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# WIDE DIFFERENCE BETWEEN THE CYTOTOXICITY OF THE 11-α- AND 11-β-CYANO ANALOGUES OF TILIVALLINE AND THEIR EPIMERIC CONVERSION

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**Abstract**—Tilivalline (TV) possesses a pyrrolo[1,4]benzodiazepine nucleus and is cytotoxic toward mammalian cells. The 11-β-cyano TV analogue (**1**) is about one hundred times more cytotoxic to mouse leukemia L1210 cells than TV itself. In contrast, the 11-α-cyano TV analogue (**2**), an epimer of **1**, has only about one-hundredth the cytotoxicity of **1**. It was found that epimerization proceeded between **1** and **2** under physiological conditions, and the cytotoxicity of **2** is thought to be caused mainly by **1** that was formed from **2** during incubation in medium.

*Key words:* tilivalline; pyrrolo[1,4]benzodiazepine; 11-cyano tilivalline analogue; epimerization; interconversion; cytotoxicity; L1210 cells

TV§ was isolated from the secondary metabolites of *Klebsiella pneumoniae* var. *oxytoca*. Its structure was determined by Mohr and Budzikiewicz [1] and is shown in Fig. 1. It is cytotoxic toward mammalian cells and possesses a pyrrolo[1,4]benzodiazepine nucleus in its structure, as is present in antitumor antibiotics such as anthramycin and tomaymycin [2]. We previously synthesized many TV analogues [3–5] and evaluated their cytotoxicity toward mouse leukemia L1210 cells [6]. Among the compounds tested, the 11-β-cyano TV analogue (**1**) was found to be the most cytotoxic ($IC_{50}$ 0.05 μg/mL), about one hundred times more cytotoxic than TV itself ($IC_{50}$ 4.7 μg/mL). In contrast, the 11-α-cyano TV analogue (**2**), an epimer of **1**, had about only one-hundredth the cytotoxicity ($IC_{50}$ 4.7 μg/mL) of **1**. We were interested in this wide difference in cytotoxicity between the two diastereomers and, in this report, provide evidence that epimeric conversion proceeds between the 11-α- and 11-β-cyano TV analogues. In addition, our findings suggested that the cytotoxicity of **2** may be far weaker than that of **1** and may be caused mainly by **1** that forms from **2** during incubation in medium.

## MATERIALS AND METHODS

### *Chemicals*

TV and its 11-α- and 11-β-cyano analogues were synthesized as previously reported [3]. The purity of these compounds was more than 99.9%, judging from HPLC.

### *Cytotoxicity (growth inhibition) assay*

A previously reported procedure was employed [7]. Briefly, mouse leukemia L1210 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37° in a humidified incubator with 5% $CO_2$ in air. Cells growing in the log phase were collected, and aliquots of $2 \times 10^5$ cells were seeded in culture dishes containing 2 mL of the medium. After the cells had been incubated for 16 hr, 10 μL of dimethyl sulfoxide solution containing an appropriate concentration of test compound (TV and its 11-α- and 11-β-cyano analogues) was added, and the cell suspension was incubated for another 48 hr. Surviving cells that excluded trypan blue dye were then counted. The concentration of each compound that inhibited cell growth to 50% of the control cells ($IC_{50}$) was deduced graphically. To determine the effect of length of exposure to the compound on cell growth, cells ($2 \times 10^5$ in 2 mL medium) were treated for 1, 3, 6, 12, 24 and 48 hr with each compound at a concentration (TV: 10 μg/mL; **1**: 0.2 μg/mL; **2**: 10 μg/mL) that reduced cell growth to approximately 10% after a 48-hr exposure. After treatment, the cell suspension was centrifuged and separated into cells and medium. Fresh medium (2 mL) was then added to the cell portion, and the suspension was incubated for a period [48 hr minus treated (T) hr]. The medium portion was added to the freshly prepared cells, and the cell suspension was incubated for 48 hr. The number of surviving cells was then counted. The experimental protocol is shown in Fig. 2A. To determine the effect of length of preincubation of the compound in medium on cell growth, each compound (TV: 10 μg/mL; **1**: 0.2 μg/mL; **2**: 10 μg/mL) was preincubated in 2 mL of medium at 37° for 3, 6, 12, 24 and 48 hr. Cells ($2 \times 10^5$) were then added to the medium, and the cell suspension was

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§ Abbreviation: TV, tilivalline.

