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Report

(–)-Isostemonamine Can Enhance the Anti-Proliferative Activity of Trichostatin A Against Human Breast Cancer MDA-MB-231 Cells

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Recent findings established (–)-isostemonamine as an anti-proliferator for estrogen receptor α-negative human breast cancer cells, MDA-MB-231, known to grow/divide at an aggressive rate. However, ST-4, a thioamide derivative of (–)-isostemonamine, is believed to not affect the viability of these cells. Epigenetic changes, such as DNA methylation and histone modification, are involved in the progression of many cancers, including breast cancer. In the present study, we investigated whether ST-4 and its related compounds (ST-3 and ST-5) can potentiate the anti-proliferative activity of the established epigenetic modifiers, 5-aza-2'-deoxycytidine (5-aza-dC; a DNA methyltransferase 1 inhibitor), trichostatin A (TSA; a class I/II histone deacetylase inhibitor), and etoposide (a DNA topoisomerase IIα inhibitor). Data obtained from this study demonstrate that, among the studied compounds, ST-4 displays the strongest enhancement of the anti-proliferative activity of TSA, against MDA-MB-231 cells (IC_{20} of TSA = 14 ± 3.4 nM versus that of TSA/ST-4 combination = 7.8 ± 1.1 nM). However, this effect was not observed at higher concentrations of above 25 nM of TSA, at which the IC_{50} values of TSA with or without ST-4 were not significantly different (30 ± 4.4 nM versus 28 ± 1.4 nM, respectively). Results from the study suggest that combining ST-4 with established anti-cancer agents could potentiate the latter's anti-proliferative activity, thereby potentially minimizing the concentration of these agents needed for optimal clinical efficacy and safety.

Key words (–)-isostemonamine, trichostatin A, MDA-MB-231 cells, *Stemona* alkaloid

INTRODUCTION

Because of their unique structure, *Stemona* alkaloids, including (±)-stemonamine and (±)-isostemonamine, have been suggested to have a potential biological activity (*e.g.*, anti-tussive activity and insecticidal activity).[1)] Biological reactions, such as ligand interaction with enzymes and receptors, are generally stereospecific in nature. Therefore, in order to identify the biological activity of *Stemona* alkaloids, it is necessary to have both of their enantiomers (– and +). We originally established the method of asymmetric total synthesis of (–)-stemonamine and (–)-isostemonamine.[2)] Among the *Stemona* alkaloid-related compounds, (–)-isostemonamine showed anti-proliferative activity selectively in the estrogen receptor α (ERα)-negative human breast cancer cells, MDA-MB-231 (IC_{50} = 9.3 μM at 48 h), which is comparable to that of etoposide (IC_{50} = 7.1 μM at 48 h).[3)] Additionally, we previously showed that ST-4, a thioamide derivative of (–)-isostemonamine, does not modulate the viability of MDA-MB-231 cells.[3)] In general, it is being widely accepted that the therapeutic benefits of anti-cancer agents are enhanced when they are dosed together,[4)] because of their ability to exert their anti-proliferative effects on the cancer cells through different mechanisms. However, whether ST-4 can ameliorate the anti-proliferative activity of the established anti-cancer agents has not been investigated.

In addition to genetic variations, epigenetic modifications (*e.g.*, DNA methylation and histone modification) are also involved in the development and progression of many cancers, including breast cancer.[5,6)] Unlike genetic variations, epigenetic modifications are reversible changes in the DNA or histones that can affect gene expression without altering the DNA sequence. In cancer, epigenetic changes, such as alteration of CpG-island methylation or histone modifications, can lead to the silencing of tumor suppressor genes and activation of oncogenes.[5,6)] Experimental evidence also strongly suggests that epigenetic modifications can lead to anti-cancer drug resistance.[7)] Thus, development of therapies selectively targeting epigenetic modifiers of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) is an important area of anti-cancer drug development. However, although such therapies are thought to be highly effective against some cancers, epigenetic modifiers can cause a broad range of DNA demethylation and histone acetylation effects that may lead to unwanted side effects.[4)] Furthermore, it is generally recognized that to minimize the side effects of anti-cancer agents, they should be dosed at the lowest concentration that can offer optimal safety

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and efficacy. Ideally, anti-cancer agents should selectively target specific DNA sequences that are aggressively involved in the regulation of tumor suppressor genes or oncogenes; however, research on development of such agents is still ongoing.

