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ORIGINAL ARTICLE

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Trierixin, a Novel Inhibitor of ER Stress-induced XBP1 Activation from *Streptomyces* sp.

I. Taxonomy, Fermentation, Isolation, and Biological Activities

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Abstract In the course of screening for an inhibitor of ER stress-induced XBP1 activation, we isolated a new member of the triene-ansamycin group compound, trierixin, from a culture broth of *Streptomyces* sp. AC 654. Trierixin was purified by column chromatography on silica gel and by HPLC. The molecular formula of trierixin is $C_{37}H_{52}N_2O_8S$. Trierixin inhibited thapsigargin-induced XBP1-luciferase activation in HeLa/XBP1-luc cells and endogenous XBP1 splicing in HeLa cells with an IC_{50} of 14 ng/ml and 19 ng/ml, respectively. Moreover, in the process of isolating trierixin, we isolated structurally related mycotrienin II and trienomycin A as inhibitors of ER stress-induced XBP1 activation from a culture broth of a trierixin-producing strain. This study provides the first observation that triene-ansamycins have a novel inhibitory effect against XBP1 activation.

Keywords trierixin, triene-ansamycin, ER stress, XBP1

Introduction

Poorly vascularized solid-tumor cells encounter a range of cytotoxic conditions such as hypoxia, nutrient deprivation and pH changes. These cytotoxic conditions in solid tumors are considered to accumulate unfolded proteins, which cause ER stress [1～3]. The ER stress response is signaled in part through the dimerization of ER membrane-localized inositol requiring enzyme-1α (IRE1α) to activate its protein kinase and endoribonuclease activities. Activated IRE1α cleaves X-box binding protein 1 (XBP1) mRNA at two sites to initiate an unconventional splicing reaction. The 5′ and 3′ fragments are subsequently joined by RNA ligase activity, thereby removing a 26-base intron. This splicing reaction creates a translational frameshift to produce an active XBP1 transcription factor [4, 5]. Activated XBP1 has been reported to induce the up-regulation of molecular chaperons such as GRP78, PDI, ERdj4, or EDEM to diminish the accumulation of unfolded proteins [6～9]. The up-regulation of these molecules might result in protecting malignant solid-tumor cells from cytotoxic conditions. Thus, the activation of XBP1 is considered to be greatly correlated with tumor growth. Indeed, it has been demonstrated that *Xbp1*-knockout cells did not form tumors when implanted into mice [6], and the expression of XBP1 was increased in several types of tumor cells such as breast cancer [10] and hepatocellular carcinoma [11]; therefore, an inhibitor of XBP1 activation would be a new class of antitumor drug.

In the course of screening for an inhibitor of ER stress-induced XBP1 activation, we found that *Streptomyces* sp. AC 654 produced a novel compound, trierixin. In this study,

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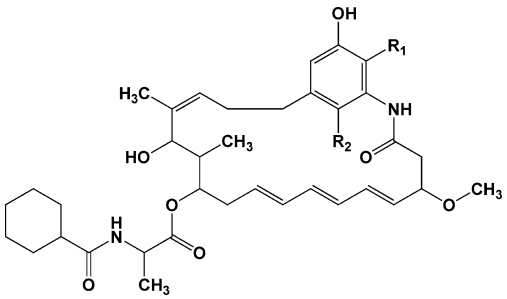

	R_1	R_2
Trierixin	SCH_3	OH
Mycotrienin II	H	OH
Trienomycin A	H	H

Fig. 1 Structures of trierixin, mycotrienin II, and trienomycin A.

the taxonomy of the producing strain, and the fermentation, isolation and biological activities of trierixin (Fig. 1) are described. The structural elucidation of trierixin will be described in an accompanying report [12].

Materials and Methods

Taxonomic Studies

The producing strain AC 654 was isolated from a grassland soil sample collected in Gunma prefecture, Japan. The morphological characteristics of the strain AC 654 were determined on yeast extract-malt extract agar (ISP medium No. 2) incubated at 27°C for 14 days.

