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INDUCTION OF ERYTHROID DIFFERENTIATION IN LEUKAEMIC K562 CELLS BY AN S-ADENOSYLHOMOCYSTEINE HYDROLASE INHIBITOR, ARISTEROMYCIN

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SUMMARY: We have isolated an unusual nucleoside, aristeromycin, from the culture filtrate of *Actinomycetes* as a compound that induces normal morphology in v-abl^{ts}-NIH3T3 cells. Aristeromycin also induced erythroid differentiation in abl-expressing human chronic myelogenous leukaemia K562 cells. It did not affect the amount of Abl or the Abl-associated tyrosine kinase activity in either v-abl^{ts}-NIH3T3 or K562 cells. As a potent inhibitor of S-adenosylhomocysteine hydrolase, aristeromycin inhibited methylation of phosphatidylethanolamine to form phosphatidylcholine in K562 cells. Among aristeromycin analogues, the activity to inhibit S-adenosylhomocysteine hydrolase was paralleled with the induction of erythroid differentiation. Thus, aristeromycin inhibits abl functions indirectly, possibly by inhibiting biological methylations.

The abl oncogene is activated by chromosomal translocation to give a bcr-abl gene. This chromosomal translocation is observed in most cases of human chronic myelogenous leukaemia (CML) associated with the Philadelphia chromosome (1). The products of activated or viral abl possess higher tyrosine kinase activity than that of unactivated cellular abl (2,3). Antisense DNA for abl (4) or a tyrosine kinase inhibitor, herbimycin (5), was shown to induce erythroid differentiation in human CML K562 cells. Herbimycin also showed antitumour activity against abl-expressing tumours in mice (6). Erbstatin, an inhibitor of tyrosine kinase that competes with the peptide substrate (7), directly inhibited Abl tyrosine kinase and induced erythroid differentiation weakly (4,8).

Because anti-Abl compounds may become useful new chemotherapeutic agents for CML, therefore we began screening culture filtrates of microorganisms for substances that induce normal morphology in v-abl-transformed NIH3T3 cells. As a result, we isolated aristeromycin from *Actinomycetes*. Aristeromycin is a potent inhibitor of S-adenosylhomocysteine hydrolase (9). In this present report, screening of aristeromycin and the effect of the compound on erythroid differentiation are described, and the mechanism of its action is discussed.

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MATERIALS AND METHODS

Materials v-abl^{ts}-NIH3T3 and K562 cells were kindly supplied by Dr. J. Wang, UCSD, San Diego and Dr. H. Tapiero, ICIG, Villejuif, respectively. Anti-abl and anti-phosphotyrosine antibodies were purchased from ICN and Oncogene Science, respectively. Formycin was a gift from Dr. T. Sawa, Institute of Microbial Chemistry, Tokyo. 3-Deazaadenosine was purchased from Sigma. [methyl-^{14}C] Methionine (55 mCi/mmol) were obtained from American Radiolabeled Chemicals Inc.

Cell culture v-abl^{ts}-NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) at the permissive temperature (33°C) or the nonpermissive temperature (39°C) in a 5% CO_2/95% air atmosphere. K562 cells were cultured at 37°C in RPMI 1640 medium containing 10% foetal bovine serum.

Isolation of aristeromycin The culture filtrate (1 liter) of *Actinomycetes* M3852-NF8 was applied onto a Diaion HP-20 column chromatography, and washed with water. Aristeromycin was eluted with 30% methanol, and the eluate was concentrated under reduced pressure. The resultant aqueous solution was adjusted to pH 2 with HCl and extracted with 1-butanol. Then the aqueous layer was adjusted to pH 8 with NaOH and extracted with 1-butanol again. The organic layer thus obtained was concentrated *in vacuo* to yield a yellow material. Final purification was carried out with Toyopearl HW-40 column chromatography with methanol to give aristeromycin as a colorless oil (17 mg).

Erythroid differentiation K562 cells were inoculated at $3x10^4$ cells/ml in a 24-well plate, and the test chemical was then added. After incubation for indicated days, the cell number was counted and erythroid differentiation was assessed by benzidine staining as previously described (10).

