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SHORT COMMUNICATIONS

Mechanistic aspects of the cytocidal action of ulithiacyclamide on mouse leukemia L1210 cells *in vitro*

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Recently considerable attention has been focused on bioactive compounds isolated from marine organisms. The structures of many of these substances have been elucidated. However, since it is often quite expensive and time consuming to obtain sufficient amounts of bioactive compounds from natural sources, more extensive testing will require producing these compounds synthetically. Shioiri and coworkers have accomplished the syntheses of a series of cytotoxic cyclic peptides of marine origin, namely, dolastatin 3 [1], ascidiacyclamide [2], patellamides (A, B, and C) [3] and ulithiacyclamide [4]. Except for dolastatin 3, these peptides possess both the thiazole and oxazoline rings, the unusual amino acid moieties, as constituents. Dolastatin 3 has thiazole rings only.

The study of the structure–activity relationship of these cytotoxic cyclic peptides and their several synthetic intermediates revealed that the oxazoline ring plays an important role in cytotoxicity [5]. Among the compounds tested, ulithiacyclamide (UTC) was found to have the most potent cytotoxic activity (IC_{50} = 0.04 μg/ml) against L1210 cells *in vitro* [5]. However, the mechanism of this activity has remained obscure. In this communication, we describe the mechanistic aspects of the cytotoxic activity of UTC, and an application for UTC as a potentiator of cytotoxicity of the anticancer drug, bleomycin.

Materials and methods

Chemicals. Ulithiacyclamide (UTC) was prepared as described in our previous report [4]. Anticancer drugs used were mitomycin C (MMC), doxorubicin hydrochloride (Adriamycin®, ADM), 5-fluorouracil (5-FU), vincristine sulfate (VCR), bleomycin hydrochloride (BLM), peplomycin (PLM), neocarzinostatin (NCS) and 1-(4-amino-2-methylpyridin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU). MMC, ADM and 5-FU were purchased from Kyowa Hakko Kogyo Co., Ltd (Tokyo), VCR from Shionogi & Co., Ltd (Osaka), BLM and PLM from Nippon Kayaku Co., Ltd (Tokyo), NCS from Yamanouchi Pharmaceutical Co., Ltd (Tokyo), and ACNU from Sankyo Co., Ltd (Tokyo).

Cell culture. Mouse leukemia L1210 cells were grown in RPMI-1640 medium (Nissui Seiyaku Co., Ltd, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (Gibco, NY) at 37° in a humidified incubator with 5% CO_2 in air as reported previously [5].

Growth inhibition (cytotoxicity) assay. Cells (5×10^5) were suspended in 5 ml of the medium in a 60 mm plastic dish and incubated at 37° for 24 hr. Then 25 μl of DMSO containing an appropriate concentration of the test compound was added to the cell suspension and the mixture was incubated for another 48 hr. Following incubation, the number of viable cells was counted [5]. For the experiments employing UTC with various anticancer drugs, growth inhibition of each anticancer drug with several doses of UTC was observed. The concentrations of the anticancer drug in combination with UTC that resulted in 50% inhibition of cell growth were determined, and an isobologram [6] was constructed. To study the effect of UTC-exposure time, 2 ml of cell suspension (3.6×10^5 cells) in a glass tube with Molton cap were used.

Inhibition of macromolecular synthesis. RNA, DNA, and protein syntheses were detected by measuring the incorporation of radioactive precursors into the acid-insoluble cell fraction. Exponentially growing L1210 cells (5×10^5 cells/ml, 2 ml) were treated with 0.05 to 1.0 μg/ml of UTC for 2 hr in a medium containing 2, 0.5, 2.5 μCi/ml of [^{3}H]-labelled uridine (Urd), thymidine (dThd), or leucine (Leu), respectively. The treated cells were then harvested, and washed with saline and 10% trichloroacetic acid (TCA) on a filter (HA, Millipore Co., MA). The radioactivity on the filter was measured using a liquid scintillation counter.