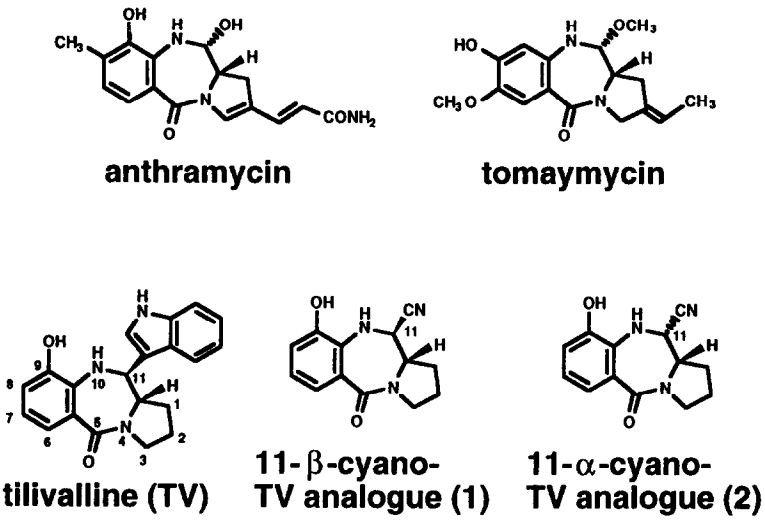


Fig. 1. Structure of tilivalline (TV), the 11-α- and 11-β-cyano TV analogues, and related compounds.

incubated for 48 hr. Surviving cells were then counted.

*Detection of TV and its* 11-α- *and* 11-β-*cyano analogues by HPLC*

A Shimadzu LC-9A HPLC apparatus equipped with a UV detector was used. The Merck LiChrospher 100RP-18(e) column (4.6 × 250 mm) used was eluted with a solution of 10 mM $NaH_2PO_4$-MeOH (13:7, v/v) at a flow rate of 0.8 mL/min. The products that eluted were detected at a wavelength of 240 and 260 nm. Retention times of the 11-α- and 11-β-cyano TV analogues were 7.3 and 8.9 min, respectively.

## RESULTS AND DISCUSSION

*Effect of exposure time of TV and its* 11-*cyano analogues on cell growth*

If the great difference in cytotoxicity between **1** and **2** is related to processes of cellular metabolism, cytotoxicity of the compound may vary in response to the length of pretreatment with the cells. Therefore, the effect on cell growth of varying the period of exposure to the compounds was examined. The experimental protocol employed is shown in Fig. 2A. Cells were treated for the indicated time (T hr) with each compound. The concentration of each compound employed for this experiment was one that reduced cell growth to approximately 10% after 48 hr of exposure and was determined from the concentration–response cell growth inhibition curve of each compound [6]. After treatment, the cell suspension was centrifuged and separated into cells and medium. Fresh medium was then added to the cell portion, and the suspension was incubated for a period (48 hr minus T hr). The medium portion was added to the freshly prepared cells, and the cell suspension was incubated for 48 hr. The number of surviving cells was then counted. With TV, cell growth decreased with exposure time, and more than 12 hr of exposure was required for TV to express close to full cytotoxicity (Fig. 2B). The cytotoxicity remaining in the medium decreased with pre-exposure time, and cell growth increased. Similarly, with the 11-β-cyano TV analogue, cell growth decreased relative to exposure time and cytotoxicity of the 11-β-cyano TV analogue remaining in the medium decreased with pre-exposure time (Fig. 2C). On the contrary, with the 11-α-cyano epimer, cell growth decreased with exposure time, but the cytotoxicity of the 11-α-cyano TV analogue remaining in the medium increased with pre-exposure time (Fig. 2D).

