FOUR-WEEK SUB-ACUTE TOXICITY STUDY OF S-(–)-9-FLUORO-2, 3-DIHYDRO-3-METHYL-10- (4-METHYL-1-PIPERAZINYL)-7-OXO-7H- PYRIDO-[1, 2, 3,-DE] [1, 4] BENZOXAZINE-6- CARBOXYLIC ACID HEMIHYDRATE (DR-3355) IN CD RATS AND CYNOMOLGUS MONKEYS

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Abstract.....S-(–)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperaziny)-7-oxo-7H-pyrido-[1, 2, 3,-de] [1, 4] benzoxazine-6-carboxylic acid hemihydrate, DR-3355, a new quinolone antimicrobial agent, was administered by oral gavage to groups of ten male and ten female CD rats at dosages of 50, 200 or 800 mg/kg/day and to groups of three male and three female cynomolgus monkeys at dosages of 10, 30 or 100 mg/kg/day. Both species were treated for four weeks. The vehicle (0.5 % sodium carboxymethyl cellulose)-treated group served as control. Rats at the high dose showed salivation, slight increases in total leucocyte and lymphocyte counts, slight changes in the plasma electrolyte balance, and minor reductions in urea concentration. The articular surfaces of the humerus and femur of rats at the high dose showed minor degenerative changes. Increased caecal weight occurred in rats at all the treatment groups. Monkeys at the high dose showed salivation, diarrhoea and lost weight. There was no microscopic change in the tissues examined. No effect levels under these conditions were established at 200 mg/kg/day in the rat, and 30 mg/kg/day in the monkey.

Key words : Subacute toxicity study, DR-3355, rat, monkey.

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INTRODUCTION

New quinolones with antibacterial activities against both gram-negative and -positive organisms have been developed. One of them, ofloxacin (OFLX), is widely used in the clinical field. Recently, S-(-)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido-[1,2,3,-de][1,4]benzoxazine-6-carboxylic acid hemihydrate (DR-3355), which is an optically active isomer of OFLX, was prepared by Hayakawa et al. (1986). This compound has been reported to be approximately twice as potent as OFLX in inhibiting the multiplication of gram-positive and -negative bacteria (Atarashi et al., 1987). Antibacterial activities of quinolones are thought to correlate with their anti-DNA gyrase activities. The inhibitory effect of DR-3355 is shown to have more excellent against bacterial DNA gyrase than one of other quinolones (Imamura et al., 1987).

Adverse effects of quinolones in laboratory animals have been reported by Christ et al. (1988) as follows : lesions in the articular cartilages in young rats, dogs and rabbits treated with all quinolones ; nephrotoxicity in rats, dogs and monkeys treated with some kinds of quinolones ; toxicity in the central nervous system of mice, cats, and dogs with fleroxacin ; ocular toxicity in rats and dogs with rosoxacin and pefloxacin, respectively ; impaired spermatogenesis and /or testicular damage in rats and dogs with pipemidic acid, norfloxacin or pefloxacin.

The present studies were performed to evaluate the toxicity of DR-3355 in rats and cynomolgus monkeys when administered by gavage for four weeks.

MATERIALS AND METHODS

Chemicals and Animals : S-(-)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido-[1,2,3,-de][1,4]benzoxazine-6-carboxylic acid hemihydrate, DR-3355, was supplied as a fine yellow crystalline powder by Daiichi Pharmaceutical Company Limited. It was prepared each day for administration as a series of suspensions of graded concentration in 0.5 % sodium carboxymethyl cellulose (CMC). Chemical analysis of the formulated material showed acceptable homogeneity, stability at room temperature and concentrations within the permitted ranges.

Specific-pathogen free (SPF) male and female CD rats (remote Sprague-Dawley origin) were obtained from Charles River U. K. Limited, England. They were three to five weeks of age, and were in the weight range 54 to 82 g. The animal were housed five per cage in a barrier-controlled animal room with positive air pressure maintained by approximately 15 air changes per hour. A 12-hour light : 12-hour dark cycle operated, and temperature and humidity were maintained at 21°C (18-25°C) and 55 % relative humidity (40-70 % RH) respectively. The rats were acclimatized to these conditions for six days before commencement of treatment. Diets and tap water were available *ad libitum*. The certified diet was supplied by Labsure

(England) and water was provided by the East Anglian Water Company (England).

Twelve male and twelve female wild-caught cynomolgus monkeys (*Macaca fascicularis*) were obtained from Charles River Research Primates Corporation, U. S. A.. During the acclimatization period one female developed a vaginal infection, and it was replaced by a monkey of similar age and weight obtained from Shamrock Farms Limited, England. The animals weighed between 1.35 and 2.00 kg on arrival, and were estimated to be two to four years old. The monkeys were kept in a purpose built primate facility with a 12-hour light /dark cycle. Negative pressure was maintained by approximately 15 air changes per hour and the temperature and relative humidity fell into the range of 19–22°C and 50–60 % RH respectively. The monkeys were acclimatized to these conditions for eight weeks (except the replaced female which was held for 11 days before the study started). Drinking water (East Anglian Water Company, England) was supplied *ad libitum*. Each animal was offered two meals daily, each of 100 g of Old World Monkey diet, (Special Diet Services, England), supplemented by a hard biscuit (Bonio, Spillers Food Ltd., England) after each dose. Fresh fruit or vegetables were offered three days in each week.

Treatment : For rats ten males and ten females were allocated to each group receiving 50, 200 or 800 mg/kg/day (dose volume of 5 ml/kg bodyweight) of DR-3355, and for monkeys three males and three females were allocated to each group, given DR-3355 at dosages of 10, 30 or 100 mg/kg/day (dose volume of 4 ml/kg bodyweight). Rats and monkeys were treated with the drug by daily gavage, for four weeks, using a flexible rubber catheter. The additional group received the vehicle (0.5 % CMC) at the same frequency and dose-volume served as control. Treatment of both rats and monkeys continued for at least 28 days.

