The Antiproliferative Effect of Acridone Alkaloids on Several Cancer Cell Lines

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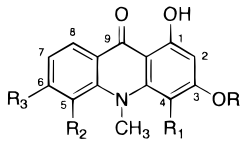
Fifteen acridone alkaloids were examined for their antiproliferative activity toward monolayers and suspension of several types of cancer and normal human cell lines. As a result, atalaphyllidine (**9**), 5-hydroxy-*N*-methylseverifoline (**11**), atalaphyllinine (**12**), and des-*N*-methylnoracronycine (**13**) showed potent antiproliferative activity against tumor cell lines, whereas they have weak cytotoxicity on normal human cell lines. The structure−activity relationship established from the results revealed that a secondary amine, hydroxyl groups at C-1 and C-5, and a prenyl group at C-2 played an important role for antiproliferative activities of the tetracyclic acridones.

Acridone alkaloids are known to have various biological activities, such as cytotoxicity,[1] antiviral[2,3] and antimalarial[4] activities, inhibition of Epstein−Barr virus activation,[5] and induction of HL-60 cellular differentiation.[6] Quite recently, acridones attracted broad attention as a component of photosensitizers used in photodynamic therapy, a newly introduced cancer treatment.[7,8]

The antitumor activity of a series of naturally occurring acridone alkaloids have been studied by determining their antiproliferative activities on several leukemia cell lines, including HL-60, the human promyelocytic leukemia cell line, and L-1210, the murine leukemia cell line.[9−13] In the present study, 15 acridone alkaloids (Figure 1), which were obtained from medicinal plants, were examined for their antiproliferative effect on monolayers and suspension of several types of cancer cell lines, that is, human lung carcinoma (A-549), melanin pigment-producing mouse melanoma (B-16 melanoma 4A5), T-cell leukemia (CCRF−HSB-2), gastric cancer cell, and lymph-node metastasized (TGBC11TKB). A-549, B-16 melanoma 4A5, CCRF−HSB-2, and TGBC11TKB have epithelial-like, fibroblast-like, lymphoblast-like, and epithelial-like morphologies, respectively. The structure−activity relationships obtained from the results are also discussed.



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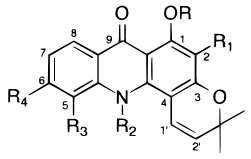
2: R=CH_3, R_1=prenyl, R_2=OH, R_3=H
3: R=CH_3, R_1=H, R_2=OH, R_3=OCH_3
4: R=H, R_1=prenyl, R_2=R_3=H
5: R=CH_3, R_1=OCH_3, R_2=OH, R_3=H



6: R=R_1=H, R_2=CH_3, R_3=OH, R_4=H
7: R=R_1=H, R_2=CH_3, R_3=OCH_3, R_4=OH
8: R=R_1=H, R_2=CH_3, R_3=R_4=OCH_3
9: R=R_1=R_2=H, R_3=OH, R_4=H
10: R=H, R_1=prenyl, R_2=CH_3, R_3=R_4=H
11: R=H, R_1=prenyl, R_2=CH_3, R_3=OH, R_4=H
12: R=H, R_1=prenyl, R_2=H, R_3=OH, R_4=H
13: R=R_1=R_2=R_3=R_4=H
14: R=R_1=H, R_2=CH_3, R_3=R_4=H
15: R=CH_3, R_1=R_2=R_3=R_4=H

Figure 1. Structures of acridone alkaloids studied. Numbering that indicates atom position in the acridones follows the literature.[16]

Results and Discussion

The alamar Blue assay has proved to be an accurate, easy, and reproducible method of evaluating cell viability. The staining with alamar Blue has shown linear correlation with the cell numbers of six cell lines examined in this study (Figure 2). The coefficients of correlation between the alamar Blue staining and the cell numbers of A-549, B-16 melanoma 4A5, CCRF−HSB-2, TGBC11TKB, human foreskin keratinocytes (HFK), and human umbilical vein endothelial cells (HUVE) are 0.993, 0.995, 0.998, 0.995, 0.999, and 0.998, respectively.

