BIOLOGICAL PROPERTIES OF RISTOCETIN-Ψ-AGLYCONE

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Ristocetin-Ψ-aglycone obtained by acid hydrolysis of ristocetin A, has a substantially greater antimicrobial activity against Gram-positive bacteria than the parent compound. None of the substances are active against Gram-negative bacteria, yeast or fungi.

The Ψ-aglycone is several times more toxic than ristocetin A when administered intravenously. Both substances are well-tolerated when given subcutaneously, intraperitoneally and perorally. Both ristocetin A and the Ψ-aglycone have a very low absorption after oral administration.

Plasma levels following intravenous administration of ristocetin A and the Ψ-aglycone are comparable, with both showing a rapid decline during the first 60 minutes followed by a somewhat slower elimination. The aggregating properties of the Ψ-aglycone could not be determined due to its low solubility at neutral pH.

Ristocetin A[1], a glycopeptide antibiotic produced by *Nocardia lurida*, belongs to the vancomycin group of antibiotics[2]. The structure of ristocetin A has recently been elucidated[3~6]. The antibiotic consists of a central heptapeptide (the aglycone) and 6 sugars (mannose (2 mole), glucose, arabinose, rhamnose and ristosamin) attached to the aglycone at 3 separate sites. Ristocetin A interferes with the formation of peptidoglycan moieties in the cell wall by a mechanism involving complexation with peptides terminating in D-Ala-D-Ala[7].

The ability of ristocetin A to cause aggregation of blood platelets contributed to the discontinuation of its use in antibiotic therapy. This effect, however, made it a useful tool for diagnosing VON WILLEBRAND's disease. Patients with this disease lack a plasma protein component which is required for ristocetin A-induced aggregation.

The Ψ-aglycone of ristocetin A consists of the heptapeptide and the aminosugar ristosamin[6]. It is prepared from ristocetin A by acid hydrolysis[8]. Several years ago, PHILIP *et al.*[9] found that acid degradation products of ristocetin A showed a substantial increase in both *in vitro* and *in vivo* activity. Recently KUWAHARA and CHAMBERS[10] were able to confirm these results. They also found that the acid hydrolysate of ristocetin A was unable to induce platelet aggregation.

This paper presents some microbiological and toxicological properties of ristocetin-Ψ-aglycone and discusses some aggregation studies.

Materials and Methods

Samples

Ristocetin A (lot 013–2 and lot 016, H. Lundbeck & Co. A/S) with a potency of 1,000 μg/mg was used. The Ψ-aglycone was prepared according to the method described by RAJANANDA *et al.*[8] and the purity was established by HPLC.

HPLC

HPLC was performed on a Waters Associates machine using reverse phase columns. Column:

Spherisorb S5 ODS, 4.6 mm ϕ, 25 cm. Eluted with 0.01 M KH_2PO_4 - MeOH, 70: 30, 1.5 ml/minute. Detection: UV 254 nm.

Antimicrobial Activity

Antimicrobial spectra were determined by 10-fold serial dilution assay. Medium: Tryptose phosphate broth (Difco). Inoculum size 10^5/ml, incubation 34°C, 24 hours.

Toxicological Studies

Male NMRI, BOM, SPF mice (body weight 23~27 g) and male WIST: MOL, SPF rats (body weight 145~160 g) were used. The animals were offered a standard laboratory pelleted diet (Rostock, new mixture). Tap water, adjusted to pH 2~3, was given *ad libitum*. Housing was conventional, with room temperature maintained at 21±1°C and the relative humidity at 55±5%. The air was changed 16 times per hour. An artificial day and night cycle of 12 hours was established. After dosing, the animals were housed individually in wire mesh cages.

The LD_{50} values in mice were determined using oral, subcutaneous, intraperitoneal and intravenous administration. The intravenous injections were given by a constant flow for 30 seconds and 5 minutes. In rats the intravenous injections were given during 5 and 30 minutes, to assay possible toxicity due to rate of injection. The injections were given manually except the 30-minute injection, which was given by means of a SAGA syringe pump with individual syringes. The animals were fixed in wire mesh tubes during the 5-minute and the 30-minute intravenous injections. The injection volume was 10 ml/kg and observation time was 24 hours.

Plasma Level Determination

Oral Absorption Study: Ristocetin A (10,000 mg/kg) and the Ψ-aglycone (8,000 mg/kg) were each administered orally to a group of ten mice. The mice were anaesthetized by chloroform and killed by vacutainer bleeding from the caudal *vena cava* 15, 30, 60, 120 and 240 minutes after the injection.

Elimination Study: Two groups of fifteen rats were given 50 mg/kg i.v. of ristocetin A and the Ψ-aglycone. The rats were anaesthetized by ether and vacutainer blood samples were obtained from the abdominal aorta at 0 (110~185 seconds), 10, 20, 40, and 120 minutes after the injection.

The concentration of the Ψ-aglycone in plasma was determined by broth dilution method as described under "antimicrobial activity". Ristocetin A was determined by agar diffusion assay using Seed Agar (BBL, Antibiotic Medium A). In both assays *M. flavus* ATCC 10240 was used as test organism. Incubation at 34°C, 24 hours.

