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Different Moieties of Tautomycin Involved in Protein Phosphatase Inhibition and Induction of Apoptosis

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ABSTRACT. The effects of tautomycin and its derivatives on protein phosphatases PP1 and PP2A and their apoptosis-inducing activity toward human leukemia Jurkat cells were examined, and the relationship between chemical structure and function was discussed. Among the compounds we examined, tautomycin was the most potent inhibitor and the most effective inducer of apoptosis. It inhibited PP1 and PP2A enzymatic activity concentration-dependently with IC_{50} values of 20 and 75 pM, respectively, in the presence of 0.01% Brij-35, and an LC_{50} value of 1 μM. Esterification of the anhydride moiety of tautomycin markedly increased the IC_{50} for the protein phosphatases. The $C_{1'}$-$C_{7'}$ fragment of tautomycin had no inhibitory effect, but the fragment containing the C_{22}-C_{26} moiety was inhibitory. These results suggest that the C_{22}-C_{26} moiety is essential for inhibition of protein phosphatase activity and that the anhydride moiety enhances the inhibition. However, the esterification of the anhydride did not decrease, nor did the inclusion of the C_{22}-C_{26} moiety increase the apoptosis-inducing activity. On the other hand, the C_1-C_{18} moiety of tautomycin was essential for induction of apoptosis, and the conformation and the arrangement of functionalities of the C_{18}-C_{26} carbon chain affected the apoptosis activity. However, modification of C_1-C_{18}, C_1-C_{21}, or C_1-C_{26} compounds had little effect on phosphatase inhibitory activity. Our results strongly suggest that different moieties of tautomycin are involved in protein phosphatase inhibition and induction of apoptosis. BIOCHEM PHARMACOL **55**;7:995–1003, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. tautomycin; antibiotic; protein phosphatase; protein phosphatase inhibitor; apoptosis; Jurkat cell

Tautomycin, an antibiotic isolated from *Streptomyces spiroverticillatus* (see Fig. 1) [1–3], is a potent and specific inhibitor of serine/threonine protein phosphatases PP1¶ and PP2A [4, 5]. Various IC_{50} values of tautomycin for PP1 and PP2A have been reported: 0.16 and 0.40 nM [4], 22 and 32 nM [5], and 0.7 and 0.65 nM [6] for the catalytic subunits of PP1 and PP2A, respectively. Here, we found that Brij-35, a non-ionic detergent, lowers the IC_{50} values for PP1 from 0.20 to 0.020 nM and for PP2A from 0.70 to 0.075 nM, suggesting that the wide variation in the IC_{50} values is due, at least in part, to differences in the assay conditions employed.

Okadaic acid is a well-known protein serine/threonine phosphatase inhibitor [7] that has apoptosis-inducing activity [8, 9]. Apoptosis-inducing activity has not been demonstrated for tautomycin. In the present study, we examined the apoptosis-inducing activity of tautomycin and found that it is a potent apoptosis inducer.

Recently, Oikawa *et al.* [10] reported total chemical synthesis of tautomycin. In the present study, we examined the biological activities, including phosphatase inhibitory activity and apoptosis-inducing activity, of tautomycin and its related compounds, such as its derivatives or its synthetic intermediates. The relationship between structure and function in the tautomycin molecule is discussed.

MATERIALS AND METHODS

Materials

The catalytic subunits of PP1 and PP2A and phosphorylase *a* were prepared from rabbit skeletal muscle as previously described [11–13]. [γ-^{32}P]ATP was purchased from Dupont/NEN Research Products. Hoechst 33342 was obtained from Sigma and Brij-35 [polyoxyethylene (23) lauryl ether] was from Wako Pure Chemical Industries. Anti-Fas antibody was a gift from Dr. Shuji Takahashi (Sapporo Medical University).

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¶ *Abbreviations:* PP1, protein serine/threonine phosphatase type 1; PP2A, protein serine/threonine phosphatase type 2A; LC_{50}, concentration that causes 50% lethality; TM, tautomycin; MPM, *p*-methoxyphenylmethyl; and MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide.

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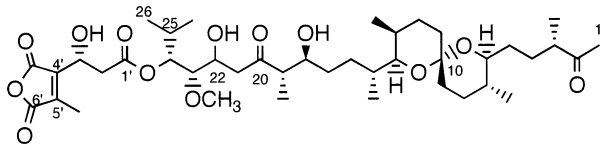

FIG. 1. Chemical structures of tautomycin and 22-epi-tautomycin.

Synthesis of Tautomycin and Its Derivatives

The structures of the compounds used in this study are shown Figs. 1 and 2. Tautomycin **1**, purified as reported from the crude extract of *S. spiroverticillatus*, was supplied by Professor Isono (Tokai University) and the Kaken Pharmaceutical Co. [3]. 22-Epi-tautomycin **2**, TM-dimethyl ester **3**, Δ_1-C_1-C_{18}-silyl ether **8a**, 2-keto-C_1-C_{18}-alcohol **8b**, Δ_1-C_1-C_{18}-aldehyde **9a**, 2-keto-C_1-C_{18}-aldehyde **9b**, (18*S*,19*R*)-C_1-C_{21}-fragment **10a**, (18*R*,19*S*)-C_1-C_{21}-fragment **10b**, and 20-keto-C_1-C_{21}-silyl ether **12a** were synthesized as described by Oikawa *et al.* [10]. Anhydrodeacyl-TM **4a**, $C_{1'}$-$C_{7'}$-methyl ester **5** and C_{19}-C_{26}-segment **6** were prepared from natural tautomycin as described by Cheng *et al.* [3]. C_{22}-$C_{7'}$-anhydride **7**, 20-keto-C_1-C_{21}-fragment **12b**, Δ_1-22-epi-C_1-C_{26}-MPM ether **13**, 18-silyl-C_9-C_{26}-MPM ether **14a**, 18-silyl-22-epi-C_9-C_{26}-MPM ether **15a**, and their desilylated analogs **14b** and **15b** were synthesized from reported materials in ways similar to those described previously [10]. C_1-C_{21}-Triol **11** was synthesized from C_{20}-C_{21}-epoxide [10] as follows. To a solution of the

