Jpn. J. Cancer Res. (Gann), 79, 1224–1229; November, 1988

Mode of Action of Estra-1,3,5(10)-triene-3,17β-diol 3-Benzoate 17-((4-(4-Bis(2-chloroethyl)amino)phenyl)-1-oxobutoxy)acetate) on Human Breast Carcinoma Xenografts in Nude Mice

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To elucidate the mode of action of busramustine (KM2210), 17β- and α-busramustine, estradiol and chlorambucil were used for experimental chemo- and endocrino-therapy against hormone-dependent (T-61) and independent (MX-1) human breast carcinomas serially transplanted into BALB/cA female nude mice. Busramustine was administered po daily for 3 weeks at doses of 12.5–300 mg/kg for the β-isomer and 25–300 mg/kg for the α-isomer. Five to 50 mg of estradiol per kg was administered im once, and 3 to 6 mg of chlorambucil per kg was administered po daily for 3 weeks. All of the compounds were effective against estrogen receptor-positive T-61 with a clear dose-response relationship, while estrogen receptor-negative MX-1 was sensitive to all of the agents except estradiol. Since the α-isomer of busramustine was effective against both tumor lines, the mode of action of 17β-busramustine may not be related to estrogenic action by estradiol released from the maternal compound. However, 17β-busramustine generated the estrogen receptor system of T-61 tumor and resulted in the endometrial hyperplasia of tumor-bearing nude mice, suggesting that this compound also has estrogenic action on transplanted human breast carcinoma and tumor-bearing host mice, besides non-estrogenic antitumor activity on human breast carcinoma xenografts.

Key words: Busramustine (KM2210) — Human breast carcinoma — Nude mouse

Busramustine, a benzoate of an estradiol-chlorambucil conjugate, is an antitumor agent which was introduced by Ohsawa *et al.*[1] This agent was initially developed as an estrogen receptor-mediated antitumor agent by conjugating an alkylating agent, chlorambucil, to a vehicle, estradiol-17β. However, we have already demonstrated that busramustine was also effective against six out of 13 human tumor xenografts without estrogen receptors (ER) serially transplanted into nude mice and that the antitumor activity of this agent was closely correlated with the accumulation of active metabolites in the tumor.[2]

Busramustine was also tested in a phase II clinical study[3] on breast carcinomas and revealed a 33.3% (12/36) efficacy rate, including complete responses.[4] In this phase II study on advanced and recurrent breast cancer, mastalgia and genital bleeding, probably due to estrogen released from busramustine, were reported.[3] Since it is well known that the growth rate of breast carcinomas with ER is enhanced by estradiol,[5,6] the estrogen released from the maternal compound might hinder control of the disease. We also can not neglect the possibility that the released estrogen might have some role in the treatment of breast cancer, in the same way as massive estrogen treatment of postmenopausal breast carcinomas.[7] To elucidate the mechanism involved, we have performed experimental endocrine therapy and chemotherapy with 17β-busramustine, 17α-busramustine, chlorambucil and 17β-estradiol (E2) on ER-positive and ER-negative human breast carcinoma xenografts serially transplanted into nude mice.

Abbreviations: Busramustine, Estra-1,3,5(10)-triene-3,17β-diol 3-benzoate 17-((4-(4-bis(2-chloroethyl)amino)phenyl)-1-oxobutoxy)acetate); E2, 17β-estradiol; ER, estrogen receptor; PgR, progesterone receptor.

Materials and Methods

Mice Female nude mice with a BALB/cA genetic background were purchased from CLEA Japan, Inc., Tokyo. The mice were maintained under specific pathogen-free conditions using an Isorack system and were fed sterile food and water *ad libitum* in our experimental animal center. Six- to eight-week-old mice weighing 20–22 g were used for the experiment.

Tumors Five human breast carcinoma xenografts, MCF-7, R-27, Br-10, T-61 and MX-1, were used for the experiments. All of these strains were common ductal carcinomas maintained by serial transfer in BALB/cA nude mice in our institute.

