R1128 SUBSTANCES, NOVEL NON-STEROIDAL ESTROGEN-RECEPTOR ANTAGONISTS PRODUCED BY A *Streptomyces*

III. PHARMACOLOGICAL PROPERTIES AND ANTITUMOR ACTIVITIES

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(Received for publication December 7, 1992)

R1128 B (1,3,6-trihydroxy-8-*n*-butylanthraquinone), a new antibiotic produced by *Streptomyces* sp. No. 1128, inhibited estrogen binding to its receptor. The IC_{50} value of R1128 B for partially purified rat uterine cytosol receptor was 1.2×10^{-7} M. However, the IC_{50} value of R1128 B against androgen-receptor binding was about 50-fold greater than that against estrogen-receptor binding. R1128 B was a competitive inhibitor against estrogen-receptor binding. R1128 B inhibited the growth of estrogen-responsive human mammary adenocarcinoma MCF-7 cells in soft agar. This inhibition, however, was reversed when estradiol was added to the culture medium. R1128 B showed antitumor activities against MCF-7 both xenografted to nude mice and implanted in subrenal capsule of mice (SRC assay). The potency of R1128 B was about 8-fold lower than that of tamoxifen both *in vitro* and *in vivo*.

New non-steroidal estrogen-receptor antagonists, R1128 A, B, C and D, were isolated from the cultured broth of *Streptomyces* sp. No. 1128[1]. The chemical structures of R1128 substances were determined on the basis of their physico-chemical properties and spectroscopic evidences as shown in Fig. 1[2]. In this paper, we describe the inhibitory activities of R1128 A, B, C and D against estrogen-receptor binding. We also show the effect of R1128 B on androgen-receptor binding. Based on the experiments measuring the colony formation of estrogen-responsive human mammary adenocarcinoma MCF-7 cells in soft agar, we show R1128 B is an estrogen-receptor antagonist. Finally, we describe the antitumor activities of R1128 B against MCF-7 both xenografted to nude mice and implanted in subrenal capsule of mice (SRC assay).

Materials and Methods

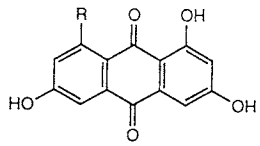
Drugs

Tamoxifen (citrate salt) and 17β-estradiol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). For *in vitro* experiments, R1128 substances, tamoxifen and estradiol were dissolved in ethanol and further diluted in buffer or medium. For *in vivo* experiments, R1128 B and tamoxifen were dissolved in sesame oil. FK-506 was suspended in saline.

Animals

Specific pathogen free (SPF) BDF_1 mice (C57BL/6 × DBA/2) (female, 6～7 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). SPF Jcl:AF-nu mice (female, 7～8 weeks old) were purchased from Japan Clea Inc. (Tokyo, Japan). SPF Sprague-Dawley rats (male and female, 7～8 weeks old) were purchased from Japan S.L.C. (Shizuoka, Japan).

Fig. 1. Structures of R1128 A, B, C and D.



R1128 A: R = $CH_2CH_2CH_3$
R1128 B: R = $CH_2CH_2CH_2CH_3$
R1128 C: R = $CH_2CH_2CH(CH_3)_2$
R1128 D: R = $CH_2CH_2CH_2CH_2CH_3$

Estrogen-receptor Binding Assay

The estrogen-receptor binding assay was described in detail in a preceding paper[1].

Androgen-receptor Binding Assay

Prostate cytosol fraction was used as the source of androgen receptors. Mature Sprague-Dawley male rats (7~8 weeks old) were castrated and 24 hours later, the rats were sacrificed. Unless specified, all the following procedures were carried out at 0~4°C. Ventral prostates excised from the rats were dissected to be free of their capsules, minced and homogenized in buffer consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol and 10 mM Na_2MoSO_4 with a Polytron homogenizer. Cytosol was obtained by centrifuging the homogenate at 108,000 × *g* for 60 minutes. Cytosol was stored at −80°C until used. The protein concentration of cytosol was determined using BioRad Protein Assay Kit (Bio-Rad, Richmond, CA, U.S.A.).