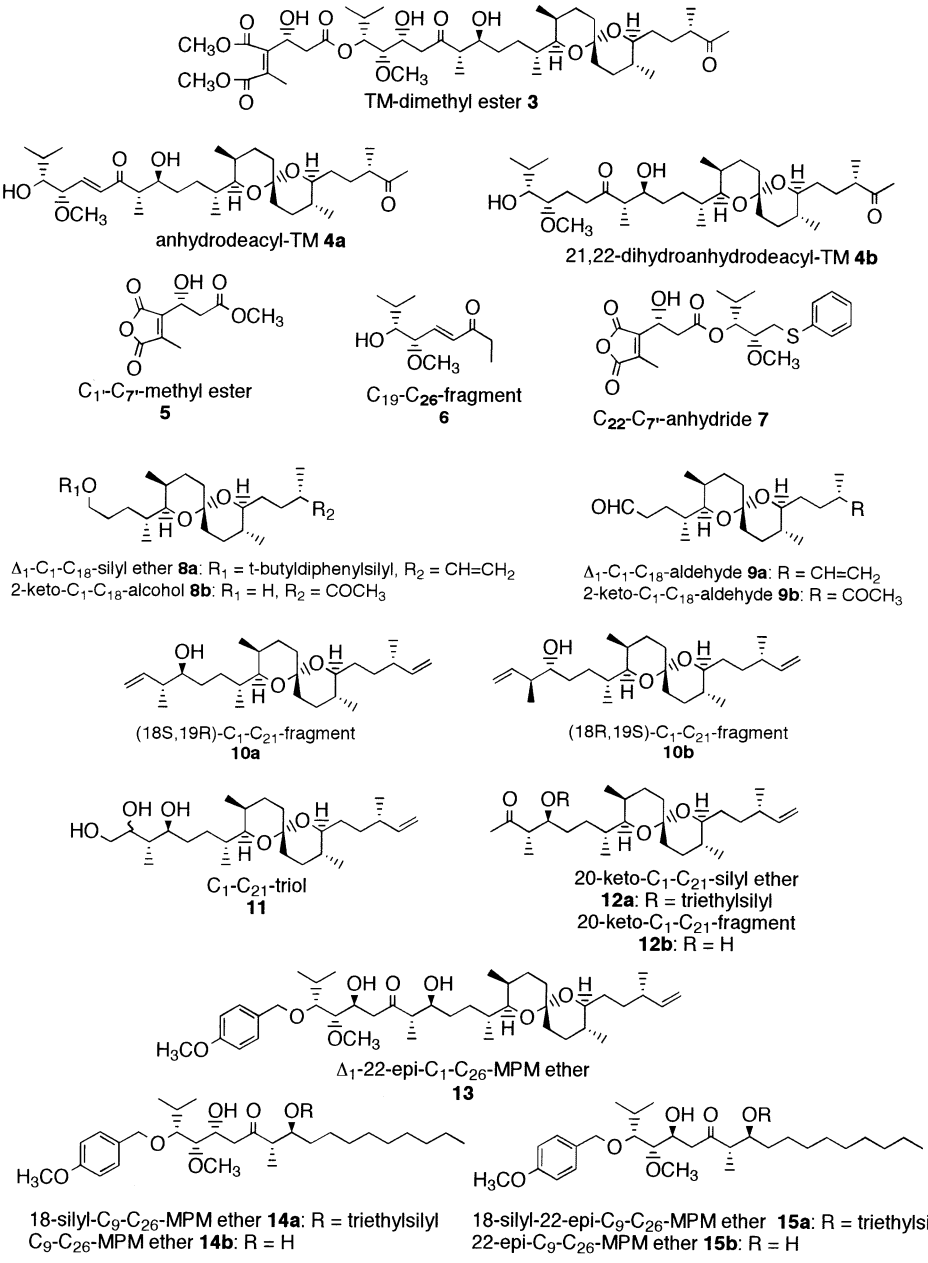

FIG. 2. Chemical structures of tautomycin derivatives and related compounds.

epoxide (59 mg, 0.14 mmol) was added 1.2 g of anion exchange resin (Amberlyst A-26, CO_3^{2-} form). The suspension was heated under reflux with stirring. After 6 hr, the resin was filtered and washed with MeOH. The filtrates were combined and concentrated *in vacuo* to give a residue, which was chromatographed with a silica gel column (chloroform:acetone, 95:5, v/v) to afford 30 mg of triol **11** (49%) and 16 mg of a starting material (27%). $[\alpha]D^{25}$ −50.8° (*c* 0.83, $CHCl_3$). IR (NaCl): 3383, 3078, 2930, 1728, 1456, 1384, 1254, 1229, 1198, 1069, 986, 909, 757 cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$): δ 5.72 (1H, ddd, *J* = 7.3, 10.2, 16.2 Hz), 4.96 (1H, br.d, *J* = 16.2 Hz), 4.91 (1H, br.d., *J* = 10.2 Hz), 3.45–3.85 (4H, m), 3.32 (1H, dd, *J* = 2.0, 9.9 Hz), 3.15 (1H, m), 1.95–2.25 (2H, m), 1.85 (1H, m), 1.1–1.9 (17H, m), 1.02 (3H, d, *J* = 5.3 Hz), 1.00 (3H, d, *J* = 6.6 Hz), 0.90 (3H, d, *J* = 6.6 Hz), 0.85 (3H, d, *J* = 6.6 Hz), 0.82 (3H, d, *J* = 6.6 Hz). EIMS *m/z*: 423 (MH^+-H_2O, 39), 391 (27), 279 (21), 236 (76), 194 (100), 167 (39), 149 (95), 137 (44), 95 (43), 55 (60). EIHRMS *m/z* 423.3445 (MH^+-H_2O, $C_{26}H_{47}O_4$ requires 423.3475). 21,22-Dihydroanhydrodeacyl-TM **4b** was prepared as follows: to a solution of anhydrodeacyl-TM **4a** (12.8 mg, 0.023 mmol) was added 7.2 mg of 5% palladium on carbon. The mixture was stirred vigorously for 4 hr under a hydrogen atmosphere and filtered through a pad of Celite. The filtrate was concentrated to give a residue, which was purified by thin-layer SiO_2 chromatography (dichloromethane:methanol, 24:1, v/v) affording **4b** (12.0 mg, 93%). $[\alpha]D^{25}$ −50.8° (*c* 0.83, $CHCl_3$). IR (NaCl): 3446, 2933, 1714, 1067, 987 cm^{-1}. ^{1}H-NMR (270 MHz, $CDCl_3$): δ 5.72 (1H, ddd, *J* = 7.3, 10.2, 16.2 Hz), 4.96 (1H, br.d, *J* = 16.2 Hz), 4.91 (1H, br.d, *J* = 10.2 Hz), 3.45–3.85 (4H, m), 3.32 (1H, dd, *J* = 2.0, 9.9 Hz), 3.15 (1H, m), 1.95–2.25 (2H, m), 1.85 (1H, m), 1.1–1.9 (17H, m), 1.02 (3H, d, *J* = 5.3 Hz), 1.00 (3H, d, *J* = 6.6 Hz), 0.90 (3H, d, *J* = 6.6 Hz), 0.85 (3H, d, *J* = 6.6 Hz), 0.82 (3H, d, *J* = 6.6 Hz). ^{13}C-NMR (67.4 MHz, $CDCl_3$): δ 216.5, 213.0, 95.6, 80.8, 77.2, 75.1, 74.9, 74.29, 74.25, 57.0, 51.2, 47.3, 38.3, 36.0, 34.8, 31.7, 30.6, 30.2, 29.7, 29.1, 28.2, 28.1, 27.6, 27.5, 26.7, 21.0, 19.1, 18.7, 18.0, 16.7, 16.2, 14.4, 10.9. EIMS *m/z*: 423 (MH^+-H_2O, 39), 391 (27), 279 (21), 236 (76), 194 (100), 167 (39), 149 (95), 137 (44), 95 (43), 55 (60). EIHRMS *m/z*: 423.3445 (MH^+-H_2O, $C_{26}H_{47}O_4$ requires 423.3475).

