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GEX1 Compounds, Novel Antitumor Antibiotics Related to Herboxidiene, Produced by *Streptomyces* sp.

II. The Effects on Cell Cycle Progression and Gene Expression

YASUSHI SAKAI, TETSUYA TSUJITA, TADAKAZU AKIYAMA[†], TATSUHIKO YOSHIDA[††], TAMIO MIZUKAMI, SHIRO AKINAGA[†], SUEHARU HORINOUCHI[††], MINORU YOSHIDA[††] and TETSUO YOSHIDA*

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6 Asahimachi, Machida-shi, Tokyo 194 -8533, Japan
[†] Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Shizuoka 411-8731, Japan
[††] Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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Six GEX1 compounds, GEX1A/herboxidiene and its related 5 novel compounds, were isolated from a culture broth of *Streptomyces* sp. GEX1 compounds induced both G1 and G2/M arrest in a human normal fibroblast cell line, WI-38. All six compounds up-regulated luciferase reporter gene expression directed by enhancer/promoter of various genes, such as cdc2, IL-2 and SV40 early genes. All GEX1 compounds showed cytotoxic activities in the same order of the up-regulating activities on gene expression, suggesting that these two activities are related. Despite the up-regulating activities on the reporter gene expression, GEX1A/herboxidiene did not enhance the expression of any endogenous genes involved in the cell cycle, proliferation and apoptosis. Although the unique effects of GEX1 compounds on cell cycle and the reporter gene expression were similar to those of trichostatin A (TSA), an inhibitor of histone deacetylase (HDAC), GEX1A/herboxidiene did not affect histone acetylation in cells. In addition, GEX1A/herboxidiene treatment gave rise to the shorter sized transcripts of the cdc25A and cdc2 genes as well as the normal sized ones. These results suggest that GEX1 compounds modulate gene expression by an unknown mechanism.

New generation of anti-tumor agents exert inhibitory activities on cell proliferation by modulating the cell cycle and gene expression, *e.g.* histone deacetylase (HDAC) inhibitors[1] and all-*trans*-retinoic acid[2]. Thus these pathways are attractive targets for anti-cancer drug discovery.

We recently isolated six structurally related antitumor antibiotics, GEX1 compounds, from the culture broth of *Streptomyces* sp.[3]. A major compound GEX1A was identified as a herbicide, herboxidiene[4], and GEX1Q1~Q5 were novel compounds (Fig. 1). GEX1 compounds had cytotoxic activity, but the mechanism of action was unknown. In this paper, we report the effects of these compounds on the cell cycle and gene expression analyzed by flow cytometry, luciferase reporter assay and RT-PCR methods, and discuss the mode of action of GEX1 compounds.

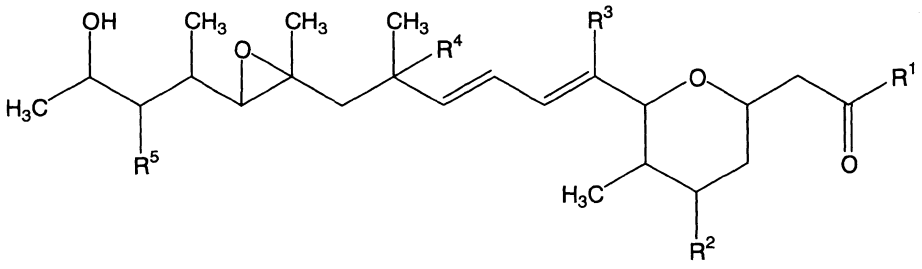
Materials and Methods

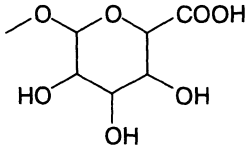
Drugs

GEX1 compounds were isolated from the culture broth of *Streptomyces* sp. GEX1[3]. Trichostatin A was purchased from Wako Pure Chemical Industry, LTD. Test samples were dissolved in dimethyl sulfoxide (DMSO), diluted

* Corresponding author: tyoshida@kyowa.co.jp

Fig. 1. Chemical structures of GEX1 compounds.



	R^1	R^2	R^3	R^4	R^5
GEX1A/herboxidiene	-OH	-H	$-CH_3$	-H	$-OCH_3$
GEX1Q1	-OH	-OH	$-CH_3$	-H	$-OCH_3$
GEX1Q2	-OH	-H	$-CH_3$	-OH	$-OCH_3$
GEX1Q3		-H	$-CH_3$	-H	$-OCH_3$
GEX1Q4	-OH	-H	$-CH_2OH$	-H	$-OCH_3$
GEX1Q5	-OH	-H	$-CH_3$	-H	-OH

properly with medium and added to cultures (final concentration of DMSO in culture; 0.1%).