Cell Culture

Human epithelial adenocarcinoma cell line HeLa was cultured in DMEM supplemented with 10% FBS.

Construction of pcDNA3/XBP1-luc Plasmid and Generation of HeLa/XBP1-luc Cells

Human XBP1 cDNA (GenBank Accession No. NM_005080) was amplified by PCR using a sense primer (5′-CCCAAG-CTTATGGTGGTGGTGGCAGCCGCG-3′) and antisense primer (5′-CCCGAATTCAAGCTTTGACACTAATCAG-CTGGGG-3′). To construct pcDNA3/XBP1-luc plasmid, human XBP1 cDNA was fused upstream of luciferase cDNA. The pcDNA3/XBP1-luc plasmid was then transfected into HeLa cells and the transfected cells were selected by supplementing medium with 600 μg/ml neomycin (SIGMA, St. Louis, MO) to generate HeLa/XBP1-luc cells.

Luciferase Assay

HeLa/XBP1-luc cells were seeded in 96-well plates at 1×10^4 cells/well, and then incubated with 0.1 μM of thapsigargin together with or without screening samples. After 24 hours of incubation, the cells were lysed in Passive lysis buffer (Promega, Madison, MA), and then luciferase activity was measured using the luciferase assay system (Promega) and a luminometer (Wallac, PerkinElmer, Waltham, MA). IC_{50} values were determined from the dose-response curves of the inhibition of XBP1-luciferase activity when the XBP1-luciferase activity of thapsigargin treatment was defined as 100%.

RT-PCR

Total RNA was extracted from HeLa cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). Aliquots 2.0 μg of total RNA were treated with M-MLV reverse transcriptase (Promega) to produce 1st strand cDNA, which was subjected to PCR with Ex-Taq polymerase (TaKaRa, Shiga, Japan) using a pair of primers corresponding to nucleotides 505～525 (AATGAAGTGAGGCCAGTGGCC) and 609～629 (CCCATGGATTCTGGCGGTATT) of XBP1 cDNA. The amplified products were separated by electrophoresis on a 8.0% polyacrylamide gel and visualized with ethidium bromide staining. The spliced XBP1 band (99 b.p.) was quantified by densitometry and IC_{50} values were determined from the dose-response curves of XBP1 splicing inhibition when the intensity of the spliced XBP1 band of thapsigargin treatment was defined as 100%.

MTT Assay

HeLa cells were seeded at 5×10^3 cells/well in 96-well plates and cultured overnight. The cells were treated with various concentrations of trierixin, mycotrienin II, or trienomycin A for 72 hours. Growth was measured by formazan formation (detected at 570 nm) after treatment of the cells with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C. IC_{50} values were determined from the dose-response curves of growth inhibition.

Results

Establishment of Screening System for an Inhibitor against XBP1 Activation

To establish a screening system for an inhibitor against XBP1 activation, we first constructed pcDNA3/XBP1-luc plasmid, in which human XBP1 cDNA was fused upstream of luciferase cDNA. This construct was designed as follows: under normal conditions, luciferase is not expressed,

