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Vacuolar-type H^+-ATPase Inhibitory Activity of Synthetic Analogues of the Concanamycins: Is the Hydrogen Bond Network Involving the Lactone Carbonyl, the Hemiacetal Hydroxy Group, and the C-19 Hydroxy Group Essential for the Biological Activity of the Concanamycins?

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Abstract—Synthetic analogue of the concanamycins, which lacks the hydrogen bond network existing in the concanamycin structure, retains vacuolar-type H^+-ATPase (V-ATPase) inhibitory activity and induces apoptosis to cancer cells that overexpressing epidermal growth factor receptors (EGFR).

The concanamycins A–F, a new class of 18-membered macrolide antibiotics, are potent and specific inhibitors of vacuolar-type H^+-ATPase (V-ATPase).[1,2] The ability of the concanamycins to disrupt the acidification of intracellular acidic organelles leads to a wide range of diverse biological activities such as antiviral, anticancer and immunosuppressant.[3–5] In addition, Yoshimoto and Imoto have recently reported that concanamycin B selectively induces apoptosis in cancer cells that overexpress epidermal growth factor receptors (EGFR) such as human epidermal carcinoma A431 cells in an EGF-dependent manner, whereas the EGF-dependent apoptosis was not induced in colon carcinoma cell lines that do not overexpress EGFR.[6] Thus, V-ATPase inhibitors such as the concanamycins have attracted attention as candidates for novel therapeutic agents for human cancers and the elucidation of the structure–activity relationship of the V-ATPase inhibitors is very important for developing a strategy for synthesizing modified inhibitors.

The X-ray crystallographic analysis and the NMR spectroscopic analysis revealed that the concanamycins and bafilomycins, 16-membered macrolide antibiotics which are also potent inhibitors of V-ATPase, have a unique hydrogen bond network involving the lactone carbonyl, the hemiacetal hydroxy group, and an intervening hydroxy group.[7–9] Based on the studies of the structure–activity relationship of the concanamycins and bafilomycins, it is proposed that the hydrogen bond network in the concanamycins and bafilomycins is very important for their biological activity.[8–10] However, these studies assessed the effect of methylation as in the hemiacetal hydroxy groups, and can not be eliminated the contribution of other structural factors such as an intervening hydroxy group and the hemiacetal moiety itself.[11] Thus, the actual contribution of the hydrogen bond network is still unclear. The first total synthesis of concanamycin F (concanolide A; **1**) was accomplished

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by Toshima et al.[12–14] This synthetic entry to the concanamycin architectures has since allowed the generation of several unique structural analogues **2**–**4**[15] which could not be obtained from natural concanamycins (Fig. 1). These analogues were prepared by desilylation (*n*-Bu_4NF, THF, rt) of the synthetic intermediates in Toshima's total synthesis of concanamycin F.[13,14] Analogue **2** lacks the hemiacetal ring moiety in the structure, and thus involves no hydrogen bond between the hydroxy groups at C-19 and C-21 and has only that between the C-1 carbonyl group and the C-19 hydroxy group. Analogue **3** has the structure of **2** that has a monomethoxytritylated hydroxy group at C-19. Thus, **3** lacks the hydrogen bond between the C-1 carbonyl group and the C-19 hydroxy group in addition to that between the C-19 and C-21 hydroxy groups. Analogue **4** is a demacrolactonized analogue of **3**. Reported here are the results of a preliminary structure–activity relationship study based upon these synthetic analogues of the concanamycins.

To examine the minimal molecular architecture of the concanamycins necessary for their biological activity, we studied the effects of the analogues on the acidification of intracellular acidic organelles.[16,17] The low pH of the intracellular acidic organelles, including lysosomes, is known to be maintained by V-ATPases, and this acidification is detected as an orange fluorescence when the cells are stained with acridine orange, a weak base fluorescent reagent. As shown in Figure 2, acidic organelles in A431 cells were stained with acridine orange, but treatment with 0.01 μM of **1** caused complete disappearance of the fluorescence, indicating that **1** at 0.01 μM inhibited V-ATPase in cultured A431 cells. Interestingly, analogue **2**, which lacks the hemiacetal ring moiety involving the C-21 hydroxy group, still maintained a V-ATPase inhibitory activity, whereas it was 1000 times lower than that of **1**. This finding suggested that the hemiacetal ring itself and the hydrogen bond between the hydroxy groups at C-19 and C-21 were all important for the V-ATPase inhibitory activity of concanamycins, however, they were not necessary for the activity. These results seemed to be in agreement with the previous studies proposing that the hydrogen bond of the hydroxy groups at C-19 and C-21 is important for the V-ATPase inhibitory activity through their contribution to the conformational stability of the concanamycin structure.[10] Surprisingly, the activity of **3** was, however, 10 times stronger than that of **2**. Treatment of the cells with 1 μM of **3** caused complete disappearance of the fluorescence. These findings indicated that although the significance of the hydrophobicity of the hemiacetal moiety could not be ruled out, both the hydrogen bond network involving the lactone carbonyl, the hemiacetal hydroxy group, and the C-19 hydroxy group, and the hemiacetal ring structure are not essential for the V-ATPase inhibitory activity of the concanamycins. On the other hand, the demacrolactonized **4** did not affect the acidification of the intracellular organelles up to 10 μM, corresponding to the report that indicated the macrolide ring of concanamycins and bafilomycins to be necessary for the V-ATPase inhibitory activity.[18]

