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

## I. Taxonomy, Production, Isolation, Physicochemical Properties and Biological Activities

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Six structurally related antitumor antibiotics named GEX1 compounds were isolated from a culture broth of *Streptomyces* sp. GEX1A was identified as a known herbicide, herboxidiene, structurally interested by the tetrahydropyran moiety and the side chain including a conjugated diene. GEX1Q1~Q5 were determined as novel compounds related to herboxidiene. All GEX1 compounds showed cytotoxicity with $IC_{50}$ values of 0.0037~0.99 $\mu$M against human tumor cell lines *in vitro*, but were not active against both Gram-positive and -negative bacteria. Though GEX1A/herboxidiene exhibited antitumor activity in murine tumor-planted mouse models, both GEX1Q3 and GEX1Q5 did not.

In the course of a screening for new antitumor antibiotics, we isolated six structurally related compounds from a culture broth of *Streptomyces* sp. Among them, GEX1Q1, GEX1Q2, GEX1Q3, GEX1Q4 and GEX1Q5 are novel compounds. The major product, GEX1A was identified as a known herbicide, herboxidiene[1]. In this paper, the taxonomy of the producing strain, fermentation, isolation, and antitumor properties of GEX1 compounds are described. The structure determination of GEX1Q1~Q5 will be reported in elsewhere.

### Materials and Methods

#### Microorganism

The producing strain GEX1 compounds was isolated from a soil sample collected in Yamanashi prefecture, Japan. The strain has been deposited at the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Tsukuba-shi, Ibaraki, Japan, as *Streptomyces* sp. GEX1 with the accession number FERM BP-5347.

#### Method for Taxonomical Characterization

Cultural and physiological characteristics of strain GEX1 were determined by the methods of the International *Streptomyces* Project[2] (ISP). Color codes were assigned to the pigments of substrate and aerial mycelium according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago). Morphology of the strain was ascertained by light microscope and scanning electron microscope HITACHI S-570. The temperature range for growth of the strain was determined after submerged cultivation with ISP No. 5 medium for 7 days. Analysis of diaminopimelic acid was performed on the hydrolysate of aerial mycelia grown on the solid medium ISP No. 4.

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Culture and Medium Conditions

A loopful of cells from the mature slant was inoculated into 50 ml of the seed medium (pH 7.2 before sterilization), which was composed of glucose 1%, soluble starch 1%, pancreatic digest of casein (Bacto-Tryptone, Difco) 0.5%, yeast extract (Nihon-Seiyaku) 0.5%, beef extract (Kyokuto) 0.3%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05%, in a 300 ml Erlenmeyer flask. The inoculated flask was incubated at 28°C for 4 days on a rotary shaker (200 rpm). Fifteen ml of the above seed culture was added to three 2-liter Erlenmeyer flasks containing 300 ml of the same medium. These flasks were incubated for 2 days on a rotary shaker (200 rpm) at 28°C. Nine hundred ml of the seed culture thus obtained was transferred into 18 liters of fermentation medium composed of soluble starch 5%, dry yeast 1.5%, $KH_2PO_4$ 0.05%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05%, in a 30-liter jar fermenter. The fermenter was operated at 25°C under agitation at 250 rpm and aeration with airflow rate of 18 liters per minute. The fermentation was continued for 72 hours without particularly regulating the pH value of medium.

Antimicrobial Activity

The *in vitro* antimicrobial activity of GEX1 compounds was determined on nutrient agar by a 2-fold serial dilution method. The lowest concentration that inhibited growth of a bacterial strain after 18 hours incubation at 37°C was recorded as the MIC.

