Spicamycin and KRN5500 Induce Apoptosis in Myeloid and Lymphoid Cell Lines with Down-regulation of Bcl-2 Expression and Modulation of Promyelocytic Leukemia Protein

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Spicamycin is a potent inducer of differentiation of human myeloid leukemia cells (HL-60) and murine myeloid leukemia cells (M1). One of the spicamycin derivatives, KRN5500, shows a broad spectrum of antitumor activity against human tumor xenografts in nude mice. In this study, we first investigated the differentiation efficacy of spicamycin and KRN5500 in HL-60 and acute pro-myelocytic leukemia cell line, NB4, and found that low concentrations of both compounds induced differentiation to a small extent in both cell lines, but markedly induced apoptosis in NB4 cells. Further investigation in a myeloid leukemia cell line, NKM-1, a lymphoma cell line, Daudi, and a multiple myeloma cell line, NOP-1, showed that high concentrations of both compounds also induced apoptosis in these cells. The 50% inhibitory concentration (IC$_{50}$) determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that myeloid cells were more sensitive to both compounds than lymphoid cells, and spicamycin was more potent than KRN5500. Western blot analysis of Bcl-2, Bcl-xL and Bax expression and immunofluorescence analysis of promyelocytic leukemia (PML) protein indicated that apoptosis induced by spicamycin and KRN5500 was associated with down-regulation of Bcl-2 expression and modulation of PML protein. Thus, spicamycin and KRN5500 may be useful for the treatment of myeloid and lymphoid neoplasms.

Key words: Spicamycin — KRN5500 — Apoptosis — Bcl-2 — PML

Spicamycin, a nucleoside-like antibiotic isolated from *Streptomyces alanosinicus* 879-MT$_3$, was first reported as a potent inducer of differentiation of a human myeloid leukemia cell line, HL-60,[1] and a murine myeloid leukemia cell line, M1.[2] The original compound is a mixture of molecules with a common spicamycin aminonucleoside (L-mannoheptopyranose plus purine; SAN), linked to many different fatty acid chains (C$_{12}$–C$_{18}$) through glycine (SAN-Gly, Fig. 1). Further studies on spicamycin and its derivative, KRN5500, have documented antitumor activity in several *in vivo* models including murine leukemia and human tumor xenografts in nude mice.[3-5] A phase I study of KRN5500 in patients with stomach, colon and lung cancers is in progress in Japan.[6]

However, there are few data as to whether spicamycin induces differentiation or shows cytotoxic activity in other hematopoietic neoplasms except HL-60, and the anti-tumor or differentiation-inducing effect of KRN5500 has not been examined. Therefore, we tested these compounds in myeloid and lymphoid cell lines and found that the differentiation-inducing efficacy of both substances was very low as compared with that of all-*trans* retinoic acid (ATRA), a commonly used differentiation inducer. However, they induced apoptosis in all cells tested, and the induction of apoptosis was associated with down-regulation of Bcl-2 expression and modulation of promyelocytic leukemia (PML) protein.

MATERIALS AND METHODS

Drugs Spicamycin and KRN5500 were provided by Kirin Brewery Co., Tokyo. Drugs were dissolved in 100% dimethylsulfoxide (Sigma, St. Louis, MO) to 1 mg/ml, stored at −20°C, and were diluted with RPMI 1640 (GIBCO-BRL, Grand Island, NY) before each experiment. Stock solution of 1 mmol/liter ATRA (Sigma) was made in ethanol.

Cell lines and cell culture NKM-1 was derived from a patient with acute myeloid leukemia (French-American-British classification, FAB, M2).[7] NOP-1 was established from a patient with multiple myeloma (IgA, κ type).[8] NB4 was isolated from a patient with acute promyelocytic leukemia (APL) (FAB, M3).[9] HL-60 (FAB, M2)[10] and the human Epstein-Barr virus positive lymphoma cell line, Daudi,[11] were obtained from the American Type Culture

Collection (Rockville, MD). All these cell lines were cultured in RPMI 1640, supplemented with 1% penicillin/streptomycin, 1 mmol/liter L-glutamine and 10% heat-inactivated fetal bovine serum (GIBCO-BRL) at 37°C in a 5% CO_2 humidified atmosphere.