*Effect on cell growth of preincubation of TV and its* 11-*cyano analogues in medium*

The 11-α-cyano TV analogue showed stronger cytotoxicity after it was preincubated in cell suspension (Fig. 2D). To determine if this was caused by the cells themselves or other factors, the cytotoxicity of each compound was examined after the compound had been preincubated for the indicated time in cell-free medium. As shown in Fig. 3, TV remained stable with a 12-hr preincubation and gradually lost activity after further preincubation, although the loss was slight. The 11-β-cyano TV analogue showed an increase in cytotoxicity up to 12 hr of preincubation, but it also showed a similar loss of activity after further incubation, as seen with TV. However, the 11-α-cyano TV analogue showed increasing cytotoxicity with preincubation time. These results strongly suggest that the 11-α-cyano TV analogue was converted to an active form by preincubation, even in the cell-free medium.

*Conversion of the* 11-*cyano TV analogue to its corresponding* 11-*cyano epimer*

The epimerization of the 11-α- and 11-β-cyano TV analogues in buffer solution was then examined. These analogues were each (20 μg/mL) incubated in PBS (pH 7.0) at 37° for an indicated time, and changes in their respective levels were measured by

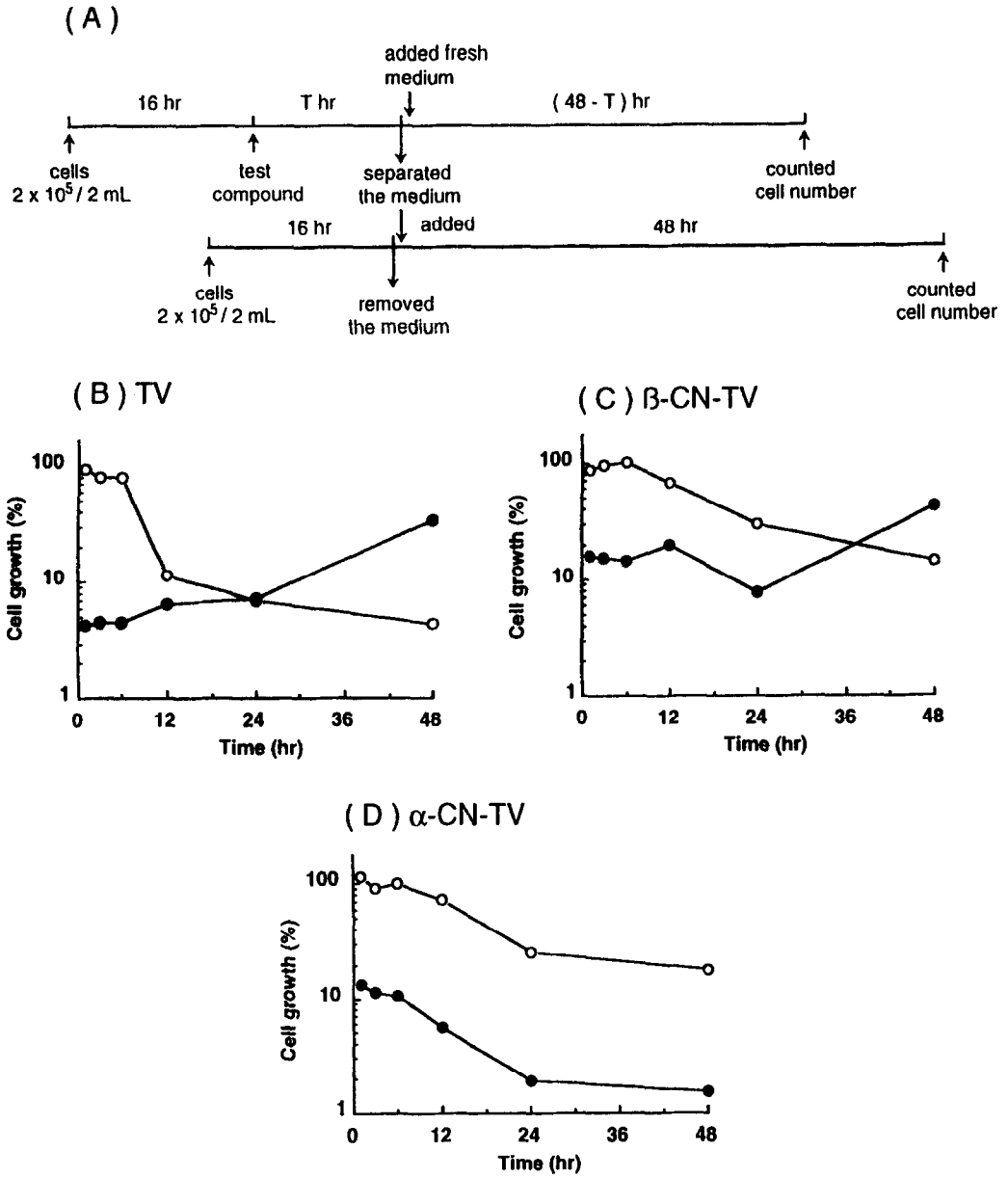


Fig. 2. Effect of length of exposure of the compound on cell growth. (A) Experimental protocol (see details in Materials and Methods); (B) TV (10 μg/mL); (C) 11-β-cyano TV analogue (0.2 μg/mL); and (D) 11-α-cyano TV analogue (10 μg/mL). Key: (○) growth of cells treated with a compound for the indicated time; and (●) growth of cell cultured in medium that had been pretreated with other cells for the indicated time.

HPLC. The 11-α-cyano TV analogue decreased with incubation time, and this decrease was accompanied by formation of the 11-β-cyano TV analogue (Fig. 4). The ratios of α to β conversion (epimerization) were 1, 2, 5 and 8% after 24, 48, 72 and 96 hr of incubation, respectively. Similarly, the 11-β-cyano TV analogue decreased with incubation, and this was accompanied by the formation of the 11-α-cyano TV analogue. The ratios of β to α conversion (epimerization) were 5, 11, 13 and 16% after 24, 48, 72 and 96 hr of incubation, respectively.