Antiproliferative effects (IC_{50}, μM) of 15 acridone alkaloids on A-549, B-16 melanoma 4A5, CCRF−HSB-2, and TGBC11TKB are summarized in Table 1. For TGBC11TKB, atalaphyllinine (**12**) demonstrated the most potent activity, showing the lowest IC_{50} value. This was followed by des-*N*-methylnoracronycine (**13**), 5-hydroxy-*N*-methylseverifoline (**11**), atalaphyllidine (**9**), 5-hydroxynoracronycine (**6**), and grandisine I (**3**). The same order of antiproliferative activity was observed with CCRF−HSB-2, which was more resistant to the acridones than TGBC11TKB. Although A-549 and B-16 melanoma 4A5 showed a higher degree of resistance to the action of the acridones, a similar pattern was observed for these cell lines, except that the order of atalaphyllidine (**9**) and 5-hydroxy-*N*-methylseverifoline (**11**) was reversed.

Acridones examined in this study could be classified into two groups: tricyclic (**1**−**5**) and tetracyclic (**6**−**15**) compounds. Roughly speaking, tetracyclic acridones had more potent antiproliferative activity than tricyclic acridones, although the tricyclic acridones are all tertiary amines. Precise comparison of the antiproliferative activity of

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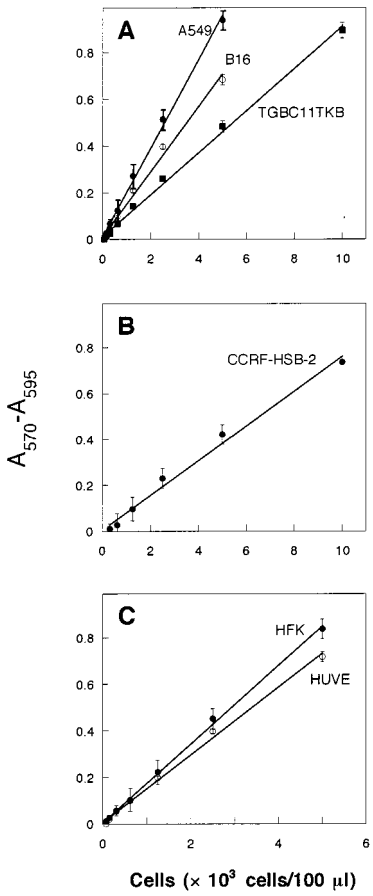

Figure 2. Comparison of alamar Blue staining with cell numbers of six cell lines. (A) Three monolayers of cancer cell lines; A-549 (●), B-16 melanoma 4A5 (○), and TGBC11TKB (■). (B) Suspension of cancer cell line; CCRF−HSB-2 (●). (C) Two monolayers of normal human cell lines; HFK (●) and HUVE (○). Each point represented the mean of triplicates of experiments. Vertical bars indicate standard deviations.

Table 1. Antiproliferative Activity (IC_{50}, μM) of Acridones Toward Several Cancer Cell Lines

compound	A-549	B-16 melanoma 4A5	CCRF−HSB-2	TGBC-11TKB
citpressine II (**1**)	> 40	> 40	> 40	> 40
glycocitrine I (**2**)	> 40	> 40	> 40	> 40
grandisine I (**3**)	24.4	> 40	29.2	19.3
glycocitrine II (**4**)	> 40	> 40	> 40	> 40
citrusinine I (**5**)	> 40	> 40	> 40	> 40
5-hydroxynoracronycine (**6**)	> 40	> 40	23.6	16.2
citracridone I (**7**)	> 40	> 40	> 40	> 40
citracridone II (**8**)	> 40	> 40	> 40	> 40
atalaphyllidine (**9**)	6.98	5.58	4.65	2.83
N-methylseverifoline (**10**)	> 40	> 40	> 40	> 40
5-hydroxy-*N*-methylseverifoline (**11**)	9.37	8.04	5.41	2.13
atalaphyllinine (**12**)	6.26	4.63	2.50	1.43
des-*N*-methylnoracronycine (**13**)	6.37	5.05	3.10	2.07
noracronycine (**14**)	> 40	> 40	> 40	> 40
des-*N*-methylacronycine (**15**)	> 40	> 40	> 40	> 40

acridones was made by the growth-inhibition against TGBC11TKB.