Growth inhibition assay Cell growth and viability were assessed in triplicate by trypan blue dye exclusion assay at indicated times. Growth inhibition was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[12] The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration that results in 50% growth inhibition.

Cell differentiation studies NB4 and HL-60 cells were seeded at 2×10^5 cells/ml in 12-well plates. After treatment with spicamycin, KRN5500 or ATRA for 72 h,
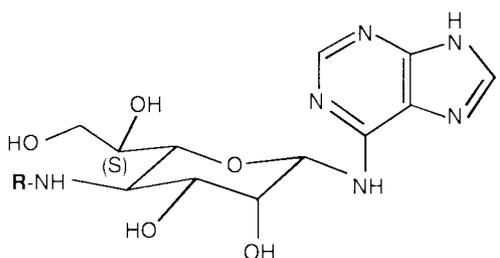

Fig. 1. Structures of SAN-Gly, spicamycin and KRN5500. R: NH_2CH_2CO-, SAN-Gly; $CH_3CH_2CH_2(CH_2)_nCONHCH_2CO$- or $(CH_3)_2CH(CH_2)_nCONHCH_2CO$-, $n=8-14$, spicamycin; $CH_3(CH_2)_8CH=CHCH=CONHCH_2CO$-, KRN5500.

the following three experiments were simultaneously performed. For nitro blue tetrazolium (NBT) reduction assays, cells were adjusted to 2×10^5 cells/ml, and incubated with 50 μg/ml NBT (Sigma) and 200 ng/ml phorbol myristate acetate (Sigma) at 37°C for 30 min. Cells containing a blue-black formazan deposit were counted as NBT-positive from a total of 200 cells under light microscopy. Expression of a monocyte-specific antigen CD14 on the cell surface was measured using direct immunofluorescence staining and flow cytometry. FITC-conjugated mouse monoclonal antibody to CD14 (TÜK4) and FITC-conjugated control mouse IgG2a were obtained from Dako (Glostrup, Denmark). The stained cells were analyzed with an EPICS Profile II (Coulter Corp., Hialeah, FL). Phagocytosis assays were performed as described by Steinkamp et al.[13] In brief, after incubation with fluorescent latex spheres, samples were analyzed with the EPICS Profile II (Coulter Corp.). Cells containing latex spheres were counted as positive cells. All these experiments were repeated at least three times.

Analysis of apoptosis Apoptosis was identified and quantified by flow cytometry with propidium iodide (PI, Sigma) staining as described before.[12] Briefly, 10^5 cells were washed twice with phosphate-buffered saline (PBS) and incubated in 70% methanol on ice. After 30-min incubation, cells were washed twice with PBS, and then 70 μl of RNase A (1 mg/ml, Sigma) and 140 μl of PI (50 mg/ml) solution were added to each sample. After 1-h incubation in the dark, DNA analysis was done using the EPICS Profile II (Coulter Corp.). Apoptosis was quantified by
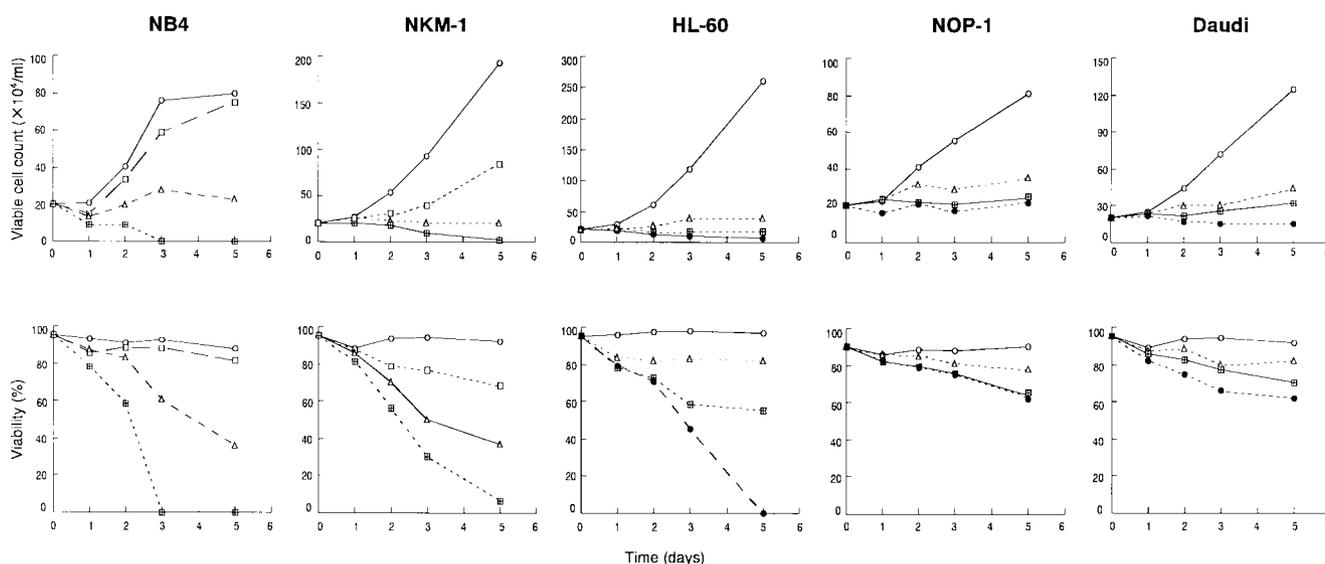

