Originals

CHRONIC TOXICITY AND HEPATOCARCINOGENICITY OF
(+) RUGULOSIN, AN ANTHRAQUINOID
MYCOTOXIN FROM *PENICILLIUM* SPECIES :
PRELIMINARY SURVEYS IN MICE.¹⁾

Yoshio UENO, Norio SATO²⁾, Teruo ITO, Ikuko UENO*,
Makoto ENOMOTO^{3)**} and Hiroshi TSUNODA***.

Department of Toxicology and Microbial Chemistry, Faculty
of Pharmaceutical Sciences, Tokyo 162, Japan.

* Department of Cancer Susceptibility, Institute of Medical
Sciences, Tokyo University, Shiroganedai, Minato-ku,
Tokyo 108.

** Department of Pathology, St. Marrianna University
School of Medicine, Kawasaki-shi.

***Department of Food Microbiology, Gunma University,
Maebashi-shi, Japan.

Accepted August 15, 1980

Abstract.....Long-term feeding experiments revealed chronic hepatocellular injury as well as hepatocarcinogenicity of (+) rugulosin in mice.

Key words : (+) rugulosin, mycotoxin, *Penicillium rugulosum*, hepatocarcinogenicity.

- 1) This work was supported by a grant-in-aid from the Ministry of Health and Welfare, Japan.
- 2) Present address : Biological Department, Tokyo Research Laboratories, Shin Nihon Jitsugyo Co. LTD., Yawara, Nerima-ku, Tokyo.
- 3) Present address : Food Drug Research Center, Fukuda-cho, Iwata-gun, Shizuoka.
Postal address : Department of Toxicology and Microbial Chemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Shinjuku-ku, Tokyo 162.

INTRODUCTION

(+) Rugulosin (RUG) ($C_{30}H_{20}O_{10}$, M. W. 542) is a bis-anthraquinoid pigment (Fig. 1) (Takeda et al., 1973) produced by various species of fungi such as *Penicillium rugulosum* Thom (Breen et al., 1955), *P. tardum* Thom (Yamamoto et al., 1956), *P. wortmannii*

連絡先 : 〒 162 東京都新宿区市ヶ谷船河原町 12 東京理科大学薬学部
毒性・微生物化学教室 上野芳夫

Fig. 1 (+) Rugulosin

Klocker (Breen et al., 1955), *P. brunneum* Udagawa (Tunoda et al., 1963), *P. concolorugulosum* Abe (Hirayama et al., 1956), *P. variabile* (Tunoda, 1956), *Endothia parasitica* (Murr.) Anderson and Anderson and *E. fluens* Shear and Stevens (sy. *E. radicalis* Fr.) (Shibata et al., 1953) and *Sepedonium ampullosporium* Daman (Shibata et al., 1957), and its chemical structure is very close to (–) luteoskyrin, a hepatocarcinogen of *P. islandicum* Sopp (Sankawa et al., 1968, and Uraguchi et al., 1972).

The acute intraperitoneal LD₅₀ values in mice and rats are 55 and 44 mg/kg, respectively, and the fatal cases occur 5–6 days after the administration (Ueno et al., 1971). The subcutaneous injection of sublethal doses of RUG for daily three days causes a maximum elevation of serum transaminase activity 4 days after the last injection (Sato and Ueno, 1977). In mice and rats intraperitoneally or subcutaneously administered with RUG, diffuse fatty degeneration of the parenchymal cells and centrilobular cell necrosis in the liver are the major pathological alteration.

The single oral LD₅₀ value in mice is over 4 g/kg, but a half of mice died when 100 mg/kg of RUG is daily intubated for 32 days (Sato and Ueno, 1977). These toxicological approaches reveal that RUG is a slow-acting hepatotoxin.

RUG is cytotoxic to *E. coli* (Yamakawa and Ueno, 1970), *Tetrahymena pyriformis* (Ueno and Saheki, 1968), Ehrlich tumor cells (Ueno et al., 1971), and cultured mammalian cells (Umeda et al., 1974). Microbial analysis in our laboratory revealed that RUG is positive both in Rec-assay with recombination-deficient mutant cells of *Bacillus subtilis* (Ueno and Kubota, 1976) and an induction of respiratory-deficient mutants of *Saccharomyces cerevisiae* (Ueno and Nakajima, 1974), but it is negative in *Salmonella*/microsome assay (Ueno et al., 1978).

This paper is a preliminary report on the chronic toxicity and carcinogenicity of RUG in mice, which showed that subchronic and long-term exposures of male mice to RUG-containing diets resulted in development of the chronic liver injury and liver tumor.

