The results are the mean ± SD of five different fields.

Figure 3. Correlation between expression levels of H-Ras and inhibition of migration by moverastin A and UTKO1

(a) Western Blot analysis of H-Ras expression levels in seven tumor cell lines. Cell lysates were separated by SDS-PAGE and then subjected to immunoblotting using anti-H-Ras antibody. Quantification of expression levels of H-Ras was analyzed by Image gauge and normalized with the level of tubulin. Percentages represent the relative expression level of H-Ras in tumor cells compared to the level in EC17 cells. (b) Simple linear correlations between two parameters were calculated. Correlation coefficients (r) are shown in this figure.

Schemes

Scheme 1. Synthesis and structures of moverastin A and B.

Scheme 2. Synthesis of UTKO1–6.

Reagents and conditions: (a) H_2, Pd-C, EtOAc (for **6**) or EtOH (for **10**, **15** and **18**); (b) KHMDS, Comin's reagent, THF, –78°C; (c) Ph_3P=CHCOMe, CH_2Cl_2 (for **9** and **14**) or

xylene (for **17**), reflux; (d) $CrCl_2$, $NiCl_2$, DMF, r.t.; (e) conc. HCl, THF, r.t.

Scheme 3. Synthesis of UTKO7–10.

Reagents and conditions: (a) Rh-Al_2O_3, EtOH; (b) DEAD, PPh_3, THF; (c) MeI, K_2CO_3, (n-Bu)$_4$N·HSO_4, EtOAc-Toluene, reflux; (d) PDC, CH_2Cl_2; (e) conc. HCl, THF, r.t.

Scheme 4. Synthesis of UTKO11 and UTKO12.

(a) O_3, CH_2Cl_2, –78%, then PPh_3; (b) $CrCl_2$, $NiCl_2$, DMF, r.t.; (c) conc. HCl, THF, r.t.

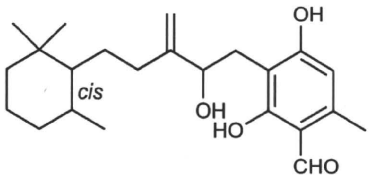
Table(s)

Table 1. Effects of UTKO compounds on cell migration, cell viability and *in vitro* FTase activity in EC17 cells.

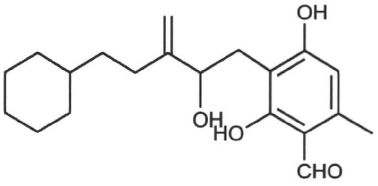
	IC_{50} [μM]		
	Cell Migration	Cell Viability[(a)]	FTase[(b)]
moverastin A	7.22	>77	14.7
UTKO1	1.98	46	>100
UTKO2	8.43	87	>100
UTKO3	7.53	>80	>100
UTKO4	30.0	>130	>100
UTKO5	10.6	45	>100
UTKO6	6.52	55	>100
UTKO7	2.12	16	>100
UTKO8	4.41	18	>100
UTKO9	2.00	18	>100
UTKO10	4.57	18	>100
UTKO11	21.4	>28	>100
UTKO12	2.17	17	>100

Figure(s)

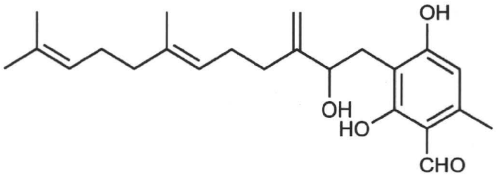
Figure 1



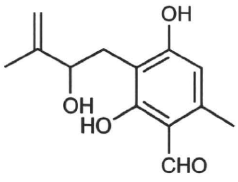
UTKO1



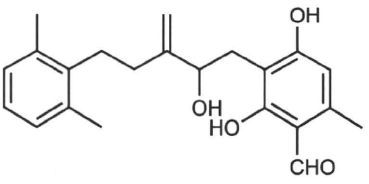
UTKO2



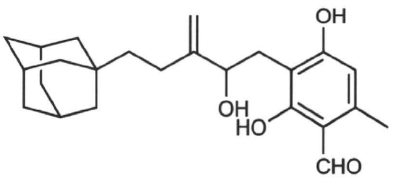
UTKO3



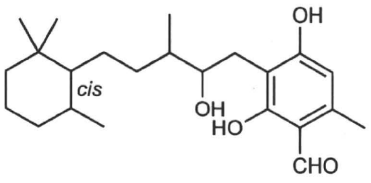
UTKO4



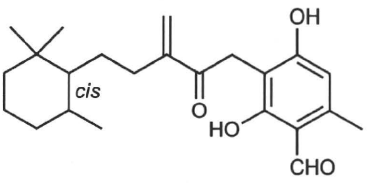
UTKO5



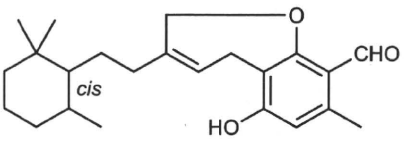
UTKO6



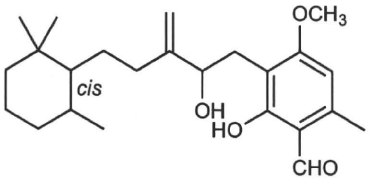
UTKO7



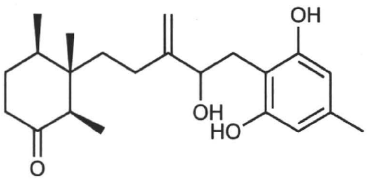
UTKO8



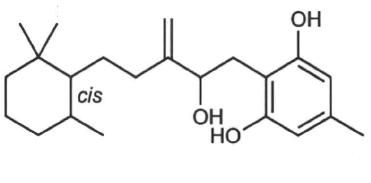
UTKO9



UTKO10

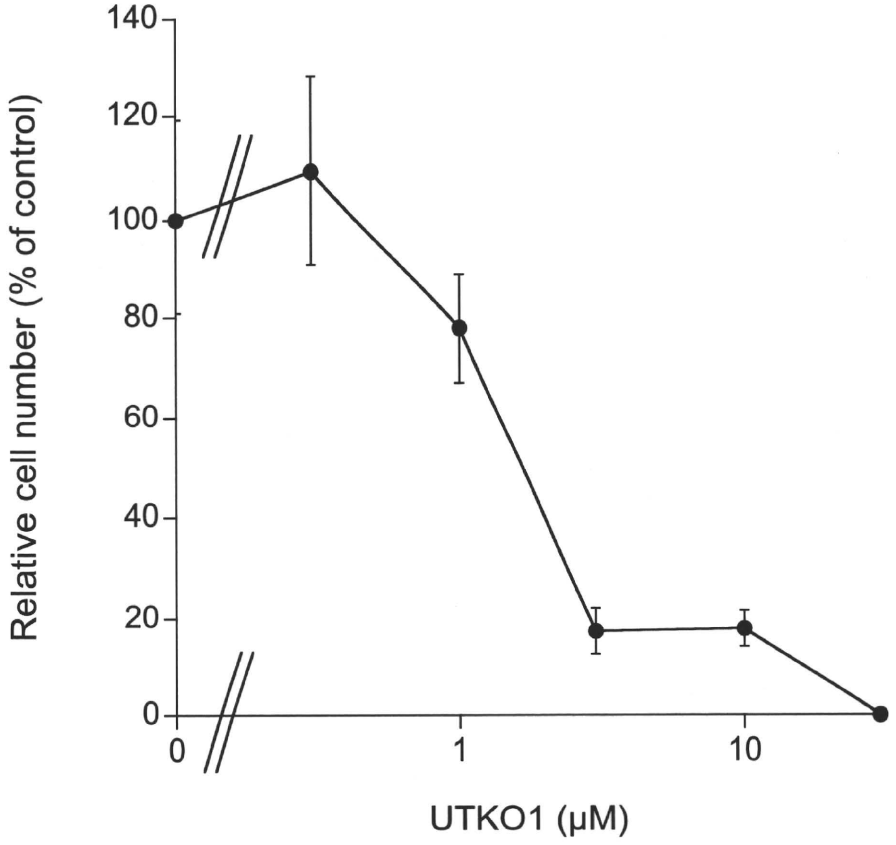


UTKO11



UTKO12

Figure 2


140
120
100
80
60
40
20
0
0
1
10
Relative cell number (% of control)
UTKO1 (μM)

