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Differentiation Inducing Activity of Anthracycline Compounds in Friend Leukemia Cells

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Several kinds of anthracyclines having γ-rhodomycinone as the aglycone were isolated from *Streptomyces cosmosus* TMF 518, and their derivatives were prepared by chemical modification. We tested their differentiation inducing activity in Friend leukemia cells and clarified their structure activity relationship as follows: 1) The aglycone, γ-rhodomycinone, had no differentiation inducing activity but was cytotoxic; 2) the compounds with two sugar chains at both C7 and C10 had more potent differentiation inducing activity than those with only a sugar chain at C-10; 3) cosmomycin C was the most favorable candidate for an anticancer agent of all anthracyclines tested, because the value of ED_{50} (cytotoxicity)/ED_{50} (differentiation) was as high as 3000; and 4) the increase in differentiation inducing activity and cytotoxicity was not always in parallel.

Anthracycline compounds have become one of the most promising drugs for the treatment of human cancers. Some of them such as adriamycin[1] and aclacinomycin[2] have been used clinically and been proved to be effective for some kinds of human cancers. The most unfavorable effect of anthracyclines is, like the other chemotherapeutic agents, its toxicity against normal proliferating tissues. Additionally, the cardiotoxic damage is serious, relating to both the total dose and the schedule of drug administration. Therapy with anthracyclines is limited by these toxic characteristics. Therefore, intensive efforts have been made to find less toxic anthracyclines with high antitumorial potency by screening from a cultured broth of *Streptomyces* and by chemical modification of naturally occurring anthracyclines. However, limited information has been available on the relationship between the structures and biological activities, and in particular, the antitumorial activity and toxicity.

Recently, differentiation-inducing activity has been considered to be a good index in the search for less toxic antitumorial drugs. Among many anticancer drugs, actinomycin D,[3] mitomycin C,[4] and bleomycin[5] are known to show highly specific inducing activity at low concentrations against Friend leukemia cells and myeloid leukemia cells, and these compounds have been the most effective in cancer treatment.

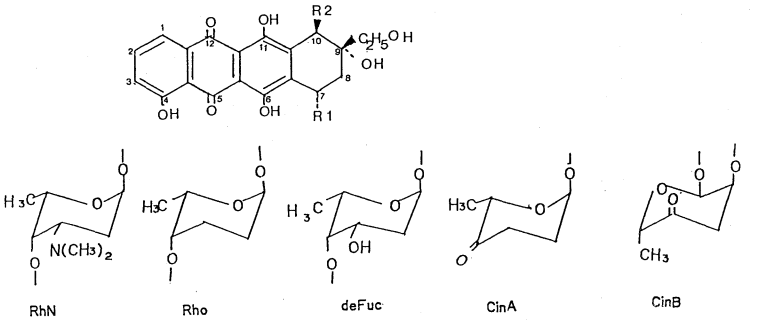
In the search for less toxic antitumorial compounds, we employed a Friend cell's differentiation system and found several novel compounds, which have been reported elsewhere. Some of these compounds, cosmomycins A, B,[6] C and D,[7] are γ- or β-rhodomycinone derivatives with one or two sugar chains at the C-7 and C-10 positions. In this paper, we describe the preparation of chemically modified compounds of cosmomycins and the structure–activity relationship, focussing our attention on the protency of differentiation inducing activity.

MATERIALS AND METHODS

Cell lines and culture conditions: Friend cell c1745a was a kind gift from Dr. K. Onodera of The University of

TABLE I. LIST OF COMPOUNDS TESTED FOR CYTOTOXICITY AND DIFFERENTIATION INDUCING ACTIVITY

	Compound	R_1	R_2
1	γ-Rhodomycinone	H	OH
2		H	O-RhN
3		H	O-RhN-deFuc
4		H	O-RhN-Rho
5	Cosmomycin A	H	O-RhN-Rho-Rho
6	Cosmomycin B	H	O-RhN-deFuc-Rho
7	Cosmomycin C	O-RhN-deFuc-Rho	O-RhN-Rho-Rho
8	Cosmomycin D	O-RhN-deFuc-Rho	O-RhN-deFuc-Rho
9		H	O-RhN-deFuc-CinA
10	Ditrisarubisin A	O-RhN-deFuc-CinB	O-RhN-deFuc-CinA
11	Ditrisarubisin B	O-RhN-deFuc-CinB	O-RhN-deFuc-CinB



Tokyo.