MATERIALS AND METHODS

(+) Rugulosin, *P. rugulosum* Thom and *P. brunneum* Udagawa were inoculated on Czapek-Dox medium and cultured at 25–27°C for 14 days. The fungal mats were dried at 60–70°C for 2–3 days and powdered. After removing lipids first with *n*-hexane in Soxhlet extractor, the pigments were extracted with acetone. The acetone-soluble pigments were dissolved in acetone, passed through a charcoal column, and RUG was eluted with acetone. After evaporation of the solvent, RUG was crystallized from acetone or methanol (Ueno et al., 1971). The yield of the mycotoxin is around 10–20 g/kg of the dried fungal mat. The purity (over 98%) was confirmed by a thin-layer chromatography (oxalic acid-treated silicagel G and *n*-hexane-acetone-water, 6 : 3 : 1.5) and spectrophotometry.

Mice. Male DDD mice were obtained in the Institute of Medical Sciences, Tokyo University, and male ddYS mice were purchased at 5-week old from Shizuoka Agricultural Co. for Experimental Animals (Hamamatsu). All the mice were caged individually and fed with a control feed for one week in our animal house and the experiments started on the beginning 7-week age.

Diets. DSS-control (Uraguchi et al., 1972) was used as basal diet. The test diets were powder, and pellets were fed to the mice in dose of 5 g/mouse/day. Water was given *ad libitum*.

Experimental design and conduct. Two independent experiments were performed : Exp. 1 ; thirty DDD mice were fed on 1.5 mg/mouse/day for 22 days and then fed the normal diet for 692 days. Nine control mice were fed on the control diet for 715 days. Exp. 2 ; Forty six ddYS male mice were divided into three groups, each receiving 5 g/day of the diets containing 0 (14 mice), 0.30 (16 mice), and 0.75 mg RUG/animal/day (16 mice) for over 800 days.

Throughout the study records were kept of body weight (weekly) and survival. Autopsy was carried out on most animals that died during the trial and on all animals killed terminally. Paraffin sections of the liver, kidney and spleen and any other organs showing gross changes to tumors were submitted to histological examinations and were stained with hematoxylin and eosin.

RESULTS

Exp. (1). Effects of the short-term exposure of DDD mice to (+) rugulosin on the incidences of liver injury and hepatoma.

Fig. 2 represents the surviving and growth curves of the nine control and 30 1.5mg RUG/animal/day for the initial 22 days of feeding. In the first 2 weeks, neither lethal cases nor growth retardation were observed in either groups. However, during the following 2 weeks, a rapid decline in the average body weight was observed in the treated mice. Therefore, we terminated the administration of RUG and changed the test diet to the normal one. Sixteen out of 30 RUG-treated mice died during 21–42 days after the

Fig. 2 Survival and growth curves of DDD male mice fed RUG for the initial 22 days. The growth curves are the mean body weights of 9 control and 30 RUG-administered mice. (H) in the Fig. represents a mouse diagnosed as hepatoma.

starting of feeding experiment.

Pathological findings in these died cases were predominantly concentrated the acute liver injuries characterized by fatty degeneration, necrosis and nuclear damage of liver cells.

During the following 43-300 days the fatal cases were very rare in both control and treated mice, and finally, all the mice died with average survival days of 412 ± 133 days and 258 ± 262 days, respectively. Pathological examination on autopsied animals revealed each one case of well-differentiated hepatocellular carcinoma (died on 481th day), lymphatic leukemia (died on 668th day), myeloproliferative disorder (died on 715th day) among 14 survivors of RUG-treated mice. In the nine control survivors no malignant findings were observed except one case which died of bronchopneumonia and papillary adenoma of lung on the 652th day.

Exp. (2). Long-term feeding of male ddYS mice with (+) rugulosin.

Fig. 3 shows the effect of RUG on survival and the influence on growth of male

ddYS mice, which were fed on the RUG-diets in doses of 0, 0.30 and 0.75 mg/animal/day. In the early stage of the feeding, a slight retardation of growth was observed in the treated mice, and the fatal cases were very rare in the following 330 days of the feeding period. All the mice died with the average survival days of 579 ± 170 days (14 control mice), 536 ± 284 days (16 low-RUG mice) and 628 ± 123 days (16 high-RUG mice). Pneumonia developed in the mice survived over 600 days.

Major pathological findings observed at autopsy revealed the following data: among 14 control mice, one leukemia (died on the 477th day) with accompanying nephrotic change was detected; among 16 low-RUG mice, pleomorphism, mitotic figure and hyperplastic area of liver cells were observed in the liver cell nuclei of four mice; and among high-RUG mice, the incidence of hepatic nodule showing liver cell hyperplasia was counted for 4 cases (died on the 542th, 664th, 726th and 833th day) (Photo. 1), and the nuclear alteration such as pleomorphism and mitotic figure of liver cells were detected in 6 mice survived over 600 days. The other noticeable findings were hepatocellular adenoma (died on the 540th day) (Photo. 2), reticuloendotheliosis of liver (died on the 741th day), atrophy of testis (died on the 611th day) and intestinal hemorrhage (died on the 647th day).