Hematological and Biochemical Examinations : *In rats*, all were observed for signs of ill-health or reaction to treatment before and shortly after treatment. A weekly calculation of food consumption was made for each cage of rats. Each animal was weighed on the day that treatment commenced and then twice a week throughout the study. Ophthalmoscopy was performed before treatment commenced, and in the fourth week, on all animals, by indirect ophthalmoscope.

Blood samples were taken during the fourth week from the retroorbital sinus of each rat. The rats were starved overnight before sampling. A portion of each sample was placed in EDTA and examined with the ELT-8 automated haematology analyzer (Ortho Diagnostics Systems Ltd., England) for packed cell volume (PCV), haemoglobin concentration (Hb), erythrocyte count (RBC), total leucocyte count (WBC), platelet count (Plat), mean cell haemoglobin (MCH), mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC). Differential leucocyte count was calculated from the total leucocyte count and the percentage found on blood smears stained with Romanowsky. Reticulocyte counts were made on smears stained with brilliant cresyl blue. Further a portion of each sample was placed in citrate to evaluate for prothrombin time (PT).

In addition, another portion of each sample was placed in lithium heparin anticoagulant. After separation the plasma was examined in respect of alkaline phosphatase (AP), alanine amino-transferase (ALT), aspartate amino-transferase (AST), creatine phosphokinase (CPK), lactate dehydrogenase (total) (LDH), urea, uric acid, creatinine, glucose, total triglycerides, phospholipids, total cholesterol, total protein, sodium (Na), potassium (K), chloride (Cl), calcium (Ca) and inorganic phosphorus (P). Also the protein fractions were estimated by electrophoresis.

At approximately the same time overnight urine samples were obtained from all animals. These samples were examined for volume, appearance, pH (by pH meter), specific gravity (SG : by Atago refractometer), protein (by sulphosalicylic acid precipitation), total reducing substances (by Clinitest) and glucose, ketones, bilirubin, urobilin, nitrite and blood by N-Multistix (Ames Company, Slough, England). After centrifugation the deposit was examined microscopically.

In monkeys, observations for clinical signs and bodyweight recordings were made in the same way as for the rats. A daily measurement of food consumption was made for each monkey. Ophthalmoscopy was performed before treatment commenced, and in the fourth week, on all animals, by direct ophthalmoscope.

Once before commencement of treatment and again 24 hours after one dose in Week 4 individual electrocardiograph tracings were recorded from each monkey, using the three standard limb leads (1, 2 and 3) and the three augmented limb leads (aVR, aVL and aVF). The signal from a portable electrocardiograph ('Cardimax', Fukuda Denshi) was fed into a computer system (Life Science Research, England) and the following were measured from the averaged waveform for Lead 2 : Heart rate, wave intervals (PR, QRS, QT, ST), and amplitudes (P, R, S, T).

Blood samples were taken before commencement of treatment and during the fourth week. Samples were withdrawn from the femoral vein. The animals were starved overnight before sampling. Haematological, blood chemical and urinary examinations were made for the same parameters as for rats. Further samples were taken into citrate, and evaluated for prothrombin time (PT) and activated thromboplastin time (PTTK).

Pathological Examination : On completion of the scheduled treatment period all animals were killed by carbon dioxide inhalation (rats) or exsanguination under sodium pentobarbitone anaesthesia (monkeys). All animals were subjected to a detailed necropsy. Special attention was paid to the articular surfaces of the humerus and femur in rats and of the scapular glenoid cavity, humerus, proximal radius and ulna, acetabulum, femur, proximal tibia and patella in monkeys.

In rats, the weights of the adrenals, brain, caecum (with and without contents), epididymides, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, thymus and thyroid (with parathyroids) were recorded. Weights relative to bodyweight were calculated. Samples of the following tissues, taken from each animal, together with any abnormality identified at necropsy were preserved in buffered 4 % formaldehyde saline for histological evaluation : adrenals, brain, bronchi, caecum,

colon, duodenum, epididymides, eyes and optic nerves (using Davidson's fixative, followed by 70 % industrial methylated spirit), femur - distal, left and right heart, humerus - distal, left and right, ileum, jejunum, kidneys, liver (two lobes), lungs (two lobes), lymph nodes - cervical and mesenteric, mammary glands, oesophagus, ovaries, pancreas, pituitary, prostate (with urethra), rectum, salivary gland (left submandibular), sciatic nerve, seminal vesicles, skin, spinal cord, spleen, sternal bone and marrow, stomach (keratinized and glandular), testes, thymus, thyroid (with parathyroids), tongue, trachea, urinary bladder, uterine cervix, uterus, vagina.

The sections from the tissues were stained with haematoxylin and eosin or oil red O for fat. Microscopy was performed on all histological sections from all rats of the control and high dose groups and from those which died at blood sampling. Rats of the low and intermediate dosage groups were examined in respect of the liver, femur and humerus.

In monkeys, in addition to those weighed for rats, the submandibular salivary glands and uterus (with cervix) were weighed: the caecum, thymus and thyroid were not weighed in monkeys. Histological examination was performed on the gall bladder and peribronchial lymph nodes in addition to the tissues examined for rats: the seminal vesicles, humerus and femur were not examined for monkeys. Microscopy was performed on all histological sections from all monkeys.

Statistical Analysis: Data from treated and control animals were analyzed statistically using Student's *t* test, or for organ weights Dunnett's test with significance assessed at the $P < 0.05$ level.

RESULTS

In rats, one male and one female at 50 mg/kg/day group and two females at 200 mg/kg/day group died during anaesthesia for blood sampling.

