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Binding of Nitrofuran Derivatives to Nucleic Acids and Protein[1]

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1) The *in vitro* reduction of ^{14}C-2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) and ^{14}C-nitrofurazone by xanthine oxidase-hypoxanthine resulted in the formation of their active metabolites capable of binding to desoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein.

2) The distribution and excretion of radioactivity in various tissues, gastrointestinal contents, feces and urine were examined after oral administration of ^{14}C-AF-2 to rats.

3) The *in vivo* binding of ^{14}C-AF-2 and ^{14}C-nitrofurazone to liver protein, DNA and ribosomal RNA, and to kidney protein was demonstrated in rats given orally these drugs.

Keywords——nitrofuran derivative; nucleic acid; protein; xanthine oxidase; binding; tissue distribution

Recent works have shown that some nitrofuran derivatives induce the prophage development[3] and the damage in desoxyribonucleic acid (DNA) of both bacteria[4] and mammalian cells,[5] and also inhibit the syntheses of DNA,[3,4] ribonucleic acid (RNA)[6,7] and protein.[6] These evidences have been considered to be important in connection with the occurrence of carcinogenic[8] and mutagenic[9] activity of nitrofuran derivatives. Such damage and synthetic inhibition seem to be mainly due to the binding of nitrofuran derivatives to macromolecules. Until now, it has been demonstrated that ^{14}C from labeled nitrofuran derivatives becomes bound to protein in the presence of nitroreductases.[10]

The present study was designed to investigate the *in vitro* binding of ^{14}C-2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) and ^{14}C-nitrofurazone to DNA, RNA and protein in the presence of xanthine oxidase-hypoxanthine system. The *in vivo* binding of these drugs to liver DNA, RNA and protein, and to kidney protein was also determined.

Materials and Methods

Chemicals——^{14}C-AF-2 (acrylamide-3-^{14}C, 4.8 μCi/mg, mp 153°), ^{14}C-nitrofurazone (formyl-^{14}C, 0.12 μCi/mg, mp 235—240° (dec.)) and the unlabeled compounds of above nitrofuran derivatives were kindly supplied by Ueno Pharmaceutical Co., Ltd. The labeled compounds were verified to be radiochemically pure by thin-layer chromatography as described previously.[11] Hypoxanthine was purchased from Nakarai Chemicals, Ltd., allopurinol [4-hydroxypyrazolo-(3,4-*d*)pyrimidine] from Sigma Chemical Co., and bovine serum albumin (fraction V from bovine plasma, BSA) from Armour Pharmaceutical Co. These preparation

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of DNA and RNA contained less than 3% and 1% of protein, respectively, when determined by the methods of Lowry, *et al.*[12] and Holden, *et al.*[13] These nucleic acids were used without further purification.

Enzyme——Purified butter milk xanthine oxidase (No. X-1875) was obtained from Sigma Chemical Co.

***In Vitro* Binding of Radioactivity to Nucleic Acids and Protein**——Reaction mixture consisted of milk xanthine oxidase (0.2 units, 0.36 mg protein), hypoxanthine (1.2 μmol), ^{14}C-nitrofuran derivative (0.3 μmol) and a macromolecule in 1/30 M phosphate buffer, pH 7.4. The final volume was 1.2 ml for BSA (10 mg) or 1.5 ml for DNA (3 mg) and RNA (3 mg or 10 mg) as macromolecule. The incubation was carried out at 37° for the periods indicated below under an atmosphere of nitrogen in the dark. The reaction mixture was dialyzed against 50 volumes of 1/30 M phosphate buffer, pH 7.4, which was renewed every 12 hr, for 60 hr at 5° using the cellulose tube. After dialysis, the inner fluids were adjusted to 5 ml with water and 1 ml each of them was counted in a toluene phosphor with BBS-3 (Beckman bio-solv No-3) for radioactivity. In order to examine the organic solvent-extractable radioactivity, the remaining inner fluids were extracted once with 5 volumes of AcOEt and then the extract was counted in a toluene phosphor. One ml of every 12 hr outer fluids was also counted in a dioxane phosphor.

