Chem. Pharm. Bull.
36(11)4462—4466(1988)

Metabolism of Barbaloin by Intestinal Bacteria

MASAO HATTORI,*[a] TOMOYUKI KANDA,[a] YUE-ZHONG SHU,[a]
TERUAKI AKAO,[b] KYOICHI KOBASHI[b]
and TSUNEO NAMBA[a]

Research Institute for Wakan-Yaku[a] and Faculty of Pharmaceutical Sciences,[b]
Toyama Medical and Pharmaceutical University,
2630 Sugitani, Toyama 930-01, Japan

(Received May 9, 1988)

The *C*-glucosyl bond of barbaloin, a major purgative principle of aloe, was cleaved with human intestinal bacteria under anaerobic conditions, yielding aloe-emodin anthrone and aloe-emodin bianthrone. Fecal flora of humans had the most potent transforming activity but those of rats and mice had less or no activity.

Keywords——aloe-emodin anthrone; barbaloin; intestinal bacteria; metabolism; purgative

Intestinal flora plays an important role in the manifestation of purgative action of popular bianthrone laxatives such as sennosides A and B from senna and rhubarb.[1-11] Sennosides are transformed to an ultimate purgative principle, rhein anthrone, through a combination of enzymic reaction by bacterial β-glucosidase and non-enzymic cleavage by reduced riboflavin-cofactors produced by bacterial enzymes in the presence of reduced nicotinamide adenine dinucleotide (NADH).[9-11] On the other hand, participation of intestinal bacteria in the action of anthrone *C*-glucoside laxatives such as barbaloin, chrysaloin and cascarosides has not yet been clarified. In addition, there has been no evidence that intestinal bacteria are capable of metabolizing anthrone *C*-glucosides. Barbaloin has been reported to have weaker purgative action than sennosides and related compounds, and there is considerable variation in response among animals.[12,13] Since intestinal flora varies from species to species,[14] this led us to speculate that such differences in the response to barbaloin would be due to differences in bacterial species and populations, capable of transforming barbaloin to aloe-emodin anthrone in the alimentary tract.

Materials and Methods

Instruments——Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Ultraviolet (UV) spectra were taken on a Shimadzu UV-210A spectrometer. Nuclear magnetic resonance (NMR) spectra were measured with JEOL JNM-FX 270 (^{1}H, 270 MHz) and FX 90Q (^{13}C, 22.5 MHz) spectrometers, and chemical shifts are given as δ values relative to tetramethylsilane as an internal standard. Mass spectra (MS) were measured with a JEOL JMS D-200 mass spectrometer at an ionization voltage of 70 eV. Densitometric profiles were recorded on a Shimadzu CS-910 dual wavelength thin-layer chromatoscanner (TLC-scanner).

Chemicals——Barbaloin was purchased from Wako Pure Chemical Industry Ltd. (Osaka) and purified by column chromatography on silica gel, followed by recrystallization from EtOH. Aloe-emodin was purchased from Carl Roth Inc. (West Germany). Aloe-emodin anthrone was prepared from barbaloin according to the method of Kinget. [15] Aloe-emodin bianthrone was obtained by air oxidation of aloe-emodin anthrone.[16]

Microorganisms——The following intestinal bacterial strains from humans were used for screening of bacteria capable of transforming barbaloin to aloe-emodin anthrone: *Bacteroides fragilis* ss. *thetaotus*, *Bifidobacterium adolescentis*, *B. breve* S-2 KZ 1287, *B. bifidum* a E319, *B. longum* IV-55, *B. pseudolongum* PNC-2-9-G, *Clostridium*

butyricum, *C. innocuum* ES 24-06, *C. innocuum* KZ 633, *C. perfringens* To-23, *Escherichia coli* 0-127, *Fusobacterium nucleatum* G-0470, *Gaffkya anaerobia* G-0608, *Klebsiella pneumoniae* ACTT 13883, *Lactobacillus acidophilus* ATCC 4356, *L. brevis* II-46, *L. fermentum* ATCC 9338, *L. plantarum* ATCC 14917, *L. xylosus* ATCC 155775, *Peptostreptococcus anaerobius* 0240, *P. intermedius*, *Proteus mirabilis* S2 and *Streptococcus faecalis* II-136.