Cell Line and Culture

Human acute T lymphoblastic leukemia Jurkat cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM of L-glutamine, 100 μg/mL of streptomycin, and 100 U/mL of penicillin. Cells were cultured at 37° in a humidified atmosphere of 5% CO_2. For induction of apoptosis, 2×10^5 cells were incubated in 1 mL of the complete medium with tautomycin or its derivatives in 24-well flat-bottom microtiter plates. Samples were withdrawn at time intervals and analyzed by fluorescence microscopy.

Preparation of Cell Extracts

Jurkat cells (2×10^6) were washed twice with 200 μL of 0.15 M of NaCl. The washed cells were homogenized in 100 μL of homogenization buffer containing 50 mM of Tris–HCl (pH 7.5), 250 mM of sucrose, 0.1% 2-mercaptoethanol, 1.0% Nonidet P-40, 0.2 μg/mL of phenylmethylsulfonyl fluoride (PMSF), 0.4 μg/mL of benzamidine, 10 μg/mL of leupeptin, 10 μg/mL of soybean trypsin inhibitor, and 4 μg/mL of antipain. The homogenates were centrifuged at 8000 *g* for 10 min at 4°, and the resulting supernatants were used as the crude extracts.

Assays of Protein Phosphatases PP1 and PP2A

The activities of PP1 and PP2A were measured by using ^{32}P-labeled phosphorylase *a* as a substrate as previously described [14]. One unit (U) of activity was defined as the amount of enzyme that catalyzes the release of 1 μmol phosphate/min.

Determination of Apoptosis

The viability of cells was determined by MTT assay [15]. Cells (2.5×10^4) were cultured for 24 hr, and then 0.5 mg/mL of MTT was added. After 4 hr, 1 vol. of 0.04 M of HCl/2-propanol was added, and the absorbance of the mixture was measured at 570 nm. Apoptotic cells were determined by fluorescence microscopy after staining with 10 μg/mL of Hoechst 33342 (Sigma) and 10 μg/mL of ethidium bromide [16]. DNA fragmentation was determined after gel-electrophoresis as follows. Cells (1×10^6) were washed with PBS and resuspended in 100 μL of lysis buffer containing 10 mM of Tris–HCl, pH 7.4, 10 mM of EDTA, and 0.5% Triton X-100. The lysate was centrifuged at 8000 *g* for 20 min. The supernatant was incubated with 40 μg of RNase A at 37° for 1 hr, and then 2 μL of 20 mg/mL proteinase K was added and the mixture was incubated for 1 hr. After the addition of 20 μL of 5 M of NaCl and 120 μL of 2-propanol, the mixture was allowed to stand overnight at −20°. After centrifugation at 8000 *g* for 20 min, the pellet was resuspended with 20 μL of TE buffer (1 mM of EDTA, 10 mM of Tris–HCl, pH 7.4), mixed with 4 μL of gel-loading buffer (40% sucrose, 0.25% Bromophenol Blue), and loaded on a 2% agarose gel containing 0.5 μg/mL of ethidium bromide, electrophoresed at 50 V for 2 hr in 2 mM of EDTA, 90 mM of Tris-borate, and photographed.

Extraction of Tautomycin and Its Related Compounds from Cells

Extraction of tautomycin and its related compounds was carried out, as follows, from Jurkat cells that had been incubated with them for 1 hr at 37°. Cells (2×10^6) were washed twice with 0.15 M of NaCl, disrupted hypotonically with 100 μL of water, mixed with 100 μL of acetone under vigorous shaking, then centrifuged at 8000 *g* for 10 min.

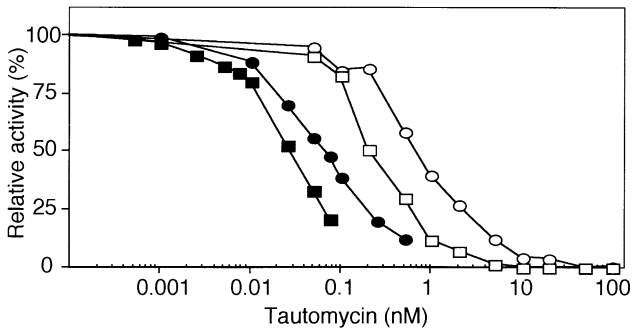

FIG. 3. Effects of Brij-35 on inhibition of PP1 and PP2A by tautomycin. PP1 (0.1 mU/mL) (■, □) and PP2A (0.08 mU/mL) (●, ○) were preincubated with the indicated concentrations of tautomycin in the presence (closed symbols) or absence (open symbols) of 0.01% Brij-35 for 15 min at 30° before phosphatase assay. The data shown are representative of three independent experiments.