Cell Cycle Analysis

WI-38 cells (3×10^5/dish), the human normal fibroblast cell line, were cultured overnight in Falcon 3003 plastic dishes, and were treated with various concentrations of GEX1 compounds for 24 hours. The cells were harvested by treatment with 0.25% trypsin, fixed with an ice-cold 70% ethanol solution, hydrolyzed with 250 mg/ml of ribonuclease A (type 1-A, Sigma Chemical Co.) at 37°C for 30 minutes, and stained with propidium iodide (Sigma) for 20 minutes. The DNA content of the cells was analyzed by an EPICS ELITE flow cytometer (Coulter). The cell cycle distribution was calculated by a MULTICYCLE program (Coulter)[5].

Construction of Plasmids

Details of the construction of plasmids for luciferase reporter cells will be described elsewhere (T. YOSHIDA *et al.*, in preparetion). Enhancer/promoter regions of the human cdc2, DNA polymerase α (pol α) and c-myc were cloned from human placental DNA (Clontech) by polymerase chain reaction (PCR) using specific primers. The amplified PCR fragments of the cdc2 (−406 to +60) and pol α (−559 to +40) enhancer/promoter sequences were inserted into the luciferase vector pluc2[6], and designated as pcdc2luc2 and ppolαluc2, respectively. Plasmid pfosluc2 was described previously[6]. The amplified PCR fragment of c-myc (−95 to +1022) was inserted into pluc2 with the addition of the spliced acceptor linker and designated as pmycluc2. Plasmid pERE1luc2 was constructed by inserting four copies of an E2F binding oligonucleotide with the core promoter of the SV40 early gene into pluc2. Plasmid pSE1lucP1 was constructed by inserting the enhancer/promoter region of the SV40 early gene into pluc2. Plasmid pIL2luc2, in which the luciferase gene was drived by the enhancer/promoter of the IL-2 promoter, was

described previously[7].

Stably Transfected Cell Lines

Details of establishment of the stably transfected cell lines will be described elsewhere (T. YOSHIDA *et al.*, in preparetion). The human osteosarcoma cell line, Saos-2, was a kind gift from Dr. R. TAKAHASHI (Kyoto university). Saos-2 cells were transfected[8] with 10 μg of pcdc2luc2, ppolαluc2, pfosluc2, pmycluc2, pERE1luc2 or pSE1lucP1, and G418-resistant cells were selected to establish the luciferase reporter cell lines, cdc2 reporter cells, pol α reporter cells, c-fos reporter cells, c-myc reporter cells, E2F reporter cells or SV40 reporter cells, respectively. The transformants were maintained in medium including 0.2 mg/ml of G418. The human T lymphocyte Jurkut cells were transfected with pIL2luc2, and hygromycin B-resistant cells were selected to establish IL-2 reporter cells[7]. IL-2 reporter cells were stimulated by incubation at 37°C with 12-*O*-tetradecanoylphorbol-13-acetate (5 ng/ml) and phytohemaglutinin (1 μg/ml) 6 hours before luciferase assay[7].

Luciferase Assay

The luciferase reporter cells were cultured (1×10^4 cells/well) in 96 well white plates (Sumitomo Bakelite) and were treated with test samples for 16 hours. Then 50 μl of lysis buffer [100 mM KH_2PO_4 (pH 7.8), 1% Triton X-100, 1 mM dithiothreitol (DTT)] and 100 μl of luciferin solution [25 mM glycylglycine (pH 7.5), 15 mM $MgSO_4$, 5 mM ATP, and 0.33 mM luciferin] were added to each well, and luciferase activity (RLU) was measured with a LB96P luminometer (EG&G Berthold). The activities of sample were presented as 'activation (fold)', which was calculated as follows; activation (fold)=RLU (treated cells)/RLU (untreated cells).

Gene Expression in cdc2 Reporter Cells

The level of gene expression was measured by competitive RT-PCR as described previously[9]. Cdc2 reporter cells (1×10^5 cells) were precultured in 15 cm dish for 24 hours in 5% CO_2-95% air atmosphere at 37°C and then treated with various doses of GEX1A/herboxidiene for 16 hours. Total RNA was isolated from the cells using the Total RNA separation kit (Clontech). The first strand cDNA was prepared from 5 μg of RNA with the Superscript Preamplification system (Gibco) in a 20 μl reaction, and was diluted 50-fold with H_2O. PCR was performed in a 40 μl reaction mixture containing 10 μl cDNA, 1 mM each primer set, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, and 2.5 units Taq polymerase (TAKARA). Temperature conditions were: one cycle at 94°C for 1 minute, 26 cycles at 94°C for 1 minute, 57°C for 1 minute, 72°C for 1 minute, and one cycle at 72°C for 7 minutes. The primers used and the expected length of PCR products were as follows (5′ primer and 3′ primer, respectively); for β-actin[10]: 5′ GAT ATC GCC GCG CTC GTC GTC GAC 3′ and 5′ CAG GAA GGA AGG CTG GAA GAG TGC 3′, for luciferase (583 bp): 5′-TGC AAA ACG CTT CCA TCT TCC AG-3′ and 5′-TTC GTC CAC AAA CAC AAC TCC TC-3′, and for cdc2 (485 bp): 5′-TGC TTA TGC AGG ATT CCA GGT TA-3′ and 5′-TTC TGG CCA CAC TTC ATT ATT GG-3′. After amplification, 15 μl aliquots were subjected to 2%-agarose gel electrophoresis. The gels were stained with ethidium bromide and were photographed under UV light.