Results and discussion

Ulithiacyclamide (UTC) (Fig. 1) is a cyclic peptide isolated from an ascidian *Lissoclium patella* and its structure was determined by Biskupiak and Ireland [7]. UTC has been chemically synthesized by Shioiri and coworkers [4] and its cytotoxicity has been evaluated [5]. UTC inhibited the cell growth of mouse leukemia L1210 cells *in vitro* effectively, IC_{50} = 0.04 μg/ml. This value is comparable or superior to those of some clinically used anticancer drugs. In order to understand the cytotoxic mechanism of UTC, firstly, the effect of exposure time on cell growth was tested. As shown in Fig. 2a, cell growth decreased in a biphasic manner, that is, the curve decreased steeply up to 3 hr with UTC-exposure and then decreased moderately until the 9 hr point was reached. Additional exposure did not result in further changes in cell growth. A steep decrease in growth even with a short period of UTC-exposure suggests that UTC might directly interact with certain cell constituent(s) to produce lethal damage. Next, the cytotoxic effect of UTC remaining in the cell suspension medium was tested. The UTC-containing medium that was used for the experiments at 0, 1, 2, 3 or 4 hr UTC-exposure was freed from the cells by centrifugation, then the separated medium was used again with freshly prepared cells and cell growth tested. The results shown in Fig. 2b indicate that the cytotoxicity of the recycled medium decreased relative to the

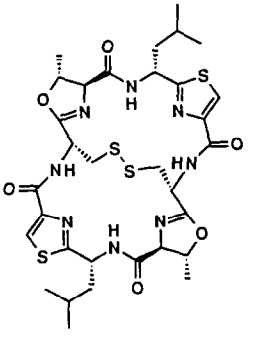

Fig. 1. Structure of ulithiacyclamide (UTC).

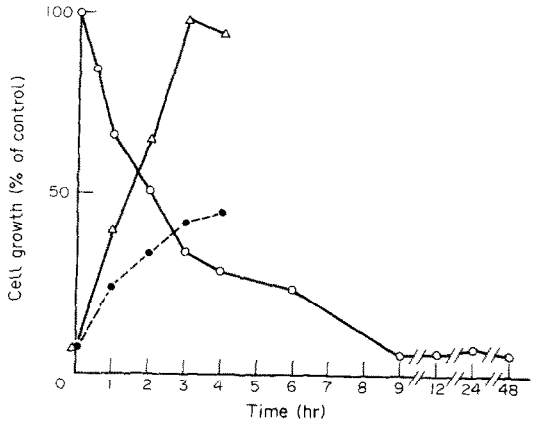

Fig. 2. Effects of the exposure time and the stability of UTC on cell growth. (a) L1210 cells (3.6×10^5) in 2 ml of medium were exposed to 0.1 μg/ml of UTC for the indicated period. After the cells were collected, washed twice with saline and re-suspended in fresh medium, each cell suspension was incubated at 37° for a total of 48 hr including the UTC-treatment time. The number of viable cells was counted and the % cell growth was calculated compared to control cell growth (average 2.4×10^6 cells/ml). (—○—). (b) The medium containing UTC that was used for the experiment shown in Fig. 2a (0, 1, 3, or 4 hrs UTC-exposure) was rendered free of cells and added to freshly prepared 3.6×10^5 cells in a tube. The cell suspension was then incubated at 37° for 48 hr. The number of viable cells was counted as described above. (—△—). (c) After UTC (0.2 μg) was incubated in 2 ml of the medium for the indicated time, the mixture was added to freshly prepared 3.6×10^5 cells in a tube, and the cell suspension was incubated at 37° for 48 hr. The number of viable cells was counted as described above. (—●—).

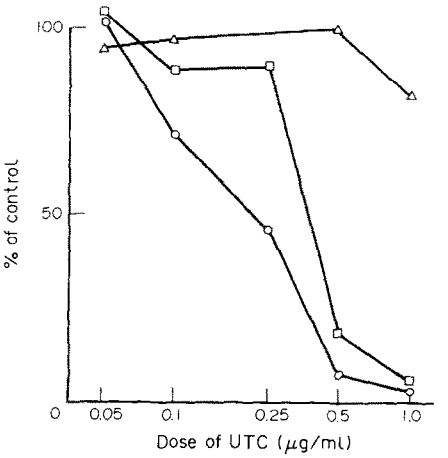

Fig. 3. Effects of UTC on macromolecular synthesis. Exponentially growing L1210 cells (5.0×10^5 cells/ml) were treated with several concentrations of UTC for 2 hr in medium containing [^{3}H]-labelled precursor (dThd, Urd, or Leu). Incorporation of radiolabelled precursor into cold TCA-insoluble cell fractions was measured. Percent inhibition was calculated by comparing the incorporation rate with that of the control cells. DNA(△), RNA(□), and protein(○) syntheses.