The supernatant was evaporated to remove acetone. Tautomycin and its related compounds were extracted three times with 100 μL of ethyl acetate from the supernatants. After removal of ethyl acetate by evaporation, the samples were dissolved with 0.01% Brij-35 and used for the inhibition assays of protein phosphatases.

RESULTS

Effects of Brij-35 on Inhibition of PP1 and PP2A by Tautomycin

In our preliminary experiments, we found that phosphatase inhibition was potentiated by preincubation of the phosphatases with tautomycin in the presence of 0.01% Brij-35 for 15 min at 30°. Figure 3 shows inhibition of PP1 and PP2A by tautomycin after preincubation in the presence or absence of Brij-35. The IC_{50} values of tautomycin for the catalytic subunits of PP1 and PP2A were 20 and 75 pM, respectively, in the presence of 0.01% Brij-35, and 200 and 700 pM, respectively, in the absence of Brij-35.

Effects of Tautomycin and Its Related Compounds on PP1 and PP2A

As shown in Fig. 4, tautomycin **1** and its related compounds inhibited activities of the catalytic subunits of PP1 and PP2A with a wide range of IC_{50} values (from 20 pM to 40 μM). The IC_{50} values of tautomycin, 22-epi-tautomycin **2**, TM-dimethyl ester **3**, C_{22}-$C_{7'}$-anhydride **7** and 20-keto-C_1-C_{21}-fragment **12b** were estimated to be 20 pM, 50 nM, 0.2 μM, 40 μM, and 5 μM, respectively, for PP1, and 75 pM, 0.1 μM, 0.5 μM, 40 μM, and 20 μM, respectively, for PP2A. It should be noted that PP1 was inhibited at slightly lower concentrations of these compounds than PP2A. Table 1 shows the IC_{50} values of all compounds examined. The magnitude of the inhibitory activity was in the order of tautomycin **1** > 22-epi-tautomycin **2** > TM-dimethyl ester **3** > 20-keto-C_1-C_{21}-fragment **12b** > C_{22}-$C_{7'}$-anhydride **7** ≈ 20-keto-C_1-C_{21}-silyl ether **12a** ≈ anhydrodeacyl-TM **4a** > C_1-C_{21}-triol **11**. 21,22-Dihydroanhydrodeacyl-TM **4b**, $C_{1'}$-$C_{7'}$-methyl ester **5**, C_{19}-C_{26}-fragment **6**, Δ_1-C_1-C_{18}-silyl ether **8a**, 2-keto-C_1-C_{18}-alcohol **8b**, Δ_1-C_1-C_{18}-aldehyde **9a**, 2-keto-C_1-C_{18}-aldehyde **9b**, (18*S*,19*R*)-C_1-C_{21}-fragment **10a**, (18*R*,19*S*)-C_1-C_{21}-fragment **10b**, Δ_1-22-epi-C_1-C_{26}-MPM ether **13**, C_9-C_{26}-MPM ethers **14a** and **14b**, and 22-epi-C_9-C_{26}-MPM ethers **15a** and **15b** did not show any inhibitory effect below 100 μM.

To examine the effects of these compounds on the activities of PP1 and PP2A in their holoenzyme forms, experiments were carried out under the same conditions except that cell extracts were used as enzyme source instead of the catalytic subunits. As shown in Fig. 5, IC_{50} values of

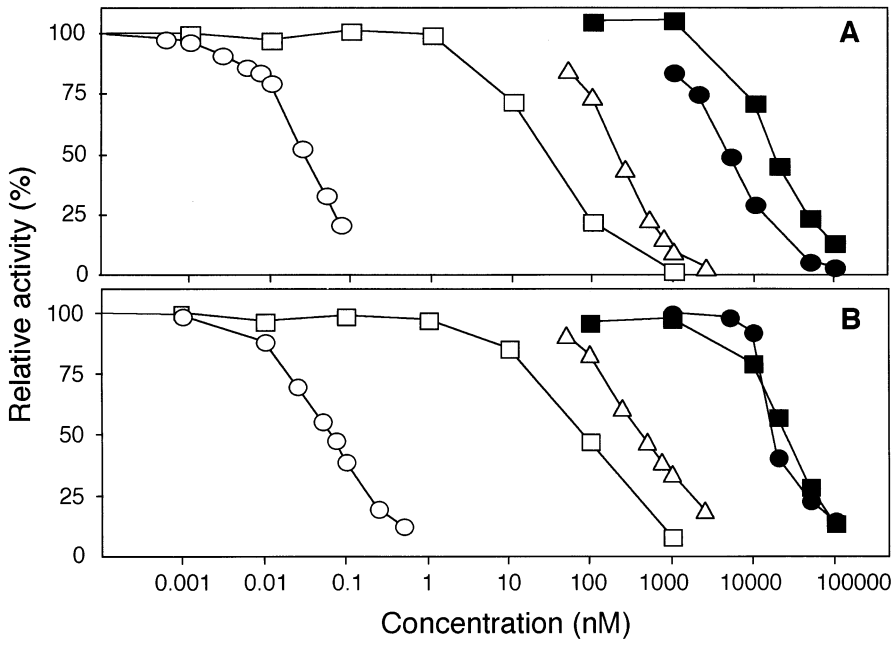

FIG. 4. Effects of tautomycin and its related compounds on the catalytic subunits of PP1 and PP2A. The phosphatase activities of the catalytic subunits of (A) PP1 (0.1 mU/mL) and (B) PP2A (0.08 mU/mL) were assayed after preincubation for 15 min with 0.01% Brij-35 and tautomycin or its related compounds. Key: (○) tautomycin; (□) compound 2; (△) compound 3; (●) compound 12b; and (■) compound 7. Data points are means of duplicate determinations.