HeLa cells (1×10^5 cells) were precultured in 15 cm dish for 24 hours in 5 % CO_2-95% air atmosphere at 37°C and then treated with 1 μg/ml of GEX1A/herboxidiene for 1, 2, 4, 8, 12 and 16 hours or various doses of GEX1A/herboxidiene for 16 hours. The primers used and the expected length of PCR products were as follows (5′ primer and 3′ primer, respectively); for E2F-1 (811 bp): 5′-ACC AAG CGC TTC CTG GAG CTG CTG AG-3′ and 5′-GGA AAC CCT GGT ACC TCC AAG CCC TG-3′, for cyclin A (378 bp): 5′-CGA GTG GTC GGC TGG GCA GAG TG-3′ and 5′-GAA TGG TGA ACG CAG GCT GTT TA-3′, for cyclin D (584 bp): 5′-CGC CCT CGG TGT CCT ACT TCA AA-3′ and 5′-GAG GAA GCG TGT GAG GCG GTA GT-3′, for c-myc (453 bp): 5′-CTG CTG CCC ACC CCG CCC CTG TC-3′ and 5′-GGG CGA GCT GCT GTC GTT GAG AG-3′, for p21/waf1 (709 bp): 5′-AGG AGG CCC GTG AGC GAT GGA AC-3′ and 5′-ACA AGT GGG GAG GAG GAA GTA GC-3′, for p27/kip1 (364 bp): 5′-CCC GGG ACT TGG AGA AGC ACT GC-3′ and 5′-TGA GTA GAA GAA TCG TCG GTT GC-3′, for cdc25A (284 bp): 5′-GAT GAT GGC TTC GTG GAC CTT CT-3′ and 5′-TGG GCC TTC TCT GGA TTA GTT GA-3′, for bcl-xL[11] (780 bp): 5′-TTG GAC AAT GGA CTG GTT GA-3′ and 5′-GTA GAG TGG ATG GTC AGT G-3′, for bax (366 bp): 5′-TCA GGA TGC GTC CAC CAA GAA GC-3′ and 5′-TGG GCG TCC CAA AGT AGG AGA GG-3′, and for c-fos (483 bp): 5′-CTA CGA GGC GTC ATC CTC CCG CT-3′ and 5′-TCT GTC TCC GCT TGG AGT GTA TC-3′. RT-PCR method was performed under the same conditions as for the cdc2 reporter cells as described above.

As to β-actin[10] and cyclin A (T. YOSHIDA *et al.*, in preparation), truncated cDNA fragments were used for internal control of PCR analysis.

Table 1. Effects of GEX1 compounds on the cell cycle progression of WI-38 cells.

compounds	μM	Cell cycle distribution (%) G1	S	G2/M
control		41.7	42.5	15.8
GEX1A/herboxidiene	0.0076	49.2	29.4	21.4
	0.046	39.5	24.4	36.0
GEX1Q1	2.8	49.3	30.6	20.1
	28	39.5	26.4	34.0
GEX1Q2	1.8	53.4	28.9	17.7
	18	41.5	21.5	37.0
GEX1Q3	0.11	50.0	32.2	17.8
	1.1	44.0	25.3	30.7
GEX1Q4	5.4	49.4	31.4	19.2
	54	41.7	21.1	37.2
GEX1Q5	0.028	51.4	28.8	19.8
	0.28	40.7	22.2	37.1

The cell cycle distributions in the DNA histogram were calculated by a MULTICYCLE program.

Detection of Histone Acetylation

Effect of GEX1A on the level of core histone acetylation was analyzed by Western blotting using an anti-acetyl-lysine antibody. After treatment with GEX1A and trichostatin A (TSA) for 3 hours, lysates were prepared from HeLa cells as discribed previously[12]. Thirty six μg of total cellular protein were subjected to SDS-12% PAGE. After being tansferred to a polyvinylidene difluoride membrane, acetylated histones were probed with an anti-acetyl-lysine polyclonal antibody (New England Biolabs, #9441) and detected using an ECL Western blotting kit (Amersham).