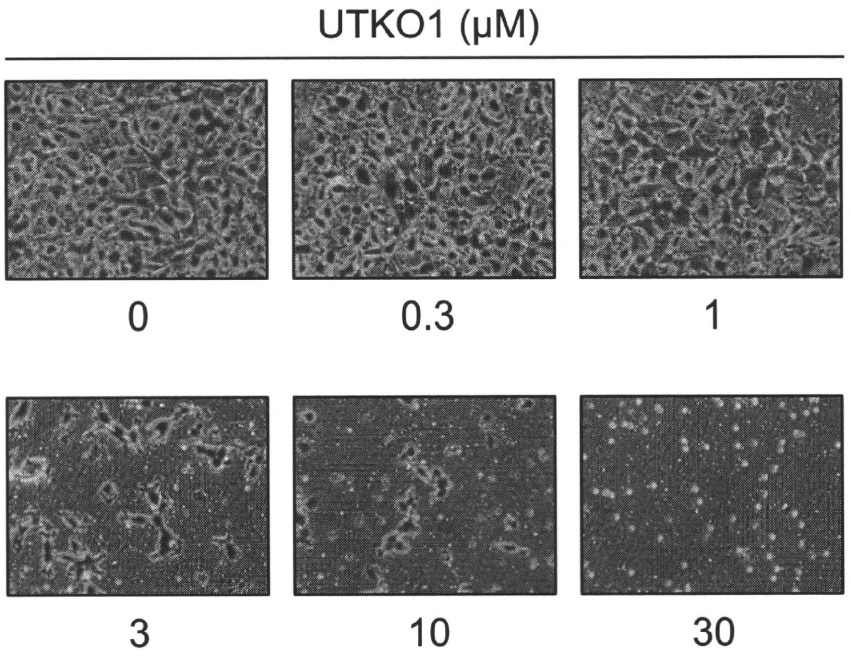
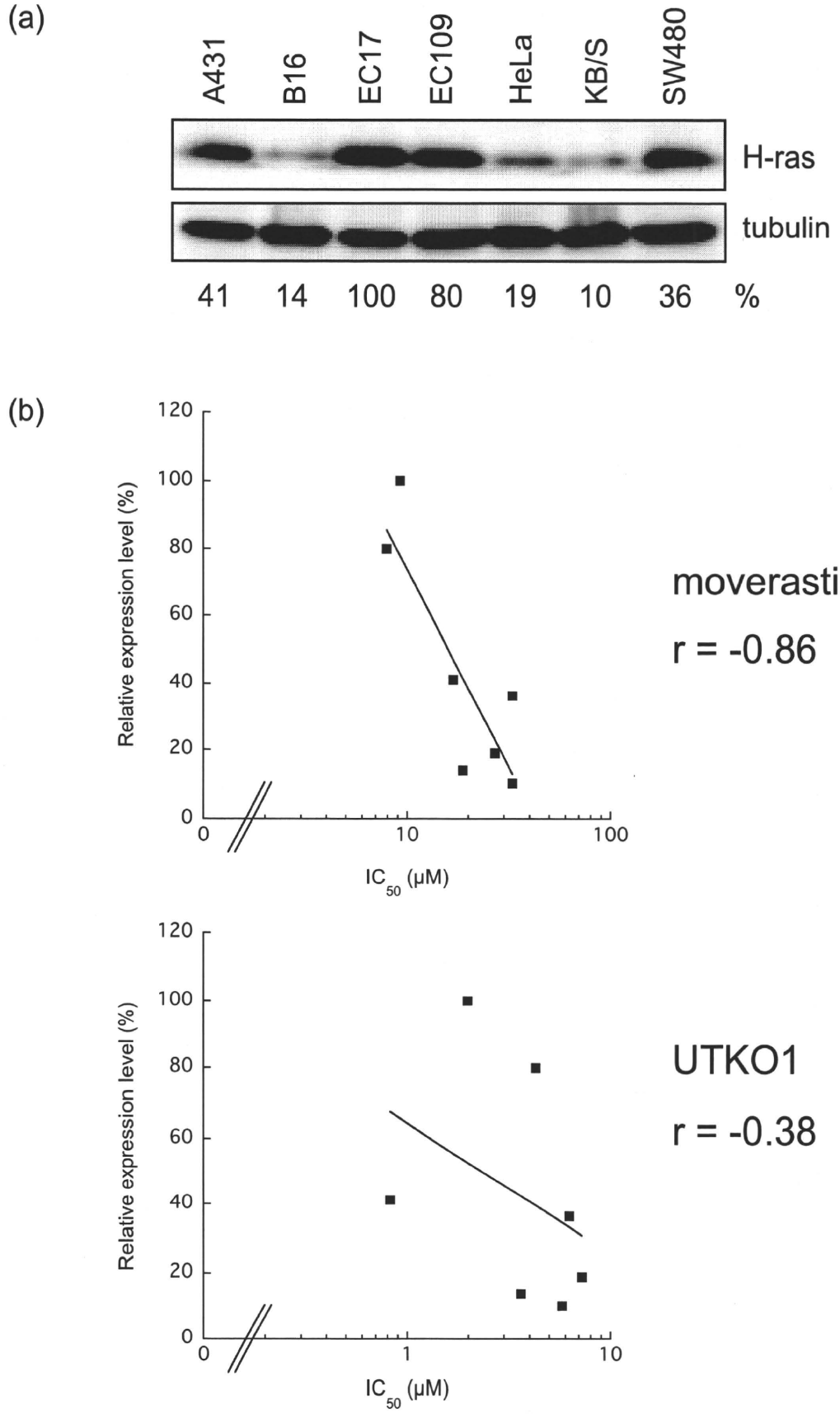
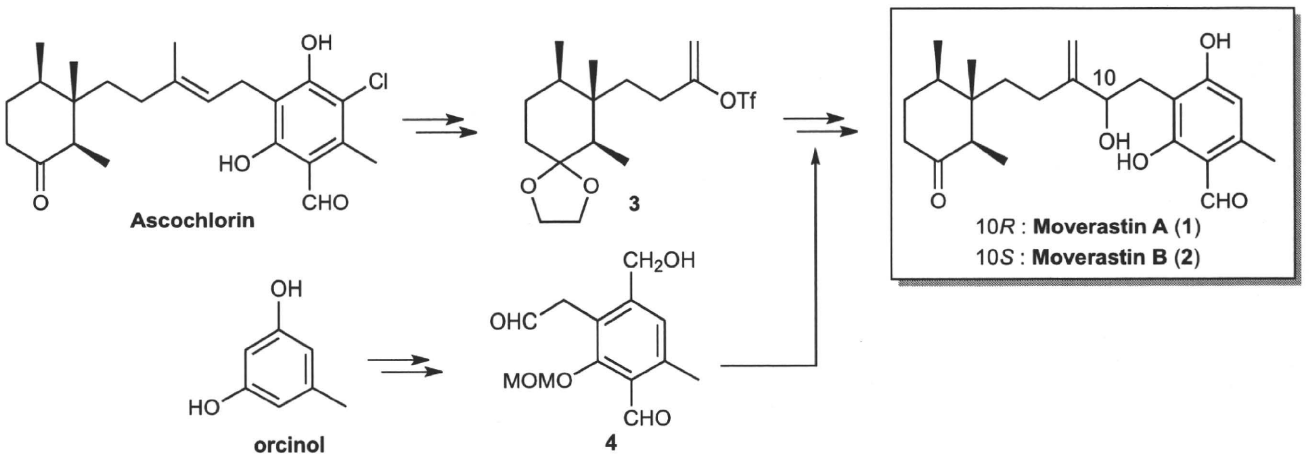

UTKO1 (μM)
0
0.3
1
3
10
30

Figure 3


(a)
A431
B16
EC17
EC109
HeLa
KB/S
SW480
H-ras
tubulin
41
14
100
80
19
10
36
%
(b)
Relative expression level (%)
120
100
80
60
40
20
0
0
10
100
IC50 (μM)
moverastin A
r = -0.86
Relative expression level (%)
120
100
80
60
40
20
0
0
1
10
IC50 (μM)
UTKO1
r = -0.38

