Notes

Inhibitory Effects of Bufadienolides on Interleukin-6 in MH-60 Cells

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Received January 24, 2004

Derivatives of bufogenin isolated from the skin of the Chinese toad, *Bufo bufo gargarizans* Cantor ("Ch'an Su"), and several semisynthetic derivatives of 20β,21β-epoxy-resibufogenin (**13**) have been evaluated for interleukin-6 (IL-6) antagonistic activity due to their growth-inhibitory activities on IL-6-dependent MH-60 cells. Among the naturally derived compounds (**1**−**17**), 20*S*,21-epoxy-resibufogenin formate (**1**) showed potent inhibitory activity on the IL-6-dependent growth of MH-60 cells. Epoxide groups at both the C-14, C-15 and C-20, C-21 positions are required to exhibit this type of activity. Compounds acetylated at the C-16 position (**7** and **9**−**11**) showed a loss of activity. An oxo group at the C-3 position (**8**, **14**, and **15**) resulted in cytotoxicity for both cell lines. Stereochemistry is important for selectivity on suppression of IL-6 activity. Among the semisynthetic derivatives (**18**−**25**) of **13**, compound **19**, with an acetyl group introduced at the C-3 position in comparison to **13**, demonstrated considerable growth inhibition of IL-6-dependent MH-60 cells.

Cytokines play important biological roles in homeostasis, defense mechanisms, and immune regulation. One multifunctional cytokine, interleukin-6,[1] is involved in the regulation of immune reactions,[2,3] hematopoiesis,[4,5] and the acute-phase response.[6,7] However, its excessive production plays a major role in cancer cachexia,[8] Castleman's disease,[9] rheumatoid arthritis,[10] hypercalcemia,[11] and multiple myeloma.[11] For this reason, modulation of this cytokine function may be useful for treating the above diseases.

We have isolated 20*S*,21-epoxy-resibufogenin-3-formate (**1**)[12] from "Ch'an Su" (obtained from the skin of *Bufo bufo gargarizans* Cantor) in the course of a screening program for IL-6 inhibitors from natural products. Compound **1** competitively suppressed IL-6 activity in a dose-dependent fashion, but it did not affect the activity of various cytokines (IL-2, IL-3, IL-4, IL-8, IL-11, TNF, NGF, LIF). Furthermore, a receptor binding assay showed an increase in unbound (free) IL-6 in a dose-dependent manner by pretreatment with **1** on the IL-6 receptor (IL-6R), suggesting that **1** suppresses binding of IL-6 to IL-6R.[13]

We were able to isolate 17 compounds from a crude extract of "Ch'an Su" (**1**−**17**) and obtained eight semisynthetic derivatives (**18**−**25**). Compounds **1**−**17** are based on the parent compound bufalin (**3**). The present study examined the inhibitory effects of these compounds and the structure−activity relationship of **1** on IL-6 activity. These compounds were tested for their anti-IL-6 activity using IL-6-dependent and IL-6-independent MH-60 cells.

Table 1 shows the inhibitory effect of these compounds on IL-6 activity. Compound **13** showed weak IL-6 inhibitory activity. When compared with **13**, compounds **4**−**6** have an epoxide group at the C-14 and C-15 or the C-20 and C-21 positions. The potency of **4** increased slightly, while the potency of **5** and **6** did not. When compared with **4**, a formate group at the C-3 position as in **17** resulted in no inhibitory activity, but a formate at the C-3 position as in **13** indicated selectivity on IL-6 activity. These findings suggest that both the epoxides at the C-14, C-15 and C-20, C-21 positions in the structure are required to exhibit inhibitory activity.