Fig. 2. Effects of spicamycin on cell proliferation and viability. Cells were incubated with various concentrations of spicamycin for 5 days. Viable cell number and cell viability were assessed by means of the trypan blue dye exclusion test. Each point represents the mean obtained from five independent experiments. ○ control, □ 10 ng/ml, △ 20 ng/ml, ⊞ 40 ng/ml, ● 80 ng/ml.

measuring signals in the hypodiploid region. Apoptosis was also confirmed by detection of fragmentation of chromosomal DNA (the classic DNA ladder) by the method previously described.[12] For morphological assessment, cells were spun down onto microslide glasses by Cytospin (Shandon, Pittsburgh, PA) and then stained with May-Grünwald-Giemsa.

Western blot analysis Cells were lysed in Laemmli sample buffer, boiled for 10 min, and centrifuged. The supernatant with 30 μg of protein was fractionated by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and electrotransferred to nitrocellulose membrane. The membrane was blocked with 3% gelatin (Bio-Rad, Richmond, CA) in Tris-buffered saline for 60 min, and then incubated for 120 min with specific anti-human Bcl-2, anti-human Bax (Medical & Biological Laboratories Co., Nagoya) monoclonal antibodies or anti-human Bcl-xL polyclonal antibody (Transduction Laboratories, Lexington, KY). These initial incubations were followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Bio-Rad) for 120 min. The immunocomplex was detected by chemiluminescence using the Immun-Blot assay kit (Bio-Rad).

Immunofluorescence analysis After incubation with spicamycin or ATRA for 24 and 36 h, cells were spun down onto microslide glasses by Cytospin and fixed with periodate-lysine-paraformaldehyde (0.01 mol/liter $NaIO_4$, 75 mmol/liter phosphate buffer and 2% paraformaldehyde) at 4°C for 20 min. A 1:100 dilution of mouse monoclonal anti-PML antibody (clone PGM3, SC-966; Santa Cruz Biotechnology, Santa Cruz, CA) was dropped on the specimen, which was incubated for 60 min. It was washed with PBS, and a 1:100 dilution of an FITC-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) was added to stain the PML protein for 60 min. The immunofluorescence was detected using a confocal microscope (MRC600, Nippon Bio-RAD Laboratories, Nagoya).

RESULTS

Inhibition of proliferation and reduction of viability Cells (2×10^5) were incubated with various concentrations of spicamycin from 10 to 160 ng/ml or KRN5500 from 20 to 320 ng/ml for 5 days. Effects of spicamycin on cell proliferation and viability are shown in Fig. 2. In NB4 and NKM-1 cells, proliferation and viability were completely inhibited by 40 ng/ml of spicamycin, while in HL-60, they were inhibited by 80 ng/ml of spicamycin. In NOP-1 and Daudi cells, spicamycin inhibited the cell proliferation, but had little effect on the viability, even at high concentrations. KRN5500 showed similar inhibitory effects on these cells, though the effective concentration was twice as high as that of spicamycin (data not shown). MTT assay after 72-h treatment showed that IC_{50}s in NB4, HL-60 and NKM-1 were much lower than those in NOP-1 and Daudi (Table I), indicating that myeloid cells were more sensitive to both compounds. It also showed that spicamycin was more potent than KRN5500 in all cells tested (Table I).