Fig. 3 Survival and growth curves of ddYS male mice fed two doses of RUG for whole observation period. The growth curves are the mean body weights of 14 control, 16 low-RUG (0.3 mg/day), and 16 high-RUG (0.75 mg/day) mice. (H) in the Fig. represents a mouse diagnosed as liver cell hyperplasia.

Photo. 1. Hyperplastic area of the liver cells with small nucleus seen in the mouse fed a diet containing RUG (0.75 mg/animal/day) for 542 days. (H. E. stain X 120)

Photo. 2. Hepatocellular adenoma developed on the mouse fed a diet containing RUG (0.74 mg/animal/day) for 540 days. (Silver stain X 100)

DISCUSSION

In the present paper, we carried out two preliminary experiments; one is a short-term exposure to RUG, and the other is a long-term feeding with one half or one fourth amount of the former experiment. In the former experiment, a half of the mice fed 1.5 mg RUG/animal/day or around 50 mg/kg/day for 22 days died from acute liver injury during 21-42 days after the start of feeding. These results indicate that RUG is very slow-acting hepatotoxin, in agreement with the previous findings that the fatal cases and an elevation of s-GOT value also occurred in the late stage of acute intoxication by RUG (Sato and Ueno, 1977; Ueno et al., 1971). Noticeable finding is that the further feeding of the survivors with a normal diet induced one hepatocellular carcinoma.

In the second experiment, in which the mice were administered with 0.3 mg RUG/animal/day (or 12 mg/kg/day) or 0.75 mg RUG/animal/day (or 25 mg/kg/day) for long term, four cases showing hyperplastic areas of liver cells in the former group and four cases of hyperplastic nodules of liver cells in the latter group were observed. Since any hyperplastic change of liver cells was not observed in control mice, it was suggested that RUG should be hepatotumorigenic to male mice.

In preceding papers (Ueno et al., 1971 : Ueno and Nakajima, 1974 : Yamakawa and Ueno, 1970), we have estimated that the i. p. LD₅₀ values, the cytotoxicity, and the mutagenicity of RUG is around 2-4 times less than those of (-) luteoskyrin. According to Uruguchi et al. (1972), (-) luteoskyrin was hepatocarcinogenic to mice and its incidences including hyperplastic areas (Hepatoma-O) and hyperplastic nodules (Hepatoma-I) in addition to hepatocellular adenoma (Hepatoma-I) and carcinoma (Hepatoma-II and III) were estimated to 16.7% (50 µg/day), 68.8% (150 µg/day), and 84.6% (500 µg/day). Furthermore, the minimum induction period for liver cell tumor was 216 days (150 µg/day of luteoskyrin) and 500-700 days (0.75 mg/day of RUG). As for RUG experiment, the incidence of evident carcinoma of liver cells was only one, but hyperplastic areas and nodules were observed in around one fourth of experimental cases. From these findings, it is presumed that the hepatocarcinogenicity of RUG is less than one tenth of (-) luteoskyrin and the induction period of RUG for liver cell tumor is far slower than that of (-) luteoskyrin. Further experiments with large number of mice and rats are planning to reconfirm the hepatocarcinogenicity of RUG.

SUMMARY

(+) Rugulosin (RUG) is bis-anthraquinoid pigment produced by fungi such as *Penicillium rugulosum* Thom, *P. tardum* and others, and its chemical structure is very close to (-) luteoskyrin, one of bis-anthraquinoid pigments of *Penicillium islandicum* Sopp and hepatocarcinogenic to mice and rats. This investigation was concerned with the subacute and chronic effects of RUG on hepatic injury and hepatocellular tumor induction in male mice fed purified diets for 22 days containing 1.5 mg/5 g diet/animal per day or whole feeding period containing 0.3 and 0.75 mg RUG/5 g diet/animal per day.

A half of male DDD mice receiving 1.5 mg RUG for the initial 22 days of feeding died 1-3 weeks thereafter from the acute hepatic injury characterized by fatty degeneration and liver cell necrosis, and hepatocellular carcinonoma developed in one out of 14 mice surviving the feeding of control diet for additional 400 days. Each one fourth of cases of male ddYS mice receiving 0.3 and 0.75 mg RUG for 500-700 days developed hyperplastic nodules of liver cells.

These data suggest that RUG is possibly a weak hepatocarcinogen for male mice with a potency less than one tenth of (-) luteoskyrin.