The reaction mixture containing 10 μl of a test sample, 100 μl of [^{3}H]mibolerone (3.22 TBq/mmol, New England Nuclear, Wilmington, DE, U.S.A., 64.3 KBq/ml) and 100 μl of rat prostate cytosol (15~20 mg protein/ml) was incubated in a test tube at 0°C for 4 hours. After incubation, 200 μl of dextran-coated charcoal solution consisting of 0.5% Norit A (Nakarai Chemicals, Ltd., Kyoto, Japan) and 0.05% Dextran T-70 (Pharmaceutical Fine Chemicals, Ltd., Uppsala, Sweden) was added to the incubation mixture and further incubated at 0°C for 10 minutes. The mixture was centrifuged at 3,000 rpm for 5 minutes and the radioactivity in 100 μl of the supernatant was counted in 10 ml of Aquazol-2 (New England Nuclear) with a liquid scintillation counter. The specific binding was calculated by subtracting the non-specific binding of [^{3}H]mibolerone in the presence of 1,000-fold molar excess of unlabeled testosterone from the total binding.

Colony Formation Assay in Soft Agar

The assay for colony formation of estrogen-responsive human mammary adenocarcinoma MCF-7 cells in soft agar was performed in 24-well tissue culture plate according to the method described by KNABBE *et al.*[3]. Briefly, 0.5 ml of DULBECCO's modified EAGLE's medium (Flow laboratories, Rockville, MD, U.S.A.) containing 4% heat-inactivated fetal bovine serum (Gibco, Gland Island, NY, U.S.A.), 50 U/ml benzylpenicillin, 50 μg/ml streptomycin and 0.5% agar (Noble agar, Difco Laboratories, Detroit, MI, U.S.A.) was poured into each well (under layer). Then, 0.5 ml of the above medium containing 0.3% agar instead of 0.5% agar, 1×10^3 MCF-7 cells and a test sample was added (upper layer). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2-95% air for 2 weeks. After incubation, colonies in soft agar of each well were counted.

Tumor Xenograft in Nude Mice

Human mammary adenocarcinoma MCF-7 was maintained subcutaneously by serial passage in Jcl: AF-nu mice with the intramuscular injection of E. P. Hormone Depo. (every week, 0.1 ml/mouse, containing hydroxyprogesterone caproate 50 mg/ml and estradiol dipropionate 1 mg/ml, Teikoku Zoki Co., Ltd., Tokyo, Japan).

Antitumor activity against xenografted tumor in nude mice was evaluated as follows. When the tumor volumes become 100~300 mm^3 (3 weeks after implantation), the drugs were administered subcutaneously at day-0, -4 and -8. Five mice were used in each group. At each time of injection, day-11 and -13, the length and width of each tumor was measured by slide caliper and tumor volume was calculated by the following formula:

$$\text{Tumor volume } (mm^3) = 1/2 \times a \times b^2$$

where (a) represents the length and (b) represents the width. The efficacy of the drug was assessed by calculating percent of growth inhibition using following formula:

$$\text{Growth inhibition } (\%) = (1 - T/C) \times 100$$

where (T) represents mean tumor volume of the treated group and (C) represents that of the control group.

Subrenal Capsule (SRC) Assay

A two-week SRC assay using the immunosuppressive agent FK-506 was performed according to the method described by NISHIMURA *et al.*[4,5]. In brief, a 1-mm^3 tumor fragment of MCF-7 maintained in nude mice was implanted under kidney capsule of BDF_1 mice and 0.1 ml of E. P. Hormone Depo. was injected intramuscularly at day-0. Drugs were administered subcutaneously at day-1, -5 and -9. FK-506 (32 mg/kg) was given subcutaneously at day-1, -2, -5, -7, -9 and -12 which completely inhibited the host immune response for rejecting the xenograft[6]. Eight mice were used in each group. Fourteen days after tumor implantation, the length and width of each tumor was measured and tumor volume was calculated by the formula mentioned above. The efficacy of the drug was also assessed by the above-mentioned calculation.

Results

Effects of R1128 Substances on Estrogen-receptor Binding

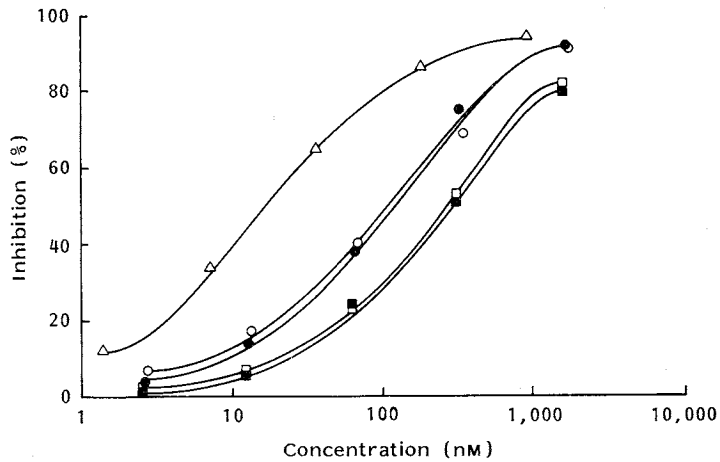
R1128 substances inhibited estrogen-receptor binding in a dose dependent manner (Fig. 2). IC_{50} values of R1128 A, B, C, D and tamoxifen were 1.1×10^{-7} M, 1.2×10^{-7} M, 2.6×10^{-7} M, 2.7×10^{-7} M and 1.6×10^{-8} M, respectively. The inhibitory activities of R1128 substances were less potent than that of tamoxifen.