Induction of differentiation in HL-60 and NB4 cells Differentiation monitored by NBT reduction activity, cell surface antigen CD14 and phagocytic function analysis showed that only a small part of NB4 cells (22% by NBT assay) and HL-60 cells (31% by NBT assay) differentiated after 72-h treatment with spicamycin or KRN5500 (Table II). The efficacy was much lower than that of ATRA (1

Table I. IC_{50} of Spicamycin (SPM) and KRN5500 (KRN) in Different Cell Lines Determined by MTT Assay as Described in "Materials and Methods"

	IC_{50} SPM (ng/ml)	IC_{50} KRN (ng/ml)
NB4	18.2±4.6	51.6±5.6
HL-60	28.6±7.3	89.7±15.9
NKM-1	23.8±6.8	66.5±17.2
NOP-1	74.9±18.2	277.0±18.9
Daudi	37.4±11.4	242.1±19.5

Table II. Differentiation Monitored by NBT Reduction Activity, Cell Surface Antigen CD14 and Phagocytic Function in NB4 and HL-60 Cells after 72-h Treatment with Spicamycin (SPM), KRN5500 (KRN) or ATRA

	NBT+ (%)		CD14+ (%)		Phagocytic cell (%)	
	NB4	HL-60	NB4	HL-60	NB4	HL-60
Control	0	0	4.2	1.0	3.4	7.7
ATRA 1 μmol/liter	90	68	12.9	2.9	32.6	16.3
SPM 20 ng/ml	22	31	8.9	2.4	5.6	13.2
SPM 10 ng/ml	10	14	4.2	1.6	6.2	11.8
KRN 40 ng/ml	15	8	4.7	1.8	6.0	8.3
KRN 20 ng/ml	11	6	4.0	1.8	6.5	10.3

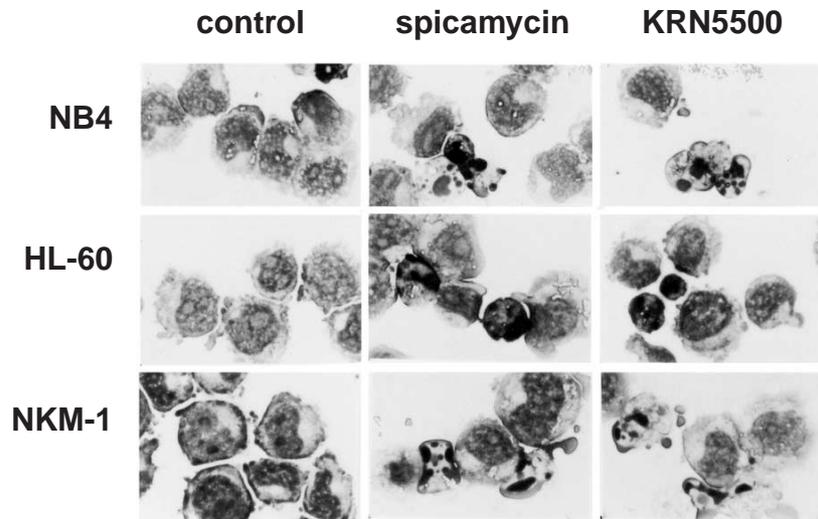control   spicamycin   KRN5500

Fig. 3.   Morphological changes of apoptosis in NB4, HL-60 and NKM-1 cells after 48-h incubation with spicamycin or KRN5500. NB4 cells were treated with 20 ng/ml of spicamycin or 40 ng/ml of KRN5500. HL-60 and NKM-1 cells were treated with 80 ng/ml of spicamycin or 160 ng/ml of KRN5500. Cells were stained with May-Grünwald-Giemsa.

μmol/liter). Prolonged incubation (5 days) gave similar results (data not shown).