To further confirm the V-ATPase inhibitory activity of analogues **2**–**4**, we next examined whether the analogues induce the EGF-dependent apoptosis in A431 cells.[6] Yoshimoto and Imoto previously reported that the V-ATPase inhibition caused EGF-dependent apoptosis in tumor cells overexpressing EGFR such as A431 cells and that there is a significant correlation between the concentration required for the induction of selective apoptosis in EGF-stimulated A431 cells and that for the

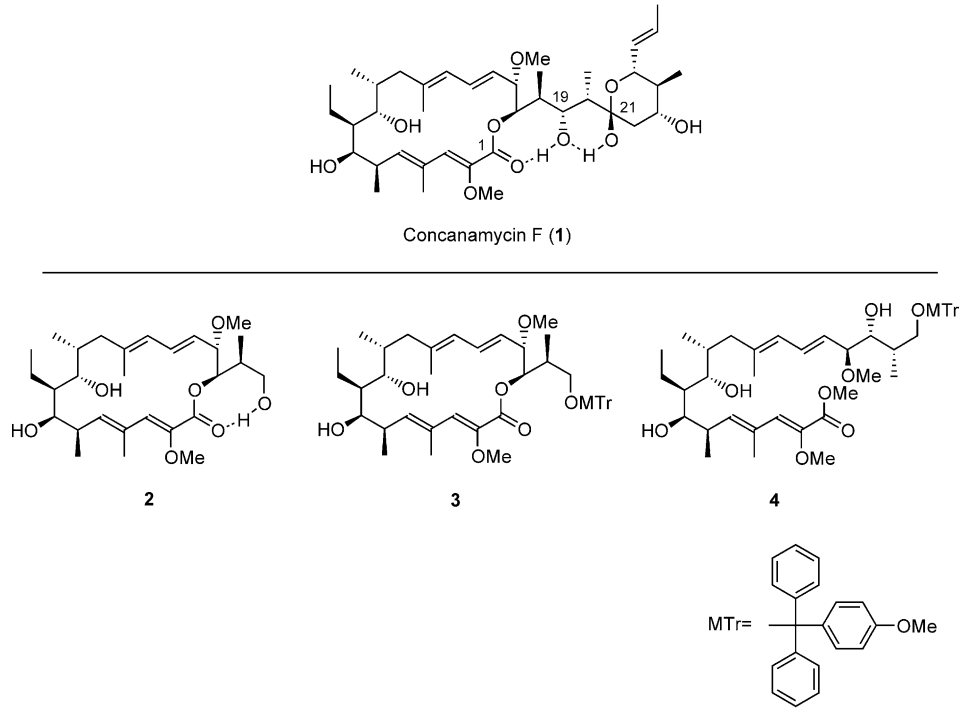

Figure 1. Structures of concanamycin F (**1**) and its synthetic analogues **2**–**4**.