REFERENCES

- BREEN, J., DACRE, J. C., RAISTRICK, H., and SMITH, G. (1955) : Studies in the biochemistry of micro-organisms. 95. Rugulosin, a crystalline colouring matter of *Penicillium rugulosum* Thom. *Biochem. J.*, 60, 618-626.
- HIRAYAMA, S., KURATA, H., SAKABE, F., INAGAKI, N., UDAGAWA, S., and IKETANI, S. (1956) : Studies on the metabolic products of fungi isolated from deteriorated rice. II. Cultivation of *Penicillium concavorugulosum* Abe. Isolation and purification of metabolic products (in Japanese). *Bull. Natl. Hyg. Lab.*, 74, 313-316.

- SANKAWA, U., SEO, S., KOBAYASHI, N., OGIWARA, Y. and SHIBATA, S. (1968) : Further studies on the structure of luteoskyrin, rubroskyrin and rugulosin. *Tetrahedron Lett.*, **53**, 5557-5560.
- SATO, N. and UENO, Y. (1977) : Hepatic injury and hepato-accumulation of (+) rugulosin in mice. *J. Toxicol. Sci.*, **2**, 261-271.
- SHIBATA, S., TANAKA, O., CHIHARA, G. and MITSUHASHI, H. (1953) : On the coloring matter produced by *Endothia parasitica* Fr. and *Endothia radicleis* Fr. *Pharm. Bull.*, **1**, 302-308.
- SHIBATA, S., SHOJI, J., OHTA, A. and WATANABE, M. (1957) : Metabolic products of fungi. XI. Some observation on the occurrence of skyrin and rugulosin. *Chem. Pharm. Bull.*, **5**, 380-382.
- TAKEDA, N., SEO, S., OGIHARA, Y., SANKAWA, U., IITAKA, I., KITAGAWA, I. and SHIBATA, S. (1973) : Studies on fungal metabolites. XXXI. Anthraquinoid colouring matters of *Penicillium islandicum* Sopp and some other fungal (-) rugulosin, (+) rugulosin and their related compounds. *Tetrahedron*, **29**, 3703-3719.
- TSUNODA, H. (1963) : Studies on the microorganisms which deteriorate the stored cereals and grains. XXXI. Studies on *Penicillium brunneum* Udagawa nov. sp., parasitic to rice storage (in Japanese). *Proc. Food Res. Inst.*, **17**, 238-248.
- TSUNODA, H. (1965) : Research on the microorganisms which deteriorate the stored cereals and grains. XXXI. On the molds parasitic to mochi (rice cake) (in Japanese). *Proc. Food Res. Inst.*, **19**, 8-14.
- UENO, Y. and SAHEKI, M. (1968) : Inhibitory effects of luteoskyrin, a hepatotoxic pigments of *Penicillium islandicum* Sopp, and chemically related pigments on a protozoon, *Tetrahymena pyriformis* GL. *Japan. J. Exp. Med.*, **38**, 157-164.
- UENO, Y., UENO, I., SATO, N., ITOI, Y., SAITO, M., ENOMOTO, M. and TSUNODA, H. (1971). Toxicological approaches to (+) rugulosin, an anthraquinoid mycotoxin of *Penicillium rugulosum* Thom. *Japan. J. Exp. Med.*, **41**, 177-188.
- UENO, Y. and NAKAJIMA, M. (1974) : Production of respiratory-deficient mutants of *Saccharomyces cerevisiae* by (-) luteoskyrin and (+) rugulosin. *Chem. Pharm. Bull.*, **22**, 2258-2262.
- UENO, Y. and KUBOTA, K. (1976) : DNA-attacking ability of carcinogenic mycotoxins in recombination-deficient mutant cells of *Bacillus subtilis*. *Cancer Res.*, **36**, 445-451.
- UENO, Y., KUBOTA, K., ITO, T. and NAKAMURA, Y. (1978) : Mutagenicity of carcinogenic mycotoxins in *Salmonella thyphimurium*. *Cancer Res.*, **38**, 536-542.
- UMEDA, M., SAITO, M. and SHIBATA, S. (1974). Comparison of cytotoxic effects of various anthraquinoids on cultured mammalian cell. *Japan. J. Exp. Med.*, **44**, 249-255.
- URAGUCHI, K., SAITO, M., NOGUCHI, Y., TAKAHASHI, K., ENOMOTO, M. and TATSUNO, T. (1972). Chronic toxicity and carcinogenicity in mice of the purified mycotoxins, luteoskyrin and cyclochlorotine. *Fd Cosmet. Toxicol.*, **10**, 193-207.
- YAMAKAWA, H. and UENO, Y. (1970) : Microbial assay of hepatotoxic anthraquinones with *Escherichia coli* F-11. *Chem. Pharm. Bull.*, **18**, 177-181.
- YAMAMOTO, Y., HAMAGUCHI, A., YAMAMOTO, I. and IMAI, S. (1956) : Studies on the metabolic products of *Penicillium tardum* Thom (in Japanese). *J. Pharm. Soc. Japan*, **76**, 1428-1430.