On the first three days of treatment, transient generalized pallor and hypothermia were observed shortly after dosing in rats receiving 800 mg/kg/day and at this dosage salivation, associated with dosing, was observed throughout the treatment period. From Week 1 of treatment orange/brown body staining was observed in rats, particularly females, receiving 800 mg/kg/day. There was a less marked effect in some females receiving 200 mg/kg/day. High dosage rats produced more faeces and larger faecal pellets than controls, but faecal consistency was similar to that of the controls.

The food consumption of treated male rats was lower than that of the control males during the first week of treatment but not thereafter. The performance of the female controls in this respect was lower than expectation resulting in apparently higher food consumption for treated females. During the first week of treatment the bodyweight gain of males receiving 800 mg/kg/day was significantly lower than that of the control males but not thereafter (Fig. 1). In neither sex was the efficiency of food utilization affected. The eyes of treated rats were similar in appearance to

Fig. 1. Changes in body weight of rats during the oral administration of DR-3355 for 28 consecutive days.

those of the controls.

There were several changes in the haematology which could be associated with treatment. Higher total leucocyte counts were found in rats receiving 800 mg/kg/day than in their controls; this was associated with high lymphocyte counts (Table 1). The neutrophil counts were low in treated females and in males receiving 50 or 200 mg/kg/day and although within the normal range for rats of this age and strain at these laboratories (0.0 to 4.2 1000/cmm for males and 0.0 to 2.6 1000/cmm for females) they were markedly lower than the mean of the background data (2.0 ± 1.1 1000/cmm for males and 1.2 ± 0.7 1000/cmm for females). However, the differences from control values were not dosage-related. The prothrombin times for rats receiving 800 mg/kg/day were slightly shorter than in control but the difference was minimal and not thought to be toxicologically significant. In the bone marrow smears (Table 3) the myeloid to erythroid ratio of female rats receiving 800 mg/kg/day was significantly higher than in controls. Treated males were unaffected.

There were minor differences in blood chemistry values between treated and control rats (Table 4). Plasma chloride concentrations were slightly lower than in controls in treated females and in males receiving 800 mg/kg/day. These differences were statistically significant but they were not dosage-related. The potassium concentrations in rats receiving 200 or 800 mg/kg/day were also slightly lower than in controls but sodium concentrations were unaffected. Phosphorus concentrations in males and females receiving 800 mg/kg/day were significantly higher than in controls. There was a dosage-related reduction in the plasma urea concentration in all groups of treated female rats and in males treated at 200 or 800 mg/kg/day. Urinalysis did not reveal any inter-group variations considered to represent an effect

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Fig. 2. Changes in body weight of cynomolgus monkey during the oral administration of DR-3355 for 28 consecutive days.

of treatment (Table 6).

There was a statistically significant dosage-related increase in the full and empty caecum weights of treated males and females receiving 200 or 800 mg/kg/day (Table 7). Females receiving 50 mg/kg/day were not clearly affected in this respect. Calculation by difference indicated that the weights of the caecal contents were substantially greater, in the affected groups, than in the controls. The changes in

Table 1. Haematology – group mean values for rats after 3 weeks of treatment.

Group / sex	Dosage (mg/kg/day)	PCV %	Hb g%	RBC mil/ cmm	MCHC %	MCV cμ	MCH pg	Total	N	L	E	B	M	Plat- lets 1000 /cmm	PT secs
1 M SD	0	47 1	15.4 0.3	6.66 0.21	33 1	70 1	23 1	14.6 4.0	1.4 0.6	13.0 3.9	0.0 0.1	0.0 0.0	0.1 0.1	856 69	14.0 0.4
2 M SD	50	46 3	15.2 0.7	6.66 0.41	33 1	70 1	23 1	13.6 1.7	0.5 ^b 0.2	13.0 1.6	0.0 0.1	0.0 0.0	0.1 0.2	804 104	13.9 0.3
3 M SD	200	46 2	15.2 0.5	6.54 0.29	33 1	70 1	23 1	14.5 1.4	0.9 0.5	13.3 1.4	0.2 0.1	0.0 0.0	0.1 0.1	855 92	14.0 0.5
4 M SD	800	47 2	15.6 0.5	6.65 0.26	33 1	71 1	23 1	18.2 ^a 3.8	1.1 0.9	16.8 ^b 2.8	0.1 0.1	0.0 0.0	0.2 0.4	746 ^a 106	13.6 ^a 0.5
1 F SD	0	48 2	15.5 0.3	6.92 0.26	32 1	69 1	22 1	12.4 3.6	1.2 0.6	10.9 3.3	0.1 0.2	0.0 0.0	0.1 0.1	793 39	14.4 0.7
2 F SD	50	48 2	15.4 0.5	6.77 0.28	32 1	71 ^b 1	23 1	12.0 3.6	0.3 ^c 0.2	11.4 3.4	0.1 0.1	0.0 0.0	0.2 0.2	832 106	14.1 0.8
3 F SD	200	49 2	15.8 0.4	7.05 0.37	32 1	70 1	22 1	12.3 2.9	0.4 ^c 0.2	11.8 2.9	0.1 0.1	0.0 0.0	0.1 0.1	847 90	14.1 0.5
4 F SD	800	49 2	15.7 0.6	7.02 0.33	32 1	70 1	23 1	15.7 ^a 4.3	0.7 ^b 0.5	14.8 ^a 4.2	0.1 0.1	0.0 0.0	0.2 0.1	791 96	13.8 0.7

SD : Standard deviation.

a : Significantly different from controls, $P < 0.05$.b : Significantly different from controls, $P < 0.01$.c : Significantly different from controls, $P < 0.001$.

Table 2. Haematology – group mean values for monkeys after 3 weeks of treatment.