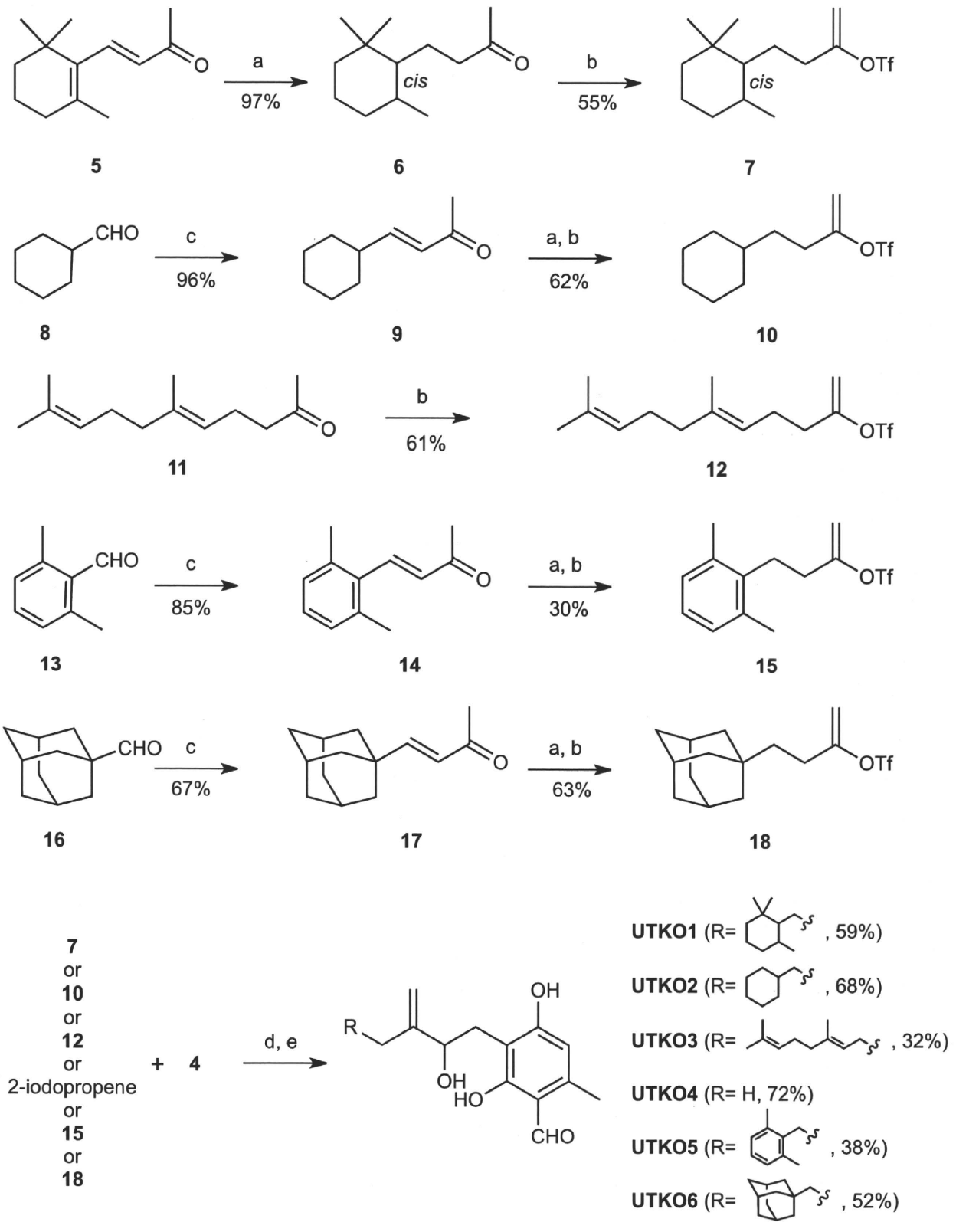
Figure(s)

Scheme 1



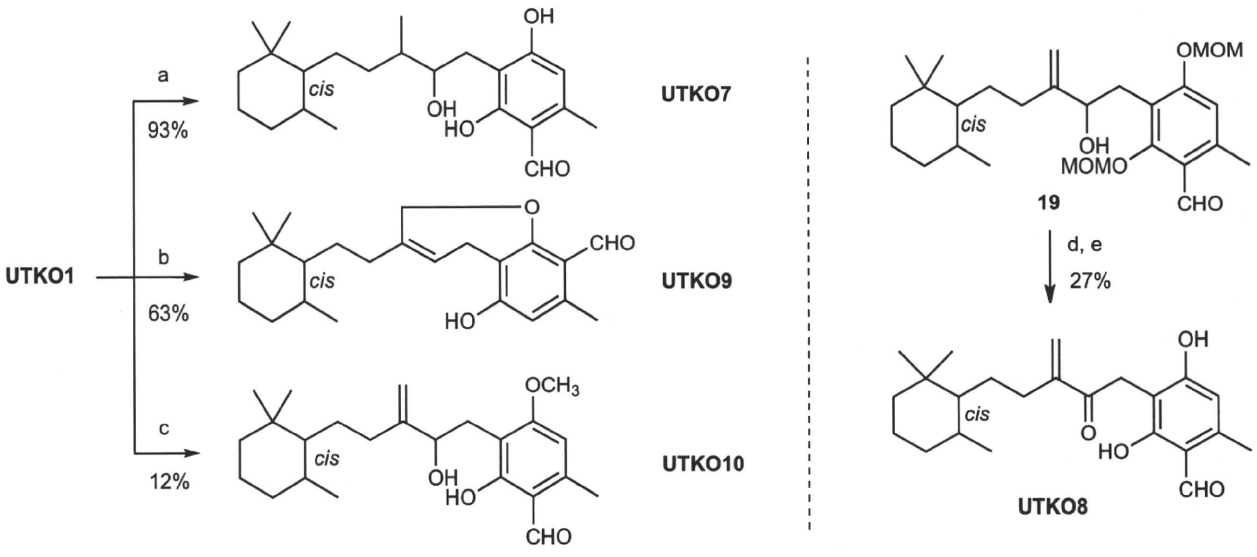
Figure(s)

Scheme 2



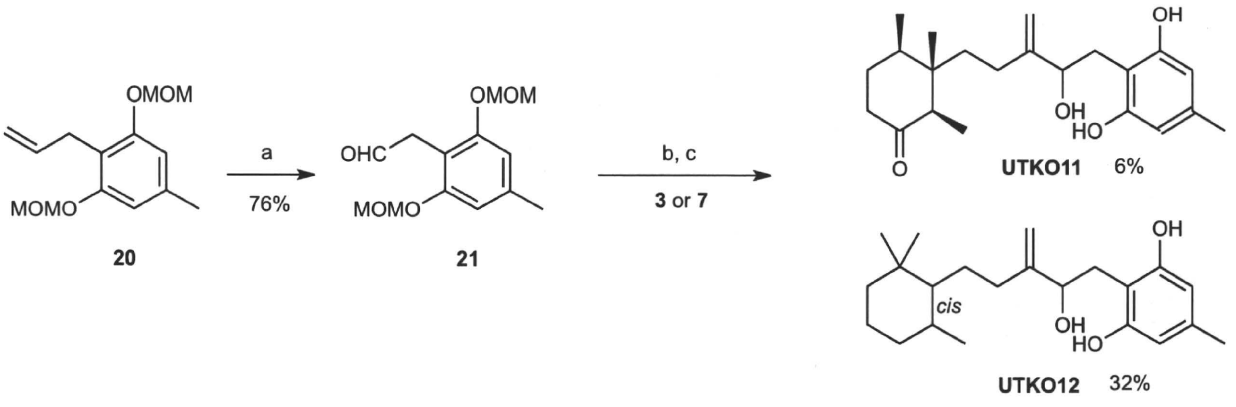
Figure(s)

Scheme 3



Figure(s)

Scheme 4



Mol Files

Click here to download Mol Files: UTKO1.mol

Biosci. Biotechnol. Biochem., **75** (2), 284–288, 2011

Quinotrierixin Inhibited ER Stress-Induced XBP1 mRNA Splicing through Inhibition of Protein Synthesis

Kohta YAMAMOTO,† Etsu TASHIRO, and Masaya IMOTO

Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan

Received August 25, 2010; Accepted November 22, 2010; Online Publication, February 7, 2011
[doi:10.1271/bbb.100622]

Quinotrierixin was isolated from microbes as an inhibitor of ER stress-induced XBP1 mRNA splicing, but its mode of action was unclear. We found that quinotrierixin is an inhibitor of protein synthesis, and that the required dose range of quinotrierixin to inhibit ER stress-induced XBP1 mRNA splicing was similar to that to inhibit protein synthesis. Furthermore, we also found that quinotrierixin inhibited the ER stress-induced increases of unfolded protein response-related genes such as GRP78, CHOP, EDEM, ERdj4, and p58^{IPK}. Thus, we showed that quinotrierixin inhibited the ER stress-induced unfolded protein response, possibly due to its inhibitory activity of protein synthesis.

Key words: quinotrierixin; ER stress; XBP1; protein synthesis inhibitor

Newly synthesized polypeptides that are secreted or localized in the plasma membrane are post- or co-translationally translocated into the lumen of the endoplasmic reticulum (ER), where they are modified, folded, and assembled correctly prior to transport to the Golgi apparatus. However, when cells are faced with cytotoxic conditions, such as hypoxia and nutrient deprivation, these folding reactions are compromised and protein aggregation occurs. The unfolded proteins are retained in the ER, and the accumulation of unfolded proteins causes ER stress.[1] As a consequence, the cell activates adaptive signaling pathways that are programmed to enhance folding capabilities and to limit the folding load on the ER. This response is called the unfolded protein response (UPR).

In mammalian cells, UPR is regulated in part by ER membrane-localized IRE1α.[2–4] IRE1α induces unconventional X-box binding protein 1 (XBP1) mRNA splicing. Twenty-six nts of XBP1 mRNA are spliced out to lead the shift in the open reading frame. Translation of spliced XBP1 mRNA produces a potent transcription factor called XBP1s (the "s" indicates spliced).[5,6] XBP1s upregulates gene expression that enhances ER protein folding capacity and quality control.[7] Furthermore, it has been reported that there is a link between XBP1 and human disease, including solid tumor and inflammatory bowel disease.[8–14] Therefore, small molecule inhibitors of ER stress-induced XBP1 mRNA splicing would be very useful in fundamental research into UPR signaling, and might eventually find clinical application.

