Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 115-120

CRITICAL MICELLE CONCENTRATION AND HEMOLYTIC ACTIVITY - A CORRELATION SUGGESTED BY THE MARINE STEROL, HALISTANOL TRISULFATE

Roger W. Moni[1], Peter G. Parsons[2], Ronald J. Quinn[1]* and Roger J. Willis[1]

[1] School of Science, Griffith University, Brisbane, Queensland 4111, Australia

[2] Queensland Institute of Medical Research, Brisbane, Queensland 4006, Australia

Received November 5, 1991

The marine natural product, halistanol trisulfate, has a relatively low critical micelle concentration of 0.001% m/v (14.5μM) and strong hemolytic potency with an EC50 of 0.00046 %m/v (6.67μM). As expected of a detergent, it inhibits the growth of gram-positive but not gram-negative bacteria. The hemolytic activity of halistanol trisulfate and other detergents has been shown to correlate with critical micelle concentration. This correlation may have important implications in the mechanism of membranolytic bioactivity.

Marine invertebrates are known to elaborate and sequester novel metabolites with diverse pharmacological actions (1-3). In particular, the hemolytic, antimicrobial and cytotoxic properties of steroidal saponins and saponin-like compounds has been well documented (4,5). These molecules have characteristic hydroxylation and alkylation patterns which confer differential biological activities.

Halistanol trisulfate (Figure 1) is a previously described C29 steroidal compound isolated from marine sponges (6,7). Halistanol trisulfate has been reported to be have hemolytic, antifungal and ichthyotoxic actions as well as the ability to inhibit the growth of gram-negative and gram-positive bacteria (6). No further details have been published. Many of the biological effects of halistanol trisulfate are now explained by its detergent properties. In seeking to further understand the biological activity of halistanol trisulfate, it was compared quantitatively with the commercially available surfactants Triton X-100 and sodium deoxycholate. We now report a

* To whom correspondence should be addressed.

0006-291X/92 $1.50

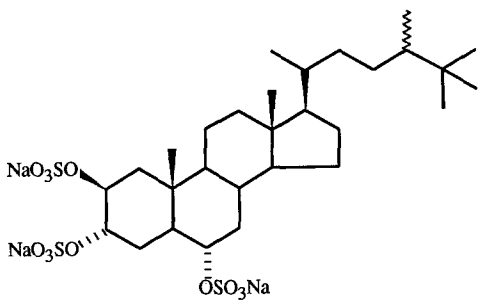

Figure 1. Halistanol trisulfate.

correlation between the physical property of critical micelle concentration and hemolytic activity.

This correlation may have implications in the mechanism of membranotropic bioactivity.

Materials and Methods

Chemicals : Halistanol trisulfate was isolated from an unidentified sponge collected on the Great Barrier Reef (Australia). The ^{1}H and ^{13}C NMR spectra and EI mass spectrum were identical to those previously reported (6) while combustion analysis confirmed the compound as the trisodium sulfate. Triton X-100; sodium deoxycholate; 8-anilino-1-napthalenesulfonic acid hemimagnesium salt; Trizma Base were from the Sigma Chemical Company. Human blood was supplied as REVERCELL (pooled red cells) by the Commonwealth Serum Laboratories, Melbourne, Australia.

Buffers : Tris-Buffered Saline : 10mM Tris base, 0.15 M NaCl, pH 7.2. Tryptic Soy Broth : 3g of Soybean Casein Digest Medium (DIFCO Laboratories) per 100 ml deionised water was sterilized in an autoclave for 15 minutes at 15 psi (121°C).

Determination of Critical Micelle Concentration : The critical micelle concentration values of halistanol trisulfate, Triton X-100 and sodium deoxycholate were determined by fluorimetry (8) both in water and tris-buffered saline. Experiments were performed using disposable PP3 vials containing aqueous/tris-buffered saline solutions of detergent and 10μM 8-anilino-1-napthalenesulfonic acid hemimagnesium salt to a volume of 3ml. After 10 minutes at 37°C, relative fluorescence yields were measured at λ_{ex} 370nm and λ_{em} 490nm against solvent/probe controls with a Perkin-Elmer LS-5 luminescence spectrometer. Duplicate results were averaged and plotted against detergent concentrations, using the formula weights of the ionic species - 689 for halistanol trisulfate 646 for Triton X-100 and 391.5 for sodium deoxycholate. Optimal linear regressions (r = 1.00) based on a minimum of four data points were fitted to graph components. Breakpoints (representing critical micelle concentration values) were then calculated by algebraic solution of regression lines.