Table 1. Cell Growth Inhibition of Bufalin-Related Compounds from "Ch'an Su" and Semisynthetic Derivatives of 20,21-Epoxy-resibufogenin

compound	IC_{50} value (μM) ind.[a]	dep.[b]
1	>58.4	8.9
2	12.9	5.6
3	>64.8	31.9
4	>65.1	22.9
5	>62.2	>62.2
6	>62.2	>62.2
7	>56.2	>56.2
8	8.4	2.4
9	>56.3	>56.3
10	>54.2	>54.2
11	>54.2	>54.2
12	45.0	18.3
13	>62.5	24.8
14	4.3	1.3
15	8.3	4.3
16	>58.7	>58.7
17	>60.7	>60.7
18	21.9	14.3
19	>56.6	5.3
20	17.5	2.0
21	>54.8	9.4
22	19.1	6.4
23	>53.2	11.6
24	34.0	18.1
25	>53.2	6.8

[a] IL-6-independent MH-60 cells. [b] IL-6-dependent MH-60 cells.

Compound **11**, equivalent to **13** with an acetoxy group at the C-16 position, showed no inhibitory activity or cytotoxicity. Similarly, no activities were observed for com-

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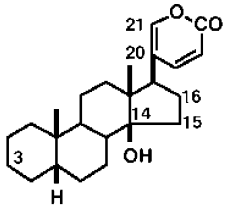
10.1021/np049950e CCC: $27.50
Published on Web 11/12/2004

Table 2.



compound	substituent (position)					
	3	14	15	16	20	21
1	βOCOH		O	H		
2	βOCOH		O	H		
3	βOH	βOH	H	H		
4	βOH		O	H		
5	βOH	βOH	H	H		
6	βOH	βOH	H	H		
7	βOH	βOH	H	αOCOCH$_3$		
8	=O		O	H		
9	βOH		O	αOCOCH$_3$		
10	βOH		O	αOCOCH$_3$		
11	βOH		O	αOCOCH$_3$		
12	βOH		O	H		
13	βOH		O	H		
14	=O		O	H		
15	=O		O	H		
16	βOCOCH$_3$		O	H		
17	βOCOH		O	H		

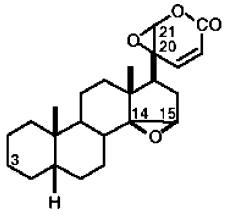
pounds **7**, **9**, and **10**, which all have an acetoxy group at the C-16 position. These results indicate that the C-16 position must be unsubstituted for inhibitory activity.

When compared with **13**, compounds **14** and **15** have a carbonyl group at the C-3 position and exhibited cytotoxic activity for both types of MH-60 cells, whereas **1** and **13** showed an obvious selectivity for IL-6 activity. These results suggest that an ester group at the C-3 position among these compounds plays an important role in determining the degree of inhibitory activity.

We also examined the activity of semisynthetic derivatives (**18**−**25**) of **12** and **13**. Table 1 shows that the introduction of an acetate group in the C-3 position resulted in potent antiproliferative activity on IL-6-dependent cells. This activity decreased according to the increase in the carbon chains of fatty acids at the C-3 position such as propionate (**20**, **21**), butyrate (**22**, **23**), and isobutyrate (**24**, **25**).

Compounds with *R* stereochemistry (**2**, **18**, **20**, **22**, and **24**) were found to possess potent cytotoxicity, not only for IL-6-dependent MH-60 cells but also for IL-6-independent MH-60 cells. In contrast, the *S* isomers at C-20 and C-21 showed a clear IL-6-selectivity. These results suggest that stereoisomerism (**1**, **19**, **21**, **23**, and **25**) is important for selectivity. We infer that there is a difference in the affinity for the IL-6 receptor or metabolism by a metabolic enzyme such as epoxidase. Further investigation is necessary to clarify this stereoselectivity in detail.