Methylation of phospholipids Cellular methylation activity was determined by monitoring the incorporation of labeled methyl groups into phospholipids. K562 cells were inoculated at $1.0x10^6$ cells/ml in 1 ml of methionine-free RPMI medium containing 10% dialyzed FBS. Then, 0.5 μCi/ml of L-[methyl-^{14}C] methionine and the chemical were added. After incubation for 3 hours the cells were collected and treated with 0.5 ml of TCA for 10 min at 4°C. The lipids were extracted with chloroform and methanol, and the extracts were spotted on a silica gel thin-layer chromatography (TLC) plate, which was developed with chloroform-methanol-acetic acid-H_2O (25:15:4:2). The TLC plate was sprayed with a sensitivity-enhancing solution (DuPont) and kept at -80°C for several days for autoradiography.

RESULTS

Culture filtrates of microorganisms were screened for induction of normal morphology in v-abl^{ts}-NIH3T3 cells, a line that shows transformed morphology at 33°C. Among 500 samples, a culture filtrate of *Actinomycetes* induced conversion of the cells to a normal flat morphology. The active principle was isolated; and the structure was elucidated spectroscopically to be aristeromycin (Fig. 1), a known nucleoside antibiotic (11).

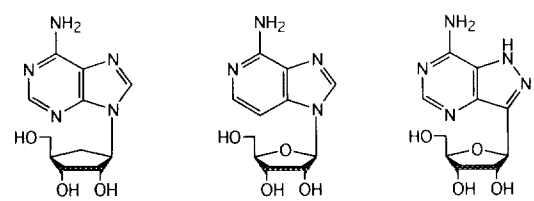

Fig. 1. Aristeromycin and related nucleosides.