Determination of Hemolytic Potency : The hemolytic actions of halistanol trisulfate, Triton X-100 and sodium deoxycholate were measured by photometry (9). Human blood was pelleted at 400g for five minutes then washed three times in 10 volumes of tris-buffered saline using a Beckman GPR centrifuge. The volume of erythrocyte suspension was adjusted with tris-buffered saline to give an absorbance reading at 545 nm (A545) of 0.8 when completely lysed by two freeze-thaw cycles. Duplicate measurements of at least eight detergent concentrations (2ml total volume) together with a control (cells plus tris-buffered saline) and 100% hemolysis standard were incubated for 30 minutes at 37°C. Vials were centrifuged at 400g for five minutes and the A545 of the supernatant recorded. Percent hemolysis was calculated relative to the standard. Averaged A545 results when plotted against detergent concentrations gave sigmoidal curves which were linearised by log-logit transformation for accurate estimation of EC50 (concentration of detergent causing 50% hemolysis).

Bacterial Screening : Two species of gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus*) and two species of gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) were cultured over 24 hours. 100µl of bacterial innoculum was plated onto sterile agar medium in Petri dishes. Six concentrations of each detergent (0.05% to 1.0%) were screened by pipetting duplicate 10µl aliquots onto sterile, blank antibiotic susceptibility discs. After a 48 hour incubation at 37°C, plates were photographed and scored for the presence or absence of bacterial growth against controls (innoculum only). Inhibition of bacterial growth was indicated by a clear annulus around the treated disc. The bacteriostatic and bacteriocidal limits were determined for sensitive bacteria. These were defined by the Minimum Inhibitory Concentration and Minimum Killing Concentration, respectively (10). 8-16 concentrations of halistanol trisulfate, Triton X-100 and sodium deoxycholate were made up in sterile PP3 tubes containing tryptic soy broth and 50 µl of gram-positive bacteria to a volume of 500 µl. Tubes were continuously shaken at 37°C for 24 hours, then scored by eye for bacterial growth. From tubes in which no growth was observed, 50 µl aliquots were transferred into 450 µl of tryptic soy broth. After a 24 hour incubation at 37°C with continuous shaking, tubes were scored against controls containing tryptic soy broth only. Only agreeing duplicate results were considered valid.

Results and Discussion

Critical micelle concentration is the narrow concentration range over which amphipaths form thermodynamically stable, isotropic colloidal aggregates (11). This property was determined because the self-association of molecules at the critical micelle concentration may correlate with the ability of compounds to interact with biological membranes.

The relative fluorescence of halistanol trisulfate in water is shown in Figure 2. The graph consists of two linear components coincident at the critical micelle concentration of 0.001%m/v. The estimated critical micelle concentration values of Triton X-100 (0.014% m/v) and sodium deoxycholate (0.16%) in tris-buffered saline (Table 1) were in good agreement with literature values (8). In both water and tris-buffered saline, halistanol trisulfate has a critical micelle concentration which is one and two orders of magnitude lower than those of Triton X-100 and sodium deoxycholate, respectively. Micelle formation was not significantly affected by the ionic and pH differences between the two solvents. It was also noted that the relative change in the

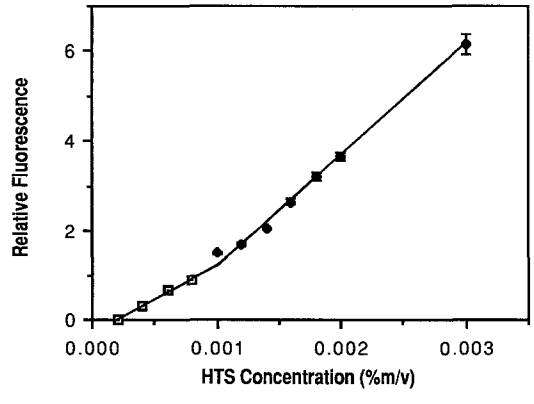

Figure 2. Fluorescence yield of halistanol trisulfate using the fluorescence probe 8-anilino-1-napthalenesulfonic acid hemimagnesium salt at 10µM. Data points represent the average of two replicates at each concentration.