Table 3.



compound	substituent (position)		
	3	20	21
18	βOCOCH$_3$		O
19	βOCOCH$_3$		O
20	βOCO(CH$_2$)$_2$CH$_3$		O
21	βOCO(CH$_2$)$_2$CH$_3$		O
22	βOCO(CH$_2$)$_3$CH$_3$		O
23	βOCO(CH$_2$)$_3$CH$_3$		O
24	βOCOCH(CH$_3$)$_3$		O
25	βOCOCH(CH$_3$)$_3$		O

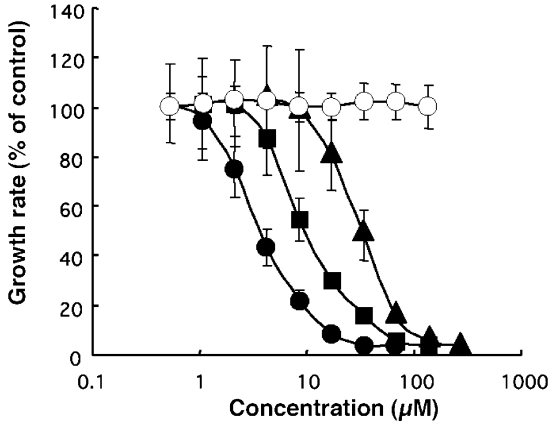


Figure 1. Effect of **19** on IL-6-dependent cell growth. IL-6-dependent MH-60 cells were incubated with graduated concentrations of **19** (●), **1** (■), or madindoline A (◆) for 72 h in the presence of rhIL-6. Independent MH-60 cells were also incubated with graduated concentrations of **19** (○).

The most promising compounds in this series were examined further for growth inhibition of IL-6-dependent MH-60 cells. Table 1 and Figure 1 show that **19** exhibited more inhibitory activity than either **1** or madindoline A, an IL-6 inhibitor isolated from cultured broth of *Streptomyces*.[14] The present results suggest that **19** might be a valuable IL-6 inhibitor and should be considered for additional biological testing because madindoline A suppressed bone resorption in our experiment that might lead to osteoporosis.[14]

Experimental Section

Reagents and Cell Lines. Recombinant human interleukin 6 (rhIL-6) was purchased from Sigma-Aldrich Corp. (St Louis, MO). Prof. T. Hirano, Osaka University (Osaka, Japan), kindly supplied IL-6-dependent MH-60 cells, which are hybridomas of mouse B cells and myeloma cells. The IL-6-independent MH-60 cell line was established by gradually decreasing rhIL-6 concentration in the medium.[14]

Measurement of Growth Inhibitory Activity. IL-6-dependent and -independent MH-60 cells were maintained in

suspension in RPMI 1640 medium supplemented with 10% fetal calf serum with or without 0.5 ng/mL of rhIL-6, respectively. Cells (0.5×10^4 cells) suspended in 200 μL of the medium with or without rhIL-6 were plated in a 96-well culture plate (Corning, Inc.) and incubated at 37 °C in a 5% CO_2−95% air atmosphere. After 24 h incubation, cells were treated for 72 h with various concentrations of compounds. Cell growth was determined by the tetrazolium salt method (MTT assay).[15] Data are represented as mean values with standard errors of three to four experiments.

Test Compounds. Bufalin (**3**) and 16 related compounds isolated from crude extract of "Ch'an Su" (**1**−**17**[16−19]) and eight semisynthetic derivatives of **1** (**18**−**25**[13,20]) were kindly supplied by Professor Y. Kamano, Kanagawa University (Hiratsuka, Japan).

These compounds were 20,21-epoxy-resibufogenin-3-formate (**1** and **2**), bufalin (**3**), resibufogenin (**4**), 20,21-epoxybufalin (**5** and **6**), bufotalin (**7**), 3-oxo-resibugogenin (**8**), cinobufagin (**9**), 20,21-epoxy-cinobufagin (**10** and **11**), 20,21-epoxy-resibufogenin (**12** and **13**), 3-oxo-20,21-epoxy-resibufogenin (**14** and **15**), resibufogenin-3-acetate (**16**), and resibufogenin-3-formate (**17**).[13]

Acknowledgment. This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to A. Fukami of the Kitasato Institute for biological evaluation.

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NP049950E