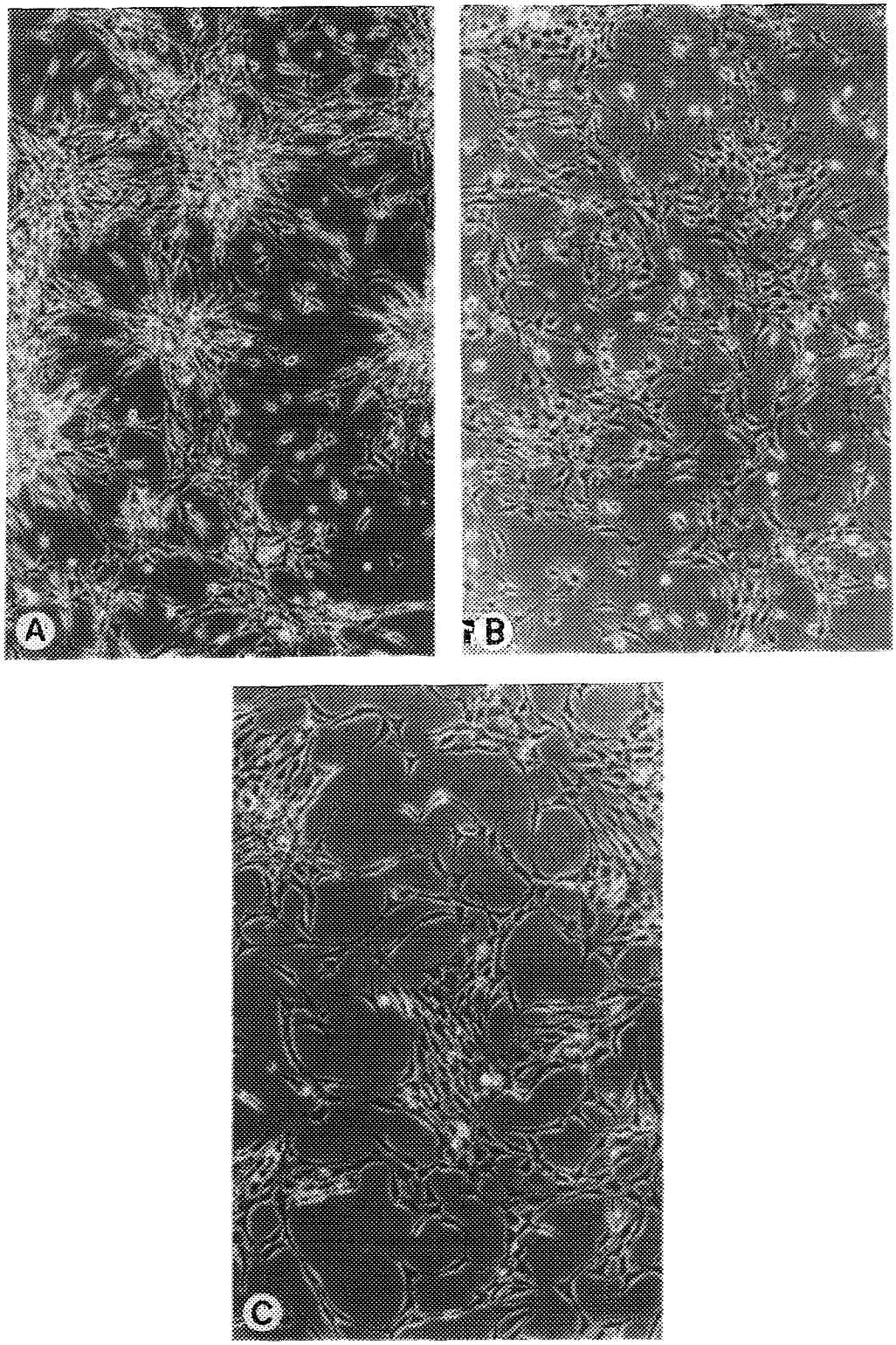

Fig. 2. Induction of normal morphology by aristeromycin in v-abl^{ts}-NIH3T3 cells. The cells were incubated at 33°C in the absence (A) or presence (B) of 1 μg/ml of aristeromycin for 24 hrs, or were incubated at the nonpermissive 39°C (C).

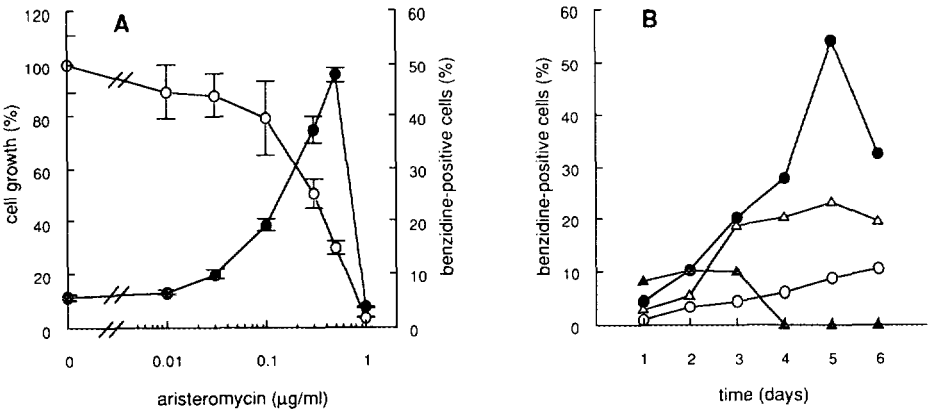

Fig. 3. Aristeromycin-induced erythroid differentiation in K562 cells. (A) The cells were incubated with aristeromycin at various concentrations for 5 days. The cell growth (○) and the erythroid differentiation (●) are shown. Each value is the mean±SD of triplicate determinations. (B) The cells were incubated with 0 (○), 0.1 (△), 0.3 (●), or 1 (▲) μg/ml of aristeromycin for various periods of time. Each value is the mean of duplicate determinations, and the difference between the two determinations was smaller than 15%.

Aristeromycin at 1 μg/ml induced a decrease in the piling up of the cells during a 24-hr incubation, as shown in Fig. 2. The IC_{50} values on the growth of v-abl^{ts}-NIH3T3 cells were 0.42 and 0.68 μg/ml at 33°C and 39°C, respectively. Aristeromycin did not change the morphology of the cells at 39°C, but induced a morphological change weakly in K-ras^{ts}-NRK cells at 33°C (data not shown).

Aristeromycin indued erythroid differentiation in K562 cells dose-dependently at 0.1-0.5 μg/ml, as shown in Fig. 3A. It inhibited the growth of the cells with an IC_{50} of 0.31 μg/ml. The erythroid cells could be detected in 3 days with 0.3 μg/ml of aristeromycin, and the maximal effect was observed on day 5 (Fig. 3B).

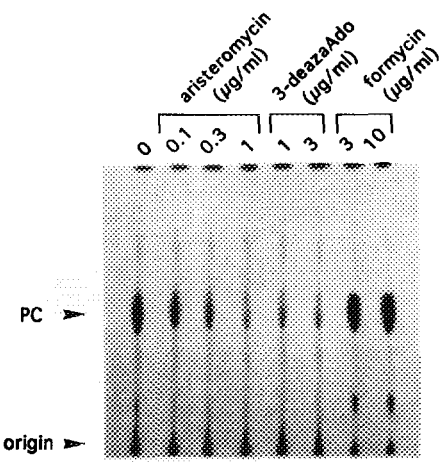

Fig. 4. Inhibition by aristeromycin and related nucleosides of methylating reaction in phophatidylcholine synthesis. K562 cells were incubated with [^{14}C] methionine and each nucleoside for 3 hrs. The result is the representative of triplicate examinations. PC, phosphatidylcholine.