MCF-7 and R-27 were kindly supplied by Dr. Y. Nomura, Kyushu Cancer Center as *in vitro* cell lines and were established as serially transplantable xenograft strains in nude mice in our institute.[8] Br-10 was established from a pleural effusion of a 43-year-old female patient with breast carcinoma by Dr. S. Hirohashi, National Cancer Center Research Institute[9] and was kindly supplied to our institute. T-61 was derived from cancerous tissue of a 54-year-old female patient with breast cancer in Frankfurt am Main and was kindly supplied by Dr. N. Brünner,[10] Copenhagen University, to our institute in 1983. MX-1 was established by Giovanella *et al.*[11] from cancerous tissue of a 29-year-old female patient and was kindly supplied by Dr. K. Inoue, Cancer Chemotherapy Center, Tokyo, to our institute in 1979.

ERs were detected in MCF-7, R-27, Br-10 and T-61, and progesterone receptors were positive in MCF-7 and R-27. The growths of MCF-7, R-27 and Br-10 were enhanced and the growth of T-61 was suppressed by exogenous E2, while that of MX-1 was unchanged.[6] T-61 and MX-1 were mainly used for the experiments reported in this paper.

Tumor Inoculations, Measurement of Tumors and Evaluation of Drug Activity One (for MX-1) or two (for the other strains) tissue fragments of approximately $3 \times 3 \times 3$ mm in size were inoculated by means of a trocar needle into the subcutaneous tissue of the backs of nude mice under ether anesthesia. Tumors were measured (length and width) with sliding calipers three times weekly by the same observer.

According to the method of Geran *et al.*,[12] the tumor weight in mg was calculated from the linear measurements using the formula: tumor weight (mg) = length (mm) × (width in mm)^2/2. When the estimated tumor weight reached 100–300 mg, tumor-bearing mice were pair-matched into control and test groups consisting of four to six mice each and the treatment was initiated.

On completion of the experiments (3 weeks after initial treatment), all mice were sacrificed, tumors, uteri, and spleens were weighed and the routine H-E sections were examined light-microscopically.

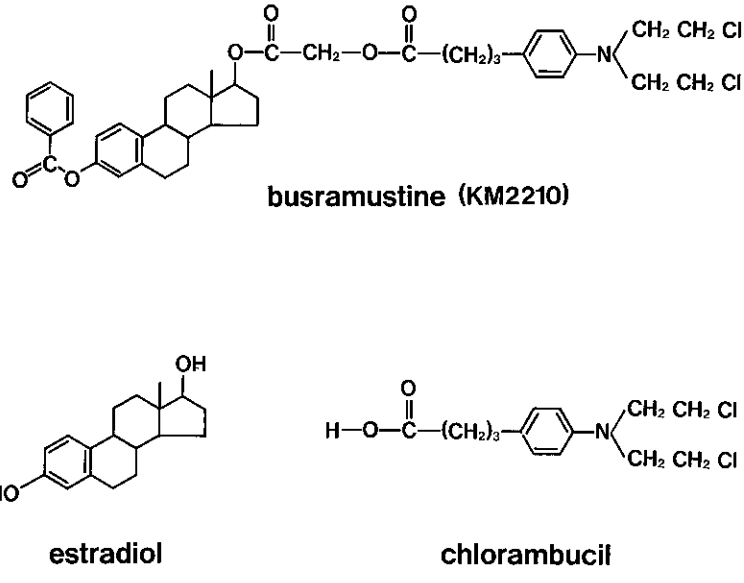

Fig. 1. Molecular structures of busramustine (KM2210), estradiol and chlorambucil.

The effects of the agents on the tumor, spleen, uterine and body weights were evaluated in terms of the ratio of the treated group to the control group as a percentage (T/C ratio).

All statistical analysis was performed by the use of Student's *t*-test.

For T-61 tumors, estrogen and progesterone receptors were assayed by the dextran-coated charcoal method[13] 24 and 48 hr after the last treatment with 17β-busramustine (300 mg/kg) or chlorambucil (3 mg/kg) po for 4 days.

Agents Estra-1,3,5(10)-triene-3,17β-diol 3-benzoate 17-((4-(4-bis(2-chloroethyl)amino)phenyl)-1-oxobutoxy)acetate (17β-busramustine), its 17α isomer (17α-busramustine) and chlorambucil were prepared in the Biomedical Laboratory, Kureha Chemical Ind., Co., Ltd. 17β-Estradiol dipropionate (E2) was purchased from Teikoku Zoki Co., Ltd., Tokyo. The molecular structures of busramustine, chlorambucil and E2 are shown in Fig. 1.