Group / sex	Dosage (mg/kg/day)	PCV %	Hb g%	RBC mil / cmm	MCHC %	MCV cμ	MCH pg	WBC 1000/cmm					Plat- elets 1000 /cmm	PT secs	PTTK secs	
								Total	N	L	E	B	M			
1 M	0	42	12.3	6.69	29	63	19	9.3	2.8	5.7	0.6	0.0	0.2	210	9.4	23.2
SD		3	0.7	0.83	1	5	2	1.6	1.6	0.8	0.5	0.0	0.2	103	0.5	1.9
2 M	10	41	12.6	6.27	30	66	20	10.0	4.8	4.9	0.2	0.0	0.1	214	9.7	21.1
SD		3	0.9	0.48	1	5	2	2.2	1.6	0.7	0.4	0.1	0.1	12	0.5	1.5
3 M	30	42	13.2	6.84	31 ^a	62	19	16.0 ^a	4.3	10.8 ^a	0.4	0.1	0.5	174	9.2	26.4 ^a
SD		3	1.1	0.57	1	3	1	5.1	2.3	3.4	0.4	0.1	0.3	10	0.1	0.7
4 M	100	39	12.1	6.35	31 ^a	63	19	11.8	4.3	7.0	0.2	0.1	0.2	209	9.5	22.3
SD		2	0.4	0.92	1	5	2	1.3	1.9	3.3	0.2	0.1	0.1	35	0.1	1.0
1 F	0	40	12.2	6.13	30	65	20	12.7	4.8	7.4	0.2	0.2	0.2	252	9.3	22.9
SD		1	0.3	0.19	1	4	1	3.4	0.4	3.3	0.1	0.2	0.3	8	0.2	1.7
2 F	10	40	12.5	6.36	31	63	20	9.6	4.8	4.4	0.1	0.0	0.2	152 ^b	9.2	24.8
SD		1	0.5	0.25	1	3	1	0.2	0.8	1.0	0.2	0.1	0.1	19	0.7	2.7
3 F	30	42	12.9	6.57	30	64	20	9.1	2.9	5.5	0.3	0.0	0.3	197	9.2	22.0
SD		2	0.5	0.45	1	2	1	1.9	0.8	1.2	0.3	0.1	0.3	25	0.2	3.0
4 F	100	41	12.8	6.56	31	63	20	10.2	5.2	4.3	0.2	0.2	0.4	211	9.7	21.9
SD		2	0.7	0.24	1	4	1	4.6	1.9	2.0	0.3	0.1	0.4	66	0.4	2.1

SD : Standard deviation.

a : Significantly different from controls, P<0.05

b : Significantly different from controls, P<0.01.

Table 3. Bone marrow - summary of findings for animals killed after 4 weeks of the treatment period.

Rats		Group and sex							
		1M	2M	3M	4M	1F	2F	3F	4F
Dosage (mg/kg/day)		0	50	200	800	0	50	200	800
Myeloid : erythroid ratio	Mean	2.0	1.8	1.7	1.8	1.8	1.9	1.9	2.4 ^c
	SD	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.4
Incidences									
Cellularity normal		9	8	10	8	10	7	5	9
Composition normal		8	8	10	8	9	7	5	6
Smear cells present		1	0	0	0	0	0	0	0
Increase in lymphoid series		1	0	0	0	1	0	0	2
Increase in myeloid series		0	0	0	0	0	0	0	3
Increase in eosinophils		0	0	0	0	0	0	0	1
Number of animals examined		9	8	10	8	10	7	5	9
Monkeys		Group and sex							
		1M	2M	3M	4M	1F	2F	3F	4F
Dosage (mg/kg/day)		0	10	30	100	0	10	30	100
Myeloid : erythroid ratio	Mean	1.3	1.2	1.8	1.5	1.4	1.4	1.5	1.4
	SD	0.1	0.3	0.5	0.3	0.2	0.1	0.1	0.2
Incidences									
Cellularity normal		3	3	3	3	3	3	3	3
Composition of cells normal		2	2	2	3	2	3	3	3
Increase in erythroid series		1	1	1	0	1	0	0	0
Number of animals examined		3	3	3	3	3	3	3	3

SD : Standard deviation.

^c : Significantly different from controls, $P < 0.001$.

Table 4. Blood chemistry – group mean values for rats after 3 weeks of treatment.