The culture medium for c1745a was Dulbecco's MEM supplemented with 10% fetal calf serum. The cells were cultured in a humidified 5% CO_2 incubator at 37°C for 3 ~ 4 days.

Detection of the differentiation inducing activity: The differentiated cells of c1745a were detected as benzidine positive cells by the hemoglobin accumulated in the cells using the benzidine staining method. After cultivating the cells for six days with a testing sample dissolved in methanol under the culture conditions already described, 0.2 ml of the cell suspension was mixed with 20 μl of freshly prepared benzidine solution (a 10 to 1 mixture of 2% 3,3-dimethoxybenzidine in 0.5 M acetic acid and 30% hydrogen peroxide). The differentiated cells were detected as benzidine positive cells and were counted under a microscope. The extent of the differentiation is expressed in terms of (benzidine positive cells/total cells) × 100. Cytotoxicity is expressed in terms of (viable cells/total cells) × 100. The viable cells were determined by the trypan blue dye exclusion method, and were counted with a Burker–Turk counter.

Cultivation of microorganisms: *Streptomyces cosmosus* TMF518 was cultured in a medium consisting of 2.0% soluble starch, 1.0% glucose, 0.7% Bact-soytone (Difco), 0.2% yeast extract (Difco), 0.1% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.1% NaCl. Fermentation was run in a 300 liters tank at 27°C for 4 days at an agitation speed of 310 rpm and air flow rate of 1/2 vvm.

Purification of compounds: The anthracycline derivatives examined in this work are listed in Table I. Compounds **5**, **6**, **7** and **8** were prepared as described previously.[6,7] From a chloroform–methanol extract of the mycelium of *Streptomyces cosmosus* TMF518, **5** and **6** were purified by silica gel chromatography developing with chloroform–methanol (5 : 1), and **7** and **8** with chloroform–methanol (1 : 1). The concentrated residues of each active fraction were separated by silica gel TLC developing with the solvent system A, chloroform–methanol (9 : 1), and B, chloroform–triethylamine (10 : 1). The *Rf* values of **5**, **6**, **7** and **8** in the solvent system A were 0.36, 0.39, 0.24 and 0.25, respectively, and 0.58, 0.36, 0.15 and 0.10, respectively, in the solvent sytem B. The yields of **5**, **6**, **7** and **8** from the chloroform–methanol extract of mycelium (2.2 kg on a wet basis) were 22, 58, 5.8 and 22 mg, respectively.

From an *n*-butanol extract of the filtered culture fluid, **10** and **11** were purified by Sephadex LH-20 column chromatography eluting with methanol, and followed by silica gel (Wako gel C-200) column chromatography eluting with chloroform–methanol (95 : 5). The concentrated residue of the active fractions was separated by prepara-

tive silica gel TLC (Whatmann) developing with chloroform–methanol (20 : 1). The *Rf* values of **10** and **11** in this solvent system were 0.40 and 0.45, respectively. The yields of **10** and **11** from 10 liters of filtrated culture fluid were 5 mg and 10 mg, respectively.

The analytical data of FD-MS, ^{13}C- and ^{1}H-NMR, and CD spectra indicated that **10** and **11** were identical to ditrisarubisin A and B.[9] The molecular weights of **10** and **11** were determined to be 1181 and 1183, respectively, by FD-MS [m/z $(M+H)^+$ 1182 and 1184, respectively]. By hydrolysis with 0.1 N HCl for 30 min at 85°C, **10** gave β-rhodomycinone as the aglycone, and rhodosamine, deoxyfucose and cinerulose A and B as the sugar moiety, while **11** gave β-rhodomycinone as the aglycone, and rhodosamine, deoxyfucose and cinerulose B as the sugar moiety. Identification of these compounds was made by an FD-MS analysis of the reaction products given by partial hydrolysis and hydrogenolysis with a catalyst (Pd/H_2SO_4) under 1 atm of H_2 for 1 hour at room temperature (data not shown). It is well known that under such reductive conditions, the sugar chain at C-7 is specifically cleaved.