In other experiment, the reaction mixture containing AF-2 and BSA was incubated in the presence of amino acid (30 μmol). After incubation, 50 μl of the reaction mixture was pipetted onto a small filter paper disc (Toyo filter paper No. 2, 2.5 cm diameter). The disc was placed in 50 ml of 5% trichloroacetic acid in a culture dish, 9 cm diameter, and then moved twenty times from one side to other side of the dish for 10 min with forceps. This procedure was repeated once more in fresh 5% trichoroacetic acid. Next, it was picked out with forceps, washed again with 5 ml of 5% trichloroacetic acid and once with 5 ml of 95% EtOH. The disc was then dried and counted in a toluene phosphor for radioactivity. The counting efficiency was about 90% in this disc. In order to determine the reduction rate of ^{14}C-AF-2, the remaining reaction mixture was extracted twice with 2 volumes of AcOEt, the combined AcOEt solution was evaporated to dryness *in vacuo* and the residue was submitted to thin-layer chromatography with $CHCl_3$–MeOH (7: 3). After development, the area corresponding to *Rf* value (0.68) of AF-2 was scraped and the radioactivity was measured in a toluene phosphor.

Tissue Distribution of Radioactivity——^{14}C-AF-2 was given orally to male Donryu rats weighing about 160 g at a dose of 15 mg/kg as suspension in 0.5% carboxymethylcellulose. The animals were immediately placed in individual metabolism cages designed for the separation of urine and feces. The animals were killed by cervical fracture. The tissues of interest were immediately excised, weighed and homogenized in 10 volumes of distilled water. Stomach, small intestine and large intestine were washed to separate from their contents, and the tissues and contents were then processed separately. Duplicate samples of the tissues, contents and feces were oxidized to $^{14}CO_2$ for counting as described previously.[11] Aliquots of urine samples were also counted in a dioxane phosphor.

Isolation of Nucleic Acids and Protein——The rats given ^{14}C-AF-2 or ^{14}C-nitrofurazone as described above, were killed, and the liver and kidney were immediately dissected out. The isolation of liver DNA and ribosomal RNA (r-RNA) was achieved as described by Irving and Veazey.[14] The protein of liver and kidney was precipitated by MeOH from the interphase which was obtained by the above procedure, and washed with MeOH and ether as described by Kuroki, *et al.*[15]

Radioactivity Measurement——Radioactivity was determined on a liquid scintillation spectrometer (Packard 3375) with automatic external standardization. The samples were counted in a toluene phosphor consisting of 4 g PPO, 0.1 g POPOP and toluene to make 1 liter, and a dioxane phosphor consisting of 60 g naphthalene, 4 g PPO, 0.2 g POPOP, 20 ml ethyleneglycol, 100 ml MeOH and dioxane to make 1 liter. The oxidation of samples to $^{14}CO_2$ and the subsequent counting were carried out as described in the previous paper.[11]

Thin-Layer Chromatography (TLC)——TLC was conducted on silica gel plate (Wako gel B-5 FM, 0.25 mm thick) developing in $CHCl_3$–MeOH (7: 3). The chromatograms were visualized under ultraviolet light (3650 Å).

Results

Table I shows the binding ratios of radioactivity to DNA, RNA and protein after incubation of ^{14}C-nitrofuran derivatives with these macromolecules in the xanthine oxidase system.

In both cases of ^{14}C–AF–2 and ^{14}C-nitrofurazone, the radioactivity was bound more to macromolecules in the presence of xanthine oxidase supplemented with hypoxanthine as a

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Table I. Binding Ratios (%) of ^{14}C-AF-2 and ^{14}C-Nitrofurazone with Nucleic Acids and Protein in the Xanthine Oxidase System

	Hypo-xanthine	DNA (3 mg)	RNA (3 mg)	RNA (10 mg)	BSA (10 mg)	Without macro-molecules
AF-2	+	10.4	4.5	10.2	30.5	4.1
	−	2.6	0.7	3.0	6.2	
Nitrofurazone	+	3.7	2.7	4.1	7.1	1.0
	−	0.1	0.2	0.2	0.6	