Table 1. Critical Micelle Concentrations and Hemolytic Effects of Halistanol Trisulfate, Triton X-100 and Sodium Deoxycholate

Compound	Critical Micelle Concentration (% m/v)		Hemolysis
	Water	Tris-buffered Saline	EC50 (%m/v)
halistanol trisulfate	0.001	0.001	0.00046
Triton X-100	0.015	0.014	0.0039
sodium deoxycholate	0.32	0.16	0.039

Standard deviation was within 5% of the mean for each data point.
Negative controls showed less than 1% hemolysis.

gradients of regression components on either side of the critical micelle concentration, could be ranked as : Triton X-100 > sodium deoxycholate > halistanol trisulfate, because the ratio of the upper to lower graph components was 10.14:1 for Triton X-100, 3.15:1 for sodium deoxycholate and 1.58:1 for halistanol trisulfate. Assuming a similar mechanism of interaction between the fluorescence probe (8-anilino-1-napthalenesulfonic acid hemimagnesium salt) and detergents, this may suggest a relatively low micellar aggregation number for halistanol trisulfate (12).

Data from hemolytic experiments with halistanol trisulfate are graphed in Figure 3. The critical micelle concentration values were good indicators of hemolytic potency - halistanol trisulfate being one and two orders of magnitude more potent than Triton X-100 and sodium deoxycholate, respectively (Table 1). EC50 values for hemolysis were well below critical micelle concentration values for all three compounds, which supports the hypothesis that detergent monomers are responsible for the disruption of plasma membrane integrity (11). The reported ichthyotoxicity of halistanol trisulfate might be explained by its surfactant and hemolytic properties.

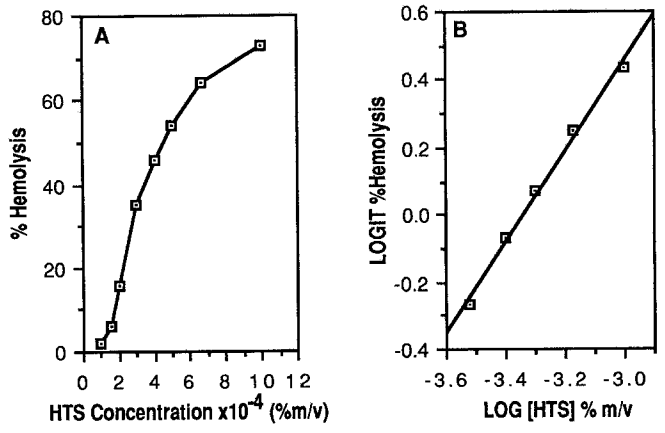

Figure 3. Lysis of human red blood cells by halistanol trisulfate at 37°C. **A.** Percentage hemolysis against concentration of halistanol trisulfate. **B.** Log-logit transformation of hemolysis data for accurate estimation of EC50. Data points represent the average of two replicates at each concentration.

Table 2. Minimum Inhibitory Concentrations (MIC) and Minimum Killing Concentrations (MKC) for Halistanol Trisulfate, Triton X-100 and Sodium Deoxycholate against *Staphylococcus aureus* and *Bacillus cereus*

	Staphylococcus aureus		*Bacillus cereus*	
Compound	MIC (% m/v)	MKC (%m/v)	MIC (% m/v)	MKC (%m/v)
halistanol trisulfate	0.1	>0.1	0.02	0.08
Triton X-100	>1	>1	0.09	0.5
sodium deoxycholate	0.05	0.7	0.07	0.4