TABLE 1. IC_{50} Values of tautomycin and its related compounds for the catalytic subunits of PP1 and PP2A

Compounds	IC_{50} (μM) PP1	PP2A
TM	20×10^{-6}	75×10^{-6}
2	50×10^{-3}	100×10^{-3}
3	0.2	0.5
4a	75	100
4b	> 100	> 100
5	> 100	> 100
6	> 100	> 100
7	40	40
8a	> 100	> 100
8b	> 100	> 100
9a	> 100	> 100
9b	> 100	> 100
10a	> 100	> 100
10b	> 100	> 100
11	50	> 100
12a	45	100
12b	5	20
13	> 100	> 100
14a	> 100	> 100
14b	> 100	> 100
15a	> 100	> 100
15b	> 100	> 100

The phosphatase activities of PP1 (0.1 mU/mL) and PP2A (0.08 mU/mL) were assayed after preincubation for 15 min with 0.01% Brij-35 and tautomycin or its related compounds.

tautomycin and 22-epi-tautomycin **2**, TM-dimethyl ester **3**, C_{22}-$C_{7'}$-anhydride **7**, and 20-keto-C_1-C_{21}-fragment **12b** were 200 pM, 80 nM, 1 μM, 35 μM, and 20 μM, respectively. The IC_{50} values of tautomycin and TM-dimethyl ester **3** for PP2A in its holoenzyme forms were 1 nM and 4.5 μM, respectively, where PP2A activity in the cell extracts was assayed in the presence of 0.2 μM inhibitor-2 (I-2) [14].

These values were at the same orders of magnitude as those for the catalytic subunits of PP1 and PP2A (Fig. 4), although those of tautomycin and TM-dimethyl ester **3** were increased slightly. These results demonstrate that tautomycin and its related compounds inhibit activities of PP1 and PP2A in their holoenzyme forms with a sensitivity similar to that in the catalytic subunits.

Effects of Tautomycin and Its Related Compounds on Apoptosis

Tautomycin and its related compounds induced nuclear fragmentation and condensation of the chromatin with reduction of nuclear size, which are considered to be typical features of apoptosis [17]. These morphological changes characteristic of apoptosis were observed in Jurkat cells treated with tautomycin derivatives (Fig. 6). DNA ladders seen in Jurkat cells treated with tautomycin **1** and TM-dimethyl ester **3** were indistinguishable from the typical one induced by anti-Fas antibody (Fig. 7). Figure 8 shows the concentration-dependent effect of TM-dimethyl ester **3** on the viability of Jurkat cells. The LC_{50} value was 1 μM. The LC_{50} values of tautomycin and its related compounds on the induction of apoptosis are shown in Table 2. Tautomycin **1** and TM-dimethyl ester **3** induced DNA fragmentation at 1 μM. 22-Epi-tautomycin **2**, Δ_1-C_1-C_{18}-aldehyde **9a**, 2-keto-C_1-C_{18}-aldehyde **9b**, C_1-C_{21}-triol **11**, (18*S*,19*R*)-C_1-C_{21}-fragment **10a**, (18*R*,19*S*)-C_1-C_{21}-fragment **10b**, and Δ_1-22-epi-C_1-C_{26}-MPM ether **13** showed not strong but marked apoptosis-inducing activity.

Inhibition of PP2A by Tautomycin and Its Related Compounds Extracted from Jurkat Cells Preincubated with Them

To evaluate the incorporation of tautomycin and its related compounds, Jurkat cells were preincubated with these compounds, and then after extraction of these compounds with ethyl acetate, PP2A inhibition by those compounds in the extracts was determined using the PP2A catalytic

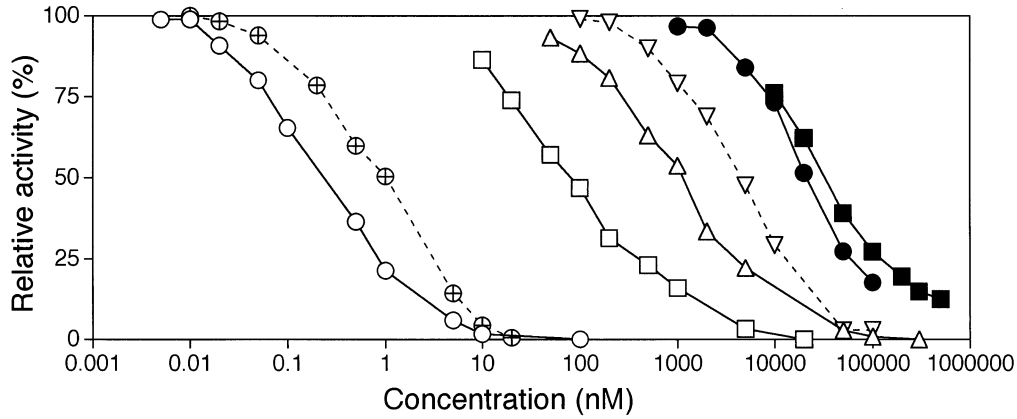

FIG. 5. Effects of tautomycin and its related compounds on PP1 and PP2A in their holoenzyme forms. Phosphatase activities were assayed in the absence (—) or presence (----) of I-2 under the same conditions as described in the legend of Fig. 4 except that cell extracts instead of the catalytic subunits were used as the enzyme source. Key: (○, ⊕) tautomycin; (□) compound 2; (△, ▽) compound 3; (●) compound 12b; and (■) compound 7. Phosphatase activity without tautomycin derivatives was 0.21 mU/mL (—) or 0.05 mU/mL (----). Data points are means of duplicate determinations.