Aggregation Test

Preparation of Platelet-rich Plasma (PRP) and Platelet-poor Plasma (PPP): Whole blood was collected from a healthy donor, diluted 9:10 with ACD-solution* and spun at 300×*g* for 10 minutes, whereafter the top layer of PRP was collected. After respinning at 2,000×*g* for 15 minutes, the top layer of PPP was collected. 100 mg ristocetin A in 3.3 ml of 0.9% NaCl solution was used as a standard. A Bryston aggregometer with recorder was used for the test. The aggregation procedure was carried out at 37°C, motor speed 1,100 rpm., aggregometer cuvette ϕ 9.5 mm. The aggregometer was calibrated with PPP and PRP to 100% and 5~15% transmission respectively. 0.95 ml of PRP was transferred to a cuvette, which was placed in the instrument and allowed to equilibrate to 37°C. 50 μl of the standard/sample solution was added whereafter aggregation was recorded for 6 minutes (final concentration of standard/sample is 1.5 mg/ml).

Results and Discussion

Antimicrobial Activity

The antimicrobial spectra of ristocetin A and the Ψ-aglycone are presented in Table 1. Table 1 shows that ristocetin A and the Ψ-aglycone are both active against Gram-positive bacteria, with no acti-

* ACD-solution (anticoagulant citrate phosphate dextrose solution): Citric acid monohydrate 3.3 g/liter, sodium citrate dihydrate 26.3 g/liter, sodium dihydrogenphosphate monohydrate 2.2 g/liter, and dextrose monohydrate 20.0 g/liter.

Table 1. Antimicrobial spectra of ristocetin A and Ψ-aglycone.

Organism	MIC (μg/ml)	
	Ristocetin A	Ψ-Aglycone
Staphylococcus aureus ATCC 6538 P	10	1
Staphylococcus epidermidis ATCC 12228	10	0.1
Streptococcus faecalis ATCC 10541	10	1
Bacillus subtilis ATCC 6633	10	1
Micrococcus flavus ATCC 10240	0.1	<0.01
Micrococcus luteus ATCC 9341	1	0.1
Corynebacterium xerosis NCTC 9755	1	0.1
Escherichia coli NCTC 86	>100	>100
Klebsiella pneumoniae ATCC 10031	>100	>100
Brucella bronchiseptica ATCC 4617	>100	>100
Pseudomonas aeruginosa NCTC 2000	>100	>100
Proteus vulgaris NCTC 10015	>100	>100
Candida albicans PD 04600	>100	>100
Penicillium chrysogenum ATCC 10002	>100	>100

Table 2. Ristocetin A and Ψ-aglycone. LD_{50} values in mice obtained after different routes of administration.

Route of administration	Mean (95% confidence limits)	
	Ristocetin A (mg/kg)	Ψ-Aglycone (mg/kg)
Intravenous 30 seconds	900	111 (84~148)**
Intravenous 5 minutes	—	199 (166~238)**
Oral	>12,500*	>8,000
Intraperitoneal	>600	>600
Subcutaneous	>1,200	>1,200

* Value from HWANG[11].

** Statistically significantly different $p<0.05$ (FINNEY: Probit Analysis, 1952)[12].

Table 3. Ristocetin A and Ψ-aglycone. LD_{50}-values in rats.

Route of administration	Mean (95% confidence limits)	
	Ristocetin A (mg/kg)	Ψ-Aglycone (mg/kg)
Intravenous 30 seconds	767 (442~1,330)	152 (126~184)
Intravenous 30 minutes	>975*	133 (111~160)

* The infusion had to be stopped due to venous occlusion. This happened when 80% of the volume had been administered.

vity against Gram-negative bacteria, yeast and fungi. Thus, the antimicrobial spectra of the 2 substances proved identical, but the activity of the Ψ-aglycone was substantially greater.

Toxicology

The LD_{50} values obtained are shown in Tables 2 and 3. By weight the Ψ-aglycone was several times more toxic than ristocetin A following intravenous administration in both mice and rats. Slow intravenous injection (5 minutes) gave a statistically significant reduction ($p<0.05$) of the toxicity of the Ψ-aglycone in mice compared to fast intravenous injection. In the study with rats the toxicity was not lowered by slow administration (30 minutes).

Like ristocetin A[11], the Ψ-aglycone had very low toxicity when given orally indicating that the absorption was very limited. Following intraperitoneal administration the LD_{50} in mice was above 600 mg/kg for both ristocetin A and the Ψ-aglycone. Doses of 1,200 mg/kg of both substances were well-tolerated when given subcutaneously to mice. Local cutaneous lesions developed, however, apparently due to irritation.

Oral Absorption Study

Absorption of the compounds was very low with less than 10 μg/ml found in the plasma after 1/4 ~ 2 hours when doses of 10,000 mg/kg of ristocetin A and 8,000 mg/kg of the Ψ-aglycone were given.

Elimination Study

Plasma levels of ristocetin A and the Ψ-aglycone were studied in rats after intravenous administration of 50 mg/kg. Initial peak levels of 400~600 μg/ml declined rapidly during the first 60 minutes to 60~100 μg/ml for both substances. After 2 hours about 50 μg/ml were found of both ristocetin A and the Ψ-aglycone. Thus, plasma levels after intravenous doses of ristocetin A and the Ψ-aglycone were quite comparable.

Aggregation

It is well known that ristocetin A causes platelet aggregation, but it was not possible to demonstrate whether the Ψ-aglycone causes aggregation or not due to its low solubility at pH 7. The observation of KUWAHARA and CHAMBERS[10] that mild acid hydrolysis of ristocetin A destroys its ability to induce platelet aggregation could not be confirmed. In our hands, the substance tested after acid hydrolysis by the method described by KUWAHARA and CHAMBERS did cause aggregation and proved to be identical with ristocetin A by HPLC.

In conclusion, the present study has demonstrated that most, if not all, of the antibiotic and toxic properties of ristocetin A are found in its Ψ-aglycone moiety. It remains to be seen whether this also applies to its ability to induce blood platelet aggregation.

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