Effects of XW630 on cell proliferation, iNOS activity, and cGMP content in human osteoblast-like cell line TE85[1]

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KEY WORDS XW630; estrogens; tetracyclines; osteoblasts; thymidine; cell count; radioimmunoassay; nitric-oxide synthase; cyclic GMP

ABSTRACT

AIM: To study the effects of 2-[3-estrone-*N*-ethyl-piperazine-methyl] tetracycline (XW630) in human osteoblast-like cell line TE85. **METHODS**: [^{3}H] Thymidine incorporation and cell count for cell proliferation, radioimmunoassay for cyclic GMP (cGMP) content, and monitoring the conversion of [^{3}H] arginine for inducible nitric-oxide synthase (iNOS) activity assay. **RESULTS**: After treatment with XW630 for 48 h, [^{3}H] thymidine incorporation and cell numbers increased by 62.7 % and 96.9 %, respectively. N^G-monomethyl-*L*-arginine (*L*-NMMA, an NOS inhibitor) induced a concentration-dependent inhibitory effect on the proliferation after treatment for 48 h. The inhibitory effect was prevented partially by XW630 (1.0 nmol·L^{-1}). After treatment with XW630 for 12 – 48 h, iNOS activity and cGMP concentration increased in time-dependent manners. **CONCLUSION**: XW630 stimulated cell proliferation, enhanced iNOS activity and cGMP content in human osteoblast-like cell line TE85.

INTRODUCTION

Deficiency of female hormone 17β-estradiol results in accelerated bone loss. As a consequence, bone mass declines after menopause, this decline is the major factor contributing to the high rate of disabling bone fractures in postmenopausal women[1]. Estrogen replacement can delay or prevent these consequences[2], but there is a consensus that estrogen replacement is limited by its side effects in many women[3,4].

2-[3-Estrone-*N*-ethyl-piperazine-methyl] tetracycline (XW630) is a new conjugate of tetracycline and estrone synthesized by School of Pharmacy, West China University of Medical Sciences, Chengdu, China (CP 93110919, 6; 93110939, 1; and USP 8338505). Tetracycline is addicted to bone, which directs the drug to the bone tissue and increases the effect of estrone on the treatment of osteoporosis. On the other hand, tetracycline stimulates bone formation by itself[5,6]. Estrone also produced by the premenopausal ovary, declines across menopause, but the reduction in circulating levels is considerably less than that of estradiol[7]. In ovariectomized rats, XW630 improves bone formation and inhibites bone resorption without affecting on reproductive system (in press)[8]. In the present study, we investigated the effects of XW630 in human osteoblast-like cell line TE85.

MATERIALS AND METHODS

Drugs and reagents XW630 was dissolved in Me_2SO with stock concentration of 0.1 mol·L^{-1}. 17β-Estradiol (E_2), *L*-NMMA, McCoy's 5A culture medium, and trypsin were obtained from Sigma. E_2 was dissolved in absolute ethanol and stored at a final concentration of 0.1 mol·L^{-1}. Human osteoblast-like TE85 cells that express estrogen receptor[9] were a gift from the University of California San Francisco cell culture facility. The cells were cultured in McCoy's 5A medium with 10 % fetal calf serum (FCS, vol/vol), benzylpenicillin 50 mg·L^{-1}, and streptomycin 50 mg·L^{-1} at 37 ℃ under a humidified atmosphere of 95 % O_2 + 5 % CO_2.

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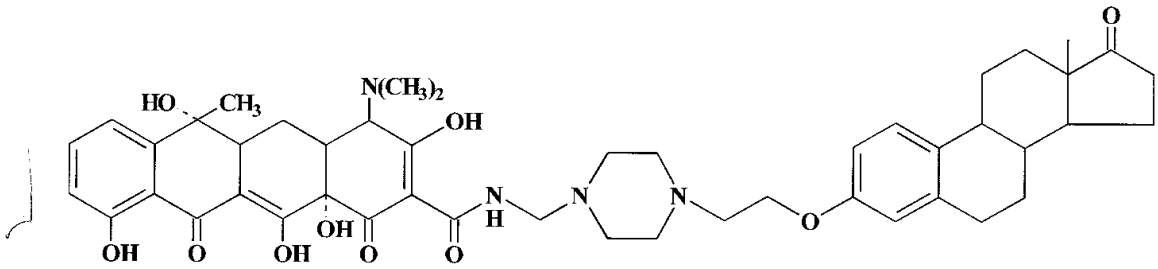
[^{3}H] thymidine incorporation Cells were seeded in 24-multiwell plastic plates at a density of 3×10^7