Preparation of an Intestinal Bacterial Mixture——Fresh feces obtained from healthy men, rats (Wistar and Donryu rats) and mice (ddY, BALB/C and C_3H mice) were thoroughly suspended in twenty-five volumes of 0.1 M phosphate buffer (pH 7.4) by bubbling with oxygen-free CO_2 gas to eliminate air. The fecal suspension was filtered with gauze and the filtrate was used as a bacterial mixture.

Incubation of Barbaloin with Intestinal Bacterial Mixture and Quantitative Analysis of Metabolites——Tubes containing barbaloin (5 mg) in dimethyl sulfoxide (DMSO) (0.8 ml) and a bacterial mixture from feces (5 ml) were incubated at intervals at 37 °C in an anaerobic jar, in which air was replaced with oxygen-free CO_2 gas in the presence of activated steel wool (steel wool method).[17] The mixture was adjusted to pH *ca.* 3 with dilute HCl, and extracted twice with AcOEt (5 ml each). The AcOEt layer was concentrated to dryness *in vacuo* and the residue was dissolved in the same solvent (0.5 ml). An aliquot (10 μl) of the solution was immediately applied to a silica gel TLC plate, which was developed with solvent A ($CHCl_3$: MeOH : AcOH : H_2O = 18 : 5 : 0.75 : 0.5). The spots of metabolites were analyzed quantitatively with a TLC-scanner at 260 nm (reference wavelength of 700 nm) by using calibration lines obtained with authentic samples. The calibration lines were linear in the ranges of 5—50, 0.5—1.5, 1—13, 1—10 μg/spot for barbaloin, aloe-emodin, aloe-emodin anthrone and aloe-emodin bianthrone.

Isolation of Aloe-emodin Anthrone and Aloe-emodin Bianthrone——Barbaloin (50 mg in 0.8 ml of DMSO) was incubated with a bacterial mixture from human feces (50 ml) for 24 h at 37 °C. The incubation mixture was acidified with HCl and extracted twice with AcOEt (50 ml each), and the AcOEt phase was washed with water, and concentrated to a small volume *in vacuo*. The mixture was applied to a preparative TLC plate of silica gel, which was developed with solvent B (toluene : cyclohexane : *n*-propanol = 3 : 6 : 2). Metabolites A-1 and A-2 were isolated.

Metabolite A-1: Yellow needles, 8 mg, mp 168 °C. UV λ_{max}^{MeOH} nm: 257, 293, 355, λ_{sh} 265. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 4.33 (2H, s, 10-H_2), 4.73 (2H, s, -CH_2OH), 6.88 (1H, br s, 2-H), 6.89 and 6.90 (each 1H, d, J = 8.0 Hz, 7-H and 5-H), 6.92 (1H, br s, 4-H), 7.48 (1H, t, J = 8.0 Hz, 6-H), 12.28 and 12.30 (each 1H, s, OH × 2). ^{13}C-NMR (22.5 MHz, $CDCl_3$) δ: 33.3 (t, C-10), 64.6 (t, -CH_2OH), 113.1 (d, C-2), 115.7 (d, C-7), 116.4 (d, C-4), 118.8 (d, C-5), 136.2 (d, C-6). MS *m/z*: 256 (M^+, base peak). The ^{1}H- and ^{13}C-NMR spectral data agreed with the reported values of aloe-emodin anthrone.[18]

Metabolite A-2: Yellow powder, 2 mg; a mixture consisting of A-2a (67%) and A-2b (33%). The peaks of A-2a (major) and A-2b (minor) in the ^{1}H- and ^{13}C-NMR spectra agreed with the reported values for the *meso* and *trans* forms of aloe-emodin bianthrone, respectively.[19]