Cytotoxicity

Cytotoxicities against human epidermoid carcinoma A431 cells, human normal fibroblast WI-38 cells, human lung carcinoma A549 cells and human colon carcinoma DLD-1 cells were measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-staining method described in the previous paper[3]. Briefly, 100 $\mu$l of a $4\times10^3$ cells/ml suspension of cells was pipetted into each well of a 96-well microtiter plate. This plate was incubated in the $CO_2$ gas incubator at 37°C for 20 hours. Then 50 $\mu$l of test compounds, which were appropriately diluted with medium, were added to each well followed by incubation in the $CO_2$ gas incubator at 37°C for additional 72 hours. After removing the culture supernatant, 50 $\mu$l of 1 mg/ml of MTT (Sigma, St.Louis, MO), which was dissolved in the medium, was added to each well. Then 5 hours later, the pigment was extracted with 150 $\mu$l of DMSO and the *A* 550 nm was measured. By comparing the absorbance of the treated cells and untreated cells, the $IC_{50}$ value was calculated.

*In Vivo* Antitumor Effect

The antitumor effect on nude mice having A549 human lung carcinoma transplanted thereinto was examined as described previously[4]. Namely, a A549 human pancreatic carcinoma was excised from a mouse for subculture and a tumor section (8 $mm^3$, 2×2×2 mm) was subcutaneously transplanted into the abdomen of BALB/c-nu/nu mouse (nude mouse) by using a trocar. Eleven days after the transplantation, the growth of the tumor was confirmed. As concerns mouse tumor, a murine sarcoma 180 and murine SV-T2 were inoculated sc into ddY mice and BALB/c mice on day 0, respectively. Then test drug was iv administered indicated in Table 5. The antitumor effect was determined in the following manner. The major axis and the minor axis of tumor are measured with calipers and the tumor volume was calculated in accordance with the formula ((major axis)×(minor axis)$^2$/2). The antitumor effect is expressed in terms of the ratio (T/C) of the tumor volume of the tested group (T) to the tumor volume of the control group (C) to which no drug was administered.

## Results

Characterization of the Producing Microorganisms

The cultural characteristics of strain GEX1 on various agar media are shown in Table 1. The vegetative mycelia grew well on various agar media except nutrient agar media. Strain GEX1 produced yellow or brown soluble pigment on ISP No. 2, No. 3, No. 4 and No. 5 media after 14 days (Table 1). No fragmentation of substrate mycelia was observed, and sclerotia, sporangia, or flagellated spores were not formed. The colors of substrate mycelia showed pale yellow to brown on the tested agar media. The aerial mycelium grew well and formed linear or curvaceous spiral chains of 10 or more short-rod spores of 0.4 to 0.7 $\mu$m by 0.7 to 1.2 $\mu$m. As shown in Fig. 1, the surface of the spore was smooth. The physiological characteristics of strain GEX1 is shown in Table 2. Analysis of whole-cell hydrolysates of the strain revealed that the cell walls contained L,L-diaminopimelic acid, and the major quinone components were hexahydrogenated menaquinone 9. These taxonomic observations indicate that the strain GEX1 belongs to the genus *Streptomyces*.

Isolation and Purification

The isolation scheme is shown in Fig. 2. The major compound of the active components is named GEX1A. The other active components were named GEX1Q1~Q5 for

Table 1. Cultural characteristics of strain GEX1.

| Medium | Amount of growth | Color of: Aerial mycelium | Substrate mycelium | Soluble pigment |
|---|---|---|---|---|
| Yeast extract-malt extract agar (ISP No.2) | Good | White - patty | Bamboo - mustard tan | Yellow |
| Oatmeal agar (ISP No.3) | Good | White - patty | Light olive - mustard brown | Yellow |
| Inorganic salt-starch agar (ISP No.4) | Good | White - citron gray | Mustard tan - dark brown | brown |
| Glycerol - asparagine agar (ISP No.5) | Good | White - covert gray | Light olive gray - mustard tan | Yellow |
| Tyrosine agar (ISP No.7) | Good | White - patty | Covert tan - mustard tan | None |
| Sucrose - nitrate agar | Good | White - olive gray | Light olive gray | None |
| Glucose - asparagine agar | Good | Parchment - mustard tan | Oat meal - covert brown | None |
| Nutrient agar | Poor | White | bamboo | None |

Fig. 1. The producing strain, *Streptomyces* sp. GEX1.