17β- and 17α-Busramustine and chlorambucil in 0.1 ml of 0.9% NaCl containing 0.5% methyl cellulose was administered po daily for 3 weeks except Sundays by using a metal gastric tube. For T-61, doses of 5, 10, 25 and 50 mg of E2 per kg dissolved in sesame oil were given once intramuscularly in the right thigh of tumor-bearing mice. Only 50 mg of E2 per kg was tested against MCF-7, R-27, Br-10 and MX-1.

Results

Antitumor activities of 17β-busramustine at a dose of 300 mg/kg and E2 at a dose of 50 mg/kg are shown in Table I. Although the growth of MCF-7 and Br-10 was significantly enhanced by exogenous E2, remarkable regression of T-61 tumor was observed with E2, while neither enhancement nor inhibition was noted for MX-1. 17β-Busramustine suppressed the growths of T-61 and MX-1, whereas no significant inhibition or enhancement was seen for MCF-7, R-27 and Br-10. Since MX-1 was sensitive to 17β-busramustine and T-61 was inhibited by both 17β-busramustine and E2, these two strains were used for further experiments to elucidate the mechanisms of antitumor activity of 17β-busramustine.

Table II shows the antitumor activities of 17β- and 17α-busramustine, chlorambucil and E2 against T-61 and MX-1, with their effects on the uterine, spleen and body weights of tumor (T-61)-bearing nude mice. 17β- and 17α-Busramustine suppressed the growth of T-61 in a dose-dependent manner. The antitumor activity of 17β-busramustine was superior to that of 17α-busramustine at all administered doses. The growth of MX-1 was also suppressed dose-dependently by 17β- and 17α-busramustine. 17β-Busramustine showed better antitumor activity against MX-1 than 17α-busramustine as in the case of T-61, and T-61 was found to be more sensitive to 17β- and 17α-busramustine than MX-1.

Although E2 revealed antitumor activity against T-61, MX-1 did not respond to E2 at a dose of 50 mg/kg. The response of T-61 to E2 was dose-dependent, and no dose which enhanced the growth of T-61 was found among the doses tested in this experiment. Chlorambucil successfully suppressed the growths of MX-1 and T-61, and T-61 was found to be more sensitive to this agent than MX-1 at doses of 3 and 6 mg/kg, where the

Table I. Antitumor Activity of Estradiol and 17β-KM2210 against Human Breast Carcinoma Xenografts

Tumor	E2[a]		17β-KM2210[b]	
	Control	Treated	Control	Treated
MCF-7	178.9±101.0[c]	367.7±127.7**	1051.5±293.0	900.0±271.3
R-27	544.0±107.5	480.8±53.8	544.0±107.5	407.8±125.3
Br-10	1714.7±821.2	2745.6±965.9*	1472.0±1112.0	2537.0±2065.0
T-61	272.2±137.2	36.4±20.7***	952.8±113.3	6.0±1.3****
MX-1	2313.3±619.2	2871.2±619.2	4111.7±2224.6	363.5±252.5***

a) Fifty milligrams of 17β-estradiol dipropionate per kg was administered im once.
b) Three hundred milligrams of 17β-KM2210 per kg was administered po daily for 3 wk except Sundays.
c) Data are given as the mean of actual tumor weight with standard deviation (in mg) at the end of experiments.
Statistically significant difference at * $P<0.05$, ** $P<0.02$, *** $P<0.01$, **** $P<0.001$.