Screening of Bacterial Strains for Ability to Metabolize Barbaloin——A defined stock strain of intestinal bacteria was inoculated into GAM broth (10 ml), followed by a 24 h anaerobic cultivation at 37 °C. The cultured broth was diluted 10-fold with the same medium and incubated for 24 h. The culture was then centrifuged at 7000 rpm for 10 min. The precipitates were washed with 0.9% NaCl solution (10 ml), collected by centrifugation and suspended in 0.1 M phosphate buffer (10 ml, pH *ca.* 7.4). A solution of barbaloin (5 mg) in DMSO (200 μl) was added to the bacterial suspension. After a 48 h incubation at 37 °C in an anaerobic jar, the mixture was acidified and extracted twice with AcOEt (10 ml each). The products were quantitatively analyzed by TLC-densitometry as described above.

Results

Metabolites of Barbaloin by Human Intestinal Bacteria

Anaerobic incubation of barbaloin with a bacterial mixture of human feces (human fecal flora) resulted in the formation of metabolites, which showed two spots (*Rf*s 0.66 and 0.60) on TLC with solvent system A. Metabolite A-1 having a higher *Rf* value showed *m/z* 256, corresponding to $C_{15}H_{11}O_4$, in the MS. The ^{1}H- and ^{13}C-NMR spectra were superimposable on those of authentic aloe-emodin anthrone. In addition, metabolite A-1 was readily oxidized to aloe-emodin and aloe-emodin bianthrone on the TLC plate when exposed to air for a long period. Metabolite A-2 having a lower *Rf* value, however, gave two spots (*Rf*s, 0.18 and 0.15, respectively) on TLC with a different solvent system (solvent system B), and was identified as a mixture of *meso* and *trans* forms (at 10–10′) of aloe-emodin bianthrone from the ^{1}H- and ^{13}C-NMR spectra. The ratio of *meso* and *anti* forms was determined to be 2 : 1 on the basis of the relative intensities of the proton signals at δ 5.82 and 4.62 in the ^{1}H-NMR spectrum. These signals were ascribed to 10, 10′-H of the *meso* and *anti* forms, respectively, as reported by Geiger.[19]

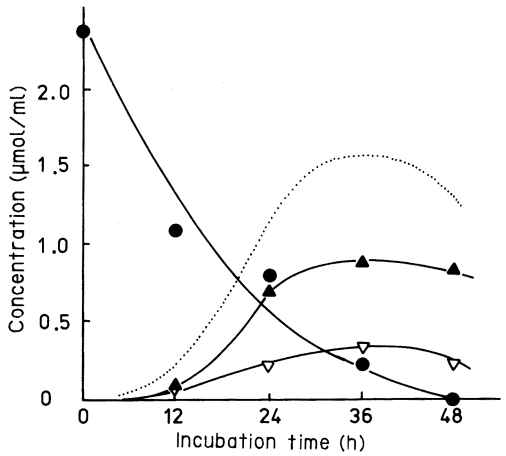

Fig. 1. Time Course of Metabolite Formation by a Bacterial Mixture from Human Feces

Barbaloin was anaerobically incubated at 37 °C with a bacterial mixture from human feces and the products were quantitatively analyzed by TLC-densitometry. (●—●), barbaloin; (▲—▲), aloe-emodin anthrone; (▽—▽), aloe-emodin bianthrone; (------), the sum of aloe-emodin anthrone and aloe-emodin bianthrone ×2.

TABLE I. Comparison of Metabolic Activity of Barbaloin among Fecal Flora from Humans, Rats and Mice

Incubation (h)	Aloe-emodin anthrone (%)		
	Source of fecal flora		
	Human	Rats[a)]	Mice[b)]
12	3.3±1.1	n.d.	n.d.
24	29.6±2.7	n.d.	n.d.
36	36.9±8.1	1.6±2.0	n.d.
48	35.3±9.7	7.1±2.5	n.d.