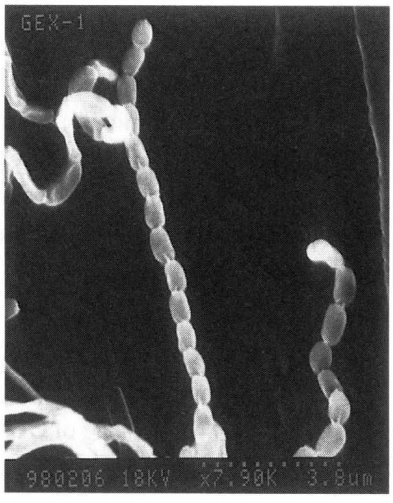


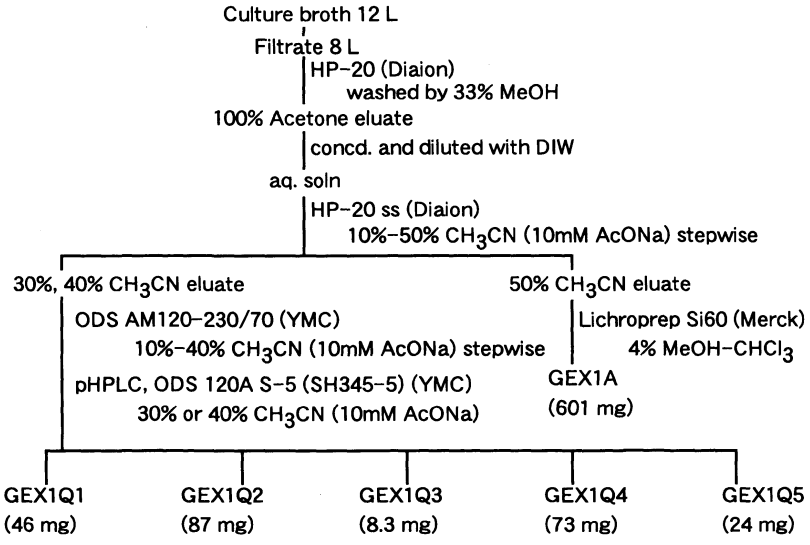

Table 2. Physiological characteristics of strain GEX1.

| Characteristics | GEX1 |
|---|---|
| Temperature for growth | 8.0 ~ 35.0 |
| Liquefaction of gelatin | Positive |
| Hydrolysis of starch | Positive |
| Coagulation of milk | negative |
| Peptonization of milk | negative |
| Formation of melanine | negative |
| Utilization of D-Glucose | Positive |
| L-Arabinose | Positive |
| D-Xylose | Positive |
| D-Fructose | Positive |
| L-Rhamnose | Positive |
| Sucrose | negative |
| Raffinose | Positive |
| D-Mannitole | Positive |
| Inositol | Positive |

the order of the elution from ODS HPLC column. As mentioned below, GEX1Q3 was not stable in methanol, so methanol was not used for elution of active components.

The culture broth obtained as described in "Materials and Methods" was filtered to separate the mycelial cake from culture filtrate. The culture filtrate was passed through a column packed with polystyrene adsorbent Diaion HP-20 (2 liters, Mitsubishi chemical industries), on which the active components were adsorbed. The impurities were removed by applying deionized water and 33% aqueous methanol, and the active components were eluted with 100% acetone (10 liters). The acetone eluate was concentrated *in vacuo* to remove acetone and passed through a column packed with a polystyrene adsorbent Diaion HP-20SS (0.4 liters, Mitsubishi chemical industries) on which the active components were adsorbed. The impurities were removed by applying to the column 10 mM sodium acetate-containing 20% aqueous acetonitrile. Then, the active components were eluted with 30% to 50% aqueous acetonitrile (10% stepwise) containing 10 mM sodium acetate and fractionated. The active fractions were concentrated, thus were obtained a fraction containing GEX1Q1 and GEX1Q3, and a fraction containing

Fig. 2. Isolation procedure of GEX1 compounds.




