Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 82-88

ENHANCEMENT OF HAEMOLYSIS AND CELLULAR ARACHIDONIC ACID RELEASE BY PYRROLOMYCINS

Kazuo Umezawa[1], Sanae Mimura[1], Taijiro Matsushima[1], Shigemi Muramatsu[2], Tsutomu Sawa[2] and Tomio Takeuchi[2]

[1] Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, and

[2] Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

Received January 19, 1982

Summary: The chlorine-containing antibiotics pyrrolomycins enhanced haemolysis induced by melittin and trichomycin. Pyrrolomycins alone were not haemolytic. Pyrrolomycin B enhanced arachidonic acid release from cellular membrane phospholipids induced by melittin and trichomycin. Pyrrolomycin B also potentiated the action of tumour promoters such as 12-O-tetradecanoylphorbol-13-acetate and teleocidin B on arachidonic acid release, but did not inhibit cellular binding of phorbol ester. Pyrrolomycin B increased hexose transport of cultured cells.

Bee venom melittin (1) and delta-haemolysin produced by *Staphylococcus aureus* (2) are polypeptides consisting of 26 amino acids. They have been shown to share certain cellular effects with phorbol ester tumour promoters. Melittin induces arachidonic acid release and prostaglandin synthesis, and enhances cell growth in agar (3). Delta-haemolysin also induces arachidonic acid and prostaglandin release, and inhibits cellular binding of epidermal growth factor as 12-O-tetradecanoylphorbol-13-acetate (TPA) (4).

We found a factor stimulating melittin haemolysis in the culture filtrate of *Streptomyces flagilis* MG303-fF8. This factor was extracted with ethyl acetate, purified by column chromatography and identified as pyrrolomycin B by elementary

Abbreviations : PBS, phosphate buffered saline ; PDBu, phorbol-12,13-dibutylate ; TPA, 12-O-tetradecanoylphorbol-13-acetate.

0006-291X/82/050082-07$01.00/0

analysis, mass spectrometry, UV- and IR- spectrometry, ^{1}H-NMR and ^{13}C-NMR. Pyrrolomycins are antibiotics recently isolated from the culture broth of *Streptomyces* strain SF-2080 (5).

We report the stimulatory effect of pyrrolomycins on haemolysis induced by melittin and on arachidonic acid release induced by melittin and tumour promoters such as TPA and teleocidin B.

Materials and Methods:

Materials: Pyrrolomycins A, C and D were kindly supplied by the Central Research Laboratories, Meiji Seika Co.,Ltd. Pyrrolomycin B was isolated from the culture filtrate of *Streptomyces flagilis* MG303-fF8 or supplied by Meiji Seika Co.,Ltd. Melittin was purchased from Sigma, and trichomycin from Fujisawa Pharmaceutical Co.,Ltd. TPA was purchased from Consolidated Midland Corporation, and teleocidin B, an indol alkaloid skin tumour promoter (6), was isolated and purified from *Streptomyces* as described by Takashima and Sakai (7). ^{3}H-Phorbol-12,13-dibutyrate (^{3}H-PDBu) and ^{3}H-2-deoxyglucose were purchased from New England Nuclear.

Methods: Haemolysis was assayed essentially as described by Kreger et al. (8). Horse erythrocytes were washed and suspended at 0.7% v/v in isotonic Dulbecco's phosphate-buffered saline (PBS) (9). The suspension was incubated with chemicals for 30 min at 37°C and centrifuged for 3 min at 1500 rpm and the OD_{574nm} of the supernatant was measured.

Arachidonic acid release was assayed as described by Mufson et al. (10). About $6x10^{5}$ C3H10T1/2 cells prelabelled with ^{3}H-arachidonic acid (78.2 Ci/mmol) were incubated in serum-free Dulbecco's modified Eagle's medium with chemicals as indicated. Then the medium was separated, acidified and extracted with ethyl acetate. The ethyl acetate extract was evaporated and mixed with 0.1 ml chloroform. Half the chloroform solution was subjected to silica-gel thin-layer chromatography (2,6-dimethylheptanone/acetic acid/0.9% NaCl, 80/40/6) with non-radioactive arachidonic acid. The spots were removed and their radioactivity was counted.

PDBu binding was assayed essentially as described before (11). About $3x10^{5}$ C3H10T1/2 cells were incubated with 3 nM of ^{3}H-PDBu (14.8 Ci/mmol) for 30 min in Dulbecco's modified Eagle's medium containing 1 mg/ml of bovine serum albumin. Then the cells were washed 3 times with the assay solution and dissolved by trypsin/EDTA/Triton X-100. The radioactivity of the cell lysate was counted. The value of non-specific binding with 3 μM PDBu was subtracted from values obtained by assay.

For hexose transport assay about $2x10^{5}$ C3H10T1/2 cells were incubated with chemicals for 3 hrs in serum free Dulbecco's modified Eagle's medium. Then, the cells were washed once with warm PBS and incubated with 2 μCi/ml ^{3}H-2-deoxyglucose (8.3 Ci/mmol) in PBS for 10 min at 37°C. The cells were washed 3 times with ice-cold PBS and solubilized. All assay data are means of duplicate samples.