Group / sex	Dosage (mg/kg/day)	AP IU/1	ALT IU/1	AST IU/1	CPK IU/1	LDH IU/1	Urea mg%	Uric Acid mg%	Creatinine mg%	Glucose mg%	Triglycerides mg%	Phospholipids mg%	Total cholesterol mg%	Plasma Proteins g%					A/G ratio	Electrolytes mEq/1					
														Alb-umin	α ₁	α ₂	β	γ		Na	K	Cl	Ca	P	
1M	0	185	27	82	161	221	27	0.1	0.5	92	98	109	62	6.9	3.8	1.0	0.3	1.5	0.2	1.3	148	3.8	99	5.3	4.8
SD		36	3	13	42	113	5	0.1	0.1	10	25	12	10	0.3	0.1	0.1	0.1	0.1	0.0	0.1	1	0.2	3	0.1	0.3
2M	50	192	29	91	149	238	25	0.3 ^b	0.5	90	76	102	59	6.8	4.0	0.8 ^b	0.3	1.4	0.2 ^a	1.5 ^b	149 ^a	3.6	98	5.2	4.8
SD		57	5	15	25	51	5	0.2	0.1	22	31	9	8	0.2	0.2	0.1	0.1	0.1	0.1	0.2	2	0.2	2	0.2	0.3
3M	200	210	28	92	152	196	22 ^a	0.3 ^b	0.5	82	73	110	64	6.6 ^a	4.0	0.8 ^c	0.4	1.4	0.2	1.5 ^c	149	3.5 ^a	99	5.1 ^b	4.7
SD		40	3	14	28	39	5	0.2	0.1	10	20	12	8	0.2	0.2	0.1	0.1	0.1	0.0	0.1	1	0.3	2	0.1	0.2
4M	800	217	49	78	166	181	19 ^c	0.2	0.4	94	94	116	70 ^a	6.8	3.8	0.9	0.4	1.5	0.2	1.3	148	3.4 ^b	97 ^a	5.3	5.4 ^c
SD		56	49	8	49	34	3	0.1	0.1	16	32	6	5	0.2	0.3	0.1	0.1	0.2	0.0	0.2	1	0.3	1	0.1	0.3
1F	0	122	22	77	133	232	38	0.4	0.5	92	71	139	70	7.0	4.1	0.7	0.3	1.6	0.3	1.4	147	3.9	97	5.3	4.3
SD		31	4	8	28	114	9	0.3	0.0	6	21	20	13	0.3	0.2	0.1	0.1	0.1	0.1	0.1	1	0.4	3	0.2	0.4
2F	50	127	23	75	135	205	28 ^c	0.5	0.5	103 ^b	71	138	69	6.9	4.2	0.7	0.4 ^b	1.4 ^b	0.2 ^b	1.6 ^a	148	3.9	93 ^b	5.3	4.3
SD		26	3	7	47	103	3	0.2	0.1	9	21	27	16	0.3	0.2	0.2	0.1	0.2	0.0	0.3	2	0.4	3	0.2	0.3
3F	200	124	26	85	157	375	27 ^c	0.5	0.5	85	62	128	74	6.9	4.4 ^a	0.6 ^a	0.3	1.4 ^a	0.2 ^b	1.8 ^c	147	3.5	95 ^a	5.4	4.4
SD		23	4	18	38	154	4	0.2	0.1	8	11	16	9	0.2	0.2	0.1	0.1	0.1	0.0	0.1	5	0.2	2	0.2	0.2
4F	800	134	27 ^a	80	176	396	25 ^c	0.3	0.5	86	69	134	74	7.1	4.5 ^b	0.6 ^a	0.4	1.5	0.2 ^c	1.8 ^c	146	3.5 ^a	94 ^a	5.0 ^b	4.7 ^b
SD		26	7	15	120	493	2	0.2	0.0	11	18	29	19	0.4	0.4	0.1	0.1	0.1	0.1	0.1	2	0.4	3	0.2	0.2

SD : Standard deviation.

a : Significantly different from controls, P<0.05.

b : Significantly different from controls, P<0.01.

c : Significantly different from controls, P<0.001.

Table 5. Blood chemistry - group mean values for monkeys after 3 weeks of treatment.

Group / sex	Dosage (mg/kg/day)	AP IU/l	ALT IU/l	AST IU/l	CPK IU/l	LDH IU/l	Urea mg%	Uric Acid mg%	Creat- inine mg%	Glu- cose mg%	Trig- lyce- rides mg%	Phos- phol- ipids mg%	Total Chole- sterol mg%	Plasma Proteins g%						A/G ratio	Electrolytes mEq/l				
														Alb- Total	umin	α1	α2	β	γ		Na	K	Cl	Ca	P
1M	0	567	44	52	50	265	52	0.0	0.9	73	56	192	143	9.1	4.7	0.8	0.7	2.3	0.7	1.1	150	4.7	107	5.0	3.8
SD		57	13	5	16	4	18	0.0	0.3	30	15	25	34	0.3	0.3	0.2	0.4	0.4	0.1	0.1	2	0.3	4	0.2	0.6
2M	10	570	33	29 ^b	49	306	53	0.0	0.8	54	90	202	151	9.1	4.4	0.9	0.7	2.3	0.7	0.9	146	4.8	108	4.9	3.7
SD		81	10	9	8	91	4	0.0	0.1	9	7	32	27	0.5	0.3	0.2	0.3	0.7	0.1	0.1	4	0.2	1	0.3	0.3
3M	30	690 ^a	31	21 ^c	43	240	45	0.0	1.0	66	67	212	158	9.2	4.3	0.8	0.9	2.5	0.7	0.9	152	5.1	109	5.2	3.3
SD		46	13	2	23	25	11	0.0	0.3	15	46	44	16	0.4	0.1	0.0	0.3	0.1	0.1	0.0	5	0.8	2	0.2	0.1
4M	100	558	19 ^a	25 ^c	39	230	49	0.0	0.8	63	82	187	112	8.8	4.9	0.6	1.2	1.5 ^a	0.6	1.2	151	4.5	108	5.2	3.4
SD		63	10	7	17	21	9	0.0	0.1	4	26	12	7	0.2	0.4	0.0	0.3	0.4	0.1	0.2	6	0.2	3	0.3	0.5
1F	0	544	24	24	67	253	37	0.0	0.8	77	65	197	153	9.1	4.2	0.7	0.9	2.5	0.7	0.9	148	4.9	110	5.0	2.8
SD		55	13	3	29	3	9	0.0	0.1	13	39	63	44	0.6	0.2	0.1	0.1	0.4	0.1	0.1	2	0.8	7	0.3	0.6
2F	10	524	138	41	80	259	52	0.0	0.9	71	71	206	161	9.1	4.5	0.7	0.7	2.4	0.7	1.0	148	5.0	110	5.1	3.2
SD		40	137	17	37	48	11	0.0	0.1	8	14	23	7	0.6	0.1	0.1	0.2	0.2	0.1	0.1	4	0.5	5	0.3	0.5
3F	30	582	39	61 ^b	332	362	50	0.0	0.9	67	71	202	147	9.0	4.4	0.8	0.7	2.3	0.8	0.9	148	5.4	106	5.1	3.4
SD		220	12	16	185	74	2	0.0	0.1	15	14	19	11	0.5	0.1	0.2	0.3	0.4	0.2	0.2	4	0.6	3	0.2	0.5
4F	100	406	27	40	497	301	41	0.0	0.9	61	60	167	118	8.6	4.3	0.7	0.9	2.0	0.7	1.0	148	4.9	105	5.0	3.0
SD		107	15	11	679	78	8	0.0	0.1	3	10	7	11	0.0	0.5	0.1	0.4	0.7	0.1	0.2	2	0.7	3	0.0	0.6

SD : Standard deviation.

a : Significantly different from controls, $P < 0.05$.b : Significantly different from controls, $P < 0.01$.c : Significantly different from controls, $P < 0.001$.