In a previous study, we established a screening system to identify small molecule inhibitors of XBP1 mRNA splicing. We have also reported that novel triene-ansamycin group compounds, quinotrierixin and trierixin, inhibited ER stress-induced XBP1 mRNA splicing in HeLa cells.[15–18] In this study, we found that quinotrierixin inhibited protein synthesis. Moreover, we investigated the relationship between quinotrierixin-inhibited ER stress-induced XBP1 mRNA splicing and protein synthesis.

Materials and Methods

Materials. Quinotrierixin, trierixin, and trienomycin A were prepared as described in our previous reports.[15,17] Cytotrienin A was kindly provided by Dr. H. Osada (RIKEN, Japan). Cycloheximide, anisomycin, and puromycin were purchased from Sigma (St. Louis, MO).

Cell culture. Human epithelial adenocarcinoma cell line HeLa was cultured in DMEM supplemented with 8% FBS.

RT-PCR analysis (XBP1 mRNA splicing assay). As reported previously,[15] HeLa cells were seeded in 12-well plates at 5×10^4 cells/well, and then incubated with 10 μg/mL of tunicamycin with and without quinotrierixin and other protein synthesis inhibitors for 4 h. Subsequently, total RNA was extracted from the HeLa cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Aliquots of 2.0 μg of the total RNA were treated with M-MLV reverse transcriptase (Promega, Madison, WI) to produce first-strand cDNA, which was subjected to polymerase chain reaction (PCR) with KOD plus polymerase (Toyobo, Osaka, Japan) using a pair of primers corresponding to nucleotides 505–524 and 609–629 of XBP1 cDNA. The amplified products were separated by electrophoresis on a 8% polyacrylamide gel and visualized by ethidium bromide staining.

Real-time RT-PCR. Real-time reverse transcription (RT)–polymerase chain reaction (PCR) was performed using SYBR Premix Ex Taq (Takara, Siga, Japan). The primer set was as follows: for GRP78, forward 5′-GCTCGACTCGAATTCCAAAG-3′ and reverse 5′-GATCACCAGAGAGCACACCA-3′; for CHOP, forward 5′-GCGCATGAAGGAGAAAGAAC-3′ and reverse 5′-TCACCATTCGGTCAATCAGA-3′; for ERdj4, forward 5′-AAAATAAGAGCCCGGATGCT-3′ and reverse 5′-CGCTTCTTGGATCCAGTGTT-3′; for EDEM, forward 5′-TGGACTGCAGGTGCTGATAG-3′ and reverse 5′-GGATTCTTGGTTGCCTGGTA-3′; for P58^{IPK}, forward 5′-CTCAGTTTCATGCTGCCGTA-3′ and reverse 5′-TTGCTGCAGTGAAGTCCATC-3′; and for GAPDH, forward 5′-AGGTCGGAGTCAACGGATTT-3′ and reverse 5′-TAGTTGAGGTCAATGAAGGG-3′.

Measurement of macromolecular synthesis. HeLa cells were seeded in 24-well plates at 5×10^4 cells/well and cultured overnight. Each culture well was refilled with fresh Dulbecco's Modified Eagle's

† To whom correspondence should be addressed. Tel: +81-45-566-1793; Fax: +81-45-566-1557; E-mail: tashiro@bio.keio.ac.jp

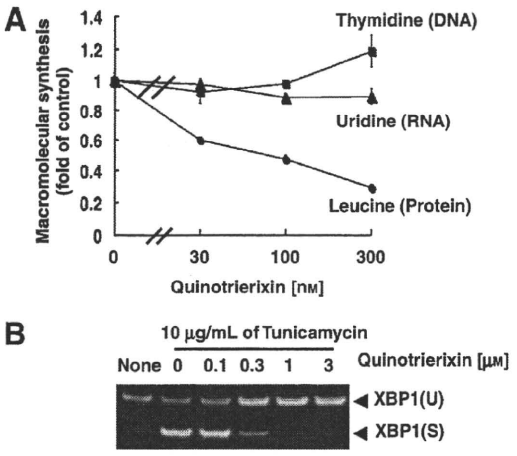


Fig. 1. Quinotrierixin, Cycloheximide, and Anisomycin Inhibited ER Stress-Induced XBP1 mRNA Splicing and Protein Synthesis.

A, Quinotrierixin inhibited protein synthesis in HeLa cells. HeLa cells were treated with the indicated concentrations of qunotrierixin with [^{3}H]-leucine, [^{3}H]-thymidine, or [^{3}H]-uridine for 1 h. The radioactivity of the macromolecular fraction was measured with a liquid scintillation counter. The representative data are the means for three independent studies. B, Quinotrierixin inhibited tunicamycin-induced XBP1 mRNA splicing. HeLa cells were treated with 10 μg/mL of tunicamycin with and without the indicated concentrations of quinotrierixin for 4 h. The cells were collected and extracted RNA was subjected to RT-PCR. Spliced and unspliced XBP1 mRNA was detected as described in "Materials and Methods." XBP1(U) and XBP1(S) indicate unspliced and spliced XBP1 mRNA respectively.

Medium (DMEM) containing 0.2% fetal bovine serum (FBS). For measurement of protein synthesis, cells were treated with 2 μCi [^{3}H]-leucine, 0.5 μCi [^{3}H]-thymidine or 0.5 μCi [^{3}H]-uridine, radioactive protein, and DNA or RNA synthesis precursor, with and without the indicated compounds for 1 h at 37 °C. After incubation, the medium was removed and the macromolecular fraction was fixed with ice-cold 10% trichloroacetic acid (TCA). Each well was washed with ice-cold 10% TCA twice, and 0.5 N NaOH was added. Radioactivity was measured with a liquid scintillation counter.

Data analysis. For determination of IC_{50} values against tunicamycin-induced XBP1 mRNA splicing, the spliced-XBP1 mRNA bands visualized with ethidium bromide staining were quantified by densitometry. IC_{50} values were determined from the dose-response curves of the inhibition of XBP1 mRNA splicing activity, where the intensity of spliced-XBP1 mRNA bands of tunicamycin treatment was defined as 100%.

IC_{50} values against protein synthesis were determined from dose-response curves, the radioactivity of non-treatment control was defined as 100%.

Real-time RT-PCR data were analyzed by paired *t*-tests to evaluate differences in means among three independent experiments, $^*p < 0.05$ and $^{**}p < 0.01$.

Results

Quinotrierixin inhibited XBP1 mRNA splicing and protein synthesis

Quinotrierixin was identified as an inhibitor of ER stress-induced XBP1 mRNA splicing, but its mode of action was unclear. Previously, cycloheximide, a well-known protein synthesis inhibitor, was reported to inhibit ER stress-induced XBP1 mRNA splicing.[19] Hence, we examined whether quinotrierixin would inhibit protein synthesis. As shown in Fig. 1A, quinotrierixin inhibited [^{3}H]-leucine uptake into the macromolecular fraction of HeLa cells in a dose-dependent manner. On the other hand, neither [^{3}H]-thymidine uptake nor [^{3}H]-uridine uptake was inhibited by quinotrierixin at up to 300 nm. These results indicate that quinotrierixin inhibits protein synthesis without affecting DNA or RNA synthesis (Fig. 1A). Because the dose range of quinotrierixin for inhibiting protein synthesis was similar to that for inhibiting tunicamycin and 2-deoxyglucose (2DG), both of which are known as ER stress inducers, -induced XBP1 mRNA splicing (Fig. 1B and Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site), another three triene-ansamycin group compounds, which inhibited ER stress-induced XBP1 mRNA splicing, were assessed for their ability to inhibit protein synthesis. As shown in Table 1, trierixin, trienomycin A, and cytotrienin A inhibited protein synthesis at almost the same concentration as for the inhibition of tunicamycin-induced XBP1 mRNA splicing.

Table 1. Inhibitory Activities of Triene-Ansamycin Group Compounds, Cycloheximide, Anisomycin, and Puromycin against ER Stress-Induced XBP1 Activation and Protein Synthesis

	XBP1 inhibition IC_{50} value [nM]	Protein synthesis inhibition IC_{50} value [nM]
Quinotrierixin	85	120
Trierixin	17	55
Trienomycin A	47	38
Cytotrienin A	190	160
Cycloheximide	530	680
Anisomycin	240	120
Puromycin	>20,000	4,400

Quinotrierixin inhibited unfolded protein response

Next, to determine whether quinotrierixin would affect other ER stress-induced unfolded protein responses, we evaluated the effects of quinotrierixin on the mRNA levels of UPR related genes, GRP78, CHOP, EDEM, ERdj4, and p58^{IPK}, under ER stress conditions. We found that quinotrierixin completely suppressed the increases in GRP78, CHOP, EDEM, ERdj4, and p58^{IPK} mRNA induced by tunicamycin or 2DG at the same concentration as for inhibition of protein synthesis and XBP1 mRNA splicing. (Fig. 2 and Supplemental Fig. 2). These results indicate that quinotrierixin did not specifically inhibit ER stress-induced XBP1 mRNA splicing, but rather that quinotrierixin would induce the shutdown of all ER stress-induced UPR.