GEX1Q1, GEX1Q2, GEX1Q4 and GEX1Q5, and a fraction containing GEX1A with high purity. Each fraction except a fraction containing GEX1A was passed through a reverse phase column packed with ODS-AM 120-230/70 (150 ml, YMC Inc.) to adsorb the active components, and the column was washed with 20% aqueous acetonitrile containing 10 mM sodium acetate to remove the impurities. Then, the active components were eluted with 30% and 40% aqueous acetonitrile containing 10 mM sodium acetate and fractionated. The active fractions thus eluated were concentrated, thus were obtained a fraction consisting essentially of GEX1Q1; a fraction consisting essentially of GEX1Q1, GEX1Q3; a fraction consisting essentially of GEX1Q2, GEX1Q4; a fraction consisting essentially of GEX1Q4; and a fraction consisting essentially of GEX1Q5.

Each fraction was subjected to a reverse phase preparative HPLC using a packed column (SH343-5, 250 mm×20 mm diameter, YMC Inc.). Except for a fraction consisting essentially of GEX1Q5, 30% aqueous acetonitrile containing 10 mM sodium acetate was used for a developing solvent. After developing (10 ml/minute), the solution of GEX1Q1 with retention time (Rt) of 20 minutes, the solution of GEX1Q2 with Rt of 26 minutes, the solution of GEX1Q3 with Rt of 33 minutes, the solution of GEX1Q4 with Rt of 40 minutes, were taken up separately with the use of the UV absorption at 237 nm as detection. For the fraction consisting essentially of GEX1Q5, 35% aqueous acetonitrile containing 10 mM sodium acetate (10 ml/minute) was used for a developing solvent. After developing (10 ml/minute), the solution of GEX1Q5 with retention time (Rt) of 50 minutes were taken up. Each solution was concentrated and passed through a column of Diaion HP-20, by which each substance was adsorbed. The column was then washed with cold water and desalted, and the active substance was eluted with acetonitrile, and concentrated to dryness to obtain finally 46 mg of GEX1Q1, 87 mg of GEX1Q2, 8.3 mg of GEX1Q3, 73 mg of GEX1Q4 and 24 mg of GEX1Q5.

The HP-20SS fraction containing GEX1A with high purity was subjected to a column chromatography on silica gel Lichroprep Si60 (150 ml, Merck). After developed with 4% methanol-chloroform (0.5% stepwise) and fractionated, the active fractions are concentrated to dryness to obtain 0.6 g of GEX1A.

## Structures of GEX1 Compounds

The physicochemical properties of GEX1 compounds are shown in Table 3. On the basis of the analysis of a variety of the spectra, the structures of GEX1 compounds were determined as shown in Fig. 3. GEX1A was identified to herboxidiene[1] which was reported as a potent, selective herbicide against a variety of weed species and wheat[1]. Herboxidiene was structurally interesting because of the tetrahydropyran moiety and the side chain including conjugated diene[5~7]. GEX1Q1 was hydroxylated derivative

Table 3. Physico-chemical properties of GEX1 compounds.