Each value represents mean of three experiments.

proton donor than in that of the enzyme only. Furthermore, when allopurinol (1.5 μmol), an inhibitor of xanthine oxidase, was added to the complete reaction mixture containing ^{14}C–AF–2 and BSA, the binding ratio of radioactivity decreased up to 6.4%. Low level of bound radioactivity ,which resulted from binding of radioactivity to the enzyme protein, was also observed when no macromolecule was added. When ^{14}C–AF–2 was incubated with 3 mg of RNA in the complete reaction mixture, the binding ratio was 4.5%, which only slightly differed from that without macromolecule. However, in the higher level of RNA (10 mg), the binding ratio increased up to 10.2%, indicating its dependence on the concentration of RNA in the reaction mixture. The similar result was obtained from the reaction between ^{14}C-nitrofurazone and RNA. In all cases, the extraction of the non-dialyzable fractions with ethyl acetate did not result in a measurable decrease of radioactivity, showing that most of the radioactivity in this fractions was firmly bound to macromolecules. These results indicate that the xanthine oxidase-proton donor system reduces nitrofuran derivatives to the active metabolites capable of binding to nucleic acids and protein.

Table II shows the influence of amino acids on the binding of ^{14}C–AF–2 to BSA in the xanthine oxidase-hypoxanthine system.

Table II. Influence of Amino Acids on Binding of ^{14}C-AF-2 to Protein in the Xanthine Oxidase-Hypoxanthine System

Amino acids	AF-2 Binding to protein nmol/g	% of control	Amino acids	AF-2 Binding to protein nmol/g	% of control
None	514	100	L-Lysine	523	102
L-Cysteine	63	12	L-Valine	494	96
Glutathione	69	13	L-Serine	554	108
L-Methionine	394	77	L-Arginine	538	105
L-Tryptophan	463	90	L-Hydroxyproline	530	103
L-Phenylalanine	370	72	L-Cystine[a)]	456	89
L-Histidine	403	78	L-Tyrosine[a)]	487	95
L-Alanine	549	107			

Each value represents mean of three experiments.
a) Incubation mixture contains 1.50 μmol of amino acid.

Addition of L-cysteine or reduced glutathione to the reaction mixture drastically decreased the binding of the drug to BSA. However, in all cases shown in Table II, the detectable amount of unchanged AF-2 was not observed in these reaction mixtures, indicating the almost complete reduction of the drug. These results suggest that the active metabolite of AF-2 may react with L-cysteine or reduced glutathione, and that the binding sites of protein for such metabolite may be the sulfhydryl groups.

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Table III shows the radioactivity in various tissues, gastrointestinal contents, feces and urine of rats given with ^{14}C–AF–2.

TABLE III. Distribution and Excretion of Radioactivity after Oral Administration of ^{14}C-AF-2 to Rats

Tissue	Time (hr) after administration					
	5(5)		24(3)		96(2)	
	% of dose	dpm/g	% of dose	dpm/g	% of dose	dpm/g
Liver	2.5	95760	1.6	61820	0.98	37650
Kidney	0.14	24150	0.10	17940	0.084	14400
Lung	0.040	8050	0.018	3620	0.004	790
Spleen	0.006	7250	0.005	6040	0.003	3620
Heart	0.008	3200	0.015	6040	0.003	1210
Testis	0.010	1210	0.013	1810	0.004	480
Brain	0.006	1610	0.003	800	0	0
Pancreas	0.033	11380	0.008	2150	0.003	1040
Fat	0.040	12080	0.013	3940	0.004	1210
Small intestine	2.4	135480	0.19	10510	0.011	620
Large intestine	1.6	188610	0.33	40210	0.014	1690
Stomach	0.24	73660	0.023	6940	0.010	
Contents of stomach	19.3		0.33			
Contents of small intestine	5.5		0.54		0.012	
Contents of large intestine	28.0		2.1		0.074	
Urine	8.8		11.8		14.4	
Feces	5.9		61.6		76.6	

Each value represents mean with the number of experiments in parentheses.