Induction of apoptosis   Morphological studies of NB4 and HL-60 cells showed that only a few cells manifested obvious features of maturation after treatment with a low concentration of spicamycin or KRN5500 (data not shown). Unexpectedly, typical apoptotic features, including cellular shrinkage, chromatin condensation and nuclear fragmentation, were observed in NB4 cells after 48-h treatment with 20 ng/ml of spicamycin or 40 ng/ml of KRN5500 (Fig. 3). The apoptotic changes were confirmed by DNA fragmentation analysis by means of agarose gel electrophoresis. A typical DNA "ladder" was detected in NB4 cells after 48-h treatment with spicamycin (Fig. 4) or KRN5500 (data not shown). The percentage of apoptotic cells evaluated by flow cytometry showed that induction of apoptosis in NB4 cells was time- and dose-dependent (Fig. 5, A and B). Remarkable changes were seen at 40 ng/ml of spicamycin and 80 ng/ml of KRN5500. In HL-60, NKM-1, Daudi and NOP-1 cells, low concentrations, which could not significantly decrease the cell viability (Fig. 2), did not induce apoptosis (data not shown). However, high concentrations of both compounds induced apoptosis in these cells (Fig. 5C). The concentrations were twice as high as those in NB4 cells. Again, myeloid cells were more sensitive to spicamycin than lymphoid cells. Morphological changes of HL-60 and NKM-1 cells are shown in Fig. 3. Typical apoptotic changes were observed after 48-h treatment with higher concentrations of spicamycin (80 ng/ml) and KRN5500 (160 ng/ml).
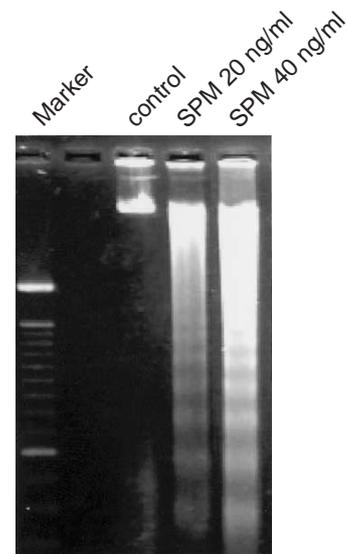

Fig. 4.   Agarose gel electrophoresis analysis of DNA fragmentation in NB4 cells after 48-h treatment with spicamycin (SPM).

Down-regulation of Bcl-2 expression   As shown in Fig. 6, spicamycin down-regulated the Bcl-2 expression at 20 and 40 ng/ml, and KRN5500 did so at 40 and 80 ng/ml in NB4 cells after 36-h treatment, while no Bcl-2 change was found in Daudi cells at these concentrations. Bcl-xL and Bax expression in NB4 showed no significant change after either spicamycin or KRN5500 treatment. Unexpectedly,
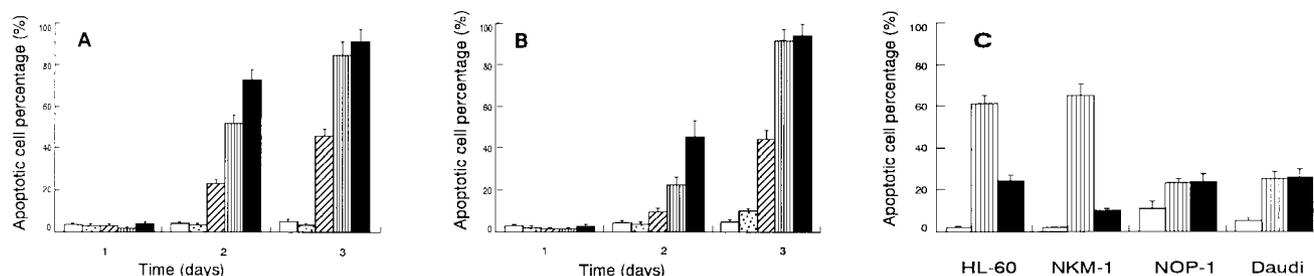
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Fig. 5. Apoptosis measured by flow cytometry after propidium iodide staining. Each value represents the mean±SD obtained from five independent experiments. (A) NB4 cells treated with spicamycin (SPM), □ control, ▨ SPM 10 ng/ml, ▨ SPM 20 ng/ml, ▥ SPM 40 ng/ml, ■ SPM 80 ng/ml; (B) NB4 cells treated with KRN5500 (KRN), □ control, ▨ KRN 20 ng/ml, ▨ KRN 40 ng/ml, ▥ KRN 80 ng/ml, ■ KRN 160 ng/ml; (C) HL-60, NKM-1, NOP-1 and Daudi cells treated with spicamycin or KRN5500 for 72 h, □ control, ▥ SPM 80 ng/ml, ■ KRN 160 ng/ml.
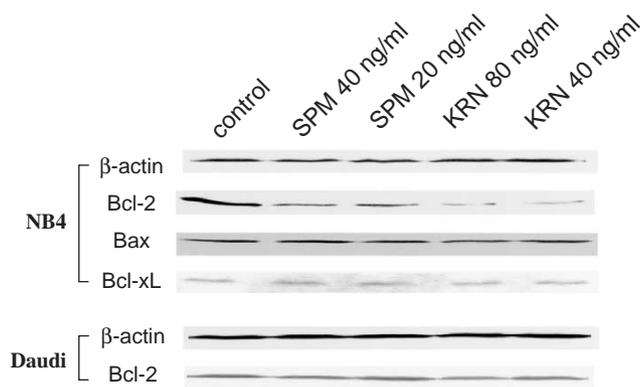

