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Metabolism of YH1885 by Rat, Dog, Monkey and Human Liver S9 Fractions

Eun Joo KIM, Jung Koo ROH and Carol GREEN[1]

Korea Research Institute of Chemical Technology. P.O. Box 107, Yusung, Taejon 305-606, Korea,

[1]SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025-3493, USA

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Abstract – YH1885 (5,6-dimethyl-2-(4-fluorophenylamino)-4-(1-methyl-1,2,3,4-tetrahydroisoquinolin-2-yl) pyrimidine hydrochloride) was developed as an antiulcer drug. The objective of this study was to examine a comparative metabolism of YH1885 in rat, dog, monkey and human liver tissues and to determine the metabolite profiles produced by the four species. YH1885 was metabolized by liver S9 fractions from all four species. Control incubations containing S9 fraction but no cofactors, contained essentially no metabolites. Metabolism of YH1885 apparently became saturated in the concentration range studied because the % of YH 1885 metabolized decreased with increasing drug concentration for all four species. Six to nine metabolite peaks were detected in the incubations and the particular profile of metabolites varied with species. The total amount of metabolites formed by liver microsomes from human and monkey were less than microsomes from rat or dog. The major metabolite peak formed by rat liver S9 fractions eluted near the solvent front on the HPLC or remained at the origin in TLC, indicating that it contained one or more polar metabolites. Dog liver S9 fractions incubations contained four major metabolites that each accounted for about 15 to 20 % of the total radioactivity at the low concentration of YH1885. The metabolite profiles of YH1885 appeared to be similar in incubations with rhesus monkey and human liver S9 fraction. The amount of metabolites formed by rhesus monkey liver preparations was greater than that of human liver that contained prominent metabolite peaks with approximate relative retention time of 0.14 and 0.43.

Keywords □ Comparative metabolism, YH1885, Antiulcer drug

YH1885 (5,6-dimethyl-2-(4-fluorophenylamino)-4-(1-methyl-1,2,3,4-tetrahydroisoquinolin-2-yl) pyrimidine hydrochloride, of which structure is shown below, was developed by Yuhan corporation, as a prototype new class of proton pump inhibitor which prossesses potent ulcer healing effect. The objective of this study was to compare metabolism of YH1885 in S9 (post-mitochondrial supernatant from centrifugation at 9,000×g) fractions prepared from rat, dog, monkey and human livers. The metabolite profiles were then determined by analyzing these samples by HPLC with radiochemical detection. The results obtained were then examined to determine which species metabolizes the drug most similarly to human.

MATERIAL AND METHODS

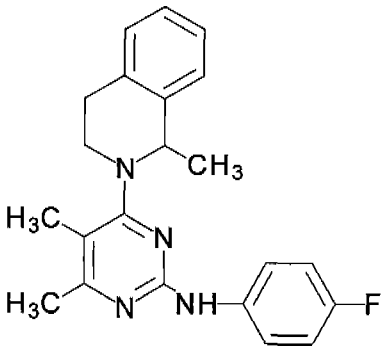
Test Articles

YH1885 and the radiolabelled compound (specific activity, 65 mCi/mg) were provided by Yuhan Research Center, Yuhan corporation.

Liver Specimens

Rat

Adult male Sprague-Dawley rats were used in this study for the preparation of liver microsomes. The liver tissue was acquired from these animals immediately after sacrifice, placed on ice, and transferred to the laboratory.



*To whom correspondence should be addressed.

Liver specimens were frozen and stored at about -80± 5℃ or below until use.

Dog

Adult male beagle dogs were used in this study for the preparation of liver microsomes. These animals were untreated controls from other studies. The liver tissues were acquired from these dogs immediately after death at necropsy, placed on ice, and transferred to the laboratory. Liver specimens were frozen and stored at -80 ±5℃ or below until use.

Monkey

Adult male and female monkeys were used for the preparation of liver S9 fraction. Untreated controls from other studies were used. The liver tissue was acquired from these animals immediately after death at necropsy, placed on ice, and transferred to the laboratory. Liver specimens were frozen and stored at -80±5℃ or below until use.

Human

Adult male and female human liver specimens were acquired through cooperation with organ procurement organizations from brain-dead human organ donors. Tissues not used for transplant were processed as for transplant. The organs were perfused in the operating room with ice-cold organ preservation solution. The tissues were then shipped to laboratory by the most expedient method. Upon receipt, the tissues were frozen in liquid nitrogen and stored at -80±5℃ or below until the preparation of S9 fraction.