In this study, we investigated the ability of certain agents to potentiate the anti-proliferative activity of established epigenetic modifiers, 5-aza-2'-deoxycytidine (5-aza-dC; a DNMT1 inhibitor) and trichostatin A (TSA; a class I/II HDAC inhibitor), along with another established anti-cancer agent, etoposide (a DNA topoisomerase IIα inhibitor). Specifically, we investigated whether ST-4 and related compounds, ST-3 and ST-5, (Fig. 2A) can positively enhance the anti-cancer/anti-proliferative properties of 5-aza-dC, TSA, and etoposide against MDA-MB-231 cells. Data obtained from the study demonstrate that the combination of TSA and ST-4 can lead to a significant reduction in the IC_{20} value of TSA (14 ± 3.4 nM of TSA alone versus 7.8 ± 1.1 nM of TSA/ST-4). However, at high concentrations of above 25 nM, the IC_{50} value of TSA is not significantly affected (30 ± 4.4 nM of TSA alone versus 28 ± 1.4 nM of TSA/ST-4). Through this study, we show that ST-4 can enhance the anti-cancer activity of epigenetic modifiers, when used in combination.

MATERIALS AND METHODS

Reagents ST-3, ST-4, and ST-5 were synthesized using our established methods.[2] These compounds were found to be ≥ 95% pure by HPLC or column chromatography. 5-Aza-dC (purity ≥ 97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). TSA (purity ≥ 99%) and etoposide (purity ≥ 98%) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The chemicals used in this study were prepared in cell culture grade dimethyl sulfoxide (DMSO).

Cell Culture Human breast cancer cell lines, MCF-7 and MDA-MB-231, were purchased from the American Type Culture Collection (Rockville, MD, USA). The cell culture conditions were based on previously reported procedures.[8]

Cell Viability Analysis Cell viability analysis was performed as described previously,[8] with minor modifications. Briefly, the MCF-7/MDA-MB-231 cells were seeded into 96-well plates at a density of 5×10^3 cells/well. For experiments corresponding to Fig. 1 and Fig. 2B, the cells were treated with 5-aza-dC, TSA, ST-3, ST-4, or ST-5, 4 h after plating. For experiments corresponding to Fig. 2C and 3, ST-3, ST-4, and ST-5 were treated at the same time as seeding, and 4 h later, the cells were treated with TSA, 5-aza-dC, or etoposide. After 48 h of treatment with the agents, cell viability was analyzed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS reagent; Promega, Madison, WI, USA).

Data Analysis IC_{20} and IC_{50} values were obtained using SigmaPlot 11 software (Systat Software, Inc., San Jose, CA, USA). Differences were considered significant for *P*-values of less than 0.05. The statistical significance of differences between the two groups was calculated using Student's *t* test. Other statistical analyses were performed by using Dunnett's post-hoc test after the ANOVA test (details are indicated in figure legends). Calculations were performed by using Statview 5.0 J software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