Comparison of IC_{50} values for the antiproliferative activities of tetracyclic acridones did reveal useful structural requirements for the observed biological activity. Among the secondary amines, only **15** was ineffective. The antiproliferative activity decreased when the secondary amine was methylated (**9** > **6**, **12** > **11**, and **13** > **14**). These observations illustrated the importance of the secondary amine for antiproliferative activity. The presence of a hydroxyl group at C-5 of acridones possessing a tertiary amine enhances their antiproliferative activities (**6** > **14** and **11** > **10**). However, the IC_{50} values of **9** and **13** were similar, indicating that the C-5 hydroxyl group has no effect on the antiproliferative activities of the secondary amines. Replacement of the C-1 hydroxyl group with a methoxyl (**15**) abolished the activity of **13**, suggesting that the phenolic hydroxyl group chelated with the 9-carbonyl group could be recognized as an important functional group for the activity. Prenylation at the C-2 position (**11** and **12**) enhanced the activity of **6** and **9**, respectively.

In tricyclic acridones, the activity-enhancing effects of the secondary amine, C-1 hydroxyl group, C-5 hydroxyl group, and C-2 prenyl group, which were observed in tetracyclic acridones, were inconclusive. Only **3** showed a perceptible activity among tricyclic acridones examined. Further study is needed to investigate the structural requirements of tricyclic acridones for the antiproliferative activity toward cancer cell lines.

Antiproliferative effects of **9**, **11**, **12**, and **13** on normal human cell lines were also examined by using HUVE and HFK. Dose−response effects of the four acridone alkaloids on the proliferation of HFK and HUVE as well as A-549, B-16 melanoma 4A5, CCRF−HSB-2, and TGBC11TKB cell lines are shown in Figure 3. Cytotoxicities of these compounds are substantially less toward normal human cell lines; these compounds exerted at least 10 times higher antiproliferative activity toward cancer cell lines than toward normal cell lines. One drawback of cytotoxic drug therapy for treatment of malignant diseases is serious toxicity. The data described above suggest that these acridones could be candidates for low-toxicity antitumor agents.

Recently, several photosensitizers, which are composed of a porphyrin linked to an acridone or related compound, have been designed as promising photodynamic therapeutic agents. Such conjugates could function as conventional therapeutic agents whose overall efficacy could be enhanced under light irradiation, and the acridone-moiety in the conjugate has been indicated to act as a DNA recognition element. Acronycine, an acridone alkaloid, exerts its antineoplastic activity by reducing nucleoside uptake by L-5178Y cells.[9]

It is natural for us to speculate that affinity of acridones toward DNA might correlate with their anticancer activities. The structure−activity relationship of free acridones would provide useful information for the design of new acridone−porphyrin conjugates, which have higher specificity toward a malignant tumor's DNA.

In summary, the present study demonstrated the structure−activity relationship in acridone alkaloids for antiproliferative activity against several cancer cell lines. Secondary amine, C-1 hydroxyl, C-5 hydroxyl, and C-2 prenyl group were important for the activity. Among acridones examined in this study, **9**, **11**, **12**, and **13** exert their antiproliferative activity toward several cancer cell lines with less toxicity on normal human cell lines.

Experimental Section

Acridones. The acridones used in these experiments are listed in Figure 1. Isolation and structure elucidation of citpressine II (**1**),[14] citracridone I (**7**),[14] citracridone II (**8**),[14] glycocitrine I (**2**),[15] glycocitrine II (**4**),[15] grandisine I (**3**),[16] citrusinine I (**5**),[17] *N*-methylseverifoline (**10**),[18] and 5-hydroxy-*N*-methylseverifoline (**11**)[18] were previously described. Other acridones used in this study,—5-hydroxynoracronycine (**6**),[18,19]