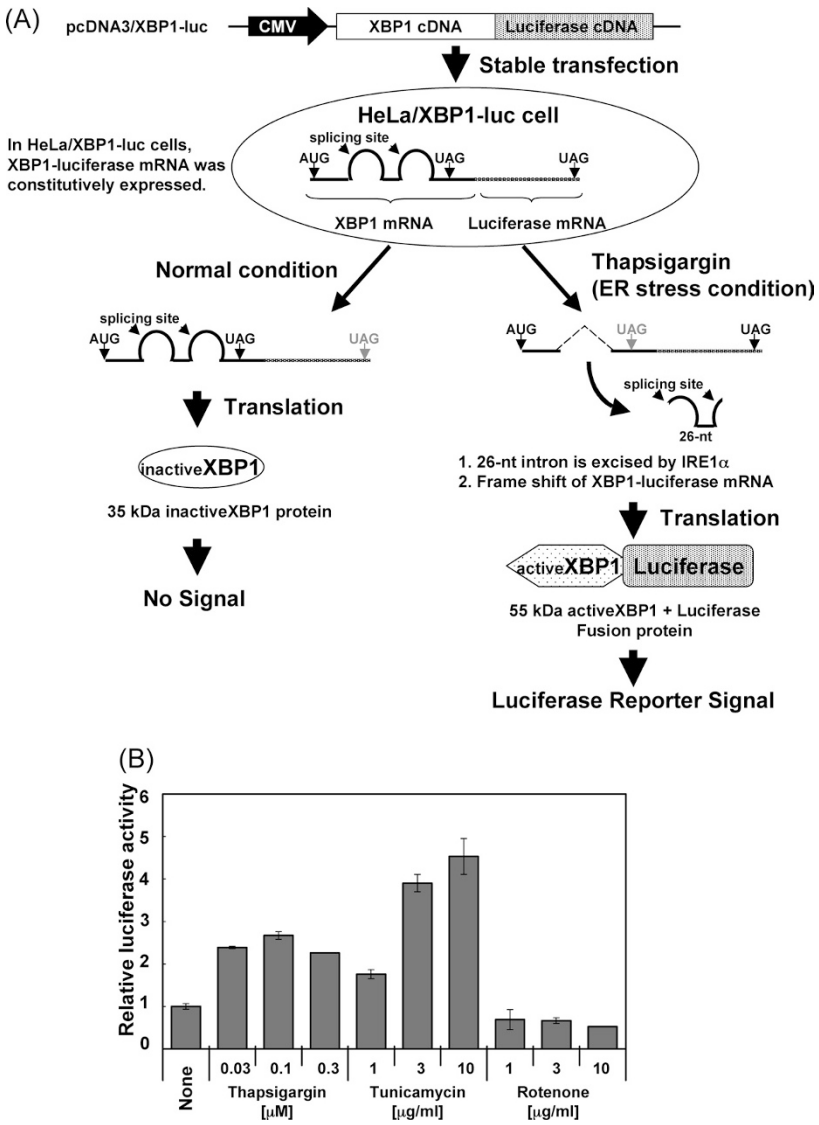

Fig. 2 Establishment of a screening system for an inhibitor against XBP1 activity.

(A) Schematic illustration of the screening system for an inhibitor against XBP1 activity as luciferase reporter signals. The schematic illustration shows mRNAs and proteins synthesized from the XBP1-luciferase construct under normal conditions and ER stress conditions.

(B) Thapsigargin and tunicamycin, but not rotenone, elevated XBP1 luciferase activities in HeLa/XBP1-luc cells. HeLa/XBP1-luc cells were treated with the indicated concentration of thapsigargin, tunicamycin, or rotenone for 24 hours. Cells were then lysed and subjected to luciferase assay to measure XBP1 luciferase activities. Data are the fold of a control sample and s.d. of a representative experiment performed in triplicate.

because the translation of XBP1-luc mRNA is terminated at the stop codon located upstream of luciferase mRNA. Under ER stress conditions, full XBP1-luciferase fused protein can be expressed because the translation of XBP1-luc mRNA is terminated at the stop codon of luciferase mRNA as the result of ER stress-induced splicing of a 26-base intron, leading to a frame shift (Fig. 2A). We next transfected pcDNA3/XBP1-luc plasmid into HeLa cells, and isolated HeLa/XBP1-luc cells. As shown in Fig. 2B, weak luciferase activity could be detected under normal conditions in HeLa/XBP1-luc cells because of the basal activity of endogenous IRE1α. On the other hand, luciferase activity was elevated about 2∼4.5-fold when HeLa/XBP1-luc cells were treated with an ER stress-inducing compound, thapsigargin or tunicamycin, for 24 hours. Luciferase activity was not elevated by treatment with rotenone which can not induce ER stress. Thus, the assay system was established, in which XBP1 activation can be easily detected as luciferase reporter signals in HeLa/XBP1-luc cells. This assay system was used for