Each value represents the mean±S.D. ($n=5$); *a*) two Wistar rats (male, 6 weeks old) and three Donryu rats (male, 4—7 weeks old); *b*) two ddY mice (male, 4 weeks old), two BALB/C mice female, 7—11 weeks old) and one C_3H mouse (female, 7 weeks old); n.d., not detected.

Time Course of the Formation of Metabolites A-1 and A-2

Figure 1 shows the time course of metabolite formation during anaerobic incubation of barbaloin with human fecal flora. Metabolites A-1 and A-2 (mixture of A-2a and A-2b) were produced starting from *ca.* 12 h and became maximal at 36 h, with a corresponding decrease of barbaloin. Since aloe-emodin bianthrone seems to be formed from aloe-emodin anthrone by air-oxidation, the net aloe-emodin anthrone produced was expected to be as much as 65% of added substrate at 36 h of incubation, as represented by the dotted line in Fig. 1.

Barbaloin-Metabolizing Activity of Fecal Flora from Humans, Rats and Mice, and of Defined Strains of Human Intestinal Bacteria

Although fecal flora of humans transformed barbaloin to aloe-emodin anthrone under anaerobic conditions, those of rats and mice showed less or no activity to metabolize it (Table I). Furthermore, a variety of intestinal bacteria from humans, including one *Bacteroides*, five *Bifidobacterium*, three *Clostridium*, four *Lactobacillus*, two *Peptostreptococcus* and other species examined (see Experimental), showed no appreciable metabolizing activity. Among them, *Bifidobacterium adolescentis* and *Clostridium perfringens* produced small amount of aloe-emodin, an oxidized product of aloe-emodin anthrone, in yields of *ca.* 1%.

Discussion

Human intestinal bacteria showed potent activity to metabolize barbaloin to aloe-emodin anthrone. The reaction included a novel type of *C*-glucosyl bond cleavage possibly through reductive processes by anaerobic bacteria. During these metabolic experiments, aloe-emodin bianthrone and aloe-emodin were also detected but these are expected to be artificially formed as in the case of the sennoside metabolism (rhein anthrone produced is readily converted to rhein and sennidin during extraction procedures). Aloe-emodin anthrone seems to be, therefore, the sole metabolite of barbaloin by intestinal bacteria (Chart 1). An attempt

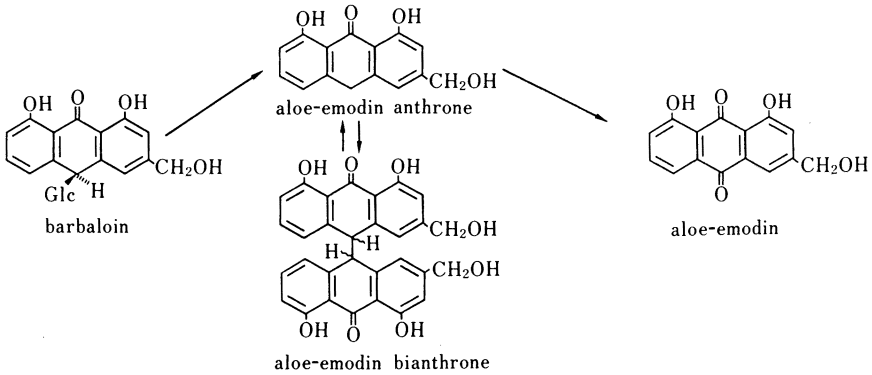

Chart 1. Possible Metabolic Pathways of Barbaloin by Human Intestinal Bacteria

to identify bacterial species capable of metabolizing barbaloin was unsuccessful (we examined 23 different species of human intestinal bacteria). This may be because (1) these bacterial strains possess little or no metabolizing activity and other intestinal bacteria responsible for the metabolism are present in the human feces, (2) these bacterial strains have lost their metabolic activity toward *C*-glucosides during long-term repeated cultivation, or (3) a combination of different bacterial species is necessary for the metabolic processes. Judging from the metabolic activities of the feces, the bacteria possessing the potent metabolizing activity are present in those of healthy human subjects but not in the feces of rats and mice.