Cycloheximide and anisomycin, but not puromycin, inhibited the unfolded protein response

To confirm that protein synthesis inhibition due to quinotrierixin results in the suppression of ER stress-induced UPR, we determined whether other protein synthesis inhibitors would inhibit ER stress-induced UPR. As shown in Figs. 3A and 4 and Supplemental Figs. 1 and 2, anisomycin as well as cycloheximide inhibited not only XBP1 mRNA splicing but also the UPR gene upregulation induced by tunicamycin and by 2DG. On the other hand, puromycin failed to inhibit the XBP1 mRNA splicing induced by tunicamycin and by 2DG under conditions in which puromycin inhibited protein synthesis, as judged by [^{3}H]-leucine uptake into the macromolecular fraction of the HeLa cells (Table 1,

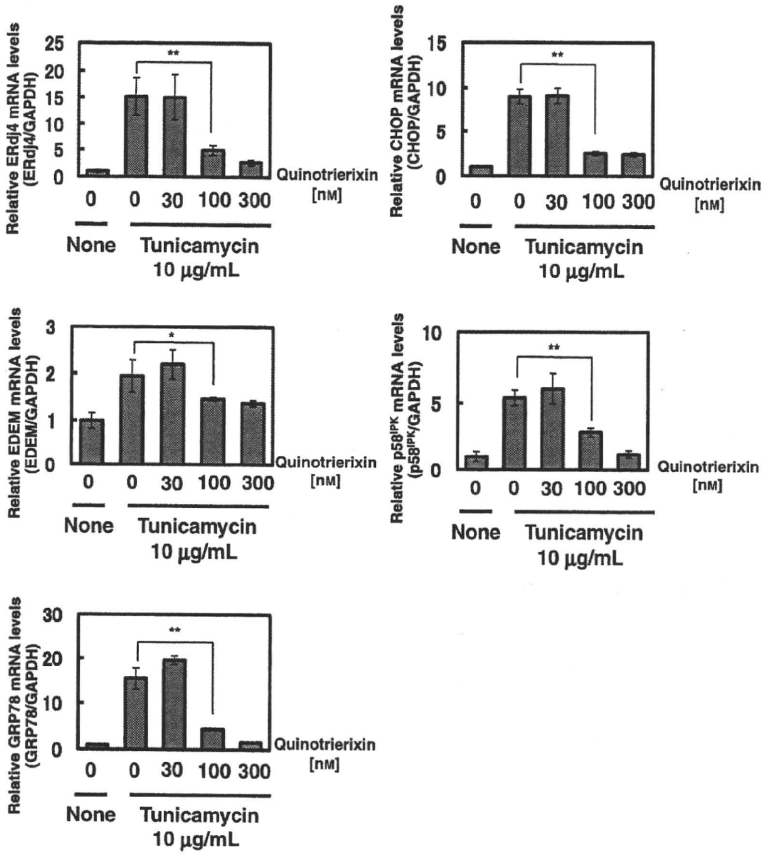


Fig. 2. Quinotrierixin Inhibited the Unfolded Protein Response.

Quinotrierixin suppressed tunicamycin-increased ERdj4, EDEM, GRP78, CHOP, and $p58^{IPK}$ mRNA. HeLa cells were treated with 10 μg/mL of tunicamycin with and without the indicated concentrations of quinotrierixin for 8 h. The cells were collected and RNA was extracted. ERdj4, EDEM, GRP78, CHOP, and $p58^{IPK}$ mRNA levels were evaluated by real-time RT-PCR. Each mRNA was normalized with the mRNA levels of GAPDH. Data are the means ± SD among three independent experiments, $^{*}p < 0.05$ and $^{**}p < 0.01$.

Fig. 3A, and Supplemental Fig. 3). Rather, consistently with a previous report,[20] single treatment of cells with puromycin induced XBP1 mRNA splicing (Fig. 3B). Both spliced and unspliced XBP1 mRNA were weakly reduced when the cells were treated with puromycin together with tunicamycin, but this was probably due to cytotoxicity, because puromycin induced cell membrane disruption at 6 μM after 4 h of treatment with puromycin together with tunicamycin (data not shown).

Discussion

In this study, we found for the first time that triene-ansamycin group compounds, including quinotrierixin, inhibited protein synthesis. Although several biological activities of triene-ansamycin antibiotics have been reported, the activity of protein synthesis inhibition has not been reported. This finding raises the possibility that some biological activities of triene-ansamycin group compounds, such as apoptosis induction,[21] inhibition of NO production,[22] and inhibition of osteoblastic resorption,[23] can be explained by inhibitory activity of protein synthesis.

Quinotrierixin and other protein synthesis inhibitors suppressed not only ER stress-induced XBP1 mRNA splicing but also other UPR, such as transcription of the UPR genes GRP78, CHOP, EDEM, ERdj4, and $p58^{IPK}$, at the same concentration as for inhibition of protein synthesis. The only exception was puromycin, which did

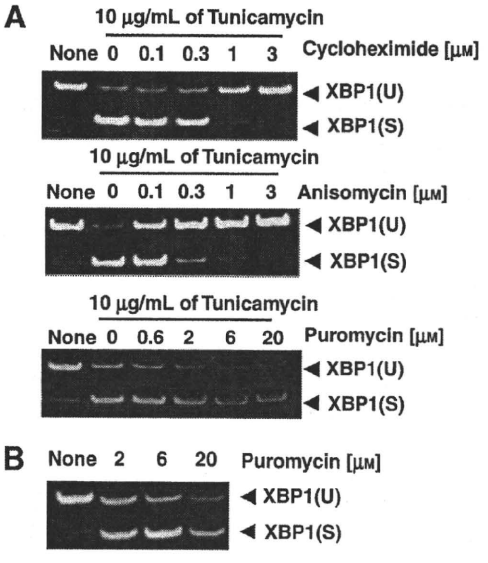


Fig. 3. Cycloheximide and Anisomycin, but Not Puromycin, Inhibited XBP1 mRNA Splicing.

A, Cycloheximide and anisomycin but not puromycin inhibited tunicamycin-induced XBP1 mRNA splicing. HeLa cells were treated with 10 μg/mL of tunicamycin with and without the indicated concentrations of cycloheximide, anisomycin, or puromycin for 4 h. B, Puromycin induced XBP1 mRNA splicing. HeLa cells were treated with the indicated concentrations of puromycin for 4 h. The cells were collected, and extracted RNA was subjected to RT-PCR. Spliced and unspliced XBP1 mRNA was detected as described in "Materials and Methods." XBP1(U) and XBP1(S) indicate unspliced and spliced XBP1 mRNA respectively.

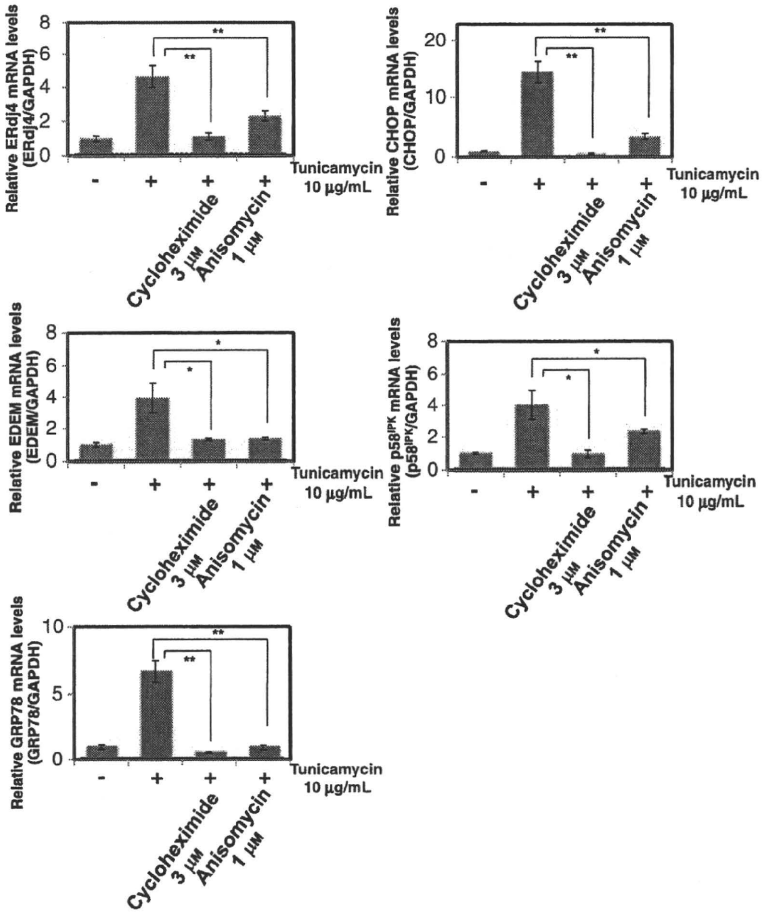


Fig. 4. Cycloheximide and Anisomycin Inhibited the Unfolded Protein Response.