Although aristeromycin induced differentiation in K562 cells, it did not affect intracellular tyrosine phosphorylation or the amount of Bcr-Abl protein in cultured K562 cells. Also it did not inhibit the tyrosine kinase activity *in vitro* of precipitated Bcr-Abl with enolase used as the substrate (data not shown).

Since aristeromycin is known to be a potent inhibitor of S-adenosylhomocysteine hydrolase (9), we looked into the effect of the compound on intracellular methylations. One of the biosynthetic pathways for phosphatidylcholine synthesis is the successive methylation of phosphatidylethanolamine. Aristeromycin inhibited formation of phosphatidylcholine by the methylating reactions, as shown in Fig. 4. 3-Deazaadenosine and formycin are known to inhibit S-adenosylhomocysteine hyrolase but more weakly than aristeromycin. 3-Deazaadenosine inhibited the phosphatidylcholine synthesis slightly more weakly, and formycin did not.

As shown in Fig. 5A, 3-deazaadenosine induced erythroid differentiation in K562 cells, but more weakly than aristeromycin. Formycin showed no effect on erythroid differentiation (Fig. 5B).

DISCUSSION

Aristeromycin, a known nucleoside antibiotic, was shown to induce normal morphology in abl-expressing NIH3T3 cells and erythroid differentiation in human CML K562 cells, but not by directly inhibiting Abl-associated tyrosine kinase.

Although it inhibited RNA synthesis in K562 cells, this activity may not be related to the induction of differentiation, since formycin did not induce the erythroid differentiation despite its inhibition of RNA sythesis.

The effect of aristeromycin on methylation was examined by means of following phosphatidylcholine sythesis. It only slightly reduced total phosphatidylcholine synthesis in terms of the incorporation of labeled phosphate (data not shown). However, using methyl-labeled methionine, a drastic reduction in the methylation of phospholipid was observed in K562 cells

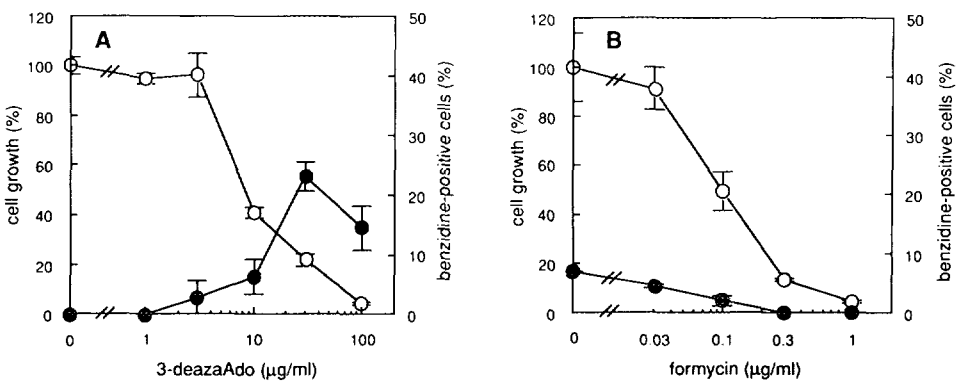

Fig. 5. Effect of 3-deazaadenosine and formycin on erythroid differentiation of K562 cells. The cells were incubated with 3-deazaadenosine (A) or formycin (B) at various concentrations for 5 days. The cell growth (○) and the erythroid differentiation (●) are shown. Each value is the mean±SD of triplicate determinations.

exposed to aristeromycin. Adenosine analogues are known to inhibit S-adenosylhomocysteine hydrolase (9). Then, accumulated S-adenosylhomocysteine acts as a feedack inhibitor, inhibiting S-adenosylmethionine-dependent methylation. Aristeromycin is an extremely potent inhibitor of the hydrolase, with a Ki of 5 nM, whereas 3-deazaadenosine and formycin have Ki values of 4 μM and 280 μM, respectively (9). Therefore, the inhibitory activity on the enzyme paralleled with the induction of differentiation.

Thus, aristeromycin may inhibit abl functions to induce erythroid differentiation by inhibiting intracellular methylations. It is likely that aristeromycin also inhibits the methylation of compounds other than phospholipids as well. The essential target of methylation for induction of erythroid differentiation is now under investigation.

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