It has been reported that the purgative potency of barbaloin significantly varies among animal species (humans, rabbits, guinea pig, rats and mice)[12,13]; mice are particularly insensitive to barbaloin. In addition, large doses of barbaloin were reported to be necessary for purgation in rats.[20] The purgative potency of powdered aloe, in which barbaloin is regarded as a major purgative principle, was also reported to be reduced by pretreatment with tetracycline,[20] suggesting a role of intestinal bacteria in the manifestation of purgative action.[20]

Our present results imply that intestinal bacteria take part in the metabolic activation of barbaloin, at least in humans, and a metabolite, aloe-emodin anthrone, is responsible for purgative action, similar to rhein anthrone in the case of sennosides.[1-6]

The *C*-glucosides widely occurring in the plant kingdom are generally resistant to acid hydrolysis and bacterial cleavage, compared to *O*-glucosides. However, we have recently observed that a *C*-glucosyl bond of homo-orientin (*C*-glycosyl flavone) is cleaved by human intestinal bacteria,[21] and the bacterial elimination of the *C*-glucosyl residue in barbaloin is the second example.

References

1) J. W. Fairbairn and M. J. R. Moss, *J. Pharm. Pharmacol.*, **22**, 584 (1970).
2) J. W. Fairbairn, *Pharm. Weekbl.*, **100**, 1493 (1965).
3) H. Ippen and T. Montag, *Arch. Exp. Path. Pharmak.*, **182**, 521 (1936).
4) K. Sasaki, K. Yamauchi and S. Kuwano, *Planta Medica*, **37**, 370 (1979).
5) J. Lemli and L. Lemmens, *Pharmacology*, **20**, 50 (1980).
6) M. Dressen, H. Eyssen and J. Lemli, *J. Pharm. Pharmacol.*, **33**, 679 (1981).
7) K. Kobashi, T. Nishimura, M. Kusaka, M. Hattori and T. Namba, *Planta Medica*, **40**, 226 (1980).
8) M. Hattori, G. Kim, S. Motoike, K. Kobashi and T. Namba, *Chem. Pharm. Bull.*, **30**, 1338 (1982).
9) Te. Akao, T. Akao, K. Mibu, M. Hattori, T. Namba and K. Kobashi, *J. Pharmacobio-Dyn.*, **8**, 800 (1985).
10) T. Akao, K. Mibu, T. Erabi, M. Hattori, T. Namba and K. Kobashi, *Chem. Pharm. Bull.*, **35**, 1998 (1987).
11) M. Hattori, T. Namba, T. Akao and K. Kobashi, *Pharmacology*, **36** (Suppl. 1), 172 (1988).
12) J. W. Fairbairn, *Pharmacology*, **14** (Suppl. 1), 48 (1976).

13) F. H. L. Van Os., *Pharmacology*, **14** (Suppl. 1), 18 (1976).
14) T. Mitsuoka, "A Color Atlas of Anaerobic Bacteria," Sobunsha, Tokyo, 1980, pp. 27—29.
15) R. Kinget, *Planta Medica*, **15**, 233 (1967).
16) Von A. Schaltegger, *Arzneim. Forsch.*, **35**, 666 (1985).
17) T. Mitsuoka, *Kansensho Gakkaishi*, **45**, 406 (1971).
18) M. Tamano and J. Koketsu, *Nihon Kagaku Kaishi*, **1982**, 1918.
19) W. Geiger, *Chem. Ber.*, **107**, 2976 (1974).
20) Y. Ishii, H. Tanizawa, C. Ikemoto and Y. Takino, *Yakugaku Zasshi*, **101**, 254 (1981).
21) M. Hattori, Y. Shu, T. Tomimori, K. Kobashi and T. Namba, *J. Nat. Products*, "in press" (1988).