Cycloheximide and anisomycin suppressed tunicamycin-increased ERdj4, EDEM, GRP78, CHOP, and $p58^{IPK}$ mRNA. HeLa cells were treated with 10 μg/mL of tunicamycin with and without the indicated concentrations of cycloheximide or anisomycin for 8 h. Cells were collected and RNA was extracted. ERdj4, EDEM, GRP78, CHOP, and $p58^{IPK}$ mRNA levels were evaluated by real-time RT-PCR. Each mRNA was normalized with the mRNA levels of GAPDH. Data are the means ± SD among three independent experiments, $^{*}p < 0.05$ and $^{**}p < 0.01$.

not inhibit ER stress-induced XBP1 mRNA splicing. As previously reported, puromycin alone induced UPR in HeLa cells. Hence a different effect on UPR by protein synthesis inhibitor appears to be dependent on the inhibitory mechanism of the protein synthesis inhibitor. Puromycin has been reported to enforce nascent polypeptides to release from the ribosome halfway through the elongation reaction.[24] Thus, un-maturated proteins might influx into the ER and accumulate, and then induce ER stress as unfolded proteins. In contrast, cycloheximide and anisomycin inhibited polypeptide release from the ribosomes due to inhibition of ribosome translocation and to inhibition of peptidyl transfer respectively.[25] This indicates that un-maturated proteins produced by cycloheximide or anisomycin cannot move to the ER, and fail to induce ER stress. This inhibitory mechanism of cycloheximide and anisomycin may explain why they suppress ER stress-induced UPR. These compounds prevent further influx of newly synthesized proteins into the ER, which results in a reduction in ER stress.

At present, the inhibitory mechanism in protein synthesis due to quinotrierixin remains unclear. However, because quinotrierixin suppressed ER stress-induced UPR as did cycloheximide and anisomycin but not puromycin, the mode of action of quinotrierixin would be similar to cycloheximide and anisomycin. Thus, it is likely that quinotrierixin blocked polypeptide release from the ribosomes. But further study is needed to elucidate the mechanism.

Acknowledgments

We thank Dr. Hiroyuki Osada (RIKEN, Japan) for kindly providing us with cytotrienin A. This study was partly supported by grants from Takeda Science Foundation and Suzuken Memorial Foundation. This work was also supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This study was supported in part by the Global COE Program for Human Metabolomic Systems Biology of MEXT, Japan.

References

1) Ellgaard L and Helenius A, *Curr. Opin. Cell Biol.*, **13**, 431–437 (2001).
2) Zhou J, Liu CY, Back SH, Clark RL, Peisach D, Xu Z, and Kaufman RJ, *Proc. Natl. Acad. Sci. USA*, **103**, 14343–14348 (2006).
3) Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, and Ron D, *EMBO J.*, **17**, 5708–5717 (1998).
4) Credle JJ, Finer-Moore JS, Papa FR, Stroud RM, and Walter P, *Proc. Natl. Acad. Sci. USA*, **102**, 18773–18784 (2005).
5) Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, and Ron D, *Nature*, **415**, 92–96 (2002).

6) Yoshida H, Matsui T, Yamamoto A, Okada T, and Mori K, *Cell*, **107**, 881–891 (2001).
7) Lee AH, Iwakoshi NN, and Glimcher LH, *Mol. Cell. Biol.*, **23**, 7448–7459 (2003).
8) Marciniak SJ and Ron D, *Physiol. Rev.*, **86**, 1133–1149 (2006).
9) Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH, and Hotamisligil GS, *Science*, **306**, 457–461 (2004).
10) Fujimoto T, Onda M, Nagai H, Nagahata T, Ogawa K, and Emi M, *Breast Cancer*, **10**, 301–306 (2003).
11) Shuda M, Kondoh N, Imazeki N, Tanaka K, Okada T, Mori K, Hada A, Arai M, Wakatsuki T, Matsubara O, Yamamoto N, and Yamamoto M, *J. Hepatol.*, **38**, 605–614 (2003).
12) Feldman DE, Chauhan V, and Koong AC, *Mol. Cancer Res.*, **3**, 597–605 (2005).
13) Romero-Ramirez L, Cao H, Nelson D, Hammond E, Lee AH, Yoshida H, Mori K, Glimcher LH, Denko NC, Giaccia AJ, Le QT, and Koong AC, *Cancer Res.*, **64**, 5943–5947 (2004).
14) Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, Tilg H, Nieuwenhuis EE, Higgins DE, Schreiber S, Glimcher LH, and Blumberg RS, *Cell*, **134**, 743–756 (2008).
15) Tashiro E, Hironiwa N, Kitagawa M, Futamura Y, Suzuki S, Nishio M, and Imoto M, *J. Antibiot.* (Tokyo), **60**, 547–553 (2007).
16) Futamura Y, Tashiro E, Hironiwa N, Kohno J, Nishio M, Shindo K, and Imoto M, *J. Antibiot.* (Tokyo), **60**, 582–585 (2007).
17) Kawamura T, Tashiro E, Yamamoto K, Shindo K, and Imoto M, *J. Antibiot.* (Tokyo), **61**, 303–311 (2008).
18) Kawamura T, Tashiro E, Shindo K, and Imoto M, *J. Antibiot.* (Tokyo), **61**, 312–317 (2008).
19) Elouil H, Bensellam M, Guiot Y, Vander Mierde D, Pascal SM, Schuit FC, and Jonas JC, *Diabetologia*, **50**, 1442–1452 (2007).
20) Croons V, Martinet W, Herman AG, and De Meyer GR, *J. Pharmacol. Exp. Ther.*, **325**, 824–832 (2008).
21) Sugita M, Natori Y, Sueda N, Furihata K, Seto H, and Otake N, *J. Antibiot.* (Tokyo), **35**, 1474–1479 (1982).
22) Kim WG, Song NK, and Yoo ID, *J. Antibiot.* (Tokyo), **55**, 204–207 (2002).
23) Feuerbach D, Waelchli R, Fehr T, and Feyen JH, *J. Biol. Chem.*, **270**, 25949–25955 (1995).
24) Azzam ME and Algranati ID, *Proc. Natl. Acad. Sci. USA*, **70**, 3866–3869 (1973).
25) Pestka S, *Annu. Rev. Microbiol.*, **25**, 487–562 (1971).

Biosci. Biotechnol. Biochem., **75** (2), 367–369, 2011

Note

A New, Convenient Cell-Based Screening Method for Small-Molecule Glycolytic Inhibitors

Mitsuhiro KITAGAWA,* Mayuko MISAWA, Seiichiro OGAWA, Etsu TASHIRO, and Masaya IMOTO†

Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan

Received September 28, 2010; Accepted October 30, 2010; Online Publication, February 7, 2011
[doi:10.1271/bbb.100693]

To counteract active glycolysis in tumors, we developed a new, convenient cell-based screening system to identify an inhibitor of glycolysis. Using this system, we searched for an inhibitor in the synthetic Carbasugar library and found two candidates. It was found that both inhibited glycolysis by suppressing the glucose uptake step in tumor cells.

Key words: inhibitor screening; tumor glycolysis; glucose uptake inhibitor; carbasugar library

It is widely recognized that solid tumors exposed to hypoxia can survive and grow aggressively despite low oxygen and limited nutrition. Active glycolysis, well known as the Warburg effect, plays an important role as a lifeline for tumor survival and growth under these conditions by fueling cancer cells with ATP energy and supplying bioorganic components, such as nucleotides and fatty acids for growth.[1,2] Hence, glycolysis inhibitors are expected to be candidate drugs for tumor treatment, and hence screening research on these inhibitors has potential for cancer therapy. Many glycolytic inhibitors have been found to be inhibitors of key glycolytic enzymes,[3–5] but it is not easy to evaluate whether these glycolytic enzyme inhibitors actually modulate glycolysis in living cells. Hence, the establishment of a new cell-based screening system is required for the discovery of small, cell-permeable molecules that induce glycolysis inhibition in living cells.

Here we propose a novel cell-based screening method for glycolytic inhibitors.

