Free Rad. Res., Vol. 23, No. 1, pp. 41–50
Reprints available directly from the publisher
Photocopying permitted by license only

SPIN-TRAPPING AND DIRECT EPR INVESTIGATIONS ON THE HEPATOTOXIC AND HEPATOCARCINOGENIC ACTIONS OF LUTEOSKYRIN, AN ANTHRAQUINOID MYCOTOXIN PRODUCED BY PENICILLIUM ISLANDICUM SOPP. GENERATIONS OF SUPEROXIDE ANION AND LUTEOSKYRIN SEMIQUINONE RADICAL IN THE REDOX SYSTEMS CONSISTED OF LUTEOSKYRIN AND LIVER NADPH- OR NADH-DEPENDENT REDUCTASES

IKUKO UENO*, MASARU SEKIJIMA+, MIKIO HOSHINO*, HIROAKI OHYA-NISHIGUCHI+ and YOSHIO UENO+

**Institute of Physical and Chemical Research, Wako, Saitama 351-01; +Department of Toxicology and Microbiological Chemistry, Science University of Tokyo, Shinjuku, Tokyo 162; ±Yamagata Technopolis Foundation, Yamagata 990, Japan*

(Received April 28, 1994)

Luteoskyrin is a hepatotoxic and hepatocarcinogenic bisdihydroanthraquinone produced by *Penicillium islandicum* Sopp. By observing the EPR spectra of DMPO-spin adducts and luteoskyrin semiquinone radical, we investigated *in vitro* whether luteoskyrin is reduced to its semiquinone radical leading to the generation of active oxygen species in redox systems catalyzed by NADPH-dependent cytochrome reductases of the liver. We found (1) the formation of luteoskyrin semiquinone radical in the NADPH-cytochrome P-450 reductase system under anaerobic conditions, (2) the generation of O_2^- in the systems composed of luteoskyrin, NAD(P)H, and either rat liver microsomal NADPH-cytochrome P-450 reductase or submitochondrial particles and (3) dicoumarol showed no effect on the O_2^- generation in the case of submitochondrial particles. From these results we proposed that luteoskyrin liver injuries are induced by the active oxygen species generated in the process of autoxidation of luteoskyrin semiquinone radical which is produced in the one-electron redox systems catalyzed by the liver NAD(P)H-dependent cytochrome reductases.

KEY WORDS: Luteoskyrin, semiquinone radical, NADPH-cytochrome P-450 reductase, NAD(P)H-cytochrome c reductase, superoxide, EPR, hepatocarcinogen, mycotoxin.

Address correspondence to: Ikuko Ueno.

Paper presented at the 4th International Symposium on Spin Trapping and Organic EPR Spectroscopy, Oklahoma City, USA, October 1993

INTRODUCTION

Luteoskyrin is an anthraquinoid hepatocarcinogen produced by *Penicillium islandicum* Sopp.[1,2,3,4] This mycotoxin specifically attacks the animal liver producing severe centrilobular necrosis at high doses[1,5] and hepatoma from benign to malignant in lower doses given long term in animals.[6,7] When luteoskyrin is given to the animals, it accumulates in the liver,[8] especially in the liver mitochondria[9] with extensive suppression of the oxidative phosphorylation.[7] The inhibitory effect of luteoskyrin on the oxidative phosphorylation was also observed with inhibition of the respiratory electron transport, *in vitro*.[10,11]

It was reported that oxygen radicals hydroxylated the 8-position of deoxyguanosine residues in DNA leading to the misreading of the gene expression.[12,13,14,15,16,17,18] In our recent studies luteoskyrin showed accelerations of both hydroxyl radical formation and the 8-hydroxylation of deoxyguanosine residues of DNA in the presence of ascorbic acid and a trace amount of iron.[19,20] The accelerated 8-hydroxylation of deoxyguanosine residues was also observed in the DNA of the Balb 3T3 cells transformed by luteoskyrin as well as in the liver cells of luteoskyrin-intoxicated mice.[21,22] In the latter case accelerated lipid peroxidation was also shown. We also observed the preventive effects of free-radical scavengers on the luteoskyrin hepatotoxicity.[20,22,23] From these observations we speculated that oxygen radicals participate in the luteoskyrin-induced hepatotoxicity and hepatocarcinogenicity.