R1128 B showed weak inhibitory activity against androgen-receptor binding as shown in Table 1. However, the IC_{50} value of R1128 B against estrogen-receptor binding was about 50 times lower than that against androgen-receptor binding. Thus, it seemed to us that R1128 B was a specific inhibitor of estrogen-receptor binding.

Lineweaver-Burk plot for inhibition of estrogen-receptor binding by R1128 B is shown in Fig. 3. This kinetic analysis suggested that R1128 B was a competitive inhibitor of estrogen-receptor binding. An apparent *Ki* value of R1128 B was calculated to be 4.1×10^{-8} M by assessing Dixon plot for inhibition of estrogen-receptor binding (data not shown).

R1128 B was the most abundant component[1] with potency similar to R1128 A, thus R1128 B was further evaluated in the following experiments.

Fig. 2. Inhibition of estrogen-receptor binding by R1128 substances and tamoxifen.
○ R1128 A, ● R1128 B, □ R1128 C, ■ R1128 D △ tamoxifen.



Effect of R1128 B on Colony Formation of MCF-7 Cells in Soft Agar

In an attempt to determine whether R1128 B was estrogen-receptor antagonist or agonist, the colony formation assay of estrogen-responsive human mammary adenocarcinoma MCF-7 cells in soft agar was performed[3]. As shown in Fig. 4, R1128 B did not stimulate the growth of MCF-7 cells, but inhibited the colony formation of them in the absence of estradiol in a dose dependent manner. Its IC_{50} value was 5.4×10^{-7} M. However, the inhibitory activity of R1128 B against the colony formation of MCF-7 cells was reversed in the presence of estradiol (10 ng/ml) (Fig. 4). These results suggested that R1128 B was estrogen-receptor antagonist just as tamoxifen. In the colony formation assay, the inhibitory activity of R1128 B was also less potent than that of tamoxifen.

Antitumor Activities of R1128 B

The antitumor activity of R1128 B against MCF-7 xenografted to nude mice was examined. When the tumor volumes became $100 \sim 300\,mm^3$, R1128 B was administered subcutaneously 3 times every 4 days at doses of 32, 100 and 320 mg/kg. The results are shown in Fig. 5. R1128 B showed strong antitumor activity against MCF-7 xenografted to

Table 1. Inhibition of estrogen- and androgen-receptor binding by R1128 B and tamoxifen.

Drugs	IC_{50} (M)	
	Estrogen-receptor	Androgen-receptor
R1128 B	1.2×10^{-7}	6.8×10^{-6}
Tamoxifen	1.6×10^{-8}	7.1×10^{-6}

Fig. 3. Lineweaver-Burk plot for inhibition of estrogen-receptor binding by R1128 B.

○ No R1128 B, ● R1128 B 8.0×10^{-8} M, ■ R1128 B 1.3×10^{-7} M.

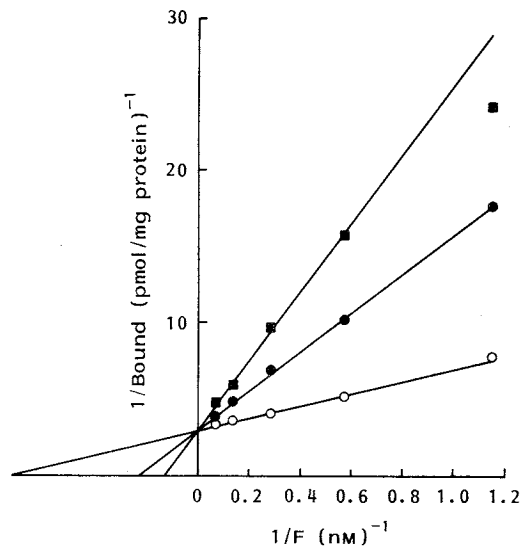


Fig. 4. Effects of R1128 B and tamoxifen on colony formation of human mammary adenocarcinoma MCF-7 cells in soft agar.