To further characterize these compounds, the epimerization in acidic (pH 2.2) and weakly alkaline (pH 8.5) solutions was examined at 37° (Fig. 5). Under acidic conditions, the 11-α-cyano TV analogue was very stable, whereas the 11-β-cyano TV analogue converted to the stable 11-α-form and other products. Unlike alkaline conditions, both the 11-α- and the 11-β-cyano TV analogues produced the 11-β- and 11-α-cyano epimer, respectively; however, both of the epimers that were formed decomposed rapidly. Although stereoselectivity is often observed with

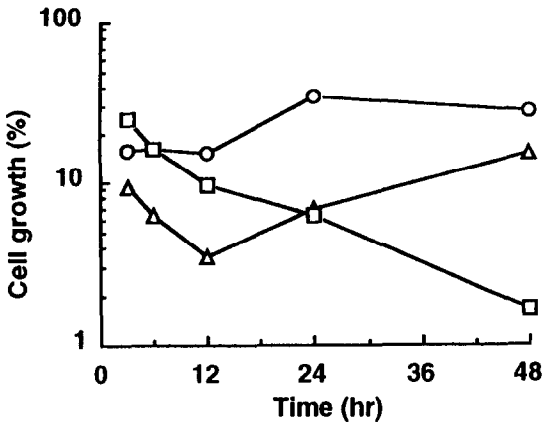


Fig. 3. Effect of length of preincubation of the compound in medium on cell growth. Each compound was preincubated in 2 mL of medium at 37° for the indicated time. Cells ($2 \times 10^5$) were then added to the medium, and the cell suspension was incubated for 48 hr. Surviving cells were then counted. Key: (○) TV; (□) the 11-α-cyano TV analogue; and (△) 11-β-cyano TV analogue. The concentration of each compound employed was 10, 10 and 0.2 μg/mL, respectively.

enzymatic reactions, it is worth noting that stereoselective epimerization of the 11-β-cyano TV analogue was found to occur under nonenzymatic acidic conditions.

*A possible mechanism for the cytotoxicity of the* 11-α-*cyano TV analogue*

Incubation of the 11-α-cyano TV analogue (20 μg/mL) in neutral PBS at 37° for 24 and 48 hr resulted in the formation of 1 and 2% of the highly cytotoxic 11-β-cyano TV analogue, respectively. The amount of the 11-β-cyano TV analogue formed from the 11-α-cyano TV analogue seemed to be sufficient to account for cytotoxicity. It may be that the 11-β-cyano TV analogue formed from the 11-α-cyano TV analogue by epimerization is mainly responsible for the cytotoxic effect.

This wide difference in cytotoxicity between the two stereoisomers, namely the 11-α- and the 11-β-cyano TV analogues, was of great interest, and a study of the mechanisms involved is now in progress.

