A NEW BIOLOGICAL ROLE OF SANGIVAMYCIN; INHIBITION OF PROTEIN KINASES

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(Received for publication July 23, 1988)

During the screening for the inhibitors of protein kinase C (PKC), we found that a streptomycete produced an inhibitor in our bleb-forming assay (Osada et al., J. Antibiotics 41:925, 1988). The inhibitor was isolated and identified as sangivamycin (4-amino-5-carboxamide-7-(2-ribofuranosyl)pyrrolo[2,3-d]pyrimidine). Biological activity of sangivamycin was compared with that of other 7-deazaadenosine group antibiotics, tubercidin and toyocamycin. Sangivamycin showed a strong inhibitory activity against bleb-formation of K562 cells and PKC. On the other hand, tubercidin and toyocamycin had only weak activities in both assays. This paper deals with a new biological activity of sangivamycin, that of an inhibitor of protein kinases, especially PKC.

Protein kinase C (PKC) plays an important role in proliferation and differentiation of mammalian cells. To search for specific inhibitors of PKC, we developed a unique assay system, named bleb-forming assay. In the course of our screening program, an inhibitor was isolated from a culture broth of a streptomycete and it was identified as sangivamycin which is known as an antifungal and antitumor antibiotic. In previous papers, the mechanism of action of sangivamycin was considered to be same as that of tubercidin and toyocamycin which have the same nucleoside skeleton, 7-deazaadenosine (Fig. 1). We found that the activity of sangivamycin was distinctly different from that of tubercidin and toyocamycin in our assay system. In this paper, we describe a unique biological activity of sangivamycin distinguishing it from tubercidin and toyocamycin.

Materials and Methods

Cell Culture and Bleb-forming Assay

K562, a human chronic myelocytic leukemia cell line was cultured in RPMI1640 medium supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY, U.S.A.). Cells were seeded into a 96-multiwell plate at a density of 1 x 10⁴ cells/well, incubated for 10 minutes at 37°C in a CO₂ incubator, and then treated with phorbol 12,13-didecanoate (PDBu) at a final concentration of 1 μg/ml. After 10 minutes incubation with PDBu, morphology of cell surface was observed under a microscope.
In Vitro Kinase Assay of PKC and Epidermal Growth Factor (EGF)-receptor

PKC was extracted from a rabbit brain and the extract was partially purified on a DEAE-cellulose column. The active fraction of PKC, eluted from DEAE-cellulose by 100 mM NaCl, was used for the assay as described in a previous paper2). EGF-receptor was prepared from the membrane of A431 cells by Thom's procedure7). Autophosphorylation of EGF-receptor was assayed as described by Carpenter et al.8). The results of phosphorylation by both kinases were observed by autoradiography after electrophoresis of ^32P-phosphorylated proteins on SDS-polyacrylamide gel9); histone was used for PKC assay as a substrate.

Chemicals

Sangivamycin was purified successively on Dowex 50, Dowex 1, silica gel, and by HPLC (Nucleosil 5C_18, 40% MeOH). Our preparation was indistinguishable from authentic sangivamycin according to the following spectroscopic data: The UV absorption spectrum (λ_max 230 and 280 nm), high-resolution fast atom bombardment mass spectrometry (HRFAB-MS) ([M+H]^+ m/z 310.1182, C_{12}H_{16}N_{5}O_{5}), and ^1H NMR.

Tubercidin10) and toyocamycin11) were isolated and stored in our laboratory. [p-^32P]ATP was purchased from ICN (Irvine, CA, U.S.A.), EGF from Collaborative Research, Inc. (Bedford, MA, U.S.A.), and PDBu from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Results

Suppression of Bleb-formation by Sangivamycin

Blebs appeared within 10 minutes on the cell surface of K562 induced by 1 μg/ml of PDBu (Fig. 2b). Staurosporine which is known as a strong inhibitor of PKC12) inhibited the bleb-formation induced by PDBu at the concentration of 1 μg/ml (Fig. 2c). We have isolated an inhibitor of bleb-formation from a culture filtrate of a streptomycete and identified it as sangivamycin. Purified sangivamycin completely suppressed bleb-formation at the concentration of 30 μg/ml (Fig. 2f). In contrast, 100 μg/ml of tubercidin and toyocamycin did not suppress the bleb-formation (Figs. 2d and 2e).

Inhibition of In Vitro Kinase Activity of PKC

Bleb-formation on K562 cell surface seemed to be closely related with the activity of PKC. Because we have shown13) that suppressors of bleb-formation (i.e., staurosporine, H7 which is an isoquinoline derivative and some isoflavones) inhibit PKC activity, the inhibitory activity of 7-deazaadenosine antibiotics against PKC was tested in vitro. Even at the concentration of 10 μg/ml, tubercidin did not inhibit phosphorylation of histone (Fig. 3a, lanes 3~6). In the case of toyocamycin, it showed no effect on PKC at
Fig. 3. Autoradiography of phosphorylated histone by PKC and of phosphorylated EGF-receptor by itself.

(a) Sodium lauryl sulfate-polyacryl amide gel (15%) electrophoresis was carried out by Laemmli’s method\(^9\).