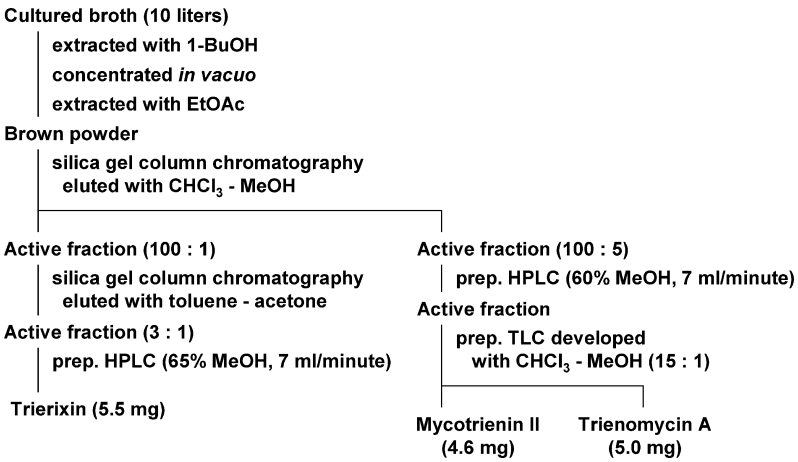

Fig. 3 Isolation procedures of trierixin, mycotrienin II, and trienomycin A from *Streptomyces* sp. AC 654.

screening an inhibitor against ER stress-induced XBP1 activation from the cultured broth of microorganisms. HeLa/XBP1-luc cells were incubated with 0.1 μM thapsigargin together with or without screening samples for 24 hours and XBP1 luciferase activity was measured to evaluate the inhibiting activity of XBP1 activation by each sample. In the course of this screening, we found that strain AC 654 produced substances that inhibit thapsigargin-induced elevation of XBP1 luciferase activity.

Taxonomy of the Producing Strain

The substrate mycelia of strain AC 654 developed well and were irregularly branched. This strain produced 10～50 or more spore chains on aerial mycelia, and the spore chains were straight or curved, rarely spiraling. The spores were cylindrical, 0.5～0.9×0.7～1.4 μm and the surfaces were smooth. Fragmentation of the substrate mycelia and sporangia was not observed. Analysis of whole-cell hydrolysates showed the presence of LL-diaminopimelic acid, indicating that the cell wall is type I. On the basis of these morphological and chemotaxonomic characteristics, strain AC 654 was assigned to the genus *Streptomyces*.

Fermentation

A thawed suspension of *Streptomyces* sp. AC 654 was used for inoculation in a 500-ml Erlenmeyer flask containing 200 ml of a seed medium composed of D(+)-glucose 1.0%, dextrin 4.0%, yeast extract 0.1%, soybean powder 2.5%, $FeSO_4 \cdot 7H_2O$ 0.05%, and $CaCO_3$ 0.8%. The inoculated medium was incubated at 24°C for 4 days on a rotary shaker (230 rpm). Aliquots of this seed culture were added to a 500-ml Erlenmeyer flask containing 200 ml of the same seed medium. The inoculated medium was incubated at 24°C for 8 days on a rotary shaker (230 rpm).

Isolation and Purification

Isolation procedures are summarized in Fig. 3. The culture broth (10 liters) of *Streptomyces* sp. AC 654 was extracted with 1-BuOH (5 liters), and the extracts were concentrated, dissolved in water (0.5 liters), and then extracted twice with EtOAc (0.5 liters). The organic layer was concentrated to dryness under reduced pressure to give a brown powder (3.61 g), a portion of which (950 mg) was applied on silica gel column chromatography (50 g, Silica gel 60, 60～230 μm, Merck), and eluted with a $CHCl_3$ - MeOH system. The active fraction (100 : 1) was subjected to a second silica gel column chromatography, and eluted stepwise with a toluene - acetone system. The active fraction (3 : 1) was further purified by preparative HPLC (CAPCEL PAK C18 UG80 S-5, 20×250 mm, SHISEIDO Co., Ltd.) to give a pure pink powder of trierixin (5.5 mg). Another active fraction was obtained in the first silica gel column chromatography (100 : 5 $CHCl_3$: MeOH). We also purified active substances with preparative HPLC followed by preparative TLC developed with $CHCl_3$ - MeOH (15 : 1) and obtained mycotrienin II (4.6 mg) and trienomycin A (5.0 mg).