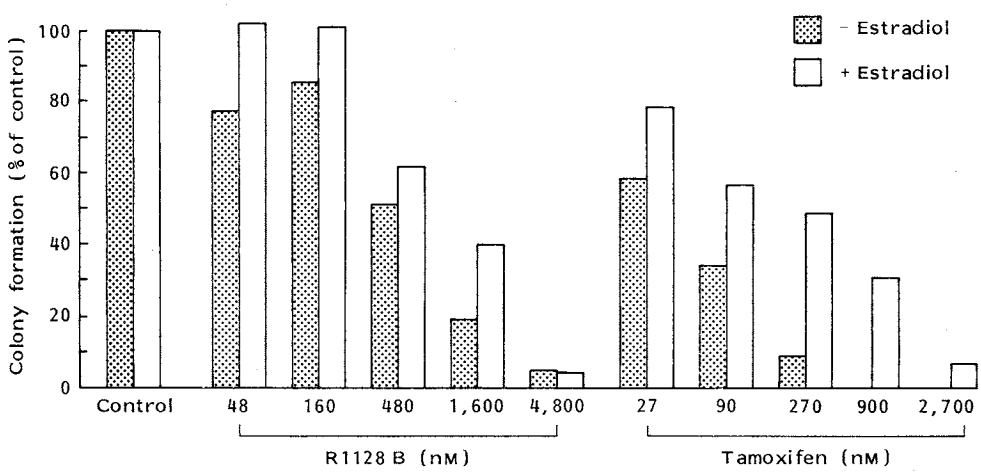


Fig. 5. Antitumor activity of R1128 B against human mammary adenocarcinoma MCF-7 xenografted to nude mice.

● Control, ■ R1128 B 32 mg/kg, ○ R1128 B 100 mg/kg, □ R1128 B 320 mg/kg.

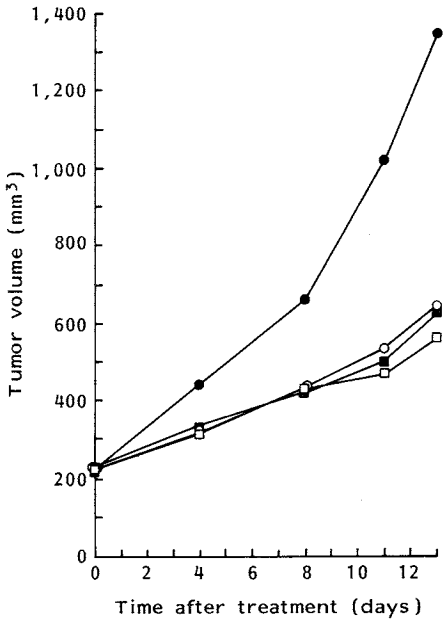
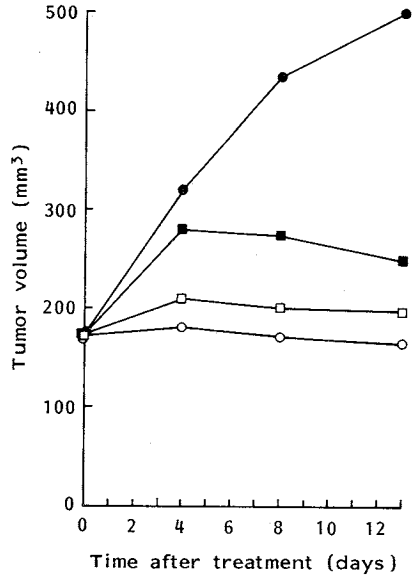


Fig. 6. Antitumor activity of tamoxifen against human mammary adenocarcinoma MCF-7 xenografted to nude mice.

● Control, ■ tamoxifen 32 mg/kg, ○ tamoxifen 100 mg/kg, □ tamoxifen 320 mg/kg.



nude mice. This inhibition manner was not a dose dependent one. In the separate experiment, tamoxifen exhibited potent antitumor activity against MCF-7, but the inhibition manner also was not a dose dependent one (Fig. 6).

So, the antitumor activity of R1128 B against MCF-7 was evaluated in the SRC assay. In this assay, many antitumor substances inhibit the growth of tumors including MCF-7 in a dose dependent manner[4,5]. As shown in Table 2, R1128 B inhibited the growth of MCF-7 in a dose dependent manner and significantly at dose of 100 mg/kg. The antitumor activity of R1128 B was also less potent than that of tamoxifen.

Table 2. Antitumor activity of R1128 B against human mammary adenocarcinoma MCF-7 in SRC assay.