We first focused on two representative epigenetic modifiers, 5-aza-dC and TSA, to study their effects on the viability of human breast cancer cells, MCF-7 and MDA-MB-231. As shown in Fig. 1A, 5-aza-dC was shown to be inactive in both, MCF-7 and MDA-MB-231 cells, even at concentrations of up to 10 μM (IC_{50} >10 μM). On the contrary, TSA effectively reduced the viability of MCF-7 and MDA-MB-231 cells in a concentration-dependent manner (Fig. 1B). When comparing the anti-proliferative efficacy of TSA on the two breast cancer cells, the highly aggressive MDA-MB-231 cells exhibited some resistance to the agent up to a concentration of 25 nM (IC_{50} = 7.2 ± 1.0 nM for MCF-7 cells and 30 ± 4.4 nM for MDA-MB-231 cells) (see also Fig. 2C). However, it should be noted that at concentrations ≥ 25 nM and ≤ 50 nM, TSA's anti-proliferative effects, which tend to favor the MCF-7 cells, appeared to diminish. A possible explanation of this phenomenon is the non-selective effect of TSA at concentrations above 25 nM, leading to cell toxicity. Because the TSA-sensitive MCF-7 cells express ERα and require 17β-estradiol activation of ERα to grow, ERα antagonists are effectively used to abrogate the proliferation of MCF-7 cells.[9] Thus, we next sought to identify the chemical(s) that can enhance TSA's anti-proliferative activity, such that it can be used at lower concentrations in the ERα-negative MDA-MB-231 cells.

We have reported that among the *Stemona* alkaloids, (–)-isostemonamine (see Fig. 2A, left panel) is an effec-

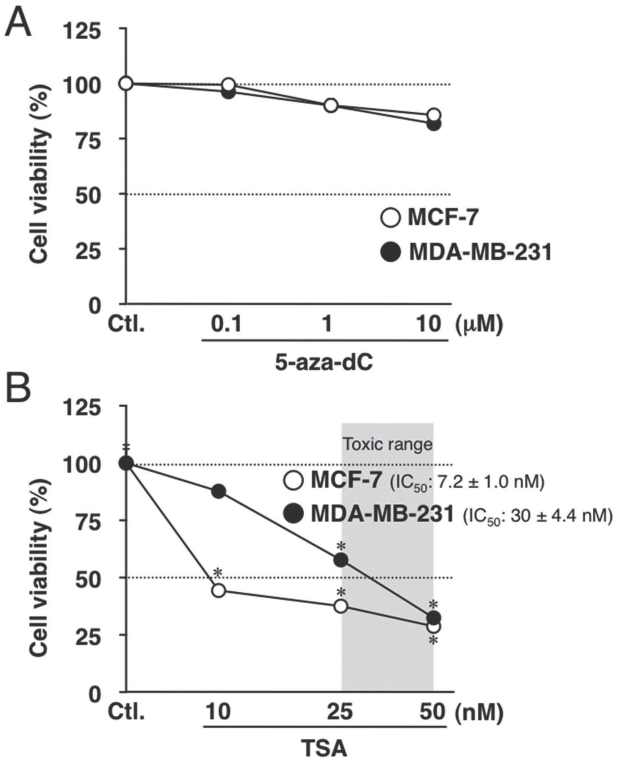

Fig. 1. Effect of 5-aza-dC and TSA on the Viability of MDA-MB-231 and MCF-7 Cells

MDA-MB-231 cells and MCF-7 cells were treated with 5-aza-dC (0.1–10 μM) (A) and TSA (10–50 nM) (B), for 48 h. Data are presented as mean ± SE (n = 6) percentage of the control group treated with vehicle alone (indicated as Ctl.). Significant differences (one-way ANOVA, followed by Dunnett's post-hoc test) from the vehicle-treated control are marked with an asterisk (*P < 0.05).