*Elucidation of the mechanisms responsible for the cytotoxicity of the* 11-β-*cyano TV analogue*

*DNA modification.* Anthramycin is known to react with the 2-amino group of guanines in cellular DNA after the carbinolamine of anthramycin undergoes reversible dehydration to form imine [2]. Therefore, we evaluated the reactivity of the 11-β-cyano TV analogue using the NBP [4-(*p*-nitrobenzyl)pyridine] assay described previously [8]. This analogue (4.5 mM) was allowed to react with NBP, and the product formed was measured by visible light (545 nm) absorption. Under conditions in which the carcinogens *N*-methyl-*N*-nitrosourea (MNU) and *N*-ethyl-*N*-nitrosourea (ENU) reacted with NBP, the 11-β-cyano TV analogue failed to do so.

*Inhibition of macromolecular synthesis.* Anthramycin is known to inhibit DNA synthesis [2]. Therefore, the effect of the 11-β-cyano TV analogue on macromolecular synthesis was examined using human T-cell acute lymphoblastoid leukemia cells (CCRF-HSB-2), as previously reported [9]. The $IC_{50}$ of the 11-β-cyano TV analogue for these cells was 0.28 μg/mL. After cells ($2 \times 10^5$) were treated with the 11-β-cyano TV analogue (0.1, 0.32, 1.0, 3.2 and 10 μg/mL) for 4 hr, a radioactive precursor (37 kBq/mL of [$^3$H]uridine, [$^3$H]thymidine or [$^{35}$S]methionine) was added, and the mixture was incubated for another hour. Incorporation of the radioactive precursor into the acid-insoluble fraction was then measured, using a liquid scintillation counter. Results showed a slight tendency toward inhibition of RNA

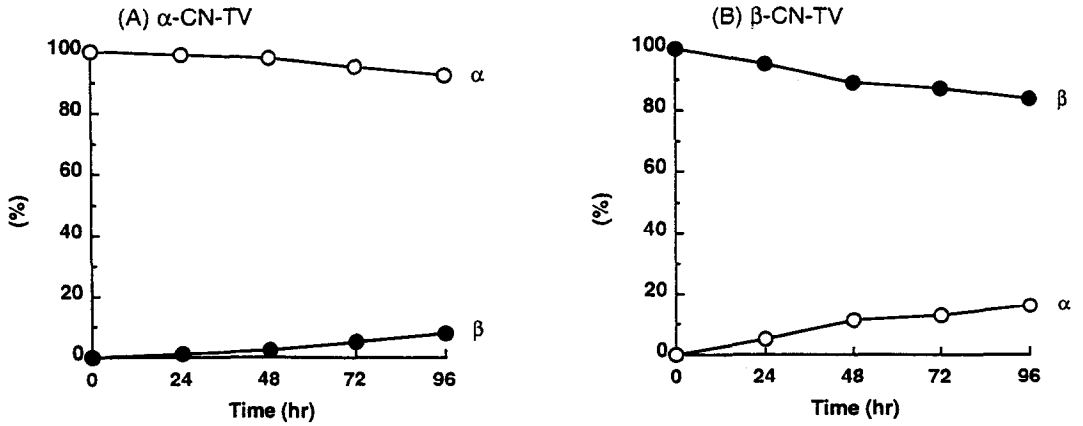


Fig. 4. Epimeric conversion of the 11-α- or the 11-β-cyano TV analogue to the 11-β- or the 11-α-cyano TV analogue, respectively. The 11-α-cyano (○) and the 11-β-cyano (●) TV analogues (20 μg/mL) were each incubated in PBS (pH 7.0) at 37° for the indicated time. The decrease in starting material and the formation of products were then analyzed by HPLC. (A) 11-α-cyano TV analogue; and (B) 11-β-cyano TV analogue.