Preparation of **1**, **2**, **3**, **4** and **9**: Compounds **1**, **2**, **3** and **4** were prepared by the partial hydrolysis of **5** or **6**. Ten mg of **5** was hydrolyzed in 5 ml of 0.1 N HCl at room temperature for 30 minutes. Saturated sodium bicarbonate solution was added to make the reaction mixture alkaline and the solution was then extracted with 3 ml of chloroform. The solvent layer was washed with water to remove the sodium bicarbonate and evaporated *in vacuo*. The dried red materials were separated by preparative silicic acid TLC developing with chloroform–methanol (9 : 1). The yield of **4** from 10 mg of **5** was 3 mg. Compounds **2** and **1** were also prepared from **5** by the same procedure except that hydrolysis was carried out for 1 hour in the case of the **2** and for 3 hours in the case of **1**. The yields of **2** and **1** were 0.5 mg and 0.3 mg, respectively, from 10 mg of **5**.

As in the case of **4**, 1 mg of **3** was prepared from 10 mg of **6** under the same hydrolysis conditions.

Compound **9** was prepared by hydrogenolysis of **10** with a catalyst (Pd/H_2SO_4) under 1 atm of H_2 at room temperature for 1 hour.

The structures of these compounds were confirmed by ^{1}H-NMR (JEOL JMN-GX-400) and FD-MS (JEOL DX-300) spectroscopy.

RESULTS

Effect of the sugar chain at C-10 on cytotoxicity and differentiation inducing activity: The aglycone, γ-rhodomycinone, had no differentiation inducing activity, but was cytotoxic as shown in Table II. Its cytotoxic effect was weak with a high ED_{50} value (2.8 μM) as compared to those of the other tested anthracyclines. In the cases of almost all the anthracyclines tested, ED_{50} for differentiation was between one fifth and one half of the ED_{50} for cytotoxicity.

The addition of a single sugar, RhN, to the aglycone, γ-rhodomycinone, caused differentiation inducing activity. The cytotoxic effect (ED_{50}) was also increased from 2.8 μM to 1.6 μM.

Further addition of a sugar moiety, deFuc or Rho, enhanced both the cytotoxicity and

TABLE II. EFFECT OF THE C-7 AND C-10 SUGAR CHAIN ON BIOLOGICAL ACTIVITIES

Compound	R_1	R_2	ED_{50} for cytotoxicity A (μM)*	ED_{50} for differentiation B (μM)*	A/B
1 γ-Rhodomycinone	H	OH	2.8	Inactive	
2	H	O-RhN	1.6	0.7	2.3
3	H	O-RhN-deFuc	0.36	0.12	3.0
4	H	O-RhN-Rho	0.45	0.15	3.0
5 Cosmomycin A	H	O-RhN-Rho-Rho	1.6	0.35	4.6
6 Cosmomycin B	H	O-RhN-deFuc-Rho	0.32	0.07	4.6
7 Cosmomycin C	O-RhN-deFuc-Rho	O-RhN-Rho-Rho	0.03	0.00001	3000
8 Cosmomycin D	O-RhN-deFuc-Rho	O-RhN-deFuc-Rho	0.07	0.012	5.8
9	H	O-RhN-deFuc-CinA	0.9	0.35	2.6
10 Ditrisarubisin A	O-RhN-deFuc-CinB	O-RhN-deFuc-CinA	0.08	0.023	3.5
11 Ditrisarubisin B	O-RhN-deFuc-CinB	O-RhN-deFuc-CinB	0.02	0.01	2.0

* ED_{50} for cytotoxicity is expressed as the dose to kill 50% of the total cells, and ED_{50} for differentiation is the dose to induce half the maximum differentiation.

differentiation inducing activity. Both the second sugars increased the cytotoxicity by 3～4 times, and the differentiation inducing activity by 5～6 times. Therefore, the degree of increase in cytotoxicity and differentiation inducing activity was almost in parallel.