TABLE IV. Binding of ^{14}C-AF-2 and ^{14}C-Nitrofurazone to Liver Protein, DNA and r-RNA, and to Kidney Protein after Oral Administration of These Drugs to Rats

(A) ^{14}C-AF-2

Macromolecule	Time (hr) after administration			
	5	24	96	168
	(nmol/g)			
Liver protein	140.2	69.4	38.3	27.5
DNA	7.2	10.3	19.1	18.0
r-RNA	8.4	5.1	2.7	3.2
Kidney protein	49.4	27.4	15.6	13.5

(B) ^{14}C-Nitrofurazone

Macromolecule	Time (hr) after administration
	24
	(nmol/g)
Liver protein	44.0
DNA	11.9
r-RNA	1.4
Kidney protein	16.8

Each value represents mean of three experiments.

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Radioactivity was predominantly excreted via feces and with lesser amount via urine. As reported previously,[11,16] a part of radioactivity excreted in feces seems to be due to its biliary excretion. The liver had the highest total radioactivity among the tissues examined 5, 24 and 96 hr after dosing. Ninety six hours after medication, the weak radioactivity was still detected in the tissues except brain, indicating the somewhat persistent remaining of AF-2 and/or its metabolites in the rat body.

Table IV shows the binding of ^{14}C–AF–2 and ^{14}C-nitrofurazone to liver protein, DNA and r-RNA, and to kidney protein after administration of these drugs to rats.

In the case of ^{14}C–AF–2, the binding of the drug to protein declined throughout the experiment in both liver and kidney, although the values in kidney were always lower than those in liver. In r-RNA, the binding declined until 96 hr similar to protein, but remained approximately constant until 168 hr after dosing. On the contrary, the binding to DNA reached maximum about 96 hr after dosing. In the case of ^{14}C-nitrofurazone, the binding of the drug to liver protein, DNA and r-RNA was also observed 24 hr after dosing, but the values in protein and r-RNA were lower as compared with ^{14}C–AF–2 at this time.

Discussion

Recent evidences indicate that the enzymes such as xanthine oxidase[10a,c,d] and NADPH-cytochrome *c* reductase[10b,d] reduce nitrofuran derivatives to give the active metabolites capable of binding to protein. Although it has been suggested that the active metabolite of nitrofurazone may also react with other macromolecules such as nucleic acids,[10a] the direct evidence has not been described. The present study shows that *in vitro* reduction of AF-2 and nitrofurazone by xanthine oxidase results in the formation of their active metabolites capable of binding to DNA and RNA as well as protein. The bound radioactivity shown in Table I is probably the result of covalent reaction between the active metabolites and nucleic acids or protein since this radioactivity was not removed either by the prolonged dialysis or by the subsequent ethyl acetate extraction. On the other hand, even when hypoxanthine was omitted from the reaction mixtures, the small amount of bound radioactivity was also observed. In our preliminary study, it was found that when ^{14}C–AF–2 (0.3 μmol) was incubated with BSA (10 mg) in 1/30 M phosphate buffer, pH 7.4 for 2 hr at 37°, 3.3% of the total radioactivity was rendered non-dialyzable. From these results, a part of AF-2 and nitrofurazone appears to react with macromolecules without enzymatic reduction.

Furthermore, the present study shows for the first time the *in vivo* binding of ^{14}C–AF–2 and ^{14}C-nitrofurazone to nucleic acids and protein in rats. So far, Stripp, *et al.*[17] reported that the binding of ^{14}C-nitrofurazone to macromolecules occurred in the tissues of mice given the drug, ip, and that the depletion of glutathione in the animals by diethyl maleate prior to the administration of the drug doubled the binding in most tissues. Also, Wang *et al.*[18] described the binding of radioactivity to cold and hot trichloroacetic acid-insoluble fractions of liver and kidney of rats after ip injection of ^{14}C–N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide. Such *in vivo* binding of nitrofuran derivatives to macromolecules appears to require the reduction of nitro group as well as their *in vitro* binding.

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