Results

Effects of GEX1 Compounds on Cell Cycle Progression

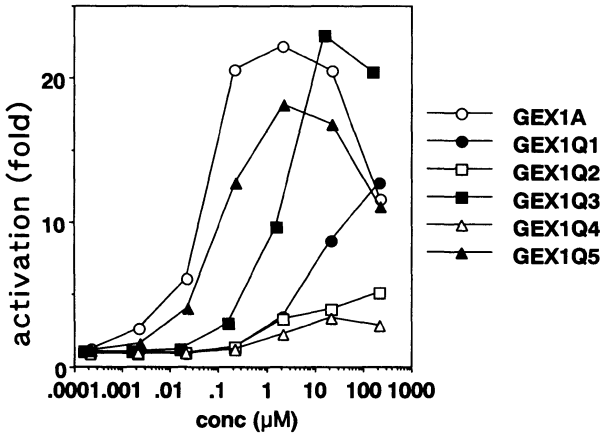
To elucidate the mode of action of cytotoxic activities of GEX1 compounds, the effects of these compounds on cell cycle progression were examined. A human normal fibroblast cell line, WI-38, was treated with GEX1 compounds and analyzed by flow cytometry (Table 1). Each compound was added at two different concentrations; the lower one was a equivalent to the IC_{50} value of cytotoxicity and the higher one was 6 to 10 fold of the IC_{50}[4]. Treatment of cells with the GEX1 compounds caused a marked decrease in the S-phase cells and an increase in G1 and/or G2/M-phase cells. This phenomenon was similar to the cell cycle arrest by trichostatin A (TSA)[13], an inhibitor of histone deacetylase (HDAC)[14].

Effect of GEX1 Compounds on Expression of cdc2 Reporter Gene

Since GEX1 compounds altered the cell cycle population, we examined the effect of GEX1 compounds on the expression of cell cycle-related genes. First, the effect of GEX1 compounds on cdc2 gene expression[15], which begins at the G1 to S phase transition, was analyzed. A reporter cell line was established by stably transfecting Saos-2, a human ostersarcoma cell line, with luciferase gene directed by the promoter region of the human cdc2 gene. As shown in Fig. 2, all GEX1 compounds markedly up-regulated the cdc2 reporter gene. Among six compounds, GEX1A/herboxidiene most effectively increased the expression of the cdc2 reporter genes. At 2 nM the up-regulation began to be observed (2 to 3 fold), and 20-fold up-regulation was observed at 2 μM. The order of up-regulating activities was as follows; GEX1A/herboxidiene>GEX1Q5>GEX1Q3>GEX1Q1>

GEX1Q2>GEX1Q4. This order was almost the same as the order of the potency of cytotoxic activity[3]. GEX1 compounds did not up-regulate the luciferase activity of the detergent-lysed cell extracts, suggesting that these compounds does not activate the luciferase enzyme reaction directly (data not shown).

Fig. 2. Effects of GEX1 compounds on expression of cdc2 in luciferase reporter cells.



The stable transformants of Saos-2 cells containing a pcdc2luc2 reporter plasmid, which include the human cdc2 promoter-driven luciferase reporter gene, were treated with GEX1 compounds: GEX1A/herboxidiene (○), GEX1Q1 (●), GEX1Q2 (□), GEX1Q3 (■), GEX1Q4 (△) and GEX1Q5 (▲). After treatment with each GEX1 compound for 16 hours, the cells were lysed and assayed for the luciferase activity. Luciferase values are reported relative to the activity without any drug.

Effect of GEX1 Compounds on Expression of Various Genes in Reporter Cells

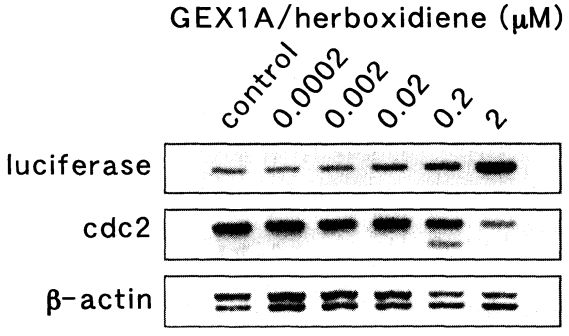
To elucidate whether GEX1 compounds up-regulate the expression of other genes than cdc2 gene, we examined the effect of GEX1 compounds on several cell cycle- related and cell proliferation- related genes, such as c-myc, c-fos and DNA polymerase α, and also IL-2 and SV40 early genes. Reporter cell lines were established by stably transfecting Saos-2 (c-myc, c-fos and DNA polymerase α) or Jurkat (IL-2). In addition, the E2F-responsive element-driven reporter gene was also introduced into Saos-2 cells. All GEX1 compounds were tested at the concentrations 0.2 nM~200 μM on these reporter cell lines, and the results were summarized in Table 2. GEX1A/herboxidiene up-regulated expression of all the genes examined with different extents (*e.g.* 5-fold for IL-2 and 70-fold for SV40 early genes at maximum), suggesting that this activity is not specific to the cell cycle- and cell proliferation-related genes but is rather general. In addition, all other GEX1 compounds than GEX1A/herboxidiene also up-regulated almost all the reporter genes examined, and the orders of potency were similar in all reporter genes. This activity correlated with the cytotoxic potential of each compound, as described above for the cdc2 gene.