Table II. Effects of 17β-KM2210, 17α-KM2210, Chlorambucil, and Estradiol against T-61 and MX-1

Agent	Dose (mg/kg)	Tumor		Uterus[e]	Spleen[e]	Body weight[e]
		T-61	MX-1			
17β-KM2210[a]	12.5[c]	34.3	ND[d]	112.1	110.9	103.7
	25	10.8	141.6	113.0	91.8	101.4
	50	2.6	93.0	109.3	70.1	106.1
	100	1.7	41.7	130.7	57.7	105.1
	200	1.1	10.9	194.8	42.2	97.0
	300	0.6	5.9	168.6	40.1	93.6
17α-KM2210[a]	25	19.5	116.9	129.6	98.3	100.5
	50	10.6	92.2	129.0	93.1	96.5
	100	3.3	53.0	46.3	52.7	88.6
	200	2.6	37.2	50.2	42.8	92.1
	300	2.0	ND	45.0	63.2	88.6
CBL[a]	3	2.3	38.2	99.4	94.5	96.0
	6	2.2	17.7	92.9	57.8	91.4
E2[b]	5	81.0	ND	116.6	95.6	103.6
	10	79.0	ND	127.7	106.3	99.0
	25	37.8	ND	143.1	104.6	99.1
	50	12.9	124.1	151.7	79.7	89.4

a) 17β- and 17α-KM2210 and chlorambucil (CBL) were administered po daily for 3 wk except Sundays.
b) Estradiol (E2) was administered im once.
c) Data are shown as T/C ratio at the end of experiments (3 wk after the initial treatment).
d) Not done.
e) Uterus, spleen and body weights are from mice bearing T-61.

antitumor activity of chlorambucil against T-61 was thought to be in a plateau phase.

T-61 treated with 17β- and 17α-busramustine, E2 and chlorambucil showed destruction of cancer nests, degeneration of tumor cells, giant tumor cell formation and fibrous changes in interstitial tissues. Histological changes due to these treatments could not be distinguished light-microscopically.

The uterine weight was elevated by 17β-busramustine with a dose-response relationship, while 17α-busramustine suppressed the uterine weight. Spleen weight of tumor-bearing mice was suppressed by both 17β- and 17α-busramustine. The body weight of treated mice was not changed significantly by 17β- or 17α-busramustine, chlorambucil or E2. Histological changes in the uterus of mice treated with 17β-busramustine showed remarkable endometrial hyperplasia similar to that seen in the case of E2 treatment.

Changes in hormone receptors of T-61 treated with 17β-busramustine or chlorambucil are shown in Table III. Control tumors of T-61 were positive for ER and PgR as reported previously.[5,6] When T-61 was treated with 17β-busramustine, ER was stable at 24 and 48 hr after the last treatment, while PgR was elevated remarkably 24 hr after the last treatment. On the other hand, PgR of T-

Table III. Changes of Hormone Receptors of T-61 Treated with 17β-KM2210 and Chlorambucil

Treatment	Hours after treatment[a]	ER[b]	PgR[c]
Control	0	24.1, 31.7	36.5
17β-KM2210	24	17.2, 27.4	113.0, 133.0
	48	23.3	46.5, 80.5
Chlorambucil	24	8.6, 26.8	13.4, 13.5
	48	12.2, 19.6	0, 0

a) Hours after the last treatment with 17β-KM2210 (300 mg/kg) or chlorambucil (3 mg/kg) po for 4 days.
b) Cytosol estrogen receptor in fmol/mg protein.
c) Cytosol progesterone receptor in fmol/mg protein.

61 treated with chlorambucil decreased 24 hr after the last treatment and no PgR was detected 48 hr after the last treatment, while ER of this tumor was found to be stable after the chlorambucil treatment.

Discussion

Busramustine was developed as a benzoate ester of 17β-E2 and chlorambucil conjugate for the purpose of targeting the agent to ER-positive tumor cells. Although the levels of E2 released in the experimental tumors and the serum of mice treated with 17β-busramustine were relatively low in our previous report,[2] it has been reported that some adverse estrogenic effects including mastalgia and vaginal bleeding appeared in patients treated with 17β-busramustine and it was also observed that these adverse effects could be controlled by tamoxifen.[3, 14] This suggested that 17β-busramustine might function as an E2 in patients with breast carcinomas, since massive E2 doses also suppressed breast carcinomas in postmenopausal patients.[7]

However, because 17β-busramustine could suppress the growth of human tumor xenografts without ER,[2] some of the antitumor activity of this agent does not depend on estrogenic action. To elucidate this other mechanism of action, 17α-busramustine was used for the treatment of MX-1 and T-61 both of which were sensitive to 17β-busramustine. Although the antitumor activity of 17α-busramustine was inferior to that of the 17β-isomer, both T-61 and MX-1 were sensitive to the 17α-isomer, with a dose-response relationship. One of the reasons for the lower efficacy of the 17α-isomer than 17β-busramustine was thought to be instability of the crystals of the 17α-isomer which were difficult to dissolve in sesame oil.