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Table 6. Urinalysis – group mean values after 3 weeks of treatment.

Species	Group / sex	Dosage (mg/kg/day)	Volume ml	pH	SG	Group / sex	Dosage (mg/kg/day)	Volume ml	pH	SG
Rat	1M	0	5.0	6.6	1043	1F	0	3.0	6.0	1059
	SD		1.5	0.2	6	SD		1.0	0.2	20
	2M	50	4.0	6.6	1050	2F	50	3.0	6.1	1062
	SD		1.0	0.3	13	SD		1.0	0.2	15
	3M	200	4.5	6.5	1052	3F	200	3.0	6.4 ^b	1055
	SD		1.5	0.3	15	SD		0.5	0.2	11
	4M	800	3.0 ^b	6.4	1072 ^c	4F	800	3.5	6.1	1063
	SD		1.0	0.3	14	SD		1.0	0.3	17
Monkey	1M	0	30	8.0	1024	1F	0	32	8.3	1026
	SD		16	0.3	7	SD		6	0.2	2
	2M	10	14	8.4	1033	2F	10	27	8.1	1027
	SD		5	0.5	3	SD		4	0.4	3
	3M	30	26	8.3	1030	3F	30	30	8.0	1026
	SD		8	0.1	3	SD		7	0.2	6
	4M	100	24	7.4	1031	4F	100	22 ^a	7.5	1027
	SD		9	1.4	12	SD		2	1.7	4

SD : Standard deviation.

a : Significantly different from controls, P<0.05.

b : Significantly different from controls, P<0.01.

c : Significantly different from controls, P<0.001.

Table 7. Absolute organ weights (g) for rats killed after four weeks of treatment (group mean values).

Dosage (mg/kg/day)		Group and sex							
		1M	2M	3M	4M	1F	2F	3F	4F
		0	50	200	800	0	50	200	800
Bodyweight	$\bar{x}$	321.7	313.6	327.3	327.9	190.9	205.1	209.9	214.1 ^b
	SD	23.7	23.5	22.1	26.3	17.9	17.6	19.6	10.7
Brain	$\bar{x}$	1.87	1.92	1.92	1.90	1.71	1.72	1.78	1.76
	SD	0.12	0.10	0.06	0.09	0.05	0.09	0.09	0.08
Pituitary	$\bar{x}$	0.008	0.009	0.008	0.008	0.009	0.010	0.011	0.011
	SD	0.002	0.002	0.001	0.002	0.002	0.003	0.003	0.002
Adrenals	$\bar{x}$	0.042	0.038	0.040	0.048	0.053	0.058	0.056	0.066 ^a
	SD	0.005	0.006	0.005	0.009	0.008	0.009	0.013	0.014
Epididymides	$\bar{x}$	0.645	0.634	0.662	0.645				
	SD	0.061	0.073	0.065	0.063				
Heart	$\bar{x}$	1.07	1.08	1.07	1.01	0.73	0.77	0.74	0.73
	SD	0.09	0.11	0.06	0.09	0.05	0.10	0.09	0.06
Kidneys	$\bar{x}$	2.81	2.79	2.80	2.91	1.71	1.82	1.83	1.82
	SD	0.22	0.29	0.29	0.39	0.16	0.16	0.10	0.15
Liver	$\bar{x}$	15.8	16.3	15.6	15.8	8.7	9.3	10.1 ^a	10.2 ^b
	SD	1.5	2.0	1.1	2.3	0.9	0.9	1.3	0.9
Ovaries	$\bar{x}$					0.070	0.077	0.073	0.071
	SD					0.007	0.024	0.008	0.014
Prostate	$\bar{x}$	0.335	0.312	0.304	0.281				
	SD	0.074	0.093	0.103	0.050				
Spleen	$\bar{x}$	0.670	0.644	0.688	0.742	0.442	0.449	0.459	0.504
	SD	0.083	0.099	0.117	0.061	0.053	0.061	0.080	0.064
Testes	$\bar{x}$	2.78	2.87	2.95	2.84				
	SD	0.16	0.15	0.17	0.21				
Thymus	$\bar{x}$	0.655	0.613	0.660	0.636	0.501	0.473	0.611	0.523
	SD	0.139	0.148	0.108	0.099	0.090	0.071	0.135	0.092
Thyroids	$\bar{x}$	0.017	0.018	0.023	0.017	0.011	0.010	0.010	0.011
	SD	0.003	0.006	0.006	0.003	0.004	0.004	0.003	0.002
Caecum (full)	$\bar{x}$	4.23	7.29 ^b	10.85 ^b	14.79 ^b	4.02	5.24	11.21 ^b	15.22 ^b
	SD	0.91	1.13	2.34	2.49	4.30	1.40	2.96	4.37
Caecum (empty)	$\bar{x}$	0.92	1.25 ^b	1.47 ^b	1.74 ^b	0.67	0.90	1.39 ^b	1.58 ^b
	SD	0.13	0.11	0.29	0.26	0.10	0.18	0.28	0.21

SD : Standard deviation.

a : Significantly different from controls, $P < 0.05$.b : Significantly different from controls, $P < 0.01$.

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Table 8. Absolute organ weights (g) for monkeys killed after four weeks of treatment (group mean values).