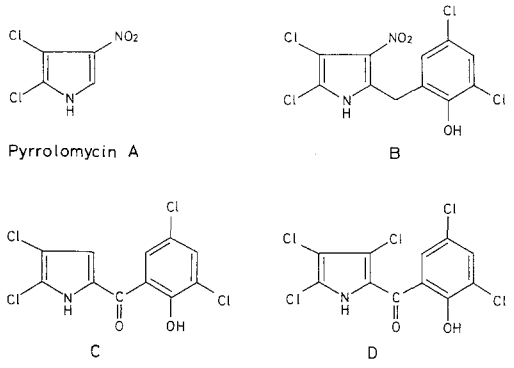

Fig. 1 Structures of pyrrolomycins

Results: The structures of pyrrolomycins are shown in Fig. 1. All pyrrolomycins contain one pyrrole ring and chlorine atoms.

Melittin at 1.1μg/ml induced about 10% haemolysis of horse erythrocytes in isotonic PBS. Addition of 2.5 μg/ml of pyrrolomycin B, C or D increased the haemolysis several fold, as shown in Fig. 2(A). Pyrrolomycin A did not enhance haemolysis and pyrrolomycin A, B, C or D alone had no haemolytic activity at concentrations below 5 μg/ml. Trichomycin, a polyene antibiotic, induced 15% haemolysis at 0.3 μg/ml. Addition of a low

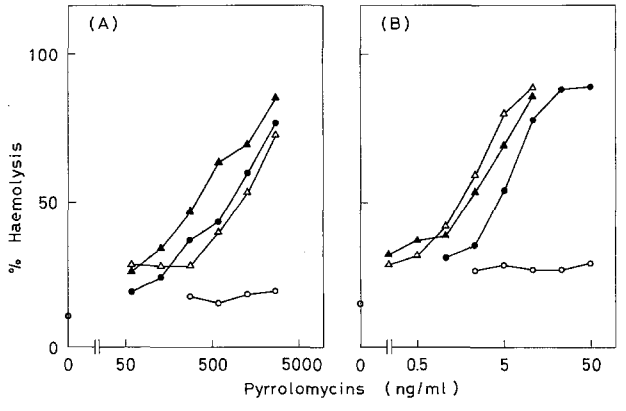

Fig. 2 Enhancement of haemolysis by pyrrolomycins.
(A) Horse erythrocytes were incubated with 1.1μg/ml of melittin and pyrrolomycin A(○), B(●), C(△) or D(▲) in isotonic PBS. Melittin alone induced 10% haemolysis. Pyrrolomycins alone at 5 μg/ml induced no haemolysis.
(B) Horse erythrocytes were incubated with 0.3 μg/ml of trichomycin and pyrrolomycins. Symbols are as indicated in (A). Trichomycin alone induced 15% haemolysis.

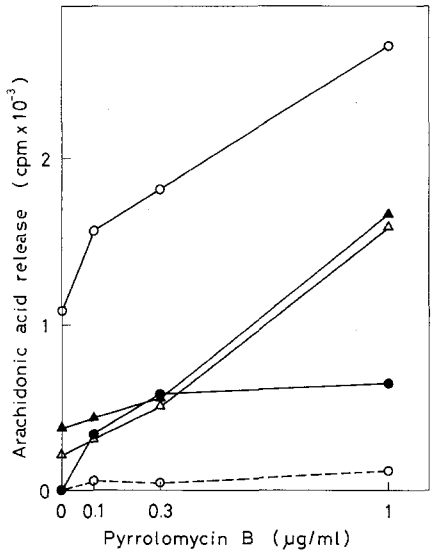

Fig. 3 Enhancement of arachidonic acid release by pyrrolomycin B. C3H10T1/2 cells were incubated for 1 hr with 0.3 μg/ml melittin (○), 0.3 μg/ml of trichomycin (●), 0.01 μg/ml of TPA (△) or 0.01 μg/ml of teleocidin B(▲) and pyrrolomycin B or with pyrrolomycin B alone (○---○).

concentration (10 ng/ml) of pyrrolomycin B, C or D enhanced haemolysis as shown in Fig. 2(B). Pyrrolomycin A again showed little stimulatory activity.

Melittin at 0.3 μg/ml induced arachidonic acid release from membrane phospholipids. Addition of 0.1-1 μg/ml of pyrrolomycin B enhanced arachidonic acid release as shown in Fig. 3. A concentration of 5 μg/ml of pyrrolomycin was cytotoxic to C3H10T1/2 cells. Trichomycin was cytotoxic at above 1 μg/ml and at 0.3 μg/ml it did not induce arachidonic acid release. However, addition of pyrrolomycin B induced arachidonic acid release. Pyrrolomycin B also synergistically enhanced arachidonic acid release induced by TPA and teleocidin B, as shown in Fig. 3. Pyrrolomycin B alone had very little effect on arachidonic acid release.

TPA and teleocidin B inhibited cellular binding of PDBu at 1-10 ng/ml, but pyrrolomycin B did not inhibit PDBu binding at concentrations of below 3μg/ml (data not shown).