Table 2. Effects of GEX1 compounds on gene expression in various reporter cells.

compounds	conc	reporter cells (activation (fold))					
	(μM)	E2F	c-myc	pol α	c-fos	IL-2	SE
GEX1A/herboxidiene	20	5.4	4.8	38	21	1.8	73
	0.2	5.5	8.1	30	21	5.7	57
GEX1Q1	20	3.8	3.0	NT[a]	NT	4.1	27
	0.2	1.1	1.0	NT	NT	1.1	1.1
GEX1Q2	20	2.3	0.9	NT	NT	2.4	15
	0.2	1.1	1.0	NT	NT	1.0	1.3
GEX1Q3	20	6.7	5.8	NT	NT	4.7	70
	0.2	1.6	1.3	NT	NT	1.0	2.4
GEX1Q4	20	2.1	0.5	NT	NT	1.3	6.4
	0.2	1.2	1.2	NT	NT	1.0	1.3
GEX1Q5	20	5.7	4.5	NT	NT	1.9	60
	0.2	4.4	4.0	NT	NT	5.0	28

[a] not tested

Fig. 3. Effect of GEX1A/herboxidiene on the expression of luciferase gene and cdc2 gene in cdc2 reporter cells (RT-PCR).



The cdc2-luciferase-transfected Saos-2 stable transformants were treated with 0 to 2 μM of GEX1A/herboxidiene for 24 hours (concentration of the compound is shown above each lane). Total RNAs were prepared from the cells and were reverse-transcribed. The first strand cDNA was PCR-amplified using either firefly liciferase (upper), cdc2 (middle) or β-actin (lower) primers, as described in the "Materials and Methods".

Effect of GEX1A/Herboxidiene on the Expression of the Exogenous Reporter and Endogenous Genes by RT-PCR

To examine whether the up-regulation of luciferase reporter activity by GEX1 compounds we observed is due to transcriptional activation, not to the stabilization or activation of the enzyme luciferase, we analyzed the amount of the exogenous cdc2-promoter-driven luciferase transcript by RT-PCR in the GEX1A/herboxidiene treated cells. After the cdc2-luciferase reporter cells had been treated 16 hours with various doses (0.2 nM~2 μM) of GEX1A/herboxidiene, the total RNA was extracted from the cells and the transcript of the luciferase gene was detected by RT-PCR. As shown in Fig. 3, GEX1A/herboxidiene increased the amount of the transcript of luciferase derived from cdc2-luciferase reporter gene in a dose-dependent manner. The increase could be detected at doses above 0.02 μM of GEX1A/herboxidiene. To confirm that GEX1A/herboxidiene up-regulates endogenously expressed transcripts as well as exogenous transcripts, we examined the level of the transcript of cdc2 derived from the endogenous cdc2 gene in the same cdc2-luciferase reporter cells. Unexpectedly, GEX1A/herboxidiene did not up-regulate the endogenous cdc2 expression and rather down-regulated the expression at the highest concentration (2 μM). Interestingly, a shorter sized transcript appeared at 0.2 μM in addition to the expected sized one. The expression of the β-actin gene, a housekeeping gene, was not affected by GEX1A/herboxidiene.