length of time the cells had been previously exposed to it. Thus the remaining cytotoxicity correlated inversely with the cytotoxicity displayed in the initial experiment. In order to determine the stability of UTC alone in the culture medium, the same experiment as described above was employed using a cell-free medium. As shown in Fig. 2c, UTC gradually lost activity, and 3 hr incubation resulted in a loss of about 40% cytotoxic activity. The remaining activity of UTC at each exposure time (0 hr, 100%; 1 hr, 63%; 2 hr, 47%; 3 hr, 24%; calculated from Fig. 2a) is less than the sum of the remaining activity shown in Fig. 2b (0 hr, 100%; 1 hr, 65%; 2 hr, 37%; 3 hr, 0%) and the activity lost spontaneously (0 hr, 0%; 1 hr, 18%; 2 hr, 28%; 3 hr, 38%; calculated from Fig. 2c). This suggests that UTC reacts with cell constituent(s) more rapidly than it undergoes spontaneous decomposition. In addition, the mechanism involved in growth inhibition is related to the inactivation of UTC itself when reacted with the target molecule(s); in other words, UTC inhibits cell growth in a self destructive manner. Furthermore, in binding-experiments, it was found that UTC might not specifically interact with cell membrane constituents. UTC (1 μg) was incubated with a membrane constituent, phosphatidylethanolamine (5 mg) or cholesterol (5 mg), in DMSO (200 μl) at 37° for 5 min and the cytotoxicity of the mixture was tested after passing through a Millipore filter. The results showed that the cytotoxicity of UTC was not changed by pretreatment with membrane constituents (data are not shown). Under these conditions, a compound that specifically interacts with cell membrane is shown to lose its cytotoxic activity [8].

The effect of UTC on macromolecular synthesis was examined using [^{3}H]-labelled precursors, thymidine (dThd), uridine (Urd), and leucine (Leu). (Fig. 3). UTC inhibited the incorporation of Leu most effectively. The 50% inhibition dose was 0.21 μg/ml under the conditions employed. Inhibition of RNA synthesis followed protein synthesis, however, the slope of the dose–response curve for RNA synthesis was steeper. DNA synthesis was only slightly inhibited by UTC even at the maximum concentration (1 μg/ml) tested. It is of interest that other cyclic peptides having anticancer properties; i.e., didemnins isolated from a Caribbean tunicate *Tridemnum solidum* [9], and RA-700 isolated from *Rubia coldifolia* [10], are also reported to inhibit protein synthesis most effectively. In addition, at higher concentrations, they were found to inhibit DNA synthesis but showed less effect on the synthesis of RNA [9, 11].

As UTC was found to have a strong inhibitory effect on protein synthesis, we considered the possibility of whether UTC could potentiate the cytotoxicity of anticancer drugs. The cytotoxicity of almost all the anticancer drugs used in clinics are based on their damaging effects on DNA itself or the systems of DNA and RNA syntheses. Therefore, the combination of anticancer drug with some other compound having a different cytotoxic mechanism(s) might yield an improved synergistic cytotoxic agent. For this purpose, the effect of UTC on the cytotoxicity of several anticancer drugs was investigated using L1210 cells *in vitro*. The drugs tested were BLM, PLM (an analogue of BLM), ACNU, MMC, NCS, VCR, ADM, and 5-FU. The effect of the UTC-drug combination was evaluated using the isobologram [6] and the results are shown in Fig. 4. The data points displayed under, on, and above the dotted line show synergism, addition, and antagonism, respectively. Among the anticancer drugs tested, only BLM and its analogue PLM showed synergistic cytotoxicity with UTC. Others