Dosage (mg/kg/day)		Group and sex							
		1M	2M	3M	4M	1F	2F	3F	4F
		0	10	30	100	0	10	30	100
Bodyweight	$\bar{x}$	2086.7	2020.0	2050.0	2000.0	1963.3	1906.7	1933.3	1956.7
	SD	166.2	158.7	177.8	26.5	231.6	181.8	210.8	147.4
Brain	$\bar{x}$	60.6	52.4	58.9	60.0	54.2	55.4	56.7	53.7
	SD	2.4	19.7	8.2	3.0	7.1	3.3	2.9	3.8
Pituitary	$\bar{x}$	0.040	0.027	0.025 ^a	0.032	0.033	0.031	0.039	0.034
	SD	0.005	0.005	0.006	0.009	0.003	0.002	0.006	0.004
Adrenals	$\bar{x}$	0.482	0.389	0.393	0.394	0.420	0.398	0.391	0.607
	SD	0.066	0.071	0.043	0.080	0.035	0.046	0.106	0.366
Epididymides	$\bar{x}$	0.53	0.43	0.50	0.41				
	SD	0.18	0.24	0.11	0.18				
Heart	$\bar{x}$	7.6	8.0	8.3	7.8	7.2	7.6	8.3	7.7
	SD	1.0	0.7	0.9	0.8	1.7	1.9	0.8	0.5
Kidneys	$\bar{x}$	8.5	9.0	7.3	9.1	8.6	8.1	7.9	9.0
	SD	1.9	0.7	0.8	0.4	0.7	0.8	2.0	0.8
Liver	$\bar{x}$	47.0	50.0	47.0	48.0	55.0	47.0	50.0	55.0
	SD	7.0	5.0	7.0	3.0	23.0	13.0	8.0	15.0
Lungs	$\bar{x}$	10.3	10.3	9.8	11.2	9.2	8.9	10.0	9.9
	SD	1.1	1.4	0.8	1.2	0.9	1.2	1.3	0.8
Ovaries	$\bar{x}$					0.228	0.156	0.231	0.198
	SD					0.012	0.019	0.093	0.060
Prostate/ urethra	$\bar{x}$	0.19	0.19	0.16	0.21				
	SD	0.11	0.07	0.04	0.04				
Salivary glands	$\bar{x}$	1.63	1.42	1.68	1.31	1.37	1.39	1.43	1.66
	SD	0.04	0.08	0.35	0.28	0.11	0.30	0.63	0.19
Spleen	$\bar{x}$	6.0	6.9	4.8	6.9	5.4	4.5	5.9	5.4
	SD	2.4	2.2	1.8	1.5	1.4	0.2	1.7	1.8
Testes	$\bar{x}$	0.87	0.67	0.86	0.72				
	SD	0.30	0.30	0.23	0.22				
Thymus	$\bar{x}$	4.31	4.60	5.22	3.75	3.40	3.01	2.11	2.89
	SD	1.85	1.28	2.13	0.39	1.60	0.40	1.58	0.64
Uterus + Cervix	$\bar{x}$					3.87	5.25	6.31	5.44
	SD					1.78	0.89	1.15	4.09
Thyroids	$\bar{x}$	0.311	0.275	0.350	0.294	0.265	0.299	0.213	0.321
	SD	0.027	0.051	0.167	0.125	0.047	0.040	0.088	0.094

SD: Standard deviation.

a : Significantly different from controls, $P < 0.05$.

caecal weight were marked ; at the high dosage the values obtained for full caeca or contents were some three times that of the controls. Although the caeca were enlarged, as evidenced by their greater weights, their appearance was not macroscopically abnormal. The absolute liver weights of females receiving 200 or 800 mg/kg/day were higher than those of the controls but there was no inter-group difference when analysis relative to bodyweight was performed.

Microscopic examination showed changes in the femur and humerus (Table 9) considered to be related to treatment. Minimal or slight articular degeneration was seen in the femur or humerus of a small number of male rats receiving DR-3355 at a dosage of 800 mg/kg/day and in the femur of a small number of females which had received 800 mg/kg/day. Acute inflammation of the left femoral condyle was seen in one male which had received 800 mg/kg/day. No similar changes of the humerus or femur were seen in the animals which had received lower dosages or in control rats. Although there were no identical changes in untreated animals, two control male rats showed minor vascularization of the articular cartilage of the femur as did one female which had received the high dosage. Minimal to slight periacinar fine vacuolation was seen in all groups of male rats which had received DR-3355 but the incidence was only statistically significant in those which had received 800 mg/kg/day. This change was not seen in any female rats. The results of Oil-Red-O staining of liver sections from animals receiving 800 mg/kg/day indicated that this vacuolation was not attributable to fat within the hepatocytes of this region. A non-statistically significant incidence of hepatocytic hypertrophy was seen in male rats which had received DR-3355 at dosages of 800 mg/kg/day and 200 mg/kg/day and females which had received 800 mg/kg/day.

In monkeys, a red colouration of the urine was seen in a few animals receiving 10 or 30 mg/kg/day, and in all females receiving 100 mg/kg/day. These events were transient ; only one animal (high dose female) was consistently affected. Although a number of these incidences could be associated with menstrual bleeding, many could not. At urinalysis in the fourth week of treatment although the samples from two monkeys were visibly brown, there was no positive reaction for the presence of blood. All monkeys receiving 100 mg/kg/day suffered diarrhoea, as did one female at 10 and another at 30 mg/kg/day. Salivation, associated with dosing, was seen in all females receiving 100 mg/kg/day. Food consumption was unaffected by treatment, but minor bodyweight losses were seen in all animals receiving 100 mg/kg/day (Fig. 2). In the case of one female this weight loss was clearly abnormal (a loss of 11 % of starting bodyweight). The eyes of treated monkeys were unaffected and their electrocardiographic traces were normal. There were no treatment-related inter-group differences in the haematology or blood chemistry examinations (Tables 2, 3 and 5).

Two high dose animals (one male, one female) had unusually acidic urine. The colour of the urine was brown in two high dosage females, and the test for blood was negative (group mean values for volume, pH and specific gravity are given in Table

Table 9. Histopathology – group distribution of findings for rats killed after four weeks of treatment.

Organ and finding description	Dosage (mg/kg/day)	Group and sex							
		1M	2M	3M	4M	1F	2F	3F	4F
		0	50	200	800	0	50	200	800

6).