| | GEX1A | GEX1Q1 | GEX1Q2 | GEX1Q3 | GEX1Q4 | GEX1Q5 |
|---|---|---|---|---|---|---|
| Appearance | Colorless solid | Colorless oily solid | Colorless oily solid | White, amorphous | Colorless oily solid | White powder |
| Formula | $C_{25}H_{42}O_6$ | $C_{25}H_{42}O_7$ | $C_{25}H_{42}O_7$ | $C_{31}H_{50}O_{12}$ | $C_{25}H_{42}O_7$ | $C_{24}H_{40}O_6$ |
| MW | 438 | 454 | 454 | 614 | 454 | 424 |
| HRFAB-MS *m/z* | 439.3085 $(M+H)^+$ | 453.2825 $(M-H)^-$ | 453.2868 $(M-H)^-$ | 613.3214 $(M-H)^-$ | 453.2843 $(M-H)^-$ | 423.2750 $(M-H)^-$ |
| Specific rotation (MeOH) | $[\alpha]_D^{25}$+5.4° (*c*0.70) | $[\alpha]_D^{25}$−13.5° (*c*0.13) | $[\alpha]_D^{25}$+1.5° (*c*0.13) | $[\alpha]_D^{26}$+3.4°* (*c*0.12) | $[\alpha]_D^{29}$−4.9° (*c*0.13) | $[\alpha]_D^{29}$+14.6° (*c*0.11) |
| UV $\lambda_{max}$ (ε) nm MeOH | 237 (25300) | 237 (26400) | 238 (26600) | 238* (24100) | 238 (35100) | 237 (27700) |
| IR (KBr) $\nu$ max $cm^{-1}$ | 3448, 2966, 2925, 1732, 1716, 1456, 1383, 1086, 1068 | 3700-2400, 3454, 1728, 1456, 1396, 1385, 1205, 1144, 1088 | 3700-2400, 3444, 1724, 1456, 1385, 1385, 1200, 1155, 1092, 1068, 1018 | 3700-2400, 3419, 1749, 1716, 1456, 1385, 1084, 1059, 1018 | 3700-2400, 3444, 1716, 1456, 1385, 1200, 1153, 1086, 1066, 1014 | 3700-2400, 3365, 1716, 1456, 1194, 1072 |

*in $H_2O$

Fig. 3. Structures of GEX1 compounds.

