544, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Effect of oxygen concentration on the metabolic pathway of anisole in rat liver microsomes

(*Received* 31 *May* 1985; *accepted* 6 *August* 1985)

The microsomal mixed-function oxidase system containing cytochrome P-450, which utilizes molecular oxygen, catalyzes the metabolism of a wide variety of endogenous and xenobiotic compounds. Recently, several groups [1–4] have reported the effects of oxygen concentration on the metabolism of several compounds. Jones [5] mentioned that many drug-metabolizing reactions require molecular oxygen either directly as a substrate or indirectly because of their dependence upon cellular energetics. His group also studied oxidation and conjugation reactions of drugs at low oxygen concentration [6–8]. However, no studies have been done on the effect of oxygen concentration on the different types of reactions in the metabolism of a substrate which are catalyzed by cytochrome P-450. In this study, we chose anisole as a substrate, because it was shown by Daly *et al.* [9, 10] that the mixed-function oxidase system metabolizes anisole by two principal oxidation pathways, O-dealkylation and aromatic hydroxylation, in spite of its simple structure, and we investigated the effect of oxygen concentration on the metabolic pathway of anisole.

Methods

Liver microsomes were prepared from male Wistar rats (*ca.* 150 g) that had been pretreated with sodium phenobarbital (60 mg/kg in saline, i.p., 3 days).

Standard gases (1.0% and 10% O_2 in N_2) were purchased from the Takachiho Chemical Industry, and we obtained gas mixtures of other desired oxygen concentrations (2.0%, 4.0% and 6.0% O_2 in N_2) with a gas divider apparatus (SGD-XC 0.5 l, STEC Inc.). From the calibration by Clark oxygen electrode measurements, the oxygen concentrations in the assay solutions equilibrated by the gases (1.0, 2.0, 4.0, 6.0, 10% O_2 in N_2 and air) were determined to be 24, 34, 54, 74, 113 and 223 μM respectively.

The formation rates of metabolites of anisole at various oxygen concentrations were determined according to the methods of Holtzman *et al.* [11], with slight modifications. The system consisted of gassing towers with rubber seals at the top, connected with stainless steel tubes. The gas was passed through the first gassing tower containing 0.1 M phosphate buffer (pH 7.4). Then the humidified gas was