Filopodia are spike-like cell membrane projections that contribute to tumor metastasis. Recently, we found that glycolytic suppression resulted in inhibition of filopodium protrusion in cancer cells only when their mitochondrial respiration was restricted.[6] This inhibition of filopodium protrusion might occur due to decreased intracellular ATP concentration caused by blocking of both glycolysis and mitochondrial respiration.[6–9] Since the test for filopodium inhibition is an easy, low-cost, quick assay, here we utilized it as a cell-based screening method for a new glycolytic inhibitor. In the assay, screening samples were added to human adenocarcinoma A431 cells co-treated with and without the mitochondrial respiratory inhibitor rotenone and

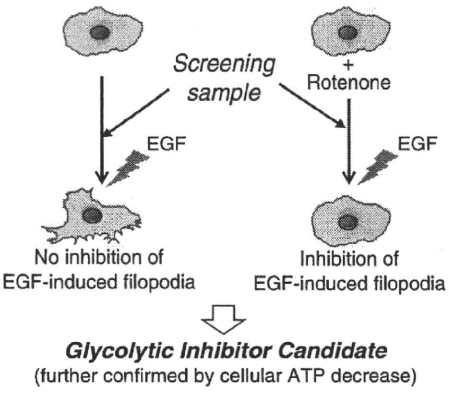


Fig. 1. Image of Screening for Glycolytic Inhibitors by EGF-Induced Filopodium Protrusion Assay.

were examined for their inhibitory effect on EGF-induced filopodium protrusion, which is easily judged by microscopic observation in 30 min (Fig. 1). A potential glycolytic inhibitor would inhibit filopodium protrusion only by co-treatment with a mitochondrial respiratory inhibitor. In the event, 2-deoxy-D-glucose (2DG), an inhibitor of glycolytic enzyme hexokinase, inhibited EGF-induced filopodium protrusion only in the presence of rotenone (Fig. 2A), indicating that this assay system works successfully. As screening source, we used a chemical library composed of 1,069 compounds, mainly cyclohexanepolyols and derivatives thereof. These compounds were carbocyclic analogs of hexopyranoses called carbasugars. The carbasugar library was originally prepared in the course of studies of the development of bioactive substances, such as antibacterial and anticancer compounds, sweeteners, and enzyme inhibitors by Ogawa *et al.* The structural features of carbasugars possibly mimic those of glucose or glycolytic metabolites, and thus are an attractive screening source for glycolytic inhibitors.

In the first screening run, 10 μg/mL of compounds (in DMSO) were tested. Hit candidates in the first run were tested again at various concentrations. We identified two compounds (sample #169 [1,4,5,6-tetra-*O*-acetyl-2-*O*-mesyl-3-*O*-benzoyl-*myo*-inositol[10]] and sample #288 [1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-tosyl-*muco*-inositol[11]]) as glycolytic inhibitors that showed filopodium inhibition only in the presence of the mitochondrial respira-

† To whom correspondence should be addressed. Fax: +81-45-566-1557; E-mail: imoto@bio.keio.ac.jp
* Present address: *Institute for Advanced Biosciences, Keio University, 246-2 Mizukami, Kakuganji, Tsuruoka, Yamagata 997-0052, Japan*
Abbreviations: EGF, epidermal growth factor; 2DG, 2-deoxy-D-glucose; RTN, rotenone

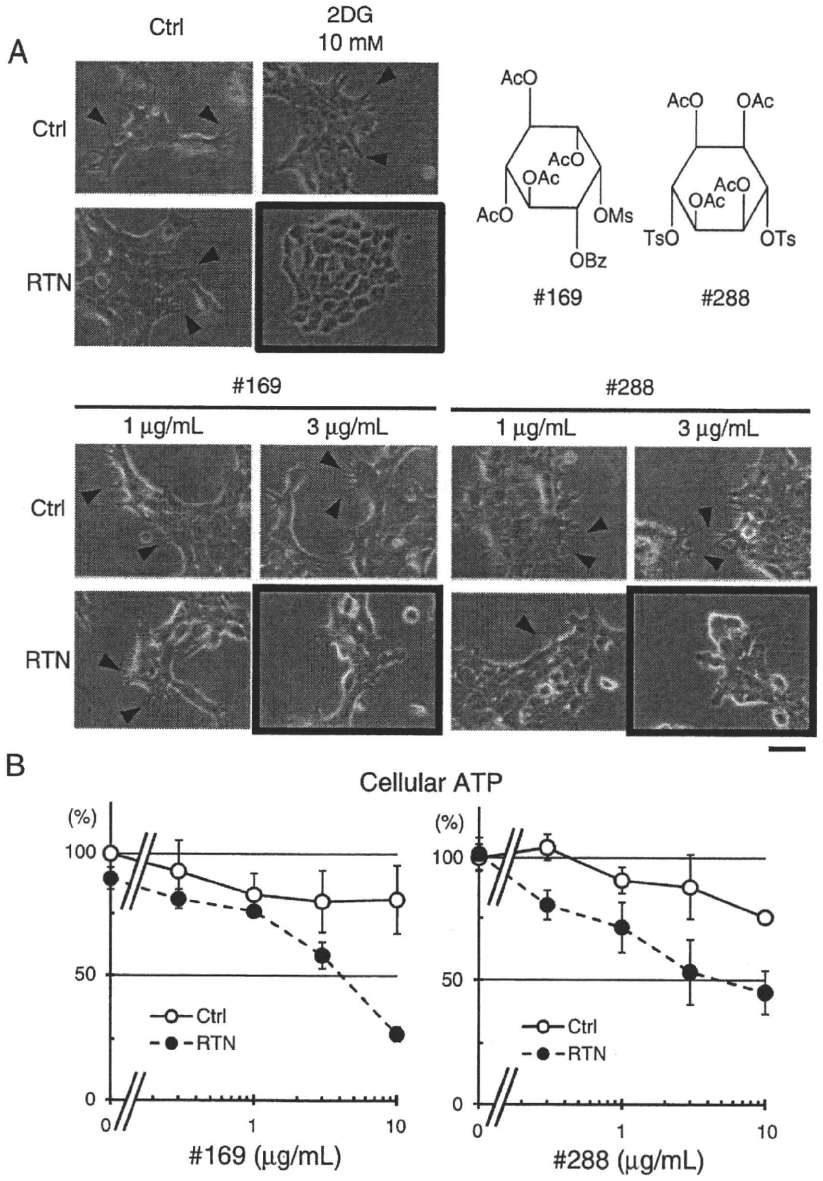


Fig. 2. Screening for Glycolytic Inhibitors in the Carbasugar Library.

A, Samples from the pseudo-sugar library were originally prepared and re-crystallized for storage by Ogawa *et al.* Two compounds, #169[10] and #288,[11] in the library, showed filopodia inhibition only in the presence of the mitochondrial respiratory inhibitor. The assay method was as described previously.[6] Hexokinase inhibitor 2-deoxyglucose (2DG, 10 mM) was used as positive control, and rotenone (RTN, 300 nM) was used to inhibit mitochondrial respiration. The arrowhead indicates filopodia, and the framed photos showed filopodium inhibition. Scale bar, 40 μm. Data represent two independent experiments. Sample purity was confirmed by LC-MS. B, Glycolytic inhibition. A431 cells were treated with the test compounds in the presence of rotenone (RTN, 300 nM) and of its solvent, ethanol, for 30 min. Cellular ATP levels were determined by luciferin-luciferase reaction (Sigma, St. Louis, MO). Bars, s.d. (n = 3). Data represent three independent experiments.

tory inhibitor rotenone (Fig. 2A). Next we tested to determine whether they would indeed suppressed glycolysis. It has been stated that glycolytic limitation caused a marked ATP decrease in tumor cells when mitochondria respiration was inhibited.[6,8] As shown in Fig. 2B, the above candidate glycolytic inhibitors slightly decreased cellular ATP in the absence of the mitochondria respiratory inhibitor. However, in combination with the mitochondria respiratory inhibitor, they did decrease intracellular ATP levels in a dose-dependent manner, indicating that they inhibited glycolysis.

To investigate further how they inhibit glycolysis, we examined their effects on the 3 steps of glycolysis: glucose uptake, hexokinase, and pyruvate kinase. It has been reported that these steps play important roles in accelerated glycolysis in tumor metabolism, and higher expression levels of the proteins facilitating these steps are frequently observed.[3,12–14] Neither #169 nor #288 affected hexokinase or pyruvate kinase reactions at up to 30 μg/mL (Fig. 3A), but they inhibited the glucose uptake step dose-dependently, with IC_{50} values of 4.5 μg/mL for #169 and 2.7 μg/mL for #288 (Fig. 3B). Inhibition of glucose uptake was similar to that due to decreasing intracellular ATP levels in the presence of rotenone, as shown in Fig. 2B, suggesting that both #169 and #288 suppressed glycolysis by inhibiting cellular glucose uptake.

In summary, here we propose a new, convenient screening assay for glycolytic inhibitors. We found two glycolytic inhibitors in a carbasugar library. They limited glycolysis by inhibiting glucose uptake at several μg/mL concentrations.