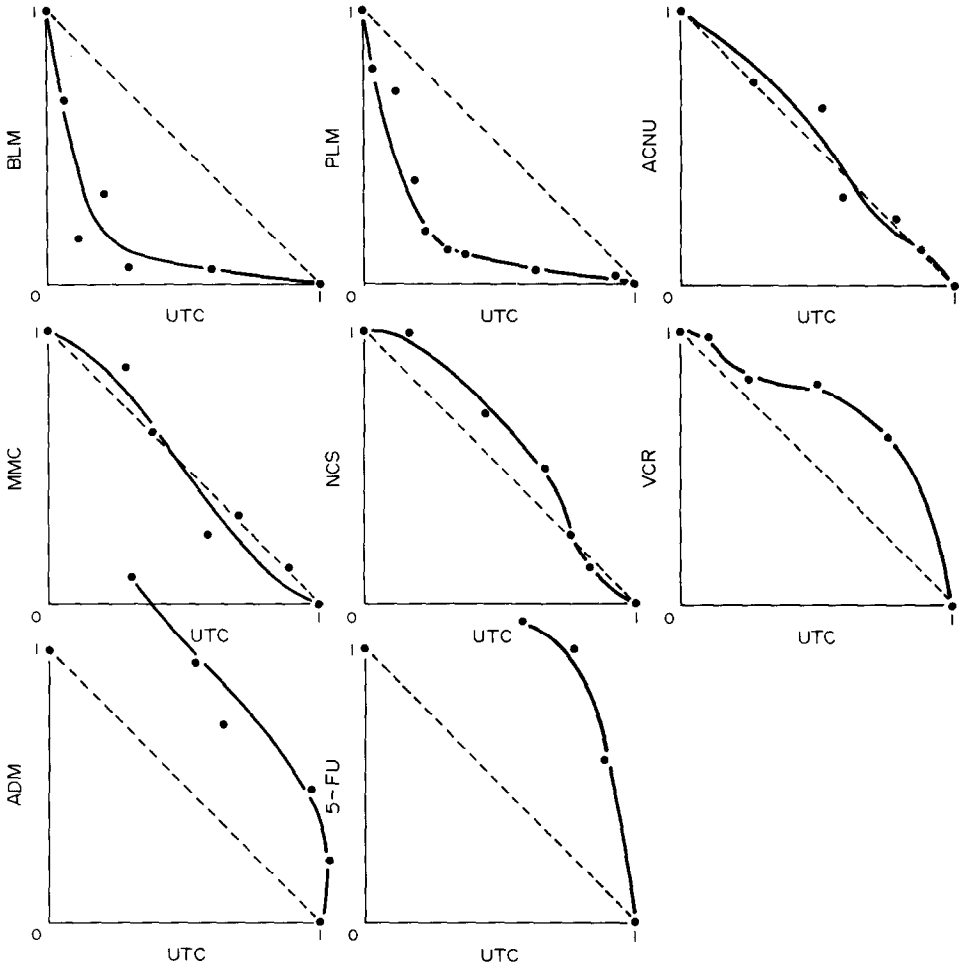

Fig. 4. Isobologram representing the cytotoxicity of the anticancer drugs combined with UTC. IC_{50} values of each drug and UTC were assumed to be unity, and the fractions of doses used in combinations resulting in 50% cell growth inhibition were plotted on appropriate axes. Values of IC_{50} (μg/ml): UTC, 0.04; BLM, 3.2; PLM, 5.3; ACNU, 3.1; MMC, 0.30; NCS, 0.04; VCR, 0.04; ADM, 0.21; 5-FU, 0.20.

were additively or antagonistically toxic with UTC. The mechanism of this synergism is under investigation. *In vivo* experiments for testing the anticancer effect of BLM with UTC are also in progress using tumor bearing mice.

In summary, we recently reported on the structure–activity relationship of a series of cytotoxic cyclic peptides of marine origin against L1210 cells *in vitro* [5]. The cytotoxic mechanism, however, remained obscure. In this communication we describe the mechanistic aspects of the cytotoxic activity of ulithiacyclamide (UTC), the most active compound tested. It was found that UTC inhibited cell growth in a self destructive manner. In addition, it inhibited protein synthesis most effectively. When UTC was used in combination with bleomycin, cytotoxicity against L1210 cells was synergistically enhanced.