Since MX-1, which was insensitive to E2, was suppressed by 17β- and 17α-busramustine, it was suggested that 17β-busramustine may have a non-estrogenic action, in addition to the release of E2 from 17β-busramustine *in vivo*. However, the estrogenic action of 17β-busramustine can not be neglected because of the increased uterine weight and endometrial hyperplasia of the uterus of mice treated with 17β-busramustine. Furthermore, changes in hormone receptors of T-61 treated with 17β-busramustine included production of PgR, which was suppressed completely by the treatment with chlorambucil. We have already reported that exogenous E2 generates the ER systems, resulting in the production of PgR,[15] and tamoxifen also causes transient generation of ER systems producing PgR.[16] From these considerations, the present findings suggested that 17β-busramustine also has estrogenic action on both the transplanted tumors and tumor-bearing host nude mice.

Chlorambucil released from 17β-busramustine might play some role in suppressing the growths of T-61 and MX-1, because chlorambucil also showed remarkable antitumor activity against these strains. Although this agent was applied to breast carcinomas in a phase II study in the United States, its efficacy was relatively low, in the 9.8–19.2% range,[17, 18] which was inferior or equal to that of busramustine in early (33.3%) and late (23%) phase II studies.[3, 14] Actually, the antitumor activity of busramustine at the dose of 300 mg/kg was superior to that of chlorambucil at the dose of 6 mg/kg against MX-1 and T-61 (this paper). In addition, from the viewpoint of adverse effect, busramustine has an advantage over chlorambucil. Although the dose-limiting toxicity of busramustine is bone marrow suppression represented by lymphocytopenia, this adverse effect is reported to be mild compared with those of other cytotoxic agents such as chlorambucil.[19]

From these findings, it was concluded that the mode of action of 17β-busramustine may not be related to estrogenic action by E2 released from the parent compound, though this agent also has some estrogenic action on transplanted human tumors and tumor-bearing host mice. When we consider that E2 itself has antitumor activity on postmenopausal breast carcinomas, and most current endocrine therapy including tamoxifen, medroxyprogesterone acetate, aromatase inhibitors and LHRH agonists has an antiestrogenic action to control the breast cancer, the estrogenic action of 17β-busramustine might be valuable as a different type of endocrine therapy for breast carcinomas.

(Received June 24, 1988/Accepted Sept. 27, 1988)