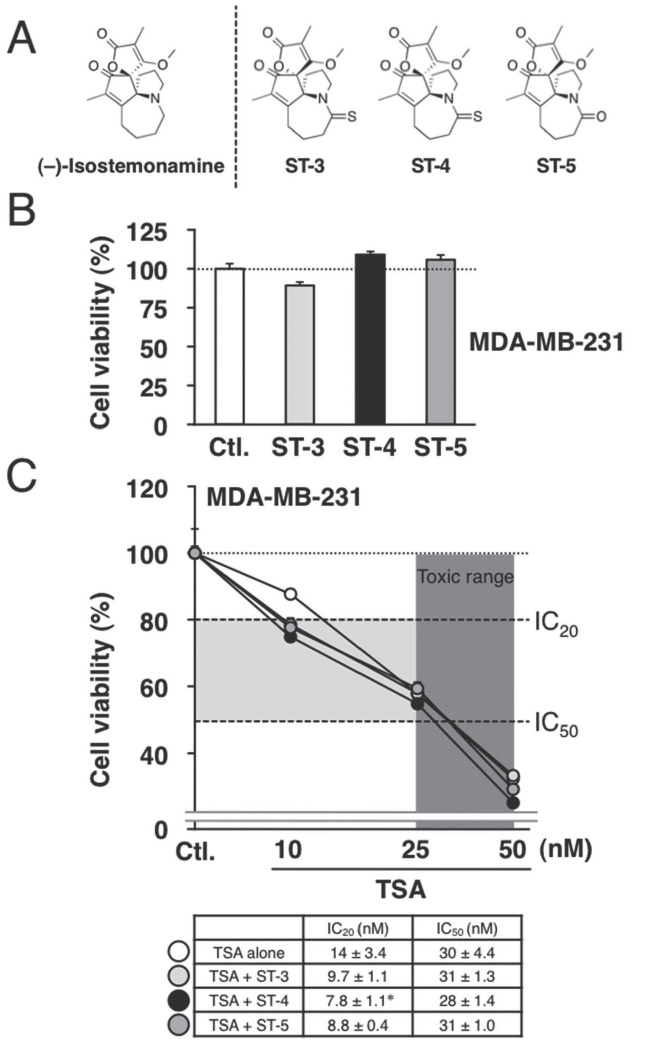

Fig. 2. ST-4 Significantly Enhanced the Anti-Proliferative Activity of TSA on MDA-MB-231 Cells

(A) Chemical structures of (–)-isostemonamine, two thioamide derivatives (ST-3 and ST-4), and one amide derivative (ST-5) are shown. (B) Effect of ST-3, ST-4, and ST-5 on the viability of MDA-MB-231 cells, measured using the MTS assay. MDA-MB-231 cells were treated with 25 μM each of ST-3, ST-4, or ST-5, for 48 h. Data are presented as mean ± SE (n = 6) percentage of the control group treated with vehicle alone (indicated as Ctl.). (C) Effect of ST-3, ST-4, and ST-5 on the anti-proliferative activity of TSA against the MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle and 25 μM each of ST-3, ST-4, or ST-5, at the same time as seeding. After 4 h of pre-incubation, the cells were treated with TSA, after which cell viability was measured using the MTS assay. (Upper panel) Data are presented as mean ± SE (n = 6) percentage of the control group treated with ST-3, ST-4, or ST-5 alone (indicated as Ctl.). (Lower panel) The calculated values of IC_{20} and IC_{50}. Significant differences (Student's t test) from the TSA alone treatment group are marked with an asterisk (*P < 0.05).

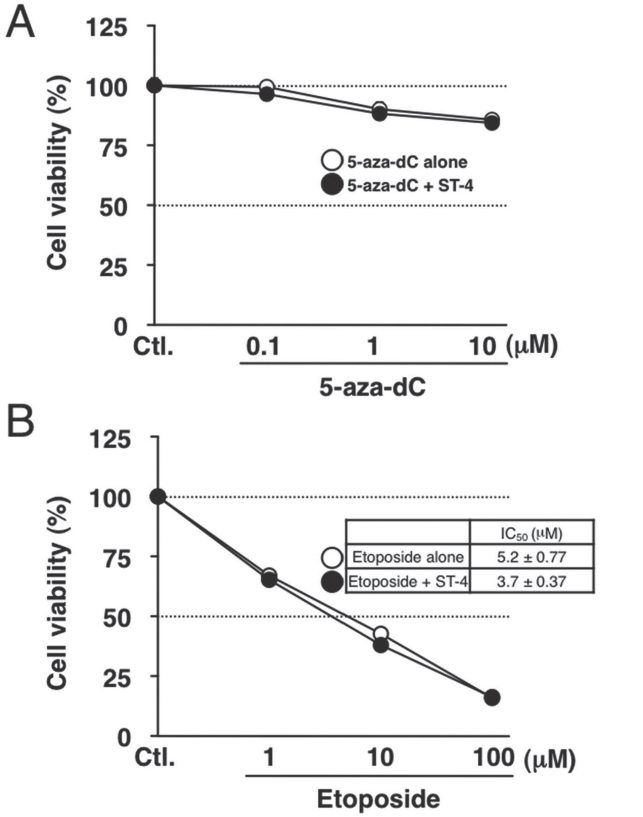

Fig. 3. ST-4 did not Modulate the Anti-Proliferative Activity of 5-aza-dC and Etoposide on MDA-MB-231 Cells