The growth of gram-negative bacteria was not affected by any of the three detergents at concentrations up to 1% m/v. All detergents inhibited the growth of gram-positive bacteria (Table 2). Contrary to the original report (6), growth of gram-negative bacteria was not inhibited by halistanol trisulfate. This finding is consistent with the known resistance of gram-negative bacteria to the action of anionic detergents (13). In addition, polyhydroxylated and sulfated sterols isolated from echinoderms are known to be active on gram-positive but not gram-negative species (5). Presumably, the lipopolysaccharide cell wall characteristic of gram-negative bacteria acts as a permeability barrier, limiting the diffusion of detergent molecules to the inner cell membrane. *Bacillus cereus* was more sensitive than *Staphylococcus aureus* to halistanol trisulfate and Triton X-100 but not to sodium deoxycholate. Halistanol trisulfate was the most potent of the three detergents in both bacteriostatic and bacteriocidal effect against *B. cereus*.

The mechanism of hemolytic natural products is usually explained by high affinity binding to specific membrane components (14, 15). To the knowledge of the authors, hemolytic activity has not previously been correlated with the physical property of critical micelle concentration. From the low critical micelle concentration but high lytic potency of halistanol trisulfate, it can be inferred that the monomers have low solubility in aqueous solutions but readily associate with erythrocyte membranes. However, and in contrast to Triton X-100 and sodium deoxycholate, high concentrations of halistanol trisulfate did not cause complete lysis of these membranes. This might be explained by the tendency of halistanol trisulfate to form micelles at lower concentrations and thereby critically reduce the number of monomers available to bind to cell membranes. Association of halistanol trisulfate with membrane components is therefore partly limited by the process of self-association. Determining the critical micelle concentration of low molecular weight amphipaths may be useful in understanding the mechanisms by which they become incorporated into and lyse membranes.

Acknowledgments

We thank the Australian Research Council for support for this research. We thank Dr. B. Patel for advice and Mrs. K. Hampson for technical assistance on bacterial screening and acknowledge the award of a Griffith University Postgraduate Research Scholarship to RWM.

References

1. Krebs, H. C. (1986) Prog. Chem. Org. Nat. Prod. 49, 151-363.
2. Faulkner, D. J. (1988) Nat. Prod. Reports 5, 613-633.
3. Garson, M. J. (1989) Nat. Prod. Reports 6, 143-170.
4. Schmitz, F. J. (1978) In Marine Natural Products: Chemical and Biological Perspectives (P.J. Scheuer, Ed.) Vol 1. pp. 241-297, Academic Press, New York.
5. Andersson, L., Bohlin, L., Iorizzi, M., Riccio, R., Minale, L., and Moreno-Lopez, W. (1989) Toxicon 27, 179-188.
6. Fusetani, N., Matsunaga, S., and Konosu, S. (1981) Tetrahedron Lett. 22, 1985-1988.
7. Makar'eva, T. N., Shubina, L. K., and Stonik, V. A. (1987) Khim. Prir. Soed. 1, 111-115 [(1987) Chem. Nat. Compd. (USSR) 23, 93-96.].
8. De Vendittis, E., Palumbo, G., Parlato, G., and Bocchini, V.(1981) Anal. Biochem. 115, 278-286.
9. Bernheimer, A. W. (1988) Methods in Enzymology 165, 213-217.
10. Calome, J. S., Kubinski, A. M., Cano, R. J., and Grady, D. V. (1986) Laboratory Exercises in Microbiology p. 95, West Publishing Company.
11. Helinius, A., and Simons, K. (1975) Biochim. Biophys. Acta 415, 29-79.
12. Makino, S., Reynolds, J. A., and Tanford, C. (1973) J. Biol. Chem. 248, 4926-4932.
13. Tortora, G. J., Funke, B. R., and Case, C. L. (1989) Microbiology, an Introduction, ed III, p 84, The Benjamin/Cummings Publishing Company Inc.
14. Weinstein, S. A., Bernheimer, A. W., and Oppenheimer, J. D. (1988) Toxicon 26, 1177-1185.
15. Dempsey, C. E. (1990) Biochim. Biophys. Acta 1031, 143-161.