It is known that certain quinoid-type anticancer drugs are metabolized to their semiquinone radicals through one-electron reduction catalyzed by microsomal NADPH-cytochrome P-450 reductase and/or mitochondrial NADH-ubiquinone (complex I) and ubiquinol-cytochrome c (complex III) reductases. The semiquinone radicals undergo reoxidation by molecular oxygen with the corresponding generation of the superoxide anion.[24,25,26,27,28,29] According to the chemical, biochemical, and toxicological properties of luteoskyrin, it is highly possible that in the redox systems catalyzed by the mitochondrial and/or microsomal reductases, the toxin is reduced to a semiquinone radical, which then reacts with oxygen to generate superoxide anion. Based on this hypothesis, first, we examined whether superoxide anion is generated by the microsomal NADPH-cytochrome P-450 reductase and/or mitochondrial NAD(P)H-cytochrome c reductase, including NADH-ubiquinone and ubiquinol-cytochrome c reductases, in the presence of luteoskyrin with or without dicoumarol, by using the spin-trapping technique with DMPO as a spin-trap. Second, we investigated whether luteoskyrin is reduced to form semiquinone radicals by the NADPH-cytochorme P-450 reductase under anaerobic conditions by direct measurement of the ESR spectra of the semiquinone radicals. Here we show both the generation of superoxide anion and the formation of luteoskyrin semiquinone radicals.

MATERIALS AND METHODS

Materials

Luteoskyrin was extracted from the dried fungal mat of *Penicillium islandicum* Sopp and purified by column chromatographies described previously.[2] Before usage, it was crystallized with acetone/n-hexane. HPLC analysis revealed purity over 98%.

5,5-Dimethyl-1-pyrroline-1-oxide (DMPO, Dojindo Chem. Co., Japan), SOD (Cu-Zn-superoxide dismutase, Boehringer-Mannheim), catalase (Seikagaku Kogyo, Tokyo), glucose oxidase (Sigma), and NADPH and NADH (both Oriental Yeast Co., Tokyo) were used. Catalase and glucose were immobilized by using oxiran acrylic beads (Euperguit, Rohm Pharma. Germany).

Enzyme Preparations

Microsomal and mitochondrial preparations were obtained according to the method described by Hogeboom,[30] from the S.D. rat liver perfused with PBS in situ and taken out under thiopental sodium anesthesia. NADPH-cytochrome P-450 reductase (NADPH-cyt P-450 reductase) was solubilized by trypsin digestion from the microsomal preparation according to the method of Omura and Takesue,[31] and utilized without further purification. Submitochondrial particles were prepared from the mitochondrial preparation described above, according to the procedure described by Takeshige *et al.*[32] NAD(P)H-cytochrome c reductase (NAD(P)H-cyt c reductase) activities of the microsomal and mitochondrial enzyme samples were 1.81 μmoles and 0.42 μmoles ferricyt c reduction/mg protein/min., respectively. Protein concentrations of the enzyme samples were determined by using the Bio-Rad protein assay kit. All the other chemicals used were analytical grade.

Spin-trapping and EPR Experiments

In the spin-trapping experiments, the following five reaction systems were used. The reaction mixture a) contained 35 mM luteoskyrin dissolved in DMSO (3.2 mM), 5 mM NADPH, 0.44 M DMPO, NADPH-cyt P-450 reductase (150 μg protein), and 100 mM potassium phosphate buffer (pH 9.0), totally 200 μl. The reaction mixtures b), c) and d) were the same as a), except submitochondrial particles (320 μg protein) were used instead of the NADPH-cyt P-50 reductase. In the reaction mixtures c) and d) 5 mM NADH was used instead of NADPH, and in the mixture d) 10^{-4} M dicoumarol was added. When luteoskyrin was omitted the same volume of DMSO was added. SOD (500 units/ml) was added to the mixture, if necessary. When NADPH-cyt P-450 reductase was used the reaction was started by adding the enzyme to the reaction mixture prepared at 24°C in a test tube. In the case of submitochondrial particules the enzyme was preincubated at 37°C for 5 minutes without NAD(P)H; then the reaction was started by adding NAD(P)H. The reaction mixture was quickly transfered into a flat quartz cell (0.3 mm thickness, 160 μl), and then the cell was fixed in an EPR spectrometer (JEOL, JES-3XG, 100 KHz field modulation (X band)). Sequential recordings were started 30 seconds after the NAD(P)H addition. Each scan took 2 min. Other experimental conditions are noted in the figure legends.