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REFERENCES

1. Hamada Y, Kohda K and Shioiri T, Proposed structure of the cyclic peptide dolastatin 3, a powerful cell growth inhibitor, should be revised! *Tetrahedron Lett* **25**: 5303–5306, 1984.
2. Hamada Y, Kato S and Shioiri T, A synthesis of ascidiacyclamide, a cytotoxic cyclic peptide from ascidian—determination of its absolute configuration. *Tetrahedron Lett* **26**: 3223–3226, 1985.
3. Hamada Y, Shibata M and Shioiri T, New methods and reagents in organic synthesis. 58. A synthesis of patellamide A, a cytotoxic cyclic peptide from a tunicate. Revision of its proposed structure. *Tetrahedron Lett* **26**: 6501–6504, 1985.
4. Kato S, Hamada Y and Shioiri T, Total synthesis of ulithiacyclamide, a strong cytotoxic cyclic peptide from marine tunicates. *Tetrahedron Lett* **27**: 2653–2656, 1986.
5. Shioiri T, Hamada Y, Kato S, Shibata M, Kondo Y, Nakagawa H and Kohda K, Cytotoxic activity of cyclic peptides of marine origin and their derivatives. Importance of oxazoline functions. *Biochem Pharmacol* **36**: 4181–4185, 1987.
6. Berenbaum MC, A method for testing for synergy with any number of agents. *J Infect Dis* **137**: 122–130, 1978.
7. Biskupiak JE and Ireland CM, Absolute configuration of thiazole amino acids in peptides. *J Org Chem* **48**: 2302–2304, 1983.
8. Yokoyama Y, Ohmori I, Kohda K and Kawazoe Y, Potentiation of the cytotoxic activity of anti-cancer drugs against cultured L1210 cells by *Bacillus thuringiensis* subs. *israelensis* toxin. *Chem Pharm Bull* **36**: 4499–4504, 1988.
9. Rinehart KL, Didemnin and its biological properties. In: *Peptides—Chemistry and Biology. Proceedings of the Tenth American Peptide Symposium* (Ed. Marshall GR), pp. 626–631. ESCOM, 1988.
10. Itokawa H, Takeya K, Mori N, Hamanaka T, Sonobe T and Mihara K, Isolation and antitumor activity of cyclic hexapeptides isolated from *Rubiae radix*. *Chem Pharm Bull* **32**: 284–290, 1984.
11. Kato T, Suzumura Y, Takamoto S and Ota K, Antitumor activity and toxicity in mice of RA-700, a cyclic hexapeptide. *Anticancer Res* **7**: 329–334, 1987.

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Ulicyclamide is cytotoxic against L1210 cells *in vitro* and inhibits both DNA and RNA syntheses

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Many bioactive compounds have been isolated from marine organisms and their structures which have been elucidated are quite varied. Cyclic peptides represent one group found, wherever ulithiacyclamide (UTC), ascidiacyclamide (AC), patellamide (PA), dolastatin 3, and didemnins, etc. are present. These compounds have recently been synthesized [1]. In a previous paper, we reported the structure–activity relationship among a series of cyclic peptides using UTC, AC, PA, dolastatin 3, and their synthetic intermediates [2]. These natural products, except for dolastatin 3, possess both the thiazole and oxazoline rings, the unusual amino acid moieties, as constituents. A study of this structure–activity relationship revealed that the oxazoline moiety has an important role in cytotoxicity. From investigations of the biological aspects of UTC, the most cytotoxic of the compounds tested, it was also found that cytotoxic activity might involve an inhibitory effect on protein synthesis [3].

Ulicyclamide (UC) (Fig. 1) is a cytotoxic cyclic peptide isolated from a tunicate *Lissoclinum patella* [4] and contains both oxazoline and thiazole moieties in its structure. It has been effectively synthesized by our group [5]. When the cytotoxicity of a synthetic UC intermediate (**2**) (Fig. 1) that has no oxazoline function in its structure, was tested against L1210 cells in culture, it was found that **2** was also cytotoxic, although the activity was less than that of UC. This contradicted our previous proposal that the oxazoline moiety is the essential constituent for cytotoxicity in the series of cyclic peptides tested [2]. Therefore, the biological aspects of UC and **2** were studied further. In this study, we report that both UC and **2** are cytotoxic and that both inhibit DNA and RNA syntheses.

Materials and methods

Chemicals. Ulicyclamide and its synthetic intermediates were synthesized according to our previous report [5].

Cell culture and growth inhibition assay. Mouse leukemia L1210 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum at 37° in a humidified incubator with 5% CO_2 in air, as reported previously [2]. For the growth inhibition (cytotoxicity) assay, several concentrations of a test compound were added to cells *in vitro* and the suspension incubated at 37° for 48 hr. Then the number of surviving cells was counted as reported previously [2].

Inhibition of macromolecular synthesis. DNA, RNA, and protein syntheses were detected by measuring the incorporation of [^{3}H]-labelled precursors, uridine, thymidine and leucine into cells which were treated for 2 hr with the test compound in the presence of a precursor as reported previously [3].

Results and discussion

Cytotoxicity of UC and its two intermediates (linear peptide, **1** and cyclic peptide, **2** that has no oxazoline function) were tested against L1210 leukemia cells *in vitro*. Dose–response curves are shown in Fig. 2. The IC_{50} of UC was 13 μg/ml, which was comparable to that previously reported [4]. Linear intermediate, **1** was not cytotoxic even at a dose of 250 μg/ml. It is worth noting that compound **2**, which has no oxazoline function, showed fairly potent cytotoxicity, IC_{50} = 35 μg/ml. This contradicted our proposal reported previously that the oxazoline moiety has an important role in cytotoxicity, which was based on our