The weights of the organs examined were unaffected by treatment with DR-3355 (Table 8), with the exception of the adrenal weights in one high dosage female, which were abnormally high. Macroscopic findings at necropsy and microscopic findings in the tissues examined revealed no changes resulting from treatment.

DISCUSSION

In rats, animals receiving DR-3355 showed salivation and a disturbance in faecal constitution. The increased size and number of faecal pellets produced by high dosage animals may be associated with their higher caecum weights. These observations may reasonably be attributed to the administration of an antibacterial agent. It has been proposed that the enlarged caecum found in animals treated with antibiotics is a consequence of the accumulation of water, induced by changes of the flora contributing to water transport (Savage and Dubos, 1968).

The reduced neutrophil counts in the blood were observed at all dosages. Examination of bone marrow smears indicated increases in the myeloid series in three high dosage females, implying an increased genesis of neutrophils; this may represent a compensating reaction to the neutropenia. However, this change was not dosage-related, and its toxicological significance is therefore in doubt. There was no other evidence of an effect of treatment on any other component of the haemopoietic system.

The plasma electrolyte balance was slightly affected by treatment in respect of potassium and phosphorus. These changes were minor in extent, and not associated with any change in urinary composition, for instance, or any evidence of renal toxicity.

There was evidence for a minor effect on the liver. This was manifested in the plasma as lower urea concentrations which may indicate reduced liver synthetic capability. The finding of periportal fine vacuolation of hepatocytes is uncertain toxicological significance especially as the vacuoles did not contain fat. This may simply represent agonal (hypoxic) change. Hepatocytic hypertrophy, seen in a number of rats treated at 200 or 800 mg/kg/day is a minor change and is not considered to be toxicologically significant.

The possibly degenerative changes in the articular surfaces were not associated with any effect on growth. They were seen at a low incidence in high dosage animals only and were of minimal or slight degree. The presence of this change is, however, considered significant because blister formation in the articular cartilage is well known to be a consequence of quinolone treatment (Kato and Onodera, 1988).

In monkeys, animals treated with DR-3355 at 100 mg/kg/day was associated with minor reductions in bodyweight, some clinical signs of limited toxicological significance, and changes in urinary composition. The reductions in bodyweight were, in general, small but statistically significant. One female was particularly

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affected, and suffered an unusual degree of weight loss. Such bodyweight reduction has also been reported in monkeys treated with NY-198, the most recent quinolone, for 13 weeks (Nomura et al., 1988).

The clinical signs of salivation and diarrhoea were non-specific. Salivation is frequently observed at dosing in oral gavage studies, and diarrhoea is not unexpected during treatment with an antibacterial material. The degree of diarrhoea was not serious or life-threatening.

The changes in urinary composition included discolouration and acidic pH. There was no biochemical or morphological change to suggest renal toxicity. Although crystalluria and morphological changes of the kidney have been reported in rats given enoxacin, norfloxacin or cinoxacin (Takemoto et al., 1984 ; Corrado et al., 1987 ; Narama et al., 1980), DR-3355 failed to induce these changes in the rat or the monkey.

High adrenal weights noted in one female were unusual, but there was no histopathological correlate, and so no toxicological significance is attached to this finding.

There are some differences in the pharmacokinetics of DR-3355 in rats and monkeys. The serum levels of the drug have been shown to attain a peak about 3 or 0.5 hours after oral administration of 20 mg/kg to monkeys or rats respectively. In addition, the maximum concentration and half-life in monkeys are about two times those in rats. Because the pharmacokinetics in monkeys resemble those in humans (unpublished data), toxicological data taken from monkeys seem to be helpful in anticipating any potential adverse effects of DR-3355 in humans.

REFERENCES

- Atarashi, S., Yokohama, S., Yamazaki, K., et al. (1987) : Synthesis and anti-bacterial activities of optically active ofloxacin and its fluoromethyl derivative. *Chem. Pharm. Bull.*, **35**, 1896-1902.
- Christ, W., Lehnert, T. and Ulbrich, B. (1988) : Specific toxicological aspects of the quinolones. *Rev. Infect. Dis.*, **10** (Suppl. 1), S141-146.
- Corrado, M. L., Struble, W. E., Peter, C., et al. (1987) : Norfloxacin : Review of safety studies. *Am. J. Med.*, **82** (Suppl. 6B), 22-26.
- Hayakawa, I., Atarashi, S., Yokohama, S., et al. (1986) : Synthesis and anti-bacterial activities of optically active ofloxacin. *Antimicrob. Agents and Chemother.*, **29**, 163-164.
- Imamura, M., Shibamura, S., Hayakawa, I., et al. (1987) : Inhibition of DNA gyrase by optically active ofloxacin. *Antimicrob. Agents and Chemother.*, **31**, 325-327.
- Kato, M. and Onodera, T. (1988) : Morphological investigation of cavity formation in articular cartilage induced by ofloxacin in rats. *Fundam. Appl. Toxicol.*, **11**, 110-119.

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- Narama, I., Sano, M., Tsuchiya, M., et al. (1980) : Acute and subacute toxicity studies of cinoxasin. *Chemotherapy*, **28** (Suppl. 4), 406–439.
- Nomura, G., Watanabe, M., Nakanishi, H., et al. (1988) : Thirteen-week oral toxicity study of NY-198 in monkeys. *Chemotherapy*, **36** (Suppl. 2), 371–390.
- Savage, D. C. and Dubos, R. (1968) : Alterations in the mouse cecum and its flora produced by antibacterial drugs. *J. Exp. Med.*, **128**, 97–110.
- Takemoto, Y., Senda, H., Nakano, S., et al. (1984) : Toxicological studies of AT-2266. II. Subacute and chronic toxicity studies in rats. *Chemotherapy*, **32** (Suppl. 3), 199–220.