For direct measurement of the EPR spectra of luteoskryin semiquinone radical, the reaction mixture contained 100 mM potassium phosphate buffer (pH 9.0), 35 mM luteoskyrin dissolved in DMSO (3.2 M), 5 mM NADPH, and NADPH-cyt P-450 reductase (375 μg protein), and 1% glucose, totally 500 μl, in a small vial. To remove the oxygen present in the vial, glucose oxidase and catalase, which were immobilized on beads (consumed 200 μmol oxygen/min) were added to the reaction mixture and the vial was sealed with a rubber stopper. After a few minutes incubation at 24°C, the reaction mixture was transfered anaerobically into the EPR flat cell, and then the cell was fixed into the cavity. EPR spectra were recorded in the similar manner as described above at 24°C.

RESULTS

Figure 1a) shows the EPR spectrum of the DMPO-spin adducts observed 30 seconds after the reaction start in a phosphate buffer solution (pH 9.0) containing luteoskyrin, NADPH, DMPO, DMSO, and the microsomal NADPH-cyt P-450 reductase (complete system). Prominent signals of DMPO-OOH together with small signals of DMPO-CH_3 appeared, exhibiting the formation of superoxide anion (O_2^-) and hydroxyl radical ($\cdot OH$). Similar spectra were continuously observed until

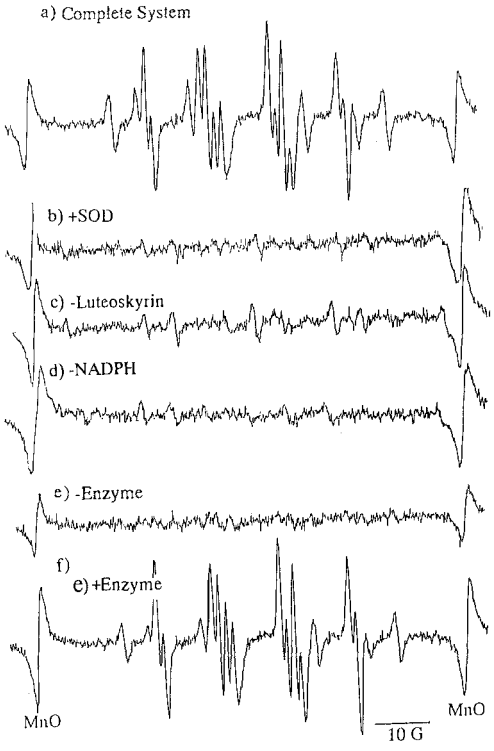

FIGURE 1 EPR spectra of DMPO-spin adducts formed in the presence of luteoskyrin, NADPH, DMSO, DMPO, and NADPH-cyt P-450 reductase, and the effect of SOD on the formation of the spin adducts. The complete system (a)) contained 100 mM phosphate buffer (pH 9.0), 35 mM luteoskyrin in DMSO (3.2 M), 5 mM NADPH, 0.22 M DMPO, and NADPH-cyt P-450 reductase (750 μg protein/ml), b) 500 units/Ml SOD was added to a). c), d), and e) were without luteoskyrin, NADPH and the enzyme from the complete system (a)), respectively. In the case of f) after the recording of the spectrum e) the reaction mixture was taken out and added with NADPH-cyt P-450 reductase, then the spectrum was recorded immediately. The recordings of the spectra a), b), c), d), and e) started 30 seconds after the addition of enzyme or water (e)) under the following instrumental conditions: Microwave power, 4 mW; microwave frequency, 9.445 GHz; modulation frequency, 100 KHz; modulation amplitude, 0.63 G; magnetic field, 3355 $\pm$ 50 G; amplitude, 1 $\times$ 1000; scan time, 2 min.; response, 0.3 s; temperature, 24°C. The EPR signals were assigned according to the previously published hyperfine splitting constants.[37]

the end of three consecutive scans, totally 7 minutes. When SOD (500 units/ml) was added to the mixture, no signals appeared (Figure 1b)), indicating that both DMPO-OOH and DMPO-CH_3 were derived from O_2^-. When either luteoskyrin, NADPH or the enzyme was omitted from the complete system, neither the signals of DMPO-OOH nor those of DMPO-CH_3 appeared (Figures 1c), d) and e)), and by adding the enzyme to e) the signals similar to those observed in a) immediately appeared (Figure 1f)). Thus, O_2^- was generated in the case of luteoskyrin and NADPH-cyt P-450 reductase, but not in the case of luteoskyrin alone, suggesting that NADPH-cyt P-450 reductase was involved in the O_2^- generation, and that O_2^- was not produced by the non-enzymatic autoxidation of luteoskyrin itself.