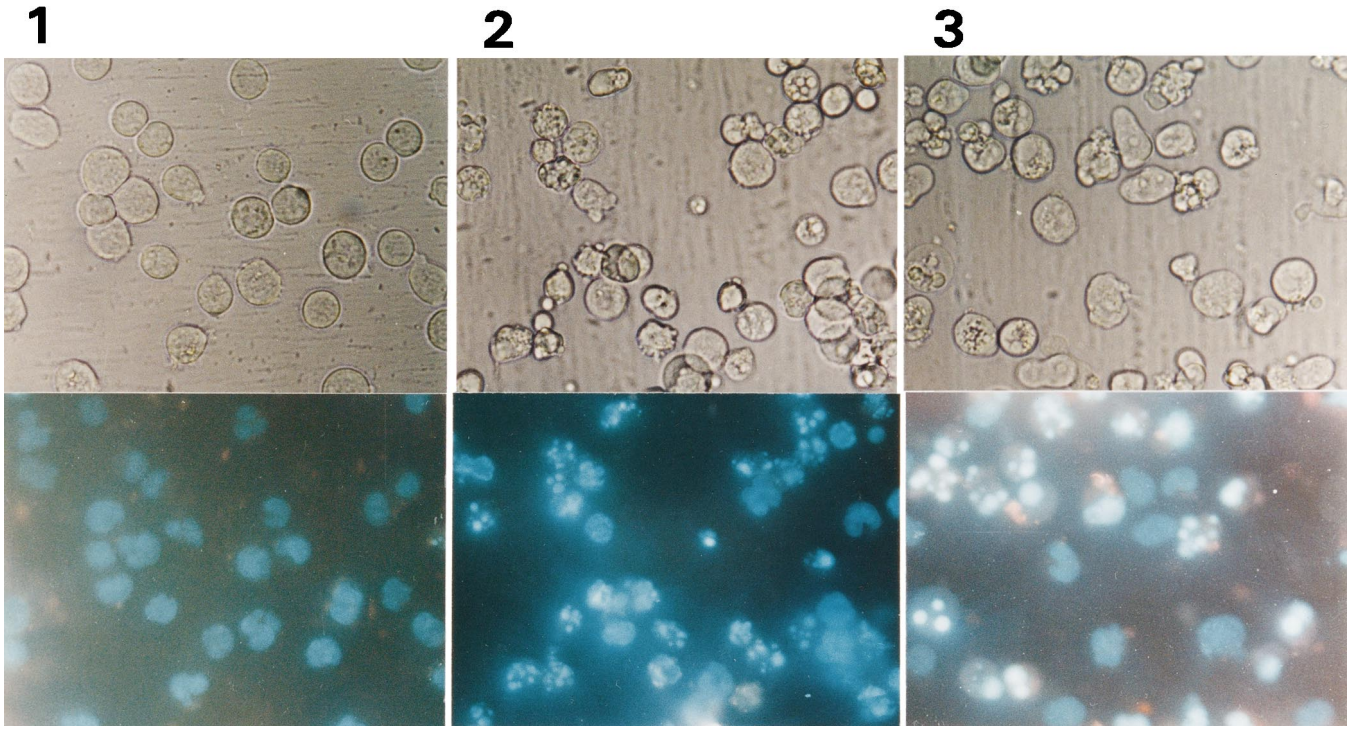

FIG. 6. Induction of nuclear fragmentation by tautomycin and its related compounds. Upper panels show phase contrast microscopy images and lower panels show fluorescence microscopy images. Cells were stained with 4 μM of Hoechst 33342 and 4 μg/mL of ethidium bromide after incubation for 6 hr with (1) the medium alone, (2) 10 μM of compound 3, and (3) 10 μM of tautomycin.

subunit. As shown in Fig. 9, IC_{50} values of TM-dimethyl ester **3** and C_{22}-$C_{7'}$-anhydride **7** in the extracts were at the same orders of magnitude as those in Fig. 4, whereas those of tautomycin and 22-epi-tautomycin **2** were 26- and 20-fold higher. The 20-keto-C_1-C_{21}-fragment **12b** extract did not show any inhibition. The IC_{50} value calculated from inhibition of the PP2A catalytic subunit by the extracted tautomycin is almost the same as that calculated from inhibition of PP1 and PP2A in the lysates of Jurkat cells preincubated with tautomycin [18]. These results demonstrate that TM-dimethyl ester **3** and C_{22}-$C_{7'}$-anhydride **7** reached a rapid equilibrium with the concentrations in the medium, whereas the incorporations of tautomycin, 22-epi-tautomycin **2**, and 20-keto-C_1-C_{21}-fragment **12b** were only 5% or less of the extracellular concentrations.

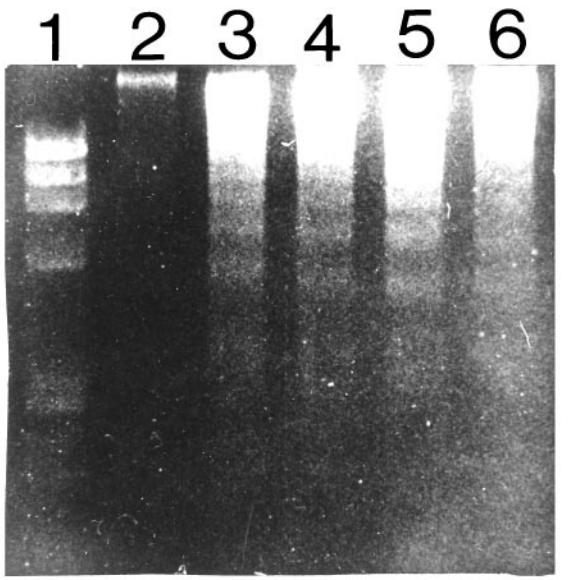

FIG. 7. DNA ladder of apoptotic cells. DNA from 2.5 × 10^5 Jurkat cells that were untreated (lane 2), or treated for 24 hr with 5 μM of tautomycin (lane 3), 5 μM of compound 3 (lane 4), 10 μM of compound 9a (lane 5), or with 10 μg/mL of anti-Fas antibody (lane 6) was loaded on a 2% agarose gel. Lane 1 is the DNA size marker (ϕx174, *Hae*III).

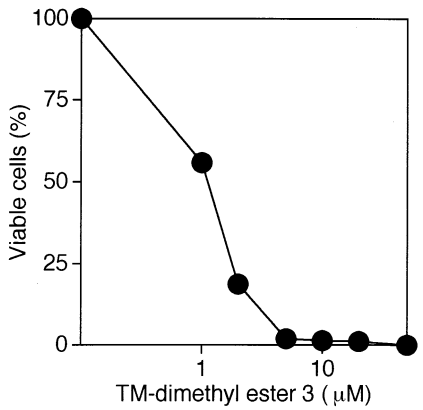

FIG. 8. Effects of TM-dimethyl ester 3 on the viability of Jurkat cells. Cells (2.5 × 10^4) were treated with TM-dimethyl ester 3 for 24 hr. Cell viability was determined by the MTT assay. The data shown are representative of three independent experiments.

TABLE 2. LC_{50} Values of tautomycin and its related compounds for Jurkat cells

Compounds	LC_{50} (μM)
TM	1
2	20
3	1.3
4a	2
4b	> 50
5	> 50
6	50
7	> 50
8a	> 50
8b	> 50
9a	15
9b	15
10a	10
10b	10
11	20
12a	> 50
12b	50
13	10
14a	ND
14b	ND
15a	ND
15b	ND

Cell viability was determined by the MTT assay. ND, not determined.