Preparation of Liver S9 FractionN

Liver tissue was thawed and rinsed with ice-cold 1.15 % KCl to remove excess blood, then finely minced. The minced tissues were added to 3 volumes of 100 mM phosphate buffer, pH 7.4 and homogenized with a Polytron tissue homogenizer for about 30 s. The homogenates were then centrifuged at 9000×g at 4±4℃ for 20 min to obtain a 25% S9 fraction. The supernatant was removed and stored at -80±5℃ or below until S9 was used in incubations with YH1885.

Preparation and Administration of test Article

YH-1885 and radiolabelled compound (specific activity, 65 mCi/mg) were provided by Yuhan Research Center, Yuhan corporation. YH1885 was dissolved in DMSO and added to the incubations such that the final DMSO concentration was equal to or less than 0.1%. The final concentrations of YH1885 were 10 and 100 μM.

Incubation of S9 with test Article

Stock solutions of the cofactors were prepared in 100 mM phosphate buffer, pH 7.4 at the concentrations listed below and stored frozen at about -20℃ until use. The incubation samples were prepared to include the following:

Components	Volume (ml)	Final Concentration
Liver S9	[a]	10 mg
100 mM phosphate buffer, pH 7.4, containing:	0.10	100 mM
8 mM $NADP^+$		0.8 mM
80 mM Glucose-6-phosphate		8.0 mM
100 mM Nicotinamide		10.0 mM
500 IU/ml Glucose-6-phosphate	0.002	1 IU
0.05 M $MgCl_2$	0.10	5 mM
100 mM phosphate buffer,		[a]
YH1885 in DMSO	0.001	10 or 100 μM
Final volume	1.0 ml	

[a]The volume of buffer was adjusted to give the correct protein concentration and final volume.

Each sample was preincubated at 37℃, with shaking at about 60-70 osc/min, for 5 min. The reaction was started by addition of the S9 fraction. Control incubations containing test article, S9 fraction and buffer, but no cofactors were also included and terminated at the final time point. Duplicate incubations with radiolabelled compound were terminated at 0, 30, and 60 min by addition of 2.5 ml of acetonitrile. The samples were centrifuged to pellet precipitated protein. The supernatants were removed and concentrated to dryness under reduced pressure. The samples were stored at -20±4℃.

Analysis of Metabolite Profiles

In the first experiment using rat liver S9 fraction, the 60 min time point samples were analyzed by both HPLC and TLC to determine which would provide better resolution of the parent compound and metabolites.

For HPLC, one of the duplicate samples was reconstituted in mobile phase and analyzed on the HPLC using the following initial conditions:

Column: Waters μ-Bondapak C18 (3.9×300 nm steel)
Mobile Phase: 0.02 N phosphate buffer, pH 7.4: methanol (80:20)
Flow Rate: 1 ml/min
Detection: Radiochemical detection; UV at 270 nm.

The other duplicate sample of the 60 min time point

sample was analyzed by TLC. An authentic standard of cold YH1885 was applied to Merck TLC (silica gel 60F 254, No. 5715, 20×20 cm) plates as a band of approximately 4 cm in width and then dried with a stream of nitrogen. Each sample was applied over the authentic standard as band of approximately 3 cm in width. The TLC plates were then placed in tanks that had been equilibrated with the mobile phase (chloroform/acetone; 1:1; v:v) and the solvent was allowed to advance to within 3 cm of the top of the plate. TLC plates were then removed from the tanks, the solvent front marked, and the mobile phase was allowed to evaporate.

The radioactivity on the TLC plates was then quantitated by using a phosphor imager (Molecular Dynamics) to determine the amount of radioactivity in each spot. The data was calculated to indicate the percentage of the total radioactivity found in spots corresponding with the parent compound or metabolite found in the entire 1 ml incubation.

The data from both analytical systems were compared. Because the HPLC method allowed the separation of more polar peaks than the TLC technique, HPLC was selected for analysis of the remainder of the samples from the other time points and other species.

Cytochrome P450 Content and Activity

As a control to indicate the presence of cytochrome P 450 associated activity, 7-ethoxycoumarin O-deethylation (ECOD) activity was determined in each preparation of S9. The preparations were incubated with 500 μM 7-ethoxycoumarin for 15 min. The incubation mixtures were assayed for hydroxycoumarin production using the fluorometric method of Greenlee and Poland. ECOD activity was calculated as the amount of hydroxycoumarin produced/min/mg protein. Protein was assayed by the dye binding technique.