When submitochondrial particles were used instead of the NADPH-cyt P-450 reductase, an EPR spectrum similar to that in Figure 1a) with DMPO-OOH and DMPO-CH_3 signals, both SOD-inhibitable, was observed (data not shown). The SOD inhibition indicated that luteoskyrin could generate O_2^- in the presence of mitochondrial NADPH-cyt c reductase, as well as in the presence of microsomal NADPH-cyt P-450 reductase. When NADH was used instead of NADPH, similar results were observed (Figure 2a)).

Because certain quinones are reduced to hydroquinones by two-electron reduction catalyzed by diaphorase, and the produced hydroquinones proportionate with original quinones to form semiquinone radicals, and then generate O_2^- in the processes of autoxidation.[29] Therefore, in order to confirm that the O_2^- generation

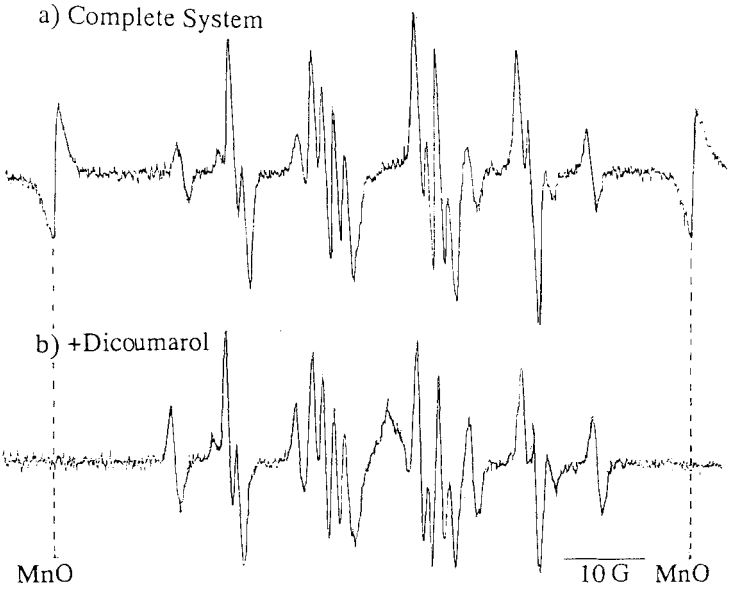

FIGURE 2 EPR spectra of DMPO-spin adducts formed in the presence of luteoskyrin, NADH, DMSO, DMPO and submitochondrial particles (a)), and the effect of dicoumarol on the formation of the spin adducts (b)). Submitochondrial particles (1600 μg/ml) were preincubated at 37°C for 5 minutes in the mixtures containing 35 mM luteoskyrin, 3.2 M DMSO, 0.44 M DMPO in 100 mM potassium phosphate buffer (pH 9.0). Then the reaction was started by adding 5 mM NADH. In the case of b), 10^{-4} M dicoumarol was added to the reaction mixture prior to the addition of NADH.