DISCUSSION

We have studied the relationship between structure and function of tautomycin, in terms of protein phosphatase inhibitory activity and apoptosis-inducing activity, by using tautomycin and its related compounds including its derivatives and the intermediates in the total synthesis (Fig. 10).

Esterification (TM-dimethyl ester **3**) of the anhydride moiety, a characteristic unit of tautomycin **1**, increased IC_{50} values four orders of magnitude (from 10^{-11} to 10^{-7} M). This indicates its importance for protein phosphatase inhibition activity. However, retention of significant activity in TM-dimethyl ester **3** suggests that the anhydride moiety is not essential for protein phosphatase inhibition but plays an important role in enhancing the activity. This proposal is supported by the fact that the protein phosphatase inhibition was observed in anhydrodeacyl-TM **4a**, C_1-C_{21}-triol **11**, 20-keto-C_1-C_{21}-silyl ether **12a** and 20-keto-C_1-C_{21}-fragment **12b**, none of which possesses the anhydride structure. In the case of the smallest unit C_{22}-$C_{7'}$-anhydride **7**, which possesses the anhydride moiety, weak inhibition was observed but this was not the case in the $C_{1'}$-$C_{7'}$-methyl ester **5**. This observation indicates that the C_{22}-C_{26} carbon chain and the anhydride moiety are minimum requirements for protein phosphatase inhibition. It has been proposed recently that the dialkyl anhydride moiety in chaetomellic acid, an inhibitor of farnesyl-protein transferase, mimics the pyrophosphate group [19]. Considering the proposal, the importance of the anhydride moiety implies its direct interaction with the phosphatase molecule. Nishiyama *et al.* [20] also pointed out the importance of the anhydride moiety in tautomycin for inhibitory action on the protein phosphatase.

Most small units possessing the spiroketal structure, such as Δ_1-C_1-C_{18}-silyl ether **8a**, 2-keto-C_1-C_{18}-alcohol **8b**, Δ_1-C_1-C_{18}-aldehyde **9a**, 2-keto-C_1-C_{18}-aldehyde **9b**, (18*S*,19*R*)-C_1-C_{21}-fragment **10a**, and (18*R*,19*S*)-C_1-C_{21}-fragment **10b**, did not exhibit the protein phosphatase inhibition, but C_1-C_{21}-triol **11** and 20-keto-C_1-C_{21}-fragment **12b** did. These results indicate the importance of the functionality in the C_{18}-C_{21} moiety. Inversion of C_{22} stereochemistry (22-epi-tautomycin **2**) caused a significant decrease of protein phosphatase inhibition, i.e. the increase of IC_{50} from picomolar to nanomolar. Compared with 20-keto-C_1-C_{21}-fragment **12b**, the three compounds anhydrodeacyl-TM **4a**, 21,22-dihydroanhydrodeacyl-TM **4b** and Δ_1-22-epi-C_1-C_{26}-MPM ether **13** possessing the correct functionality between C_1 and C_{20} showed very weak or no inhibition. The fact that the IC_{50} values were at the same orders of magnitude between Figs. 5 and 4 demonstrates that tautomycin and its related compounds inhibit the PP1 and PP2A activities similarly in terms of sensitivity either

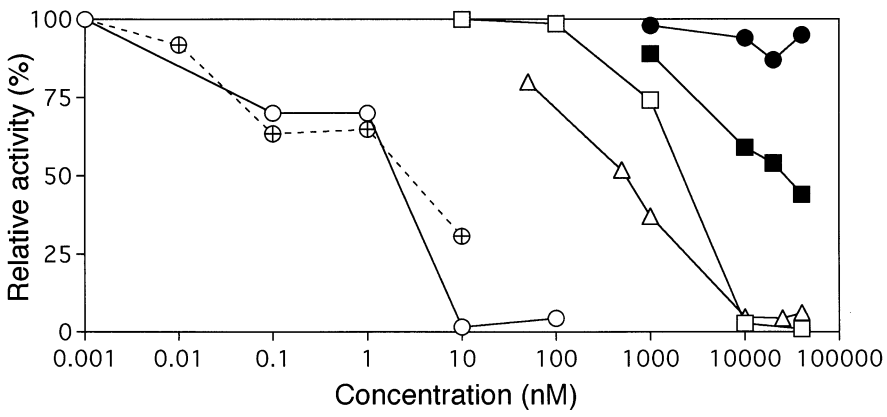

FIG. 9. Inhibition of PP2A by tautomycin and its related compounds extracted from cells preincubated with them. Tautomycin and its related compounds were extracted, as described in the text, from Jurkat cells that had been incubated with these compounds for 1 hr at 37°. Phosphatase activities were assayed under the same conditions as described in the legend of Fig. 4B except that the data in symbol ⊕ were obtained by using the cell extract as the enzyme source (0.20 mU/mL) instead of the PP2A catalytic subunit. Key: (○, ⊕) tautomycin; (□) compound 2; (△) compound 3; (●) compound 12b; and (■) compound 7. The data of tautomycin shown are representative of six independent experiments. Other data shown are representative of two independent experiments.