The total cytochrome P450 content was determined from the dithionite difference spectra of carbon monoxide-treated samples, using the extinction coefficient of 91 mM^{-1} cm^{-1} for P450, according to the method of Omura and Sato.

RESULTS AND CONCLUSIONS

The data on the individual human liver donors is shown in Table I and the results of ECOD activity and cytochrome P450 content for the rat, dog, monkey and human liver microsomes preparation that were used in this study, are summarized in Table II. YH1885 was metabolized by liver S9 fraction from all four species. Control incubations containing S9 fraction but no cofactors, contained essentially no metabolites. Rat liver S9 fraction incubations at the 60 min time point were examined by both HPLC and TLC with radiochemical detection. Both techniques showed that a large percentage of the radioactivity was converted to polar forms that eluted very early from the HPLC (metabolite R-A) and stayed at the origin of the TLC plates (spot 1, origin). By HPLC, 9 separate peaks were detected including YH 1885. TLC analysis detected 14 spots including the orgin and YH1885. The amount of YH1885 detected by the two methods was similar. Based on these results, it was decided that either technique could be used to study the metabolite profile of YH1885 and HPLC technique was selected for the additional analysis. The major metabolite (R-A) formed by rat liver S9 fraction is very polar based on its early elution from in the chromatogram and constituted as much as 35 to 40% of the total radioactivity after an 1 hr incubation.

Seven other metabolite peaks were detected and quantitated. Metabolite R-C was the next most abundant metabolite, with as much as 7% of the total radioactivity

Table I. Human Liver Specimen Information

Specimen No.[a]	Cause of Death	Ethnic Information[b]	Sex	Age
H65 (H1)	Motor vehicle accident	C	M	24
H71 (H2)	Intracranial bleed	C	F	63

[a]The first specimen number is the one used for the liver bank. The number in parentheses is the code number used to identify this specimen in this particular study. [b]C=Caucasian.

Table II. Characteristics of Liver S9 Fraction

Species	Specimen No.	Protein (mg/ml)[a]	ECOD (pmol/mg protein/min)	P450 (pmol/mg protein)
Rat	R1	22.7	266.7	267
	R2	33.2	275.0	210
Dog	D1	29.8	788.6	119
	D2	27.1	908.3	97
Monkey	RM1	31.7	703.8	245
	RM2	33.2	682.0	87
Human	H1	22.8	108.9	32
	H2	23.8	77.7	49

[a]The protein values presented indicated the concentration of S9 protein in the preparations before dilution for this study. Incubations with YH1885 were conducted with diluted S9 fraction to gave final protein concentrations of 10 mg/ml.

Table III. Summary of Metabolism of YH1885 by Rat Liver S9 Fraction

	Time (min)	Metabolite or parent Compound (% of total radioactivity[2])								
		[1]R-A	R-B	R-C	R-D	R-E	R-F	R-G	R-H	YH1885
10 μM	0	2.82	0.00	0.00	0.95	0.00	0.00	0.00	0.00	94.54
	30	26.64	2.79	0.80	1.79	2.88	6.43	1.20	2.16	52.59
	60	37.20	2.94	7.12	0.89	2.89	5.66	2.24	0.00	32.54
Control	60	5.48	0.57	0.00	0.00	0.00	1.73	0.00	0.00	88.93
100 μM	0	0.85	0.00	0.00	1.01	0.00	0.00	0.34	0.00	94.80
	30	14.05	4.01	0.00	1.41	1.58	3.94	0.27	0.55	71.31
	60	26.15	2.53	5.35	0.62	1.77	3.80	1.88	1.72	49.91
Control	60	4.06	0.00	0.00	0.64	0.00	0.00	0.00	0.00	92.28

[1]R=Rat. [2]Total radioactivity was expressed in dpm.