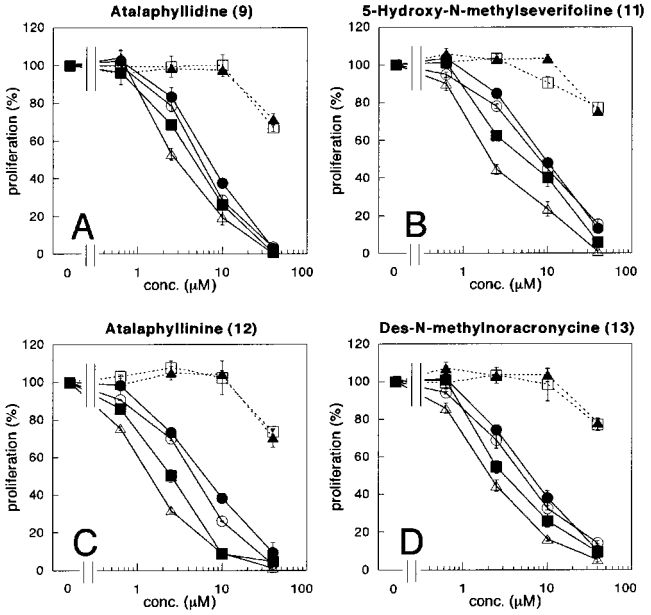

Figure 3. Dose−response of acridone alkaloids on clonal proliferation of several cell lines. Growth (% of control) of A-549 (−●−), B-16 melanoma 4A5 (−○−), CCRF−HSB-2 (−■−), TGBC11TKB (−△−), HUVE (···□···), and HFK (···▲···). The solid lines represent cancer cell lines, and the dotted lines represent normal human cell lines. Each point represented the mean of triplicates of experiments. Vertical bars indicate standard deviations.

atalaphyllidine (**9**),[18,20] atalaphyllinine (**12**),[14,21] des-*N*-methylnoracronycine (**13**),[18,22] noracronycine (**14**),[18,22] and des-*N*-methylacronycine (**15**)[18,22]—were also isolated and identified from the root barks of some Rutaceae plants. Each was dissolved in DMSO at 10 mM to create a stock solution.

Cells. Cancer cell lines used in this study were obtained from the Riken Gene Bank (Tsukuba, Japan) and are as follows: human lung carcinoma (A-549), melanin pigment-producing mouse melanoma (B-16 melanoma 4A5), T-cell leukemia (CCRF−HSB-2); human gastric cancer cell, and lymph-node metastasized (TGBC11TKB). A-549 and B-16 melanoma 4A5, TGBC11TKB, and CCRF−HSB-2 were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), DMEM supplemented with 5% FBS, and RPMI1640 medium supplemented with 10% FBS, respectively. Cultural kits of normal HUVE and normal HFK were purchased from the Morinaga Institute of Biological Science (Yokohama, Japan). The maximum concentration of DMSO in the culture medium (0.4%) did not influence cell growth or differentiation.

Cell Proliferation Assay. The level of cell proliferation was measured by using alamar Blue (Biosource International, Lewisville, TX), an oxidation−reduction indicator. The level of proliferation was measured for each cell line when grown in 96-well microtiter plates. On staining with alamar Blue, the absorbance linearly correlated with cell numbers of each cell line (Figure 2). To each well 2×10^3 cells/100 μL of A-549, B-16 melanoma 4A5, TGBC11TKB, HUVE, or HFK, or 10^4 cells/100 μL of CCRF−HSB-2 cell suspension was added, grown for 24 h, and then mixed with 100 μL of medium containing serial dilutions of samples to be assayed. After 3 days of incubation, 20 μL of alamar Blue was asceptically added to each well, and incubated for 6 h (for monolayer-cultured cell lines, i.e., A-549, B-16 melanoma 4A5, TGBC11TKB, HFK, and HUVE) or 24 h (for suspension-cultured cell line, i.e., CCRF−HSB-2). Cellular proliferation (% of untreated control) was calculated with the following equation:

$$\text{Proliferation (\%)} = \frac{[(A_{570} - A_{595}) \text{ of test agent dilution}] - [(A_{570} - A_{595}) \text{ of blank}]}{[(A_{570} - A_{595}) \text{ of positive growth control}] - [(A_{570} - A_{595}) \text{ of blank}]} \times 100$$

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