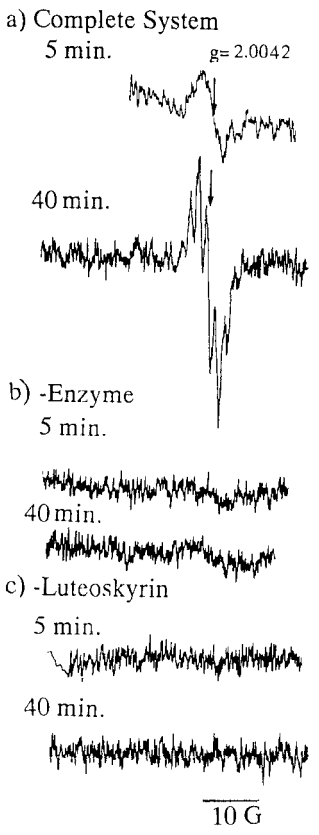

FIGURE 3 EPR spectra of luteoskyrin semiquinone radical formed in the presence of luteoskyrin, NADH, DMSO, and NADPH-cyt P-450 reductase under anaerobic conditions. a) complete system, b) without enzyme, and c) without luteoskyrin, 5 and 40 minutes after mixing, respectively. The reaction mixture contained 100 mM potassium phosphate buffer (pH 9.0), 35 mM luteoskyrin dissolved in DMSO (3.2 M), 5 mM NADPH, and cyt P-450 reductase (750 μg protein/ml), and 1% glucose, and glucose oxidase and catalase immobilized on beads (consumed 200 μmol oxygen/min), totally 500 μl. EPR spectra were recorded under the following conditions: Microwave power, 5 mW; microwave frequency, 9.445 GHz; modulation frequency, 100 KHz; modulation amplitude, 0.8 G; magnetic field, 3355 ± 100 G; amplitude, 7.9 × 1000; scan time, 32 min.; response, 1.0; temperature, 24°C. Other experimental conditions were noted in Materials and Methods.

observed in the presence of luteoskyrin is caused by one-electron reduction catalyzed by either NADH-ubiquinone reductase or ubiquinol-cyt c reductase, we investigated the effect of dicoumarol, a well known diaphorase-inhibitor, on the DMPO-OOH formation.[24] As shown in Figure 2b), no effect of dicoumarol was observed with added dicoumarol, suggesting that diaphorase did not participate to the O_2^- generation observed in the presence of luteoskyrin and the submitochondrial particles. Thus, by the enzymatic reaction catalyzed by either the microsomal NADH-cyt P-450 reductase or mitochondrial NAD(P)H-cyt c reductase, including NADH-ubiquinone and ubiquinol-cyt c reductases, luteoskyrin may undergo one-electron reduction to form the semiquinone radical, which then reacts with oxygen to generate O_2^- .

In order to obtain evidence supporting this hypothesis, we investigated the formation of luteoskyrin semiquinone radical in the presence of NADPH and NADPH-cyt P-450 reductase, by measurements of EPR spectra of the semiquinone radical under anaerobic conditions. As shown in Figure 3a), 5 minutes after the start of reaction a broad EPR spectrum at g = 2.0042 was observed. In 40 minutes, the spectrum at g = 2.0042 showed five lines (splitting about 1.7 G). When either the enzyme (Figure 3. b)), luteoskyrin (Figure 3c)), or NADPH (data not shown), was omitted, both of the spectra were not observed, indicating that the formation of the free radicals was dependent on luteoskyrin, the enzyme and NADPH. From the g-values (g = 2.0042) of the two spectra in Figure 3a) and the hyperfine splitting (1.7 G), the two spectra are assigned as luteoskyrin semiquinone radical. Five lines in Figure 3a) may be attributed to approximately equivalent hyperfine splitting from one ring hydrogen and three methyl hydrogens.

DISCUSSION

Antitumor quinones are known to be reduced reversibly to semiquinone radicals in the reaction catalyzed by certain enzymes such as NADPH-cyt P-450 reductase, with generation of oxygen radicals, and those radicals formed at the critical sites in the tumor cells are postulated to be responsible for their antitumor activities.[28] According to the chemical structure and biochemical and toxicological characteristics disclosed so far, we predicted that in the animal liver, luteoskyrin is enzymatically reduced to its semiquinone radical, which enhances the O_2^- generation in the cells. Hydroxyl radical may be site-specifically produced from the O_2^- and thus damages liver cell functions. In this work by detecting the EPR spectra of DMPO-OOH and luteoskyrin semiquinone radical, we demonstrated both the formation of luteoskyrin semiquinone radical and the O_2^- generation in the presence of luteoskyrin and microsomal NADPH-cyt P-450 reductase. The O_2^- generation was also observed in the presence of mitochondrial NAD(P)H-cyt c reductase. These results suggest that oxygen is reduced to O_2^- in the reoxidation process of luteoskyrin semiquinone radical which has been formed in the one-electron reduction catalyzed by the NAD(P)H-dependent oxidoreductases present in the liver microsome and/or mitochondria.