Fig. 6. Western blot analysis for Bcl-2, Bcl-xL and Bax protein following treatment with spicamycin (SPM) and KRN5500 (KRN) in NB4 and Daudi cells. Spicamycin down-regulated the expression of Bcl-2 at concentrations of 20 and 40 ng/ml, and KRN5500 at 40 and 80 ng/ml in NB4 cells.

KRN5500 caused stronger suppression of Bcl-2 expression than spicamycin did.

Modulation of PML staining pattern The PML staining pattern in untreated NB4 cells exhibited a microspeckled pattern (Fig. 7A), and was restored to a normal speckled pattern upon treatment of the cells with 1 μmol/liter ATRA for 24 h (Fig. 7B). After treatment with 20 ng/ml of spicamycin for 24 (Fig. 7C) or 36 h (Fig. 7D), the microspeckled pattern of PML staining changed to a speckled pattern, followed by a reduction in the number of PML speckles. The untreated HL-60 cells displayed a normal distribution of PML, 10–20 speckles in each nucleus (Fig. 7E). ATRA treatment did not cause any significant change (data not shown). However, the number of PML speckles was slightly reduced after treatment with 40 ng/ml of spicamycin (Fig. 7F).
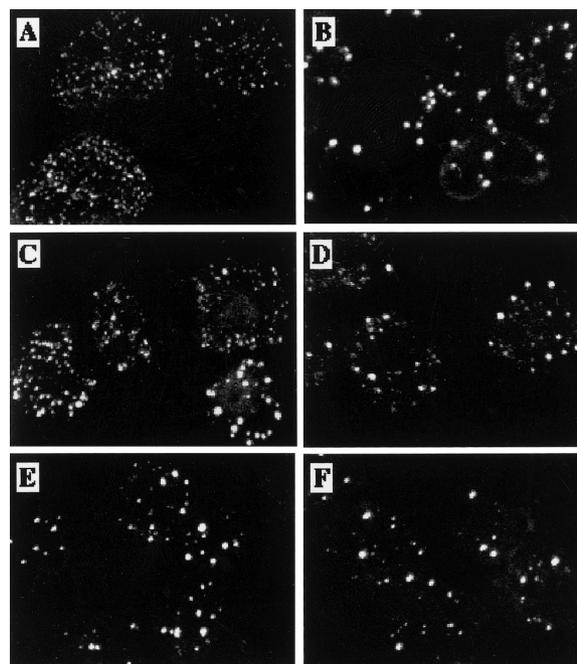

Fig. 7. Immunofluorescence analysis of the subcellular localization of PML in NB4 and HL-60 cells. (A) NB4 cells; (B) NB4 cells treated with 1 μmol/liter of ATRA for 24 h; (C) NB4 cells treated with 20 ng/ml of spicamycin for 24 h; (D) NB4 cells treated with 20 ng/ml of spicamycin for 36 h; (E) HL-60 cells; (F) HL-60 cells treated with 40 ng/ml of spicamycin for 24 h.