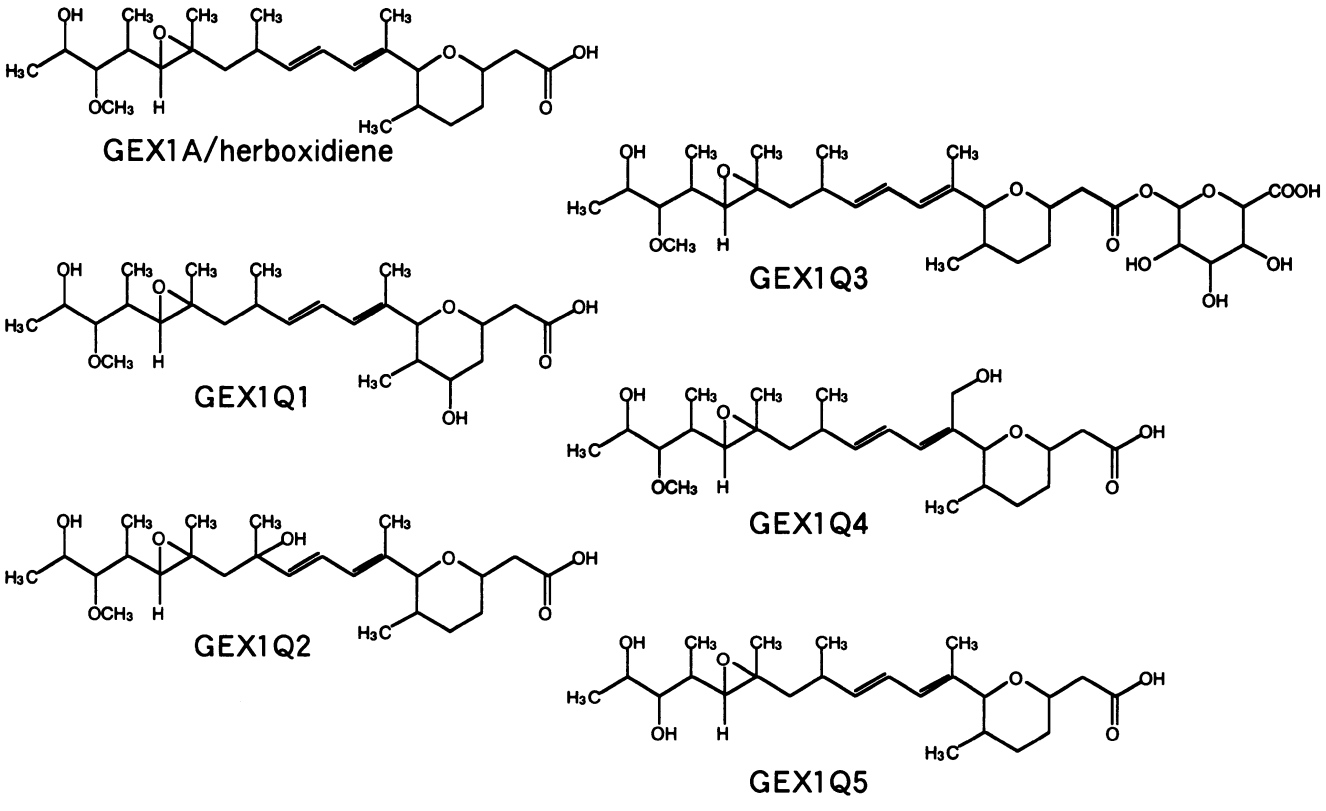


Table 4. Cytotoxicities of GEX1 compounds.

| | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| compounds | A431 | A549 | DLD-1 | WI-38 |
| GEX1A (herboxidiene) | 0.0037 | 0.021 | 0.051 | 0.0076 |
| GEX1Q1 | 0.93 | ND[a] | ND | 2.8 |
| GEX1Q2 | 0.51 | ND | ND | 1.8 |
| GEX1Q3 | 0.033 | 0.26 | 0.70 | 0.11 |
| GEX1Q4 | 0.99 | ND | ND | 5.4 |
| GEX1Q5 | 0.013 | 0.080 | 0.13 | 0.028 |

[a] ND; Not determined

at tetrahydropyran moiety of herboxidiene. GEX1Q2 and GEX1Q4 were hydroxylated derivatives at the side chain of herboxidiene. GEX1Q3 was glucuronide of herboxidiene. GEX1Q5 had an additional hydroxy group instead of the methoxy group of other GEX1 compounds. Among six GEX1 compounds, only GEX1Q3 was soluble in water. Details of structural elucidation studies will be described elsewhere.

### Antimicrobial Activity

The antimicrobial activities of GEX1 compounds were estimated by a 2-fold serial dilution method. All GEX1 compounds were inactive against *Staphyrococcus aureus*, *Enterococcus hirae*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgari*, *Shigella sonnei* and *Candida albicans* less than 100 μg/ml. GEX1A/Herboxidiene was also reported to have no antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Gaeumannomyces graminis* var. *tritici*, *Pyricularia grisea*, *Candida albicans*, *Saccharomyces cerevisiae*[1].

### *In Vitro* Cytotoxicity

As shown in Table 4, all GEX1 compounds exhibited cytotoxicity against human tumor cell lines, epidermoid carcinoma A431 cells, human lung carcinoma A549 cells and human colon carcinoma DLD-1 cells, with various $IC_{50}$ value. Against A431 cells, GEX1A/herboxidiene was the strongest, and the order of the strength of the cytotoxicity is as follows; GEX1A/herboxidiene>GEX1Q5>GEX1Q3>GEX1Q2>GEX1Q1>GEX1Q4. This tendency was supported as to human normal fibroblast WI-38. GEX1A/herboxidiene showed the one~fourth to one~second times lower $IC_{50}$ value than that of GEX1Q5, and as one~tenth times as GEX1Q3 against all four cell lines tested (Table 4).