Luteoskyrin induces karyorexis in the poisoned animals[1] and inhibits RNA synthesis in the luteoskyrin-treated cell.[34] Previously, by using EPR spin-trapping with DMPO and DMSO, and the detection of 8-hydroxylation of guanosine residue in DNA, we showed that luteoskyrin and related hydroxyanthraquinones generated ·OH in the presence of ascorbic acid and trace amount of iron.[19,20] The accelerated 8-hydroxylation of the deoxyguanosine residues was also observed in the DNA of the liver cells of mice exposed to luteoskyrin as well as in the DNA of the tissue-cultured cell lines treated with this mycotoxin.[21,22] Based on these observations site-specific generation of oxygen radicals, probably ·OH, is expected to take place in the liver nuclei of the luteoskyrin-intoxicated animals.

In the nuclei of liver and other organs of animals, the presence of NADPH-cyt P-450 reductase together with formation of antibiotic free radicals and superoxide anions has been reported.[35,36] Furthermore, intercalation of antitumorigenic anthraquinoid drugs between the base pairs of DNA has been attributed to the increased formation of ·OH by the drugs.[28] Luteoskyrin has a strong chelating action with various divalent metals including ferrous ion and binds to DNA with formation

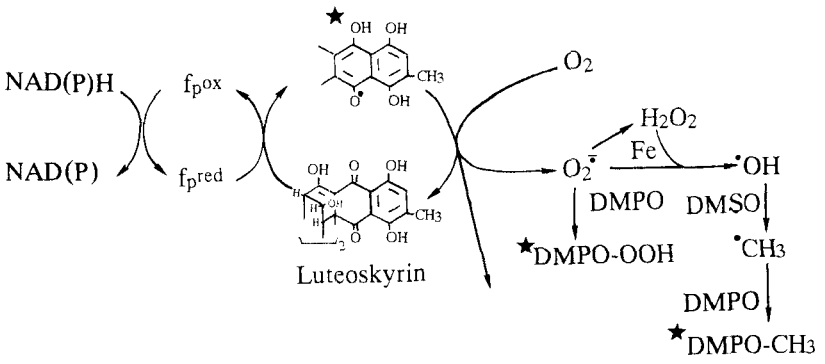

FIGURE 4 A possible mechanism for the formations of luteoskyrin semiquinone and superoxide anion radicals in the redox system catalized by the liver NAD(P)H-dependent reductases.

of a DNA-Mg^{++}-luteoskyrin complex in which luteoskyrin is oriented in parallel with the axis of DNA double helix according to the flow dichroism analysis.[37,38] Therefore, if luteoskyrin accelerates the generation of O_2^- in the nuclei, or the O_2^- generated by the system of luteoskyrin and the NAD(P)-dependent reductases in microsome and/or mitochondria can enter into the nuclei it may change easily to ·OH. In these cases karyorexis or 8-hydroxylation of the deoxyguanosine residues in DNA may occur as observed in the liver cells of luteoskyrin-intoxicated animals.

In this experiment we demonstrated the one-electron reduction of luteoskyrin to its semiquinone radical and the generation of O_2^-, which changes to ·OH, in the redox system catalyzed by the liver NAD(P)H-dependent reductases. (Figure 4). Based on these results we propose that in the liver of the luteoskyrin-intoxicated animals O_2^- is generated and converted to a more reactive oxygen species, probably ·OH, which attacks the cell constituents to induce acute liver injuries as well as hepatocarcinoma.