DISCUSSION

Since the first report in 1983,[1] spicamycin has been regarded as a potent differentiation inducer of human myeloid leukemia cells. However, results obtained in the present study showed that spicamycin and KRN5500 induced differentiation to a small extent in both HL-60

and NB4 cells, and only when their concentrations were low. The efficacy was very low as compared with that of ATRA. Unfortunately, we could not directly compare our results with those obtained in the previous study,[1] because they did not mention the method used for monitoring differentiation. Our study showed that both compounds markedly induced apoptosis, rather than differentiation, in NB4 cells. Subsequent investigations with higher concentrations of both compounds showed that they also induced apoptosis in HL-60, NKM-1, NOP-1 and Daudi cells.

KRN5500 was shown to inhibit protein synthesis in tumor cells after being hydrolyzed to yield SAN-Gly.[3] A study of spicamycin-resistant sublines also demonstrated that intracellular conversion of spicamycin to SAN-Gly was the major mechanism contributing to the cytotoxicity of spicamycin.[14] Kamishohara et al.[3] reported that this conversion was catalyzed by an enzyme called "acylase" (acyl amide hydrolase), and the enzyme activity was observed in the cell membrane fraction. They speculated that the level of this enzyme in tumor cells might be higher than that in normal cells. In the present study, we found that myeloid cells were more sensitive to spicamycin and KRN5500 than lymphoid cells. This might be due to different levels of acylase in the cells.

Although the IC_{50} was different in each cell line, apoptosis was induced in all cells. What were the pathways through which spicamycin induced apoptosis? Many studies have suggested that apoptosis can be induced by antitumor agents through various signaling pathways, such as the regulation of p53,[15] Fas[16] or Bcl-2.[17] Among these, Bcl-2 may be one of the key factors in the common final pathway of apoptosis.[18] Western blotting showed that Bcl-2 expression was down-regulated following treatment with spicamycin and KRN5500, while expression levels of the other Bcl-2 family members Bcl-xL and Bax were not changed. This suggests that the apoptosis induced by spicamycin was possibly mediated by down-regulation of Bcl-2 expression. Spicamycin was more potent than KRN5500 as judged from MTT assay. However, KRN5500 caused stronger suppression of Bcl-2 expression. This discrepancy indicated that other factors were probably also involved in the process of spicamycin-induced apoptosis.

An interesting finding in this work was that spicamycin caused modulation of PML in the APL cell line NB4. APL is characterized by a chromosomal translocation t(15;17). This translocation generates a fusion protein PML-retinoic acid receptor α (PML-RARα).[19] The fusion protein het-erodimerizes with PML, alters the normal localization pattern of PML from speckled pattern to microspeckled pattern, and blocks cell differentiation.[20] ATRA, which induces clinical remission in APL through inducing differentiation, is well known to restore the normal speckled pattern of PML by the release of sequestered PML from the heterodimer through the degradation of PML-RARα.[21] Spicamycin also restored the microspeckled pattern of PML to a speckled pattern, followed by a reduction in the number of PML speckles. These changes were similar to those induced by arsenic trioxide, a novel inducer of apoptosis and partial differentiation in APL cells.[22, 23] Modulation of PML by arsenic trioxide is reported to be due to the hypermodification of both PML and PML-RARα by PIC-1/SUMO-1 protein.[24] However, the mechanism of modulation caused by spicamycin is unclear. It may work in the same way as arsenic trioxide. Although the biological function of PML restoration is still unclear, PML suppression activity is reported to correlate with nuclear localization.[25] Recent reports indicate that PML is involved in apoptotic pathways.[25–29] PML over-expression induces rapid cell death,[25, 26] while antisense PML oligonucleotides increase cell survival under serum deprivation conditions,[25] indicating that PML is directly involved in the apoptotic activity. Restoration of PML by spicamycin probably modulates the function of PML, and results in the induction of apoptosis.

In summary, we have demonstrated that low concentrations of spicamycin and KRN5500 induced differentiation of NB4 and HL-60 cells to a small extent, but markedly induced apoptosis in NB4, and high concentrations of both compounds induced apoptosis in all myeloid and lymphoid cells tested. The induction of apoptosis by spicamycin and KRN5500 was associated with down-regulation of Bcl-2 expression and modulation of PML protein. Therefore, spicamycin and KRN5500 may be useful for the treatment of myeloid and lymphoid neoplasms.

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