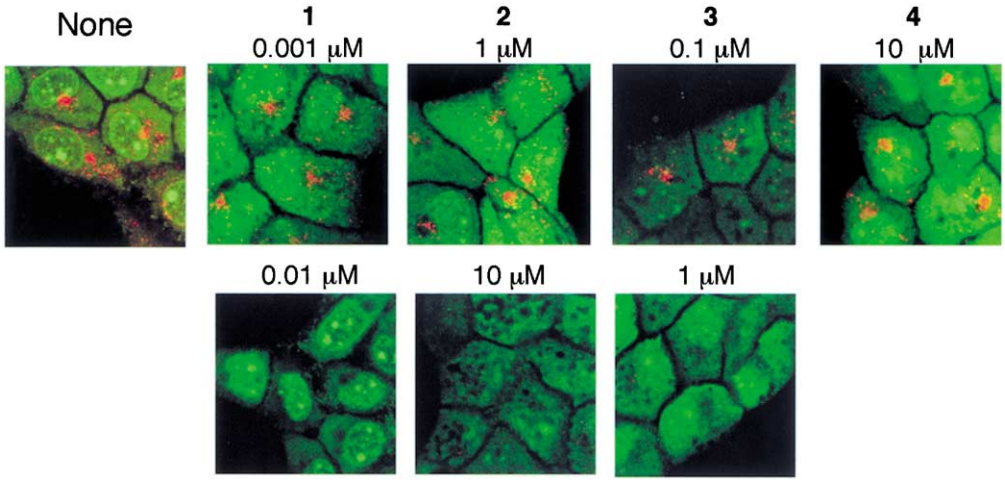

Figure 2. Effect of concanamycin F (**1**) and its synthetic analogues **2**–**4** on acidification of intracellular acidic organelles in A431 cells. Cells were left untreated (none), or treated with the indicated concentrations of the analogues for 4 h, and then stained with 1 μM acridine orange for 30 min. After being washed, the cells were observed under a laser-scanning confocal microscope.

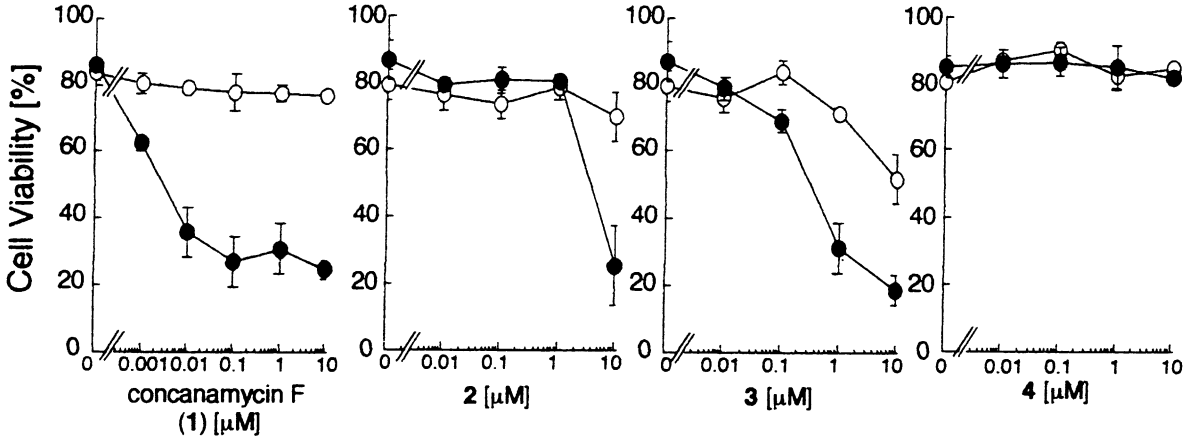

Figure 3. Effect of concanamycin F (**1**) and its synthetic analogues **2**–**4** on cell viability in A431 cells. Cells were treated with the indicated concentrations of the analogues in the presence (●) or absence (○) of 30 ng/mL EGF for 48 h. The cell viability was assessed by trypan blue dye exclusion assay. 'Cell Viability (%)' indicates the percentage of trypan blue-impermeable cells. Values are means for four independent determinations; *bars,* SD.

inhibition of V-ATPase.[6] The cells stimulated with or without EGF were incubated with various concentrations of the analogues for 48 h, and their viability was assessed by trypan blue dye exclusion assay (Fig. 3). Concanamycin F (**1**) did not affect the viability of unstimulated cells up to 10 μM, whereas it induced cell death at 0.01 μM when the cells were stimulated with EGF. Thus, as reported previously, the concentration required for the induction of the EGF-dependent apoptosis corresponds with that of the complete inhibition of V-ATPases, as evaluated by the disappearance of the fluorescence. Analogues **2** (10 μM) and **3** (1–10 μM) also induced cell death only when A431 cells were stimulated with EGF as well as the V-ATPase inhibition in A431 cells. On the other hand, as expected, the demacrolactonized **4** did not affect cell viability up to 10 μM, whereas the cells were stimulated with EGF.

Concanamycin F is an aglycon of the concanamycins and inhibits the V-ATPase activity. So far, studies of the structure–activity relationship of the concanamycins have largely paid attention to the hydrogen bond network involving the lactone carbonyl, the hemiacetal hygroxy group, and the C-19 hydroxy group, and the effect of substituents on the hemiacetal ring moiety. This is the first report on analogues that lack the hemiacetal ring moiety. In this report, we found, for the first time, that the analogues **2** and **3** still retained V-ATPase inhibitory activity. Moreover, the new finding that the V-ATPase inhibitory activity of **3**, which does not have the hydrogen bond network at all, is 10 times stronger than that of **2**, suggesting the possibility that the hemiacetal ring moiety could be replaced by another chemical structure and that this position seems to be a promising site for chemical modifications. Further study on substituents at C-19 might provide insight into how to develop useful analogues of the concanamycins.

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