References

1. K. Uraguchi (1971) Pharmacology of mycotoxins. In *International Encyclopedia of Pharmacology and Therapeutics Section 71, Part V* (ed. H. Raskova), Pergamon Press, Oxford, pp. 143–198.
2. K. Uraguchi, T. Tatsuno, F. Sakai, M. Tsukioka, Y. Sakai, H. Yonemitsu, H. Ito, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiko (1961) Isolation of two toxic agents, luteoskyrin and chlorine-containing peptide, from the metabolities of *Penicillium islandicum* Sopp, with some properties thereof. *Japanese Journal of Experimental Medicine*, **31**, 19–46.
3. S. Shibata (1973) Some recent studies on the metabolites of fungi and lichens. *Pure and Applied Chemistry*, **33**, 109–128.
4. N. Takeda, S. Seo, Y. Ogihara, U. Sankawa, I. Iitaka, I. Kitagawa and S. Shibata (1973) Studies on fungal metabolites – XXXI. Anthraquinoid colouring matters of *Penicillium islandicum* Sopp and some other fungi. (−) Luteoskyrin, (−) rubroskyrin, (+) rugulosin and their related compounds. *Tetrahedron*, **29**, 3703–3719.
5. M. Enomoto and I. Ueno (1974) *Penicillium islandicum* (Toxic yellowed rice) – Luteoskyrin – Islanditoxin – Cyclochlorotine. In *Mycotoxins* (ed. I.F.H. Purchase), Amsterdamm-Oxford-New York, pp. 303–326.
6. K. Uraguchi, M. Saito, Y. Noguchi, K. Takahashi, M. Enomoto and T. Tatsuno (1972) Chronic toxicity and carcinogenicity in mice of purified mycotoxins, luteoskyrin and cyclochlorotine. *Food and Cosmetics Toxicology*, **10**, 193–207.

7. Y. Ueno and I. Ueno (1978) Toxicology and biochemistry of mycotoxins. In *Toxicology, Biochemistry and Pathology of Mycotoxins* (eds. K. Uraguchi, M. Yamazaki), Kodansha LTD, Tokyo and John Wiley & Sons, New York, pp. 107–188.
8. K. Uraguchi, I. Ueno, Y. Ueno and T. Komai (1972) Absorption, distribution and excretion of luteoskyrin with special reference to the selective action on the liver. *Toxicology and Applied Pharmacology*, **21**, 335–347.
9. I. Ueno, T. Hayashi and Y. Ueno (1974) Pharmacokinetic studies on the hepatotoxicity of luteoskyrin. (I) Intracellular distribution of radioactivity in the liver of mice administered with ^{3}H-luteoskyrin. *Japanese Journal of Pharmacology*, **24**, 535–542.
10. I. Ueno (1966) Action of luteoskyrin, a hepatotoxic pigment of *Penicillium islandicum* Sopp on the oxidative phosphorylation of rat liver mitochondria, *Seikagaku*, **38**, 74–747.
11. I. Ueno, Y. Ueno, T. Tatsuno and K. Uraguchi (1964) Mitochondrial respiratory impairment by luteoskyrin, a hepatotoxic pigment of *Penicillium islandicum* Sopp. *Japanese Journal of Experimental Medicine*, **34**, 135–152.
12. H. Kasai and S. Nishimura (1986) Hydroxylation of guanine in radical-forming agents. *Environmental Health Perspectives*, **67**, 35–40.
13. K. Kohda, M. Tada, H. Kasai, S. Nishimura and Y. Kawazoe (1986) Formation of 8-hydroxyguanine residues in cellular DNA exposed to the carcinogen 4-nitroquinoline 1-oxide. *Biochemical and Biophysical Research Communications*, **139**, 626–632.
14. H. Kasai, S. Nishimura, Y. Kurokawa and Y. Hayashi (1987) Oral administration of the renal carcinogen, potassium bromate, specially produces 8-hydroxydeoxyguanosine in rat target organ DNA. *Carcinogenesis*, **8**, 1956–1961.
15. R.A. Floyd (1990) The role of 8-hydroxyguanine in carcinogenesis, *Carcinogenesis*, **11**, 1447–1450.
16. B. Halliwell and O.I. Aruoma (1991) DNA damage by oxygen-derived species, *FEBS Letters*, **281**, 9–19.
17. Y. Kuchino, F. Mori, H. Kasai, H. Inoue, S. Iwai, K. Miura, E. Otsuka and S. Nishimura (1987) Misreading of DNA templates containing 8-hydroxyguanosine at the modified base and at adjacent residues, *Nature*, **327**, 7–79.
18. S. Shibutani, M. Takeshita and A. Grollman (1991) Insersion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxo dG. *Nature*, **349**, 431–434.
19. I. Ueno, M. Hoshino, T. Maitani, S. Kanegasaki and Y. Ueno (1993) Luteoskyrin, an anthraquinoid hepatotoxin, and ascorbic acid generate hydroxyl radical *in vitro* in the presence of a trace amount of ferrous iron. *Free Radical Research Communications*, **19**, S95–S100.
20. S. Akuzawa, H. Yamaguchi, T. Masuda and Y. Ueno (1991) Radical-mediated modification of deoxyguanine and deoxyribose by luteoskyrin and related anthraquinones. *Mutation Research*, **266**, 63–69.
21. N. Miyasaka, H. Yamaguchi, T. Kato and Y. Ueno (1989) Accelerated formation of 8-hydroxydeoxyguanosine in DNA in the presence of luteoskyrin, an anthraquinoid mycotoxin. In Abstracts of the 16th annual meeting of the Japanese society of toxicological sciences, Yokohama, p. 40.
22. Y. Ueno, W. Habano, H. Yamaguchi, T. Masuda, S. Morimura, K. Nemoto, S. Kojima and F. Tashiro (1991) Transformation of mammalian cells by luteoskyrin. *Fd Chemical Toxicology*, **29**, 607–613.
23. I. Ueno, T. Horiuchi and E. Enomoto (1980) Effects of chemical agents on the hepatotoxicity and hepatic accumulation of luteoskyrin. *Toxicology and Applied Pharmacology*, **52**, 278–284.
24. N.R. Bachur, S.L. Gordon and M.V. Gee (1977) Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. *Molecular Pharmacology*, **13**, 901–910.
25. N.R. Bachur, S.L. Gordon and M.V. Gee (1978) A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Research*, **38**, 1745–1750.
26. G. Powis and P.L. Appel (1980) Relationships of the single-electron reduction potential of quinones to their reduction by flavoproteins. *Biochemical Pharmacology*, **29**, 2567–2572.
27. G. Powis, B.A. Swinger and P.L. Appel (1981) Quinone-stimulated superoxide formation by subcellular fractions, isolated hepatocytes, and other cells. *Molecular Pharmacology*, **20**, 387–394.
28. G. Powis (1989) Free radical formation by antitumor quinones. *Free Radical Biology and Medicine*, **6**, 63–101.
29. P.L. Gutierrez (1989) Mechanism(s) of bioreductive activation. The example of diazequine (AZQ). *Free Radical Biology and Medicine*, **6**, 405–445.
30. G.H. Hogeboom (1955) Fractionation of cell components of animal tissue In *Methods in Enzymology* (eds. S.P. Colowick, N.O. Kaplan), Academic Press, New York, pp. 16–19.
31. T. Omura and S. Takesue (1970) A new method for simultaneous purfication of cytochorome b_5 and NADPH-cytochroem reductase from rat liver microsomes. *Journal of Biochemistry* (Tokyo), **67**, 249–257.