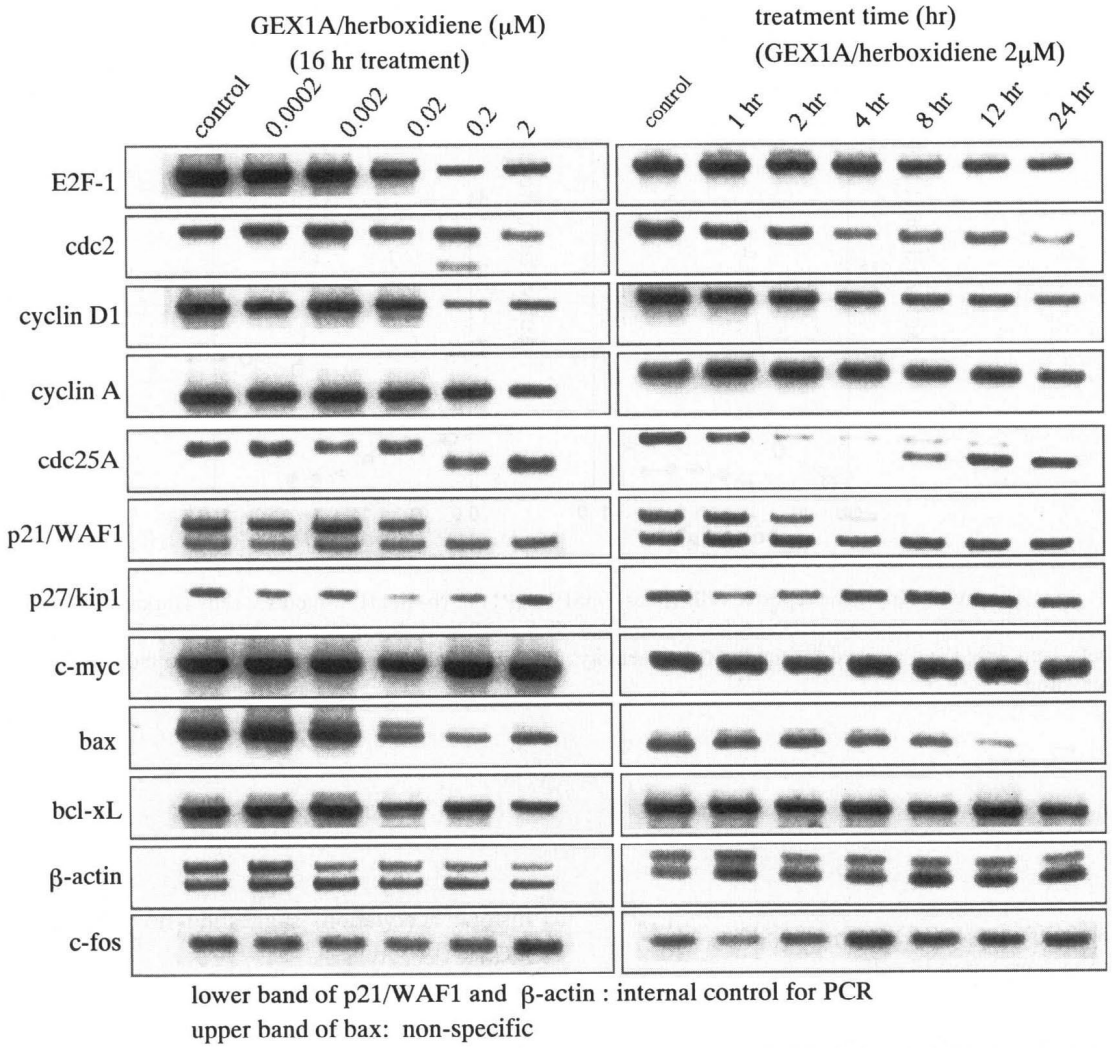
Effect of GEX1A/Herboxidiene on the Expresssion of Various Endogenous Genes

In addition to the cdc2 gene, we also examined the effect of GEX1A/herboxidiene on the expression of endogenous genes including cell cycle-, cell proliferation- and apoptosis-related genes by RT-PCR. The genes examined were as follows; E2F-1 as a cell cycle related transcriptional factor gene, cdc2, cyclin D1 and cyclin A as the cell cycle related kinase genes, p21/waf1 and p27/kip1 as the inhibitors of cell cycle related kinase genes, cdc25A as the cell cycle related phosphatase gene, bcl-xL and bax as apoptosis-related genes, and c-myc and c-fos as the cell proliferation-related genes. As shown in Fig. 4 (left panel), GEX1A/herboxidiene did not increase the expression of any endogenous genes examined except c-fos in HeLa cells. At the higher concentration (0.2 or 2.0 μM) GEX1A/herboxidiene down-regulated the expression of several genes including E2F-1, cdc2, cyclin D1, p21/waf1, cyclin A, bax, bcl-xL and cdc25A rather than activation. We examined the time course of gene expression during GEX1A/Herboxidiene treatment (Fig.4, right panel). The expression of p21/waf1 and cdc25A genes was down-regulated as early as 2 hours, whereas the expression of E2F-1, cdc2, cyclin D1, cyclin A, bax, and bcl-xL was down-regulated later than 8 hours. Like in Saos-2 cells, 0.2 μM concentration of GEX1A/herboxidiene gave rise to a shorter form of the cdc2 transcript in HeLa cells (Fig. 4). In addition, a novel shorter length PCR product of cdc25A also appeared 8 hours after treatment at 0.2 μM concentration or more (Fig. 4).

The Difference in the Effect on Reporter Gene Expression between GEX1A/Herboxidiene and Trichostatin A

The biological activity of GEX1A/herboxidiene was quite similar to trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor. TSA is known to induce accumulation of cells at G1 and G2 phase[13]. TSA has also been reported to up-regulate some reporter genes, *e.g.* c-fos[7], p21/waf1[16,17] and LDL-receptor[18]. GEX1A/herboxidiene was compared with TSA in regard to the action on reporter gene expression. GEX1A/herboxidiene up-regulated SV40 early

Fig. 4. Effect of GEX1A/herboxidiene on expression of various endogenous genes (RT-PCR).