Biological Activities

Trierixin Inhibits ER Stress-induced XBP1 Activation

The inhibitory activity of trierixin was first assessed using HeLa/XBP1-luc cells. As shown in Fig. 4A, treatment of HeLa/XBP1-luc cells with 0.1 μM thapsigargin for 24 hours elevated XBP1 luciferase activities about 2.5-fold more than the control in HeLa/XBP1-luc cells. Trierixin inhibited thapsigargin-induced XBP1 luciferase activation in a dose-dependent manner with an IC_{50} value of 14 ng/ml. To examine whether trierixin also inhibited thapsigargin-induced enodogenous XBP1 splicing in HeLa cells, we next

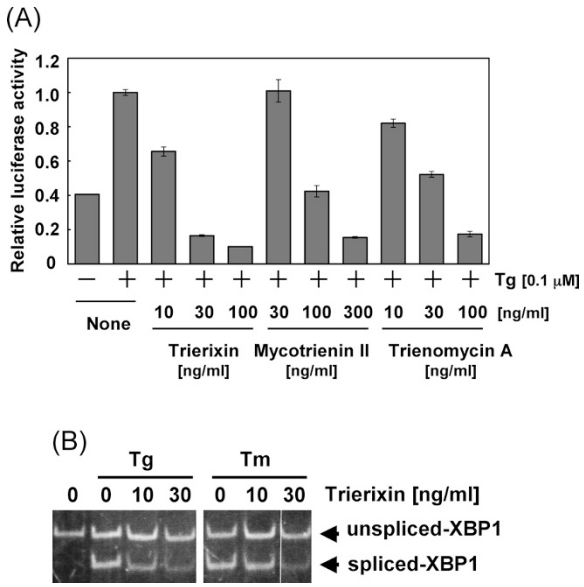

Fig. 4 Trierixin, mycotrienin II, and trienomycin A inhibited ER stress-induced XBP1 activation.

(A) Effect of trierixin, mycotrienin II, or trienomycin A on thapsigargin-induced XBP1 activation assessed by luciferase assay. HeLa/XBP1-luc cells were treated with the indicated concentration of trierixin, mycotrienin II, or trienomycin A in the presence or absence of 0.1 μM of thapsigargin (Tg). After 24 hours, the cells were lysed and subjected to luciferase assay. Data are the fold of a thapsigargin-treated sample and s.d. of a representative experiment performed in triplicate.

(B) Effect of trierixin on thapsigargin- or tunicamycin-induced endogenous XBP1 splicing assessed by RT-PCR. HeLa cells were treated with the indicated concentration of trierixin in the presence or absence of 0.1 μM of thapsigargin (Tg) or 10 μg/ml of tunicamycin (Tm) for 8 hours. The cells were collected and RNA was extracted. Spliced- or unspliced-XBP1 mRNA was detected as described in Materials and Methods.

performed RT-PCR analysis of RNA isolated from trierixin-treated or -untreated HeLa cells using PCR primers for the region encompassing the splice junction of XBP1. Treatment with 0.1 μM thapsigargin for 8 hours increased the expression of spliced XBP1 mRNA (Fig. 4B), indicating that thapsigargin induced endogenous XBP1 splicing. Trierixin inhibited thapsigargin-induced endogenous XBP1 splicing in a dose-dependent manner with an IC_{50} value of 19 ng/ml. Trierixin also inhibited both tunicamycin-induced XBP1-luciferase activation (data not shown) and endogenous XBP1 splicing (Fig. 4B). These results indicated that trierixin is an inhibitor of ER stress-induced XBP1 activation. Trierixin did not influence the viability of HeLa cells up to 30 ng/ml after 24 hours, suggesting that the inhibition of ER stress-induced XBP1 activation by trierixin was not due to the cytotoxic effect of trierixin. In the same manner, the structurally related compounds

Table 1 Effects of trierixin, mycotrienin II, and trienomycin A on the cell growth of HeLa cells