32. K. Takeshige and S. Minakami (1979) NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. *Biochemical Journal*, **180**, 129–135.
33. T. Omura, P. Siekevitz and G.E. Palade (1967) Turnover of constituents of the endoplasmic reticulum membranes of rat hepatocytes. *Journal of Biological Chemistry*, **242**, 2389–2396.
34. Y. Ueno, I. Ueno, K. Ito and T. Tatsuno (1967) Impairment of RNA synthesis in Ehrlich ascites tumor by luteoskyrin, a hepatotoxic pigment of *Penicillium islandicum* Sopp. *Experientia*, **23**, 1001–1004.
35. G.M. Bartok, T. Galeotti and A. Azzi (1977) Production of superoxide anions and hydrogen peroxide in Ehrlich ascites turmor cell nuclei. *Biochimica et Biophysica Acta*, **497**, 622–626.
36. N.R. Bachur, M.V. Gee and R.D. Friedman (1982) Nuclear Catalyzed antibiotic free radical formation. *Cancer Research*, **42**, 1078–1081.
37. Y. Ueno, A. Platel and P. Fromageot (1967) Interaction entre pigments et acides nucleiques. II. Interaction *in vitro* entre la luteoskyrine et le DNA thymus de veau. *Biochimica et Biophysica Acta*, **134**, 27–36.
38. Y. Ueno, I. Ueno and K. Mizumoto (1968) The mode of binding of luteoskyrin, a hepatotoxic pigment of *Penicillium islandicum* Sopp, to deoxyribonucleic acid. *Japanese Journal of Experimental Medicine*, **38**, 47–64.
39. B.E. Britigan, M.S. Cohen and G.M. Rosen (1987) Detection of the production of oxygen-centered free radicals by human neutrophils using spin trapping techniques: A critical perspective. *Journal of Leukocyte Biology*, **41**, 349–362.