Left panel: Dose response. HeLa cells were treated with 0 to 2 μM of GEX1A/herboxidiene for 24 hours (concentration of the compound is shown above each lane). Changes in the amounts of the endogenous genes (the name of genes were shown on the left side of the panel) were analyzed by RT-PCR.

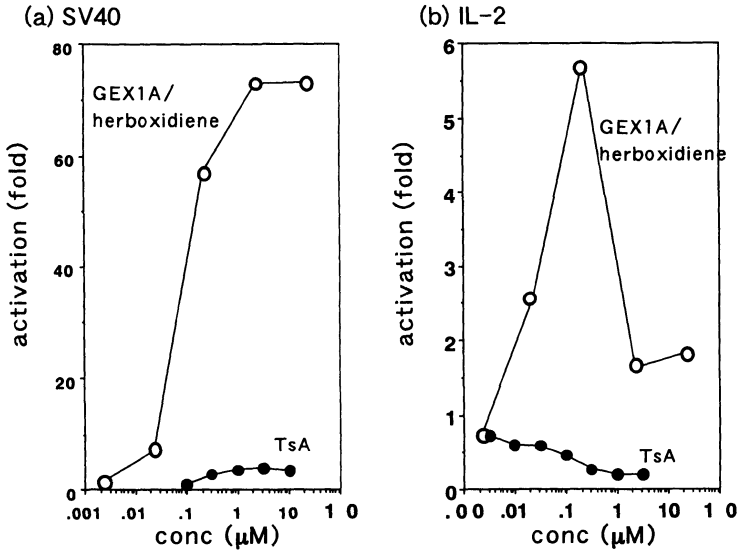
Right panel: Time course. HeLa cells were added with 2 μM of GEX1A/herboxidiene at time 0, cultured for the indicated times above each lane. Changes in the amounts of the endogenous genes were analyzed by RT-PCR.

gene promoter-driven luciferase 70-fold in Saos-2 cells, whereas TSA did only 2-fold at most (Fig. 5a). As for IL-2 reporter gene expression, the effects were quite different between the two drugs (Fig. 5b). While GEX1A/herboxidiene up-regulated the expression of the IL-2 reporter gene (5.7 fold), TSA[7] down-regulated the expression to one-third.

The Effect of GEX1A/Herboxidiene on Acetylation of Histones in HeLa Cells

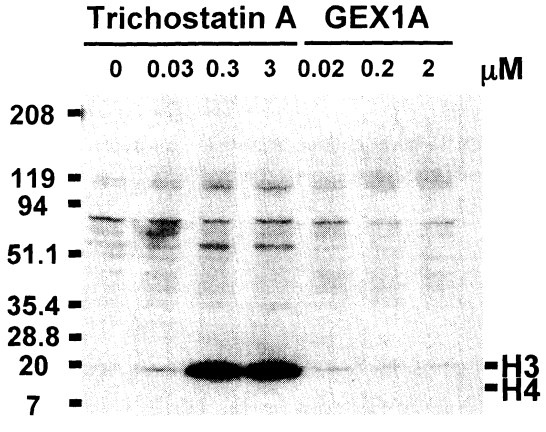
To examine whether GEX1A/herboxidiene have HDAC inhibitory activity like TSA, we treated HeLa cells with various concentration of GEX1A/herboxidiene or TSA, and analyzed the level of histone acetylation by Western blotting using an anti-acetylated lysine polyclonal antibody (Fig. 6). TSA at the concentration of 0.3 μM the accumulated acetylated histone H3. On the other hand, GEX1A/herboxidiene did not cause accumulation of

Fig. 5. Effects of GEX1A/herboxidiene and TSA on SV40 early and IL-2 reporters.



(a) The SV40 early gene reporter cells (Saos-2/pSE1lucP1) or (b) the IL-2 reporter cells (Jurkat/pIL2luc2) were treated with 0.002~20 μM of GEX1A/herboxidiene or TSA[7]. After treatment with each compound for 16 hours, the cells were lysed and assayed for the luciferase activity. Luciferase values are reported relative to the activity without any drug.

Fig. 6. Effects of GEX1A/herboxidiene on histone acetylation in cells.



HeLa cells were cultured for 3 hours in the presence of 0~3 μM of TSA or 0~2 μM of GEX1A/herboxidiene. Cell lysates were analyzed by Western blotting using an anti-acetyl-lysine antibody.

histone acetylation, suggesting no inhibitory activity on histone deacetylase.