### *In Vivo* Antitumor Activity

*In vivo* antitumor effect of GEX1A/herboxidiene, GEX1Q3 and GEX1Q5 against murine tumors and human tumor were examined in the mouse models. Against the SV-T2 murine fibrosarcoma, when administered by single intraperitoneally injection on day 0, GEX1A/herboxidiene showed significant antitumor activity (T/C 0.47) on day 4 at dose of 1 mg/kg (Table 5). By consecutive injection of GEX1A/herboxidiene (0.13 mg/kg), four of five mice died within 11 days. No significant antitumor effects of GEX1A/herboxidiene, GEX1Q3, GEX1Q5 were detected both in the murine sarcoma 180 model and in the human tumor A549 xenograft model. Mice injected with these three compounds exhibited a weight-loss within 7 days but then recovered. In all the case of died mice injected with GEX1A/herboxidiene, decreases in white blood cells and platelets were observed on day 4 (data not shown).

## Discussion

GEX1A/herboxidiene showed most effective cytotoxicity among the GEX1 compounds. The effect of GEX1Q5, which have the hydroxy group in spite of methoxy group near the end of side chain of GEX1A/herboxidiene, was as one~third times weak as GEX1A/herboxidiene. GEX1Q2,

Table 5. Evaluation of antitumor activities of GEX1 compounds *in vivo*.

| compounds | Tumor | Dose (mg/Kg) | Frequency [a] | T/C (on Day) | Mortality [c] |
|---|---|---|---|---|---|
| GEX1A/herboxidiene | SV-T2 | 1.0 | x1 | 0.41 [b] (4) | 0/5 |
| | SV-T2 | 0.13 | x5 | 0.23 (7) | 4/5 |
| | Sarcoma180 | 1.0 | x1 | 0.78 (11) | 0/5 |
| | Sarcoma180 | 0.13 | x5 | 0.80 (11) | 0/5 |
| | sarcoma180 | 0.25 | x5 | 0.81 (11) | 1/5 |
| | A549 | 0.25 | x5 | 0.75 (11) | 0/5 |
| GEX1Q3 | Sarcoma180 | 2.5 | x1 | 0.69 (11) | 0/5 |
| | Sarcoma180 | 5.0 | x1 | 0.54 (11) | 2/5 |
| | Sarcoma180 | 1.3 | x5 | 0.72 (11) | 1/5 |
| | A549 | 0.65 | x5 | 0.52 (18) | 3/5 |
| GEX1Q5 | SV-T2 | 4.0 | x5 | 0.83 (11) | 0/5 |
| | Sarcoma180 | 50 | x1 | 0.69 (11) | 0/5 |
| | Sarcoma180 | 100 | x1 | 0.90 (11) | 4/5 |
| | Sarcoma180 | 50 | x5 | 0.73 (11) | 0/5 |
| | A549 | 50 | x5 | 0.59 (7) | 0/5 |

[a] Compounds were administered i.v., by single injection on day 0( x1), or consecutive injection on day 0-4(x5).

[b] T/C(%)<0.5 and $p<0.01$ by Mann-Whitney's U-test as compared with control group.

[c] Number of died mice on the day when antitumor activity was evaluated.

GEX1Q4 and GEX1Q1, which possess a hydroxy group near the diene moiety or tetrahydropyran, showed dramatically weaker activity, suggesting these moieties are critical to the cytotoxicities.

GEX1A/herboxidiene showed antitumor effect against murine SV-T2 by single injection. TAN-1609, the identical compound with GEX1A/herboxidiene, was reported in patent[8] to inhibit murine colon 26 and M5076. GEX1A/herboxidiene and related compounds showed hematotoxicities *in vivo* in this paper. It is necessary to increase selective cytotoxicity to tumor cells for development of these compounds as clinical drugs for cancer therapy.

To elucidate the mode of action of GEX1 compounds, we analyzed the effect of these drugs on cell cycle progression and on the expression of various genes[9]. In the course of the analysis, GEX1 compounds were shown to activate gene expression in various reporter cells. Details of these biological activities are described in the succeeding paper[9].

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