Drugs		Tumor volume (mm^3)	Inhibition (%)
Control (+E. P. hormone)		55.4±10.5	0.0
Control (−E. P. hormone)		4.2±0.4***	92.4
R1128 B	3.2 mg/kg	57.3±12.1	−3.5
	10.0	46.4±3.5	16.3
	32.0	34.5±4.8	37.7
	100.0	30.2±3.3*	45.5
Tamoxifen	3.2 mg/kg	38.4±6.5	30.7
	10.0	30.4±6.6	45.1
	32.0	16.7±2.2**	69.9
	100.0	14.5±3.2**	73.8

For statistical significance, the STUDENT's *t*-test was analyzed against the group of control (+E. P. hormone), * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Discussion

Tamoxifen, the non-steroidal estrogen-receptor antagonist, is now the first choice of anti-hormone treatment for advanced breast cancer (pre- and post-menopausal)[7]. In spite of its effectiveness, the long-term use of tamoxifen frequently results in tumor resistance to itself and its related compounds which have the triphenylethylene moiety.

In considering the mechanisms that might explain such resistance, KATZENELLENBOGEN proposed 4 major categories[8]; (1) alterations in estrogen-receptor structure and function, (2) alterations in post-receptor interactions, (3) changes in paracrine interactions, and (4) pharmacologic alterations.

Among these 4 categories, the mechanisms of resistance directly related to tamoxifen itself are (1) and (4). In the case of (1), mutant receptor[9] might interpret tamoxifen as estrogen, resulting in tamoxifen-stimulated growth of tumor cells[10]. In the case of (4), breast cancer might acquire resistance by decrease in the uptake of tamoxifen, or by expression of an active efflux system of tamoxifen. In these cases, tamoxifen treatment would be ineffective. Furthermore, breast cancer cells might also metabolize tamoxifen to inactive forms or to estrogenic forms[11]. In the latter case, tamoxifen metabolites might stimulate rather than inhibit tumor growth.

In the aforesaid cases, new chemical structure estrogen-receptor antagonists different from tamoxifen and related compounds with triphenylethylene moiety could overcome such resistances, since the mechanisms responsible for resistance to tamoxifen strongly depend on its chemical structure.

Consideration mentioned above led us to screen for new non-steroidal estrogen-receptor antagonists from microbial products which did not have the triphenylethylene moiety.

As a result of the screening, we isolated novel non-steroidal estrogen-receptor antagonists, R1128 A, B, C and D, from the cultured broth of *Streptomyces* sp. No. 1128[1]. They were found to have novel structures as shown in Fig. 1[2]. The currently known non-steroidal estrogen-receptor antagonists are triphenylethylene derivatives exemplified by tamoxifen and diphenyldihydronaphthalene derivatives exemplified by nafoxidine[12]. R1128 substances, however, do not belong to these groups and are therefore a novel type of non-steroidal estrogen-receptor antagonists.

Among R1128 substances, the ranking of potency for estrogen-receptor binding inhibition was R1128 A > B > C > D. It seemed to us that smaller 8-alkyl compounds had more potent estrogen-receptor binding inhibition.

The IC_{50} value of R1128 B against estrogen-receptor binding was about 50 times lower than that against androgen-receptor binding. So, R1128 B was considered to be a specific inhibitor of estrogen-receptor binding.

R1128 B inhibited not only estrogen-receptor binding, but also the colony formation of estrogen-responsive human mammary adenocarcinoma MCF-7 cells in soft agar. R1128 B did not stimulate the growth of MCF-7 cells. These activities were also obtained with R1128 A, C and D (data not shown). Furthermore, R1128 B showed *in vivo* antitumor activities against MCF-7 both xenografted to nude mice and implanted in subrenal capsule of mice (SRC assay). Based on the experiments mentioned above, it was concluded that R1128 A, B, C and D were estrogen-receptor antagonists.

The potency of R1128 B was about 8-fold lower than that of tomoxifen both *in vitro* and *in vivo*. However, R1128 substances, because of their different chemical structure, may overcome the resistance of breast tumors to tamoxifen. Furthermore, chemical modification of R1128 substances might lead to more potent estrogen-receptor antagonists than tamoxifen. Detailed studies on the effects of R1128 substances and chemical modification of them on tamoxifen-resistant tumor cells are currently in progress.

Until now, several estrogen-receptor agonists of microbial origin have been reported, such as zearalenone[13], BE-14348 substances[14] and WS-7528[15]. To the best of our knowledge, however, these are the first reports of estrogen-receptor antagonists from microbial products.

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