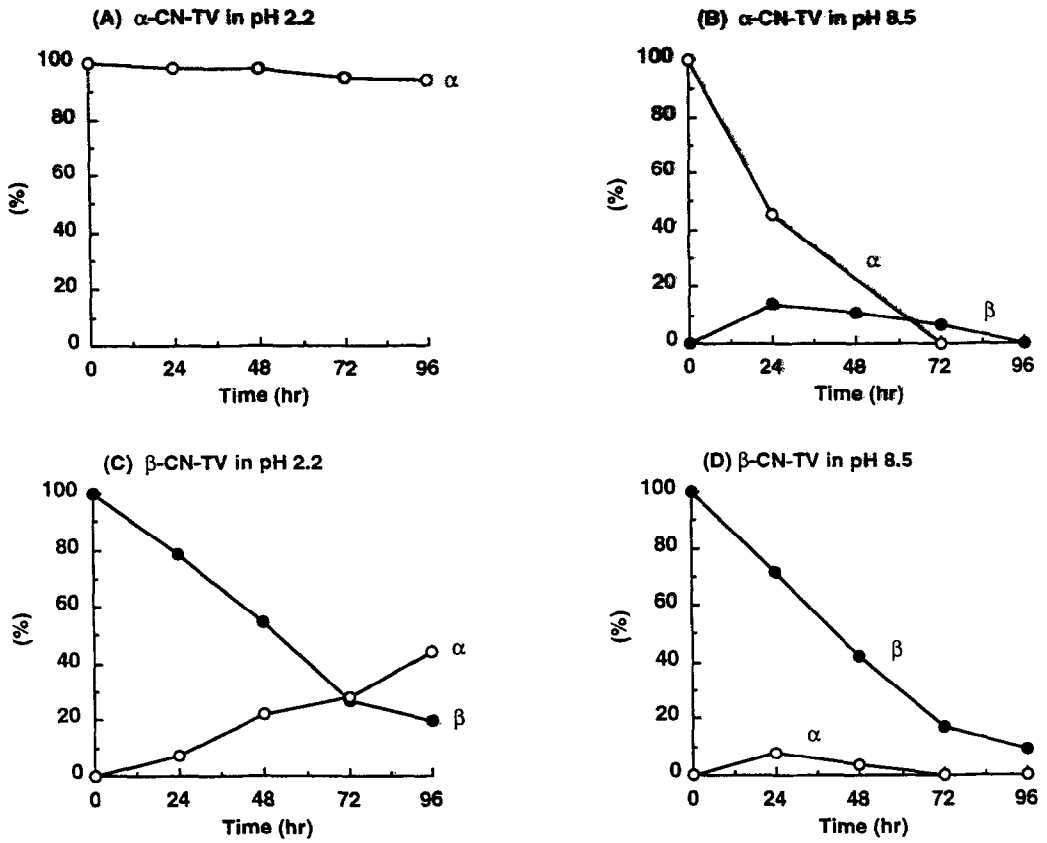


Fig. 5. Epimerization and stability of the 11-α- and 11-β-cyano TV analogues under acidic (pH 2.2) and weakly alkaline (pH 8.5) conditions. Each compound (20 μg/mL) was incubated in a solution at 37° for the indicated time. Then the decrease in starting material and the formation of products were analyzed by HPLC. Shown are the 11-α-cyano TV analogue in acidic (A) and weakly basic (B) solutions; and the 11-β-cyano TV analogue in acidic (C) and weakly basic (D) solutions. Key: (○) 11-α-cyano TV analogue; and (●) 11-β-cyano TV analogue.

and protein syntheses at the highest concentration of 10 μg/mL, but it was not significant.

*Inhibition of cell cycle.* The effect of the 11-β-cyano TV analogue on the cell cycle was examined using a FACScan. After mouse leukemia L1210 cells were treated with 0.05 μg/mL of the 11-β-cyano TV analogue for 0. 8, 16, 24 and 48 hr, cells were collected, fixed with MeOH, hydrolyzed with RNase, and stained with propidium iodide. Cell cycle analysis was then carried out with a FACScan. Results showed that there was some tendency toward $G_2$/M arrest, but it was not significant.

If the cyano group of the 11-β-cyano TV analogue was eliminated to form imine, as proposed for anthramycin, the 11-β-cyano TV analogue should express chemical and biological responses similar to anthramycin. However, results of this study suggest that the cytotoxicity of the 11-β-cyano TV analogue may differ from that of anthramycin in terms of its mode of action, and further study including investigation of the wide difference in cytotoxicity between the α- and β-forms is required.

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