Table IV. Metabolism of YH1885 by Rat Liver S9 Fraction

Spot on TLC	YH1885 (% of total radioactivity[1])	
	10 μM	100 μM
Origin	28.14	17.59
2	2.38	1.52
3	5.01	5.06
4	1.49	1.16
5	1.78	0.00
6	6.32	4.52
7	2.54	2.98
8	4.39	3.11
9	6.13	3.09
YH1885	33.89	55.89
11	2.11	1.22
12	4.40	3.59
13	1.27	1.06
14	0.00	0.00

[1]Total radioactivity was expressed in dpm.

found in the peak (Table III and IV). Table V presented the metabolite profile from dog liver S9 fraction incubations that contained 11 peaks including YH1885. At 10 μM YH1885, four metabolite peaks, D-A, D-B, D-C and D-G, were found to contain more than 10% of the total radioactivity after 60 min. YH1885 was metabolized rapidly by dog liver S9 with approximately 15% of the total radioactivity remaining after 60 min at the low concentration. Table VI showed that nine peaks, including YH1885 were found in incubations with rhesus monkey liver S9 fraction. The metabolite profile was also evenly distributed with five peaks containing 3 to 5% of the total radioactivity of the low concentration after 60 min. Approximately 55% of the radioactivity remained as YH1885 at the end of 60 min incubation with 10 μM of the drug. Table VII showed that seven peaks, including YH1885 obtained from human liver microsomes. The major metabolites were H-A, H-E and H-F, about 3% of the total radioactivity was found in each of these peaks after 60 min incubation with the low concentration, 80 to 90% of the total radioactivity remained as the parent compound at the end of 60 min. The total amount of metabolites formed by liver microsomes from human and monkey were less than microsomes from rat or dog. Metabolism of YH1885 by all four species apparently became saturated between 10 and 100 μM as evi-

Table V. Summary of Metabolism of YH1885 by Dog Liver S9 Fraction

	Time (min)	Metabolite or parent Compound (% of total radioactivity[2])										
		[1]D-A	D-B	D-C	D-D	D-E	D-F	D-G	D-H	D-I	D-J	YH1885
10 μM	0	1.28	1.14	0.72	0.40	0.74	0.00	0.00	0.34	0.28	0.26	93.29
	30	11.67	14.84	12.52	0.00	2.02	3.52	19.84	3.85	1.70	0.00	27.78
	60	20.66	22.74	14.48	2.46	2.23	2.69	13.79	4.67	1.55	0.36	13.54
Control	60	2.09	1.54	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.00	94.03
100 μM	0	3.35	0.82	0.00	0.26	1.40	0.00	0.00	0.00	0.00	0.00	93.07
	30	6.23	8.91	6.54	0.00	1.92	1.57	10.61	1.65	0.00	0.00	61.04
	60	10.52	13.93	9.08	0.96	1.60	1.92	10.62	2.46	0.00	0.00	48.18
Control	60	1.83	0.78	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	94.73

[1]D=Dog. [2]Total radioactivity was expressed in dpm.

Table VI. Summary of Metabolism of YH1885 by Rhesus Monkey Liver S9 Fraction

	Time (min)	Metabolite or parent Compound (% of total radioactivity[2])								
		[1]M-A	M-B	M-C	M-D	M-E	M-F	M-G	M-H	YH1855
10 μM	0	0.95	0.00	0.55	0.00	0.94	0.00	0.00	0.00	95.32
	30	2.76	1.55	3.12	1.52	3.30	2.48	0.57	0.41	76.19
	60	5.36	5.05	6.73	2.35	4.67	2.74	0.77	0.70	54.26
Control	60	2.84	0.76	0.00	0.00	0.73	0.51	0.00	0.89	92.94
100 μM	0	1.44	0.12	0.00	0.00	1.00	0.00	0.00	0.00	94.77
	30	3.34	0.98	1.84	1.13	1.84	1.86	0.39	0.19	80.76
	60	3.72	2.97	2.94	1.23	2.15	1.78	0.58	0.24	71.98
Control	60	0.98	0.30	0.00	0.00	0.00	0.00	0.00	0.00	97.29

[1]M=Monkey. [2]Total radioactivity was expressed in dpm.