References

1) Ohsawa, N., Yamazaki, Z., Wagatsuma, T. and Isurugi, K. Bestrabucil; a possible target-oriented anticancer agent. *Jpn. J. Cancer Chemother.*, **11**, 2115–2124 (1984) (in Japanese).
2) Kubota, T., Kawamura, E., Suzuki, T., Yamada, T., Toyoda, H., Miyagawa, T. and Kurokawa, T. Antitumor activity and pharmacokinetics of bestrabucil in human tumor xenografts serially transplanted into nude mice. *Jpn. J. Clin. Oncol.*, **16**, 357–364 (1986).
3) Abe, O., Izuo, M., Watanabe, H., Enomoto, K., Ohsawa, N. and Kuno, K. Early phase II study of bestrabucil on advanced and recurrent breast cancer. *Oncologia*, **18**, 158–166 (1986) (in Japanese).
4) Kubota, T., Yamada, Y., Kawamura, E., Toyoda, H., Miyagawa, T. and Kurokawa, T. Two cases of recurrent breast carcinoma achieving CR with KM2210. *Jpn. J. Cancer Chemother.*, **14**, 2556–2559 (1987) (in Japanese).
5) Kubota, T., Kubouchi, K., Koh, J., Ishibiki, K., Abe, O. and Kawamura, E. Experimental endocrine therapy of human breast carcinomas transplanted into nude mice. *In* "Immune Deficient Animals," ed. B. Sordat, pp. 385–388 (1984). Karger, Basel.
6) Kubota, T., Fukutomi, F., Isobe, Y., Kikuyama, S., Ishibiki, K. and Abe, O. Experimental endocrinotherapy of hormone-dependent human breast carcinomas transplanted into nude mice. *In* "Immune-deficient Animals in Biomedical Research," ed. J. Rygaard, N. Brünner, N. Gram and M. Spang-Thomsen, pp. 352–355 (1987). Karger, Basel.
7) Hawkins, R. A., Robert, M. M. and Forrest, A. P. M. Oestrogen receptors and breast cancer: current status. *Br. J. Surg.*, **67**, 153–169 (1980).
8) Kubota, T., Kubouchi, K., Koh, J., Enomoto, K., Ishibiki, K. and Abe, O. Human breast carcinoma (MCF-7) serially transplanted into nude mice. *Jpn. J. Surg.*, **13**, 381–384 (1983).
9) Hirohashi, S., Shimosato, Y., Kameya, T., Nagai, K. and Tsunematsu, R. Hormone dependency of a serially transplantable human breast cancer (Br-10) in nude mice. *Cancer Res.*, **37**, 3184–3189 (1977).
10) Brünner, N. Spang-Thomsen, M., Skovgaard, S. A., Engelholm, A., Nielsen, A. and Vindelov, L. Endocrine sensitivity of receptor-positive T-61 human breast carcinoma serially grown in nude mice. *Int. J. Cancer*, **35**, 59–64 (1985).
11) Ovejera, A. A., Houchens, D. P. and Barker, A. D. Chemotherapy of human tumor xenografts in genetically athymic mice. *Ann. Clin. Lab. Sci.*, **8**, 50–56 (1975).
12) Geran, R. I., Greenberg, N. H., Macdonald, M. M., Schumacher, A. M. and Abott, B. J. Protocols for screening chemical and natural products against animal tumors and other biological systems. *Cancer Chemother. Rep.*, **3**, 51–61 (1972).
13) Yamasaki, T. Estrogen receptor in malignant and benign breast tumors and normal tissues of Japanese patients. *Jpn. J. Clin. Oncol.*, **8**, 37–47 (1978).
14) Abe, O., Izuo, M., Watanabe, H., Enomoto, K., Ohsawa, N. and Kuno, K. Phase II study of bestrabucil on advanced and recurrent breast cancer. *Oncologia*, **19**, 100–111 (1986) (in Japanese).
15) Kubouchi, K. Experimental and clinical study on endocrine therapy for breast carcinoma with special reference to nuclear estrogen receptor. *Jpn. J. Surg.*, **85**, 1405–1417 (1984) (in Japanese).
16) Fukutomi, T., Ikeda, T., Nishiumi, T., Kikuyama, S., Isobe, Y., Nakamura, A., Shimada, M., Inada, T., Ishii, S., Koh, J., Enomoto, K., Ishibiki, K., Abe, O. and Kubota, T. Effect of hydroxy tamoxifens against human breast carcinomas transplanted into nude mice. *J. Jpn. Soc. Cancer Ther.*, **21**, 50–59 (1986) (in Japanese).
17) Moore, G. E., Bross, I. D. J., Ausman, R., Nadler, S., Jones, R., Jr., Slack, N. and Rimm, A. A. Effects of chlorambucil (NSC-3088) in 374 patients with advanced cancer. *Cancer Chemother. Rep.*, **52**, 661–666 (1968).
18) Goldenberg, I. S., McMahan, C. A., Escher, G. C., Volk, H., Ansfield, F. J. and Olson, K. B. Secondary chemotherapy of advanced breast cancer. *Cancer*, **31**, 660–663 (1973).
19) Kamei, H., Takenaka, K., Goto, T., Suga, S., Fugiwara, A., Nakao, I., Agatsuma, T., Fujita, K., Isurugi, K., Kubota, T., Kawamura, E., Mori, H., Mori, M., Yamazaki, Z., Kondo, T., Inoguchi, K., Ohta, K., Taguchi, T., Ohsawa, N., Saito, T. and Kimura, K. Phase I study of bestrabucil (KM2210). *Jpn. J. Cancer Chemother.*, **13**, 2208–2245 (1986) (in Japanese).