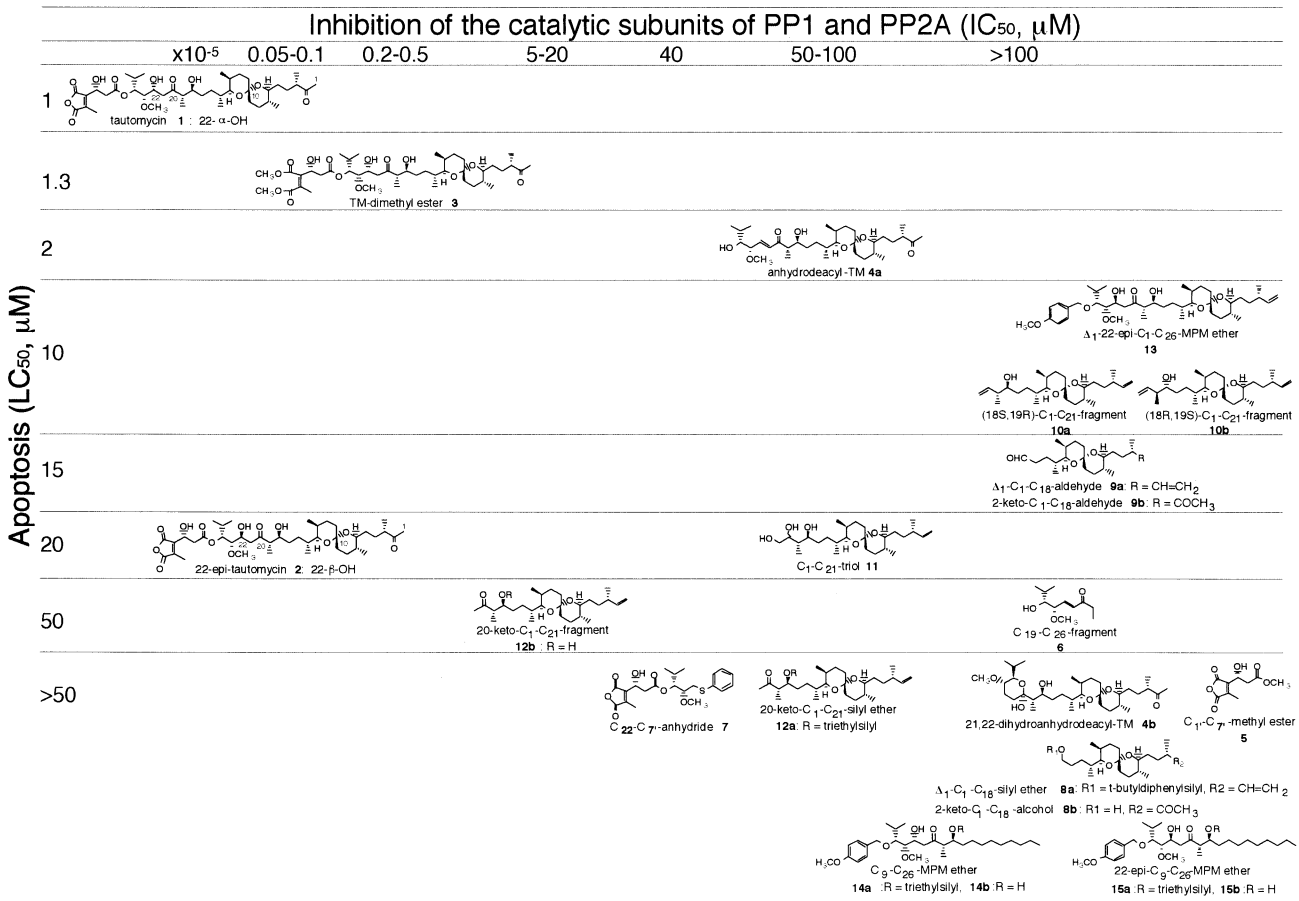

FIG. 10. Relationship of structures of tautomycin and its related compounds to the inhibitory activity of the PP1 and PP2A catalytic subunits and apoptosis-inducing activity.

in the catalytic subunits or in their holoenzyme forms. These data suggest that the conformation of the C_{22}-C_{26} moiety is very important for protein phosphatase inhibition. Similarly, the importance of the 22-hydroxy group in tautomycin for protein phosphatase inhibition has been pointed out by Isobe's group [21].

In most cases, the compounds having inhibitory activity showed higher inhibition against PP1 than PP2A. C_{22}-$C_{7'}$-anhydride **7** is the exceptional case since it showed the same IC_{50} value against the two enzymes. This implies that affinity for the enzymes is determined by the right-half structure of tautomycin.

It is well known that okadaic acid shows apoptosis-inducing activity in addition to the inhibitory effect on protein phosphatase [8, 9]. Here, we found that tautomycin also not only inhibits protein phosphatase but also induces apoptosis. The LC_{50} values of tautomycin and TM-dimethyl ester **3** were 1 and 1.3 μM, respectively. It should be noted that the IC_{50} value of TM-dimethyl ester **3** was four orders of magnitude higher than that of tautomycin in spite of similar LC_{50} values. This fact demonstrates that the maleic anhydride moiety of tautomycin is critically important in its phosphatase inhibitory activity but not in its apoptosis-inducing activity. Since the LC_{50} values of tautomycin **1**, TM-dimethyl ester **3**, and anhydrodeacyl-TM **4a** are similar, the correct functionality and conformation of the C_{22}-C_{26} moiety are not essential factors for apoptosis. Simple spiroketals Δ_1-C_1-C_{18}-aldehyde **9a** and 2-keto-C_1-C_{18}-aldehyde **9b** possessing aldehyde at C-18 induced apoptosis, but similar spiroketals Δ_1-C_1-C_{18}-silyl ether **8a** and 2-keto-C_1-C_{18}-alcohol **8b** did not. Thus, the functionality at C_{18} is important, and the replacement of the 2-keto group to C_1-C_2-olefin does not affect the apoptosis-inducing activity. Most of the compounds possessing the spiroketal moiety showed apoptosis activity except for 21,22-dihydroanhydrodeacyl-TM **4b**, 20-keto-C_1-C_{21}-silyl ether **12a**, and 20-keto-C_1-C_{21}-fragment **12b**. Tautomycin and 22-epi-tautomycin **2** showed only small incorporation into cells but induced clear apoptosis. TM-dimethyl ester **3** showed high incorporation but induced apoptosis at concentrations similar to that of tautomycin. These results demonstrated that the apoptosis-inducing activity is affected by both permeability for the cells and sensitivity for the phosphatase inhibition. However, it should be noted that the left wing, C_{22}-$C_{7'}$-anhydride **7** showed high incorporation but no apoptosis-inducing activity, whereas 20-keto-C_1-C_{21}-fragment **12b**, a derivative of C_1-C_{21} fragment, showed less incorporation but clear apoptosis-induc-

ing activity. These results demonstrate that the C_1-C_{18} moiety of tautomycin is essentially required for induction of apoptosis and that the conformation and arrangement of functionalities of the C_{18}-C_{26} carbon chain do affect apoptosis activity.

From these results, it is strongly suggested that each partial structure of tautomycin plays a different role in protein phosphatase inhibition and the induction of apoptosis. Tautomycin strongly inhibited protein phosphatase activities and also caused nuclear condensation, DNA fragmentation, and cell morphological changes. However, our present results with various tautomycin-related compounds strongly suggest that a variety of biological functions of tautomycin are induced through different mechanisms that should be elucidated further.

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