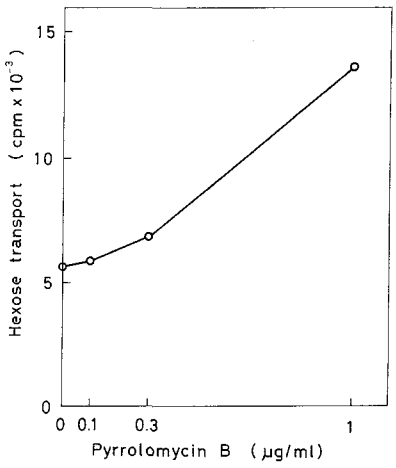

Fig. 4 Stimulation of hexose transport by pyrrolomycin B. C3H10T1/2 cells were incubated with pyrrolomycin B for 3 hrs.

Pyrrolomycin B itself enhanced hexose transport, as shown in Fig. 4. Tumour promoters also increase hexose transport (11,12), but their effects were not synergistic with that of pyrrolomycin B.

Discussion: Pyrrolomycin A, B, C and D are anti-bacterial agents which are effective on *Staphylococci* and *Bacilli* at 0.03 μg/ml (D) - 3 μg/ml (A). Pyrrolomycin A and D are also effective on fungi such as *Cryptococci* and *Trichophyton* (5). Only pyrrolomycin B, C and D are active in enhancing of haemolysis, indicating that both chlorinated pyrrole and phenyl rings are essential for this effect. Since pyrrolomycins enhance haemolysis induced by the chemicals with completely different structure, melittin and trichomycin, they are unlikely to bind to haemolytic reagents, but they may bind to the cell membrane directly.

Melittin facilitates phospholipase A_2-catalyzed hydrolysis (13) and release of arachidonic acid from the cell membrane (3). Pyrrolomycin B stimulated melittin-induced arachidonic acid release, and also potentiated arachidonic acid release by the tumour promoters TPA and teleocidin B. It has been suggested

that induction of release of arachidonic acid (10) or its metabolite prostaglandins (14) is correlated with tumour promoting activity. Thus, pyrrolomycins may accelerate tumour promotion in *in vivo* carcinogenesis.

Specific cellular binding of phorbol esters has been shown using PDBu (15), and it has been suggested that the phorbol ester receptor is located on the cell surface (16). Since pyrrolomycin B did not inhibit cell binding of PDBu, it must bind to a different receptor.

Melittin, TPA and teleocidin B all have amphipathic characters; i.e. they have both a hydrophobic and a hydrophylic portion. Pyrrolomycins are active on the membrane and have an aromatic structure containing chlorine. An interesting problem for further study is how pyrrolomycins act on the cell membrane to increase haemolysis or arachidonic acid release.

Acknowledgements: The authors wish to thank the Central Research Laboratories, Meiji Seika Co.,Ltd. for supplying pyrrolomycins. This work was supported in part by grants from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

References:

1. Habermann, E. (1972) Science, 177, 314-322.
2. Fitton, J.E., Dell, A. and Shaw, W.V. (1980) FEBS Letters, 115, 209-212.
3. Mufson, R.A., Laskin, J.D., Fisher, P.B. and Weinstein, I.B. (1979) Nature, 280, 72-74.
4. Umezawa, K., Weinstein, I.B. and Shaw, W.V. (1980) Biochem. Biophys. Res. Commun., 94, 625-629.
5. Ezaki, N., Central Research Laboratories, Meiji Seika Co. Ltd., personal communication.
6. Fujiki, H., Mori, M., Nakayasu, M., Terada, M., Sugimura T. and Moore, R.E. (1981) Proc. Natl. Acad. Sci. USA, 78, 3872-3876.
7. Takashima, M. and Sakai, H. (1960) Bull. Agr. Chem. Soc. Japan, 24, 647-651.
8. Kreger, A.S., Kim, K.-S., Zaboretzky, F. and Bernheimer, A.W. (1971) Infection and Immunity, 3, 449-465.
9. Dulbecco, R. and Vogt, M. (1954) J. Exp. Med., 99, 167-182.
10. Mufson, R.A., DeFeo, D. and Weinstein I.B. (1979) Molec. Pharmacol., 16, 569-578.
11. Umezawa, K., Weinstein, I.B., Horowitz, A., Fujiki, H., Matsushima, T. and Sugimura, T. (1981) Nature, 290, 411-413.

12. Driedger, P.E. and Blumberg, P.M. (1977) Cancer Res., 37, 3257-3265.
13. Yunes, R., Goldhammer, A.R., Garner, W.K. and Cordes, E.H. (1977) Arch. Biochem. Biophys., 183, 105-112.
14. Levine, L. and Hassid, A. (1977) Biochem. Biophys. Res. Commun., 79, 477-484.
15. Driedger, P.E. and Blumberg, P.M. (1980) Proc. Natl. Acad. Sci. USA, 77, 567-571.
16. Horowitz, A.D., Greenebaum, E. and Weinstein, I.B. (1981) Proc. Natl. Acad. Sci. USA, 78, 2315-2319.