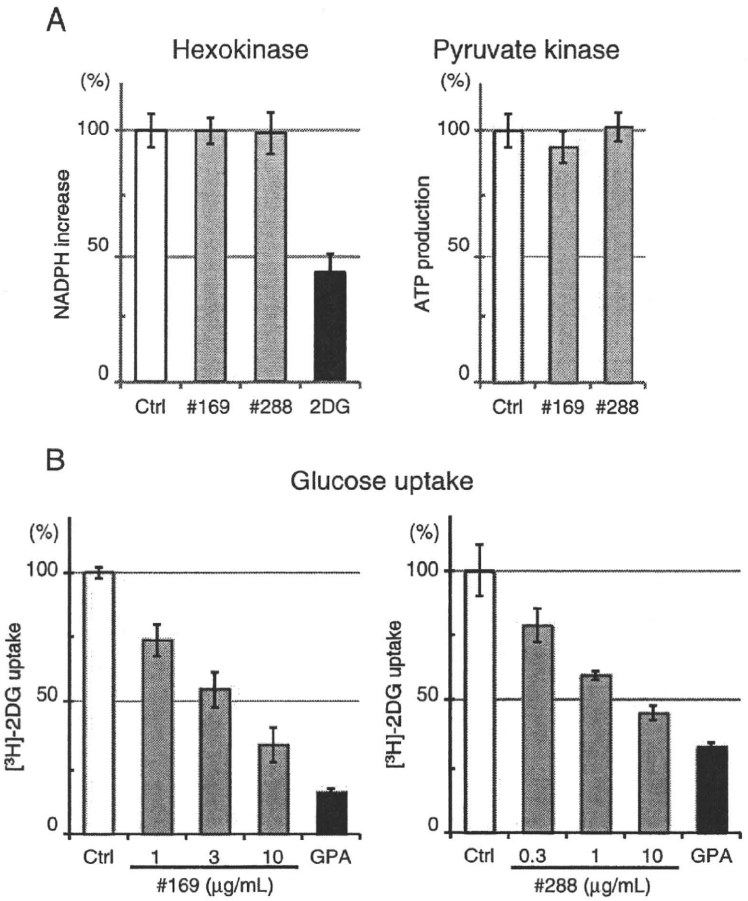


Fig. 3. Inhibition of the Glucose Uptake Step to Explain Glycolytic Suppression by #169 and #288.

A, Effects of compounds on the activities of two of the rate-limiting enzymes *in vitro*. Hexokinase activity was determined as described previously.[6] For pyruvate kinase activity, purified recombinant human pyruvate kinase 1 was used. The complete human pyruvate kinase 1 from HeLa cDNA was cloned into *E. coli* expression vector pRSETc. The reaction (3 μM ADP and 0.3 μM F1, 6P for the substrate in PK buffer, containing 5 mM $MgCl_2$, 0.1 mg/mL BSA, 2 mM DTT, 1 mM EDTA, 50 mM KCl, 50 mM Tris pH 7.7) for 30 min was quenched with TCA and neutralized. Enzymatic activity was calculated by ATP production. Bars, s.d. (n = 3). B, Inhibition of glucose uptake by #169 and #288. The assay method was as described previously,[6] and glucopiericidin A (GPA, 10 ng/mL) was used as positive control.[6] Bars, s.d. (n = 3). Data represent three independent experiments.

Further studies of structural modification to develop more potent inhibitors and analysis of structure-activity relationships are now under investigation.

Acknowledgments

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1) Deberardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, and Thompson CB, *Proc. Natl. Acad. Sci. USA*, **104**, 19345–19350 (2007).
2) Hsu PP and Sabatini DM, *Cell*, **134**, 703–707 (2008).
3) Madhok BM, Yeluri S, Perry SL, Hughes TA, and Jayne DG, *Am. J. Clin. Oncol.*, in press.
4) Shanmugam M, Mcbrayer SK, and Rosen ST, *Curr. Opin. Oncol.*, **21**, 531–536 (2009).
5) Vander Heiden MG, Christofk HR, Schuman E, Subtelny AO, Sharfi H, Harlow EE, Xian J, and Cantley LC, *Biochem. Pharmacol.*, **79**, 1118–1124 (2010).
6) Kitagawa M, Ikeda S, Tashiro E, Soga T, and Imoto M, *Chem. Biol.*, **17**, 989–998 (2010).
7) Le Clainche C and Carlier M-F, *Physiol. Rev.*, **88**, 489–513 (2008).
8) Molitoris BA, Geerdes A, and Mcintosh JR, *J. Clin. Invest.*, **88**, 462–469 (1991).
9) Witke W, *Trends Cell Biol.*, **14**, 461–469 (2004).
10) Suami T, Lichtenthaler F, and Ogawa S, *Bull. Chem. Soc. Jpn.*, **39**, 170–178 (1966).
11) Ogawa S, Oki S, and Suami T, *Bull. Chem. Soc. Jpn.*, **52**, 1095–1101 (1979).
12) Airley RE and Mobasheri A, *Chemotherapy*, **53**, 233–256 (2007).
13) Mathupala SP, Ko YH, and Pedersen PL, *Oncogene*, **25**, 4777–4786 (2006).
14) Mazurek S, Boschek CB, Hugo F, and Eigenbrodt E, *Semin. Cancer Biol.*, **15**, 300–308 (2005).

ORGANIC LETTERS

2010
Vol. 12, No. 21
5068−5071

Total Synthesis of Incednam, the Aglycon of Incednine

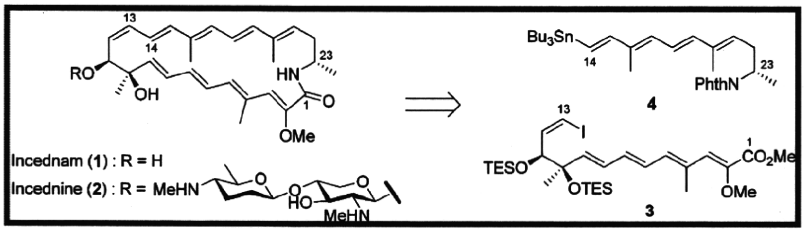
Takashi Ohtani,[†] Shinya Tsukamoto,[†] Hiroshi Kanda,[†] Kensuke Misawa,[†] Yoshifumi Urakawa,[†] Takahiro Fujimaki,[‡] Masaya Imoto,[‡] Yoshikazu Takahashi,[§] Daisuke Takahashi,[†] and Kazunobu Toshima*[,†]

Department of Applied Chemistry and Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan, and Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

toshima@applc.keio.ac.jp

Received October 5, 2010

ABSTRACT



The first total synthesis of incednam (1), the aglycon of antibiotic incednine (2), is described. Incednine has been reported to exhibit significant inhibitory activity against the antiapoptotic oncoproteins Bcl-2 and Bcl-xL. The synthesis of 1 commenced with the preparation of the C1−C13 subunit 3 and the C14−C23 subunit 4. The construction of the novel 24-membered macrocycle was achieved by the application of a Stille coupling between 3 and 4, followed by macrolactamization.

Incednam (**1**) is the aglycon of the 24-membered macrolactam glycoside antibiotic incednine (**2**), which was isolated from *Streptomyses* sp. in 2008.[1] It was demonstrated that **2** exhibits significant inhibitory activity against the antiapoptotic oncoproteins Bcl-2 and Bcl-xL, with a mode of action distinctly different from those of other compounds that inhibit the binding capacity of Bcl-xL to the pro-apoptotic protein Bax. In addition, it is known that these proteins are overexpressed in many cancer cells, resulting in the expansion of a transformed population and the advancement of the multidrug-resistant stage.[2−4] Therefore, **2** is now expected to be a compound in the development of novel antitumor drugs. Furthermore, **2** is likely to be a useful tool for the further study of Bcl-2 and Bcl-xL functions. The identification of its target protein could provide insight into the antiapoptotic mechanism of the Bcl-2 family proteins. From a structural perspective, **1** and **2** contain unique salient features: an α-methoxy-α,β-unsaturated amide moiety and two independent conjugated polyene systems embedded in the 24-membered macrolactam ring. As a result of the nature of the highly conjugated polyene subunits, **1** and **2** are light- and acid-sensitive. Although **1** was also isolated from *Streptomyses* sp.,[1] its semisynthesis from **2** has not been realized, in part, because of the inherent chemical instabilities mentioned above. Furthermore, the stereochemical configuration at C23 was postulated on the basis of computational modeling studies, thus the configuration has not been conclusively defined. Because of its important biological

[†] Department of Applied Chemistry.
[‡] Department of Biosciences and Informatics.
[§] Institute of Microbial Chemistry.

(1) Futamura, Y.; Sawa, R.; Umezawa, Y.; Igarashi, M.; Nakamura, H.; Hasagawa, K.; Yamasaki, M.; Tashiro, E.; Takahashi, Y.; Akamatsu, Y.; Imoto, M. *J. Am. Chem. Soc.* **2008**, *130*, 1822.
(2) Tsujimoto, Y.; Finger, L. R.; Yunis, J.; Nowell, P. C.; Croce, C. M. *Science* **1984**, *226*, 1097.
(3) Reed, J. C.; Cuddy, M.; Slabiak, T.; Croce, C. M.; Nowell, P. C. *Nature* **1988**, *336*, 259.
(4) Gross, A.; McDonnell, J. M.; Korsmeyer, S. J. *Gene Dev.* **1999**, *13*, 1899.

10.1021/ol102400c
Published on Web 10/12/2010