Inhibitor	IC_{50} (ng/ml)
Trierixin	10
Mycotrienin II	58
Trienomycin A	30

mycotrienin II and trienomycin A exhibited inhibitory activities against thapsigargin-induced XBP1-luciferase activation with IC_{50} values of 86 and 32 ng/ml, respectively, without affecting cell viability (Fig. 4A). Furthermore, trienomycin A inhibited endogenous XBP1 splicing induced by thapsigargin or tunicamycin as well as another ER stress-inducing compound, 2-deoxyglucose (data not shown). Thus, mycotrienin II and trienomycin A are also inhibitors of ER stress-induced XBP1 activation.

Trierixin Inhibits Tumor Cells Growth

We next examined the effects of trierixin, mycotrienin II, and trienomycin A on the cell growth of HeLa cells. As shown in Table 1, trierixin strongly inhibited cell growth with an IC_{50} value of 10 ng/ml as evaluated by MTT assay. In the same manner, mycotrienin II and trienomycin A also inhibited cell growth with IC_{50} values of 58 and 30 ng/ml, respectively.

Discussion

In the present study, we isolated a new member of triene-ansamycin antibiotic, trierixin, containing an SCH_3 group from *Streptomyces* sp. AC 654 as an inhibitor of ER stress-induced XBP1 activation. Although many triene-ansamycin antibiotics, such as trienomycins [13], mycotrienins [14], cytotrienins [15], thiazinotrienomycins [16], TMC-135s [17], and UCF116s [18] have been isolated from the cultured broth of *Actinomycete* strains, they do not have a SCH_3 group in their structures; therefore, trierixin is a new member of the triene-ansamycin family.

Several biological activities of triene-ansamycin antibiotics have been reported. Mycotrienin I and II were reported to inhibit tyrosine kinase activity of $pp60^{src}$ [19], UCF116s was reported to be an inhibitor of farnesyltransferase [18], and trienomycin G was revealed to be an inhibitor of NO production [20]. Moreover, cytotrienin A was reported to induce apoptosis in tumor cells through the proteolytic activation of MST/Krs and JNK activation [21], and thiazinotrienomycin B was reported to interfere with EGF

receptor-mediated signal transduction in human tumor cells [22]. However, so far, triene-ansamycin antibiotics that modify ER stress response have not yet been reported; therefore, trierixin is the first example in a series of triene-ansamycin antibiotics, demonstrating that the compound inhibits XBP1 activation in response to ER stress. In addition, we found that trienomycin A and mycotrienin II also inhibited ER stress-induced XBP1 activation.

Versipelostatin, a novel 17-membered macrocyclic compound produced by *Streptomyces* [23], has been reported to inhibit splicing of XBP1 mRNA induced by 2-deoxyglucose, but not by tunicamycin [24]. On the other hand, triene-ansamycins inhibit XBP1 activation induced by not only 2-deoxyglucose but also thapsigargin and tunicamycin. Therefore, the inhibition mechanism of ER stress-induced XBP1 activation by triene-ansamycins seems to be different from that by versipelostatin.

There is now abundant evidence to suggest that the ER stress response is activated in various tumors [11, 25～27] to protect tumor cells from apoptosis [24, 28]. As XBP1 is a major regulator of the ER stress response, inhibition of XBP1 activation would be a new type of therapeutic strategy for cancer treatment. Indeed, we demonstrated that trierixin as well as mycotrienin II and trienomycin A showed an antitumor effect on HeLa cells, and the IC_{50} value of each compound to inhibit tumor cell growth is in the same range as that to inhibit XBP1 activation. Furthermore, *in vitro* and *in vivo* antitumor effects of triene-ansamycins have been reported; however, their mechanisms remain obscure. Our results raise the possibility that inhibitory effects against XBP1 activation could explain the antitumor activities of triene-ansamycins. Detailed studies of the biological activities of trierixin *in vivo* and *in vitro*, and identification of a molecular target of trierixin would provide valuable information not only on the mechanism of the antitumor effect induced by triene-ansamycins, but also on the role of XBP1 in tumor development.

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