Table VII. Summary of Metabolism of YH1885 by Human Liver S9 Fraction

	Time (min)	Metabolite or parent Compound (% of total radioactivity[2])						
		[1]H-A	H-B	H-C	H-D	H-E	H-F	YH1885
10 μM	0	0.68	0.00	0.00	0.00	0.77	0.00	95.07
	30	1.62	0.08	0.17	0.22	1.06	2.01	93.25
	60	2.37	0.14	1.01	0.34	3.21	3.28	88.29
Control	60	1.18	0.00	0.00	0.00	0.26	0.00	97.83
100 μM	0	0.72	0.00	0.00	0.00	1.19	0.00	96.49
	30	1.88	0.00	0.24	0.00	1.80	1.45	93.23
	60	1.71	0.54	0.51	0.31	2.35	2.10	92.40
Control	60	1.12	0.00	0.00	0.00	0.28	0.00	98.13

[1]H=Human. [2]Total radioactivity was expressed in dpm.

denced by a decrease in the % of the total radioactivity in the metabolite peaks and an increase in the % of the total radioactivity remaining as the parent compound with the higher drug concentration. There was essentially no evidence of metabolism in the control samples which contained S9 fraction and YH1885, but no cofactors. Fig. 1 shows the example chromatogram from each species. Relative retention times were calculated to correlate individual peaks from each species although there was some variability in these values for the different runs. Therefore letters were assigned to each peak but it is not possible from these experiments to definitively determine whether a peak found in incubations with one species is the same metabolite formed by another species. The major metabolite peak formed by rat liver S9 fraction eluted near the solvent front on the HPLC or remained at the origin in TLC, indicating that it contained one or more polar metabolites, possibly glucuronide or sulfate conjugates. All other metabolites formed by rat liver S9 were relatively minor in comparison to R-A. The metabolite profile for the other three species also contained early-eluting peaks, suggesting that this metabolite or metabolites may be produced in all four species. Dog liver S9 fraction incubations contained two peaks, D-A and D-B, that had relative retention times similar to the rat metabolite, R-A. Two other significant metabolites were formed by dog liver S9 fraction, D-C and D-G, each containing a-

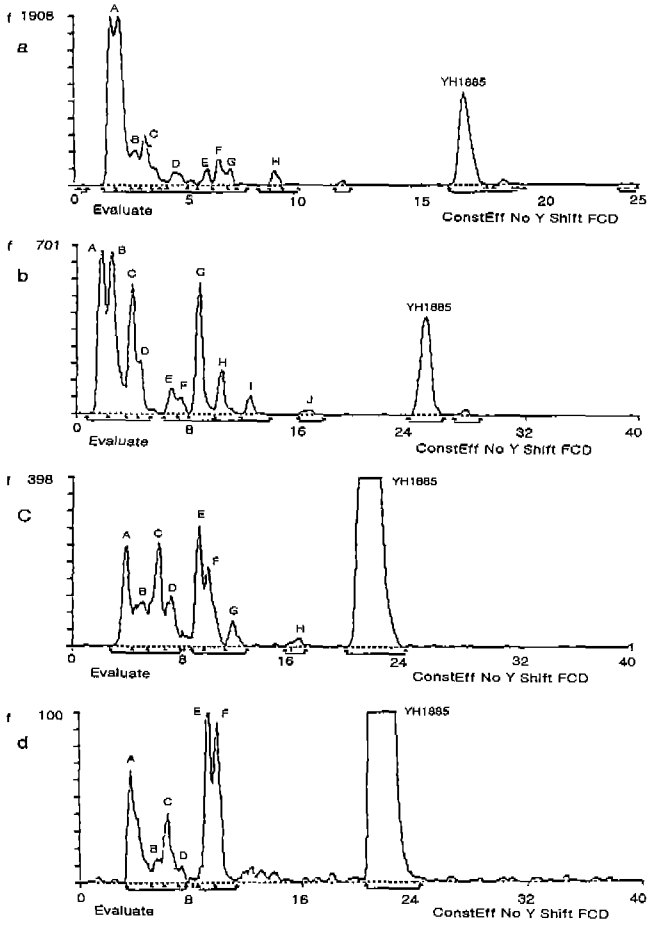


Fig. 1. Example chromatograms from incubations of YH1885 with S9 from rat (a) dog (b), rhesus monkey (c) and human (d) liver.

bout 15% of the radioactivity in the incubations with 10 μM YH1885. The metabolite profiles of YH1885 appeared to be similar in incubations with rhesus monkey and human liver S9 fraction. The amount of metabolites formed by rhesus monkey liver preparations was greater than that of human liver but both species exhibited profiles that contained prominent metabolite peaks with approximate relative retention times of 0.41 (M-E or H-E) and 0.43 (M-F and H-F). The relative quantities of the metabolites varies in these two species but the same 6 metabolites were the most significant for both species. However, additional studies would be require to definitively determine whether the same metabolites are formed by both species.

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