Discussion

In the course of our screening for new anti-tumor antibiotics, we isolated six structurally related compounds from a culture broth of *Streptomyces* sp. GEX1 compounds showed cytotoxic activity *in vitro* and anti-tumor activity *in vivo*[3]. GEX1A, the compound with the strongest activity among six GEX1 compounds we identified, was also reported by other groups as an herbicide, herboxidiene[4], and also as an anti-tumor agent, TAN-1069[19]. However, the mechanisms for the herbicidal and anti-tumor activity is unknown.

In this report we found that GEX1 compounds have quite unique property of modulating gene expression. GEX1 compounds up-regulated luciferase reporter expression directed by various gene promoters, including cell cycle- and cell proliferation-related genes (Fig. 2 and Table 2). All GEX1 compounds showed cytotoxic activity, which correlated well with the up-regulating activities on gene

expression, suggesting that this up-regulating activity may be involved in the cytotoxic activity. We observed the reporter gene up-regulating activity of GEX1 compounds using the cell lines, which are stably transfected with reporter gene constructs. We also observed the same phenomena in the cells transiently transfected with reporter gene constructs (unpublished results). In spite of this up-regulating activity on reporter gene expression, GEX1 compounds did not up-regulate any endogenous gene expression except c-fos (Fig. 4). In the course of this research, GEX1A/herboxidiene was also reported to activate LDL receptor-luciferase reporter to 40-fold[18]. Although this report described that the amount of LDL bound to the cells was also increased by herboxidiene treatment, this increase level was only 30%. Therefore, their observation appears consistent with ours. The mechanism by which GEX1A/herboxidiene differently affect gene expression between reporter genes and endogenous genes is unknown. The status of chromatin structure around each gene may be responsible for the different response to GEX1.

Recent progress in research for the chromatin structure of eukaryotic cells gave us a large amount of knowledge on the regulatory mechanism of transcription[20]. The regulation of histone acetylation is one of the most important processes for change in chromatin structure[21]. Several inhibitors of histone deacetylase (HDAC) were isolated from microorganisms such as trichostatin A (TSA)[14] and FR901228[22]. TSA was shown to up-regulate several reporter genes including c-fos[7], LDL receptor[18] and p21/waf1 genes[16,17]. FR901228 was also up-regulated the SV40 early promoter-driven CAT reporter gene[22]. These compounds have anti-cancer activity *in vivo* against some human cancer[1]. These characters of HDAC inhibitors are similar to those of GEX1 compounds. In addition, the ability of GEX1 compounds to induce accumulation of both G1 and G2/M phase population (Table 1) resembled to that of TSA[13], although GEX1A/herboxidiene had stronger activity to up-regulate reporter genes than TSA (IL-2 and SV40 early genes, Fig. 5). However, we showed that GEX1A/herboxidiene had no activity on acetylation of histones (Fig. 6). These results suggest that GEX1A/herboxidiene modulates transcription by a novel mechanism different from histone acetylation.

We found that GEX1A/herboxidiene treatment in higher concentrations than 0.2 μM caused the irregular sized PCR-products of cdc25A and cdc2 by RT-PCR (Fig. 3 and Fig. 4). The amplified fragments (284 bp for cdc25A and 485 bp for cdc2), which are expected by the primer sequences, include three (cdc25A) and four (cdc2) exons, respectively, according to the genomic structures of the human cdc25A and cdc2 genes. The sizes of shorter amplified fragments we observed may be explained if one of the exons is skipped in the course of the splicing (212 bp for cdc25A and 314 or 321 bp for cdc2). It seems interesting to explore the possibility that GEX1A/herboxidiene causes an abnormality in splicing. In addition, it also seems important to examine if the shorter transcripts forming activity and/or the endogenous genes down-regulating activity described above are correlated to the cytotoxicity of GEX1 compounds.

FR901464 and its related compounds were reported to be isolated from a culture broth of *Pseudomonas* sp.[23]. These compounds had strong cytotoxicity against tumor cell lines[23] and antitumor activity in human and mouse tumor-planted mouse models[24]. FR901464 induced G1 and G2/M arrest in human MCF-7-derived cells[24]. These compounds activated the SV40 gene promoter in the CAT reporter system but did not induce endogenous gene expression[24]. FR901464 did not cause accumulation of acetylated histone species, suggesting its inability to inhibit HDAC[22]. These effects of FR901464 and its related compounds resembled to those of GEX1 compounds. Although the mechanism of action is unclear, it is possible that GEX1 compounds affect gene expression and cell cycle progression by targeting the same pathway as FR901464. Since the eukaryotic transcription is tightly regulated by chromatin structure, further experiments should be designed to examine whether nucleosome and chromatin structure are changed in GEX1-treated cells. Identification of the target molecule of GEX1 compounds may give us a new candidate for the drug screening, especially in the oncology field. We would also like to note that the up-regulating activity of GEX1 compounds selective to transfected genes could be applicable to gene therapy and efficient production of useful proteins in mammalian cells.

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