The third sugar of Rho, which was added to -RhN-deFuc and -RhN-Rho to give cosmomycins B and A, respectively, was not always effetive for the enhancement of cytotoxicity or differentiation inducing activity. As compared with **6** to **3**, the third sugar Rho did not enhance the cytotoxicity (almost the same) and slightly enhanced the differentiation (1.7 times). In the other cases, namely with **5** and **4**, the third sugar Rho decreased the cytotoxicity to about one quarter and the differentiation inducing activity to about a half.

Effect of the trisaccharide added at C-7: In addition to the trisaccharide chain at C-10, **7**, **8**, **10** and **11**, with another trisaccharide moiety at C-7 had potent biological activities as compared to their corresponding precursors (Table II).

A comparison of the cytotoxicity between **7** and **5**, and **8** and **6**, indicates that the compounds with RhN-deFuc-Rho at C-7 were more toxic than the compounds without a C-7 sugar chain. A similar effect of the C-7 sugar chain on cytotoxicity was also observed in the case with the addition of RhN-deFuc-CinB; namely, **10** was about ten times more toxic than **9**. In all the cases tested, the addition of the C-7 sugar chain made the compounds with only the C-10 sugar chain more cytotoxic.

As for the differentiation inducing activity, the compounds with two trisaccharide chains at both C-7 and C-10 were more potent than the compounds with only a sugar chain at C-10. Most remarkably, **7** was 35,000 times more potent than **5**.

The addition of RhN-deFuc-Rho to C-7 altered the biological properties of **5** and **6**, especially in the case of **5**. The structural difference between **5** and **6** was only at the second sugar in the C-10 sugar chain; however, **6** was five times stronger than **5** in both cytotoxicity and differentiation inducing activity. The addition of the same trisaccharide, RhN-deFuc-Rho, at C-7 reversed the biological activities of these compounds.

Except for the cases of **5** and **7**, the increase in cytotoxicity and differentiation inducing activity occurred almost in parallel with the addition of RhN-deFuc-Rho, or RhN-deFuc-CinB at C-7. By adding RhN-deFuc-Rho at C-7 of **6**, both the cytotoxicity and differentiation inducing activity were increased by about five times. Also, the addition of RhN-deFuc-CinB at C-7 of **9**, increased the cytotoxicity about by ten times, and increased the differentiation inducing activity by fifteen times. However, in the case of **5**, the addition of RhN-deFuc-Rho at C-7 increased the cytotoxicity by 50 times and the differentiation inducing activity by 35,000 times. The increasing ratio was not in parallel. From the present results, the combination of RhN-deFuc-Rho at C-7 and RhN-Rho-Rho at C-10 was most effective for increasing the selectivity in favor of differentiation inducing activity.

DISCUSSION

Many anthracyclines have been discovered in the culture broths of *Streptomyces*. Some of these have been reported to be inducers of differentiation in tumor cells. However, the relationship between chemical structure and differentiation inducing activity has not been well analyzed.

Antitumorial agents for clinical use should be selectively active and effective on cancer cells but not cytotoxic to normal cells. It may be preferable that antitumorial compounds have potent differentiation inducing activity and weak cytotoxicity. Therefore, the ED_{50} ratio of cytotoxicity/differentiation may be an index for screening and evaluating the drug candidate. It is necessary to test the *in vivo* antitumorial activity of the anthracyclines possessing potent differentiation inducing activity.

An example of a desirable antitumorial compound is cosmomycin C, whose ED_{50} ratio of cytotoxicity/differentiation can be as large as 3,000. In most cases, the addition of a

trisaccharide chain at C-7 increased the biological activity of the anthracyclines, and both the differentiation inducing activity and cytotoxicity were markedly increased to the same extent. Therefore, the ED_{50} ratio of cytotoxicity/differentiation remained unchanged. The only exception was cosmomycin C, whose ED_{50} for differentiation was increased by 35,000 times as compared to a 50-fold increase in the ED_{50} value for cytotoxicity.

Anthracyclines are known to intercalate to DNA, inhibiting DNA or RNA synthesis. The finding of an independent increase in ED_{50} ratio in the case of cosmomycin C suggests that the binding sites of the anthracyclines to DNA for cytotoxicity and induction of differentiation may be different. The structure of a sugar chain is considered to play a role in determining the binding specificity. The mechanisms of differentiation induced by anthracyclines may be related to the inhibition of DNA or RNA synthesis.

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