Effect of ST-4 on the anti-proliferative activity of 5-aza-dC or etoposide against MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or 25 μM ST-4 at the time of seeding. After 4 h of pre-incubation, the cells were treated with 5-aza-dC (A) or etoposide (B), and then cell viability was measured by using the MTS assay. Data are presented as mean ± S.E. (n = 6) percentage of the control group treated with ST-4 alone (indicated as Ctl.).

tive abrogator of MDA-MB-231 cell proliferation, with an IC_{50} value of approximately 9 μM, while (–)-stemonamine, a congener of (–)-isostemonamine, is inactive.[3] Interestingly, ST-3 and ST-4, thioamide derivatives of (–)-stemonamine and (–)-isostemonamine, respectively, did not display any modulatory effects on the viability of MDA-MB-231 cells, up to concentrations of 25 μM.[3] The goal of this study was to identify chemicals that could potentiate the anti-proliferative activity of TSA without exhibiting any cell growth-modulation. Here, we focused on the two thioamide derivatives of ST-3 and ST-4, and one amide derivative of (–)-stemonamine, ST-5 (Fig. 2A).[2,3] As shown in Fig. 2B, the derivatives of ST-3, ST-4, and ST-5 were found to be inactive against the MDA-MB-231 cell viability at 25 μM. Based on this finding, we sought to study the effect of combining these agents at 25 μM with TSA. When increasing the TSA concentration up to 50 nM, TSA reduced the viability of MDA-MB-231 cells in a concentration-dependent manner, and the IC_{20} and IC_{50} values were determined to be 14 ± 3.4 and 30 ± 4.4 nM, respectively (Fig. 2C, upper and bottom panels). When MDA-MB-231 cells were treated with TSA in combination with ST-3, ST-4, and ST-5, these three derivatives displayed a tendency of enhancing the TSA activity, wherein it showed a lower IC_{20} value compared with that of TSA alone (Fig. 2C, upper and bottom panels). Among the three derivatives, ST-4 exhibited the highest enhancement of TSA activity, as suggested by its lowest IC_{20} value (IC_{20} = 7.8 ± 1.1 nM, P < 0.05). On the contrary, there was no difference in the IC_{50} values of TSA alone and TSA in combination with the three derivatives (Fig. 2C, upper and bottom panels), indicating that TSA at higher concentrations above 25 nM may cause cytotoxicity (indicated as 'Toxic range' in Fig. 2C, upper panel).

It is important to determine if the ST-4-mediated enhancement of the anti-proliferative activity is specific to TSA. As demonstrated in Fig. 3A, there was no positive interaction between ST-4 and 5-aza-dC, another epigenetic modifier, and this lack of interaction was also observed in the case of etoposide, an established anti-cancer agent (Fig. 3B). Because (–)-isostemonamine (Fig. 2A, left panel) can be racemized to (+)-isostemonamine in the presence of protic solvents,[2] it could be suggested that the isostemonamine's anti-proliferative activity is altered in the experimental settings. It is known

that the thioamide moiety in the chemical structure of ST-4 enables it to restrict its racemization.[2] Although we could not definitively determine the molecular mechanisms underlying ST-4-assisted potentiation of TSA's anti-proliferative action in MDA-MB-231 cells, the findings obtained here suggest that ST-4 should be studied as a potentiator with epigenetic modifiers used against cancer, such as HDAC inhibitors, in clinical trials for the treatment of breast cancer. Studies to uncover the mechanisms underlying ST-potentiation of TSA action are ongoing.

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Conflict of interest The authors declare no conflict of interest.

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