Lanes 1 and 2: Control (1: reaction mixture contains PDBu (10 \(\mu\)g/ml), 2: without PDBu), 3~14: with PDBu and the following antibiotics, 3~6: tubercidin, 7~10: toyocamycin, 11~14: sangivamycin at a concentration of 10, 3, 1 and 0.3 \(\mu\)g/ml, respectively.

(b) Lanes 1 and 2: Control (1: stimulated with EGF (10 \(\mu\)g/ml), 2: without EGF), lanes 3~14: treated with EGF and the same inhibitors as in experiment (a).

Concentrations up to 3 \(\mu\)g/ml (Fig. 3a, lanes 8~10). In contrast, sangivamycin inhibited PKC activity at the concentration of 0.3 \(\mu\)g/ml. This inhibition was almost equal to that shown by toyocamycin at the concentration of 10 \(\mu\)g/ml. These results are consistent with our previous observation on the correlation between the suppression of bleb-formation and inhibition of PKC activity.

Inhibition of Autophosphorylation of EGF-receptor

PKC phosphorylates serine/threonine of proteins and its activity was inhibited by sangivamycin. As EGF-receptor is known to be a tyrosine-specific protein kinase, the inhibitory activity by sangivamycin for EGF-receptor was tested. Autophosphorylation of EGF-receptor is enhanced by the addition of EGF (5 ng/ml), therefore this inhibition experiment was carried out in presence of EGF. Tubercidin and toyocamycin showed weak inhibitory activity against autophosphorylation in a dose-dependent manner (Fig. 3b, lanes 3~10). Sangivamycin showed strong inhibition against tyrosine kinase activity: It was more than 10-fold as strong as the other two antibiotics (Fig. 3b, lanes 11~14). Sangivamycin inhibited the in vitro activity of PKC at a lower concentration than that required for tyrosine kinase activity of EGF-receptor.

Discussion

Sangivamycin is one of the three 7-deazaadenosine antibiotics produced in streptomycetes. Previous papers\(^4,13\) discussed the mechanism of action of deazaadenosine antibiotics. According to
these papers, it was suggested that these antibiotics were phosphorylated in the target cell and incorp- 
ated into RNA as ATP analogs, resulting in the inhibition of RNA and protein syntheses. From this point of view, there is no difference between these antibiotics. Actually, we observed that sangivamycin inhibited protein and RNA syntheses of HL60 cells (a human promyelocytic leukemia cell) by pulse label experiments (data not shown).

However, the observation reported here suggests that the inhibition of protein kinases is an im-
portant activity of sangivamycin, because it inhibits very rapidly (10 minutes) bleb-formation induced 
by PDBu. Sangivamycin showed an activity distinct from that of other deazaadenosine antibiotics, 
tubercidin and toyocamycin. Compared with the other two antibiotics which show strong antibac-
terial and antifungal activities, sangivamycin only shows a weak antimicrobial activity10).

7-Deazaadenosine skeleton is common to the three antibiotics and may mimic a part of ATP 
which is a substrate of protein kinases. Therefore, the antibiotic may be able to inhibit kinase ac-
tivities of PKC and EGF-receptor competing with ATP. However, the deazaadenosine skeleton is 
not sufficient to inhibit PKC activity, because tubercidin and toyocamycin showed only slight inhibitory 
effect on bleb-formation of K562 cells and in vitro PKC activity. It was concluded that the carbamoyl 
group on C-7 of the 7-deazaadenosine skeleton is important to suppress bleb-formation induced by 
PDBu and to inhibit PKC activity.

We also observed that incorporation of [3H]thymidine into quiescent mammalian cells induced 
by EGF was blocked by sangivamycin (details will be published elsewhere). These data indicate that 
sangivamycin will prove to be a useful tool for studying ATP-related reactions in cells. Originally, 
sangivamycin was discovered as an antitumor agent3), and now the antibiotic was shown to inhibit 
PKC. However, the direct relationship between antitumor activity and the inhibitory activity of 
PKC is not yet known. The present study suggests that the screening of potent inhibitors of PKC may 
provide possible antitumor agents. It is worth testing whether sangivamycin inhibits tumor-promo-
tion in vivo.

It is to be noted that the findings described in this paper were obtained as the results of our screen-
ning for PKC inhibitors from microbial secondary metabolites. After publishing these results(14), we 
found a paper by LOOMIS and BELL describing the mode of action of sangivamycin15). They tested 
several known antitumor nucleoside compounds for inhibition of PKC and found that sangivamycin 
showed the strongest inhibitory activity among the nucleosides tested. They calculated $K_i$ between 
sangivamycin and PKC as $10 \mu M$ (ca. 3 $\mu g/ml$); this value is comparable to our result shown in Fig. 3a.

Acknowledgments

We thank Dr. MASAKAZU URAMOTO for helpful discussion. We gratefully acknowledge the expert technical 
assistance provided by Ms. CHINATSU WATANABE. This work was carried out as a part of the project research 
for Life Science promoted by The Institute of Physical and Chemical Research (RIKEN).

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