2-[3-Estrone-*N*-ethyl-piperazine-methyl] tetracycline (XW630)

cells·L^{-1} with fresh medium containing 7.5 % dialyzed and charcoal-stripped fetal calf serum (CS-FCS). Cells were incubated with XW630 or E_2 at concentrations of 0.01, 0.1, 1, 10 nmol·L^{-1} for 42 h or 66 h. Medium was then removed and fresh medium containing [^{3}H] thymidine (18.5 GBq·L^{-1}) and XW630 was added. Cells were cultured for another 6 h. Supernatant was aspirated and ice-cold 10 % trichloroacetic acid (TCA) was applied for 10 min. The cells were then washed twice with this solution to remove excess [^{3}H] thymidine, dissolved in NaOH 0.1 mol·L^{-1}, and neutralized with 5 % acetic acid. Radioactivity was determined by scintillation counting.

Measurement of iNOS activity Cells were grown in McCoy's 5A medium with 10 % FCS. When they were subconfluent, the medium was discarded and fresh medium with 10 % CS-FCS and XW630 or E_2 was added. After 12, 24, and 48 h, the supernatant was discarded and the cell layer was washed twice with PBS. The cells were detached with a rubber scraper. Then, using ultrasonic disrupter in water bath at 0 ℃ cells were disrupted for 7 s 3 times. The specimen was collected and centrifuged at 150 000 × *g* at 4 ℃ for 45 min. The iNOS activity was determined in the supernatants by the method of monitoring the conversion of [^{3}H]-arginine to [^{3}H] citrulline in duplicate tubes containing enzyme extract, 100 nmol·L^{-1} *L*-[^{3}H] arginine and buffer (HEPES 50, NADPH 1.0 mmol·L^{-1}) at 25 ℃ for 15 min[10,11]. The reaction was terminated by adding HEPES buffer 20 mmol·L^{-1} (pH 5.5, with edetic acid 2 mmol·L^{-1}). Samples were applied to columns of Dowex AG 50 W × 8-H^+ resin that had been converted to Na^+ form with NaOH 1 mol·L^{-1}. Each sample was eluted with deionized, distilled water and the level of [^{3}H] citrulline was measured by liquid scintillation counting.

Extraction and measurement of cGMP activity Cells were treated with XW630 for 12, 24, and 48 h, then the medium was removed. Ice-cold TCA 10 % was added. The cells were heated for 2 min in water bath at 80 ℃ and centrifuged at 3000 × *g* for 10 min. The supernatants were extracted with water-saturated ether 4 times to eliminate TCA, and then the extract was dried at 60 ℃. The dried residue was dissolved again with sodium acetate 50 mmol·L^{-1} (pH 6.2) for the measurement of cGMP content with radioimmunoassay[12].

Statistical analysis Results were expressed as $\bar{x} \pm s$ and compared by *t*-test.

RESULTS

Effects of XW630 on proliferation in osteoblast-like cell line TE 85 Treatment with XW630 0.1 nmol·L^{-1} for 48 and 72 h increased the number of cells by 96.9 % and 83.5 %, respectively, compared with control culture ($P < 0.01$). The same concentration of E_2 induced an increase by only 44.5 % and 26.8 %, respectively (Tab 1).

Tab 1. Effects of XW630 or E_2 (0.1 nmol·L^{-1}) on cell proliferation (10^{-7} × cells·L^{-1}) in human osteoblast-like cell line TE85. $n = 8$ experiments. $x \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* E_2.

	48 h	72 h
Control	65.3 ± 1.2	97 ± 5
E_2	94.4 ± 2.5^c	123 ± 3^c
XW630	128 ± 4^{cf}	178 ± 5^{cf}

In the presence of [^{3}H] thymidine, treatment with XW630 0.01 to 10 nmol·L^{-1} for 72 h increased the [^{3}H] thymidine incorporation into DNA in a concentration-dependent manner (Tab 2).

Effects of XW630 on *L*-NMMA inhibition on

Tab 2. Concentration-dependent effects of XW630 and E_2 (nmol·L^{-1}) on [^{3}H] thymidine incorporation (Bq/min) in human osteoblast-like cell line TE85. $n = 8$ experiments. $x \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* E_2.

Concentration/ nmol·L^{-1}	[^{3}H] thymidine incorporation/Bq·min^{-1} XW630	E_2
Control	21.2 ± 2.2	22.6 ± 5.5
0.01	25.9 ± 4.4	23.9 ± 3.4
0.1	28.3 ± 3.1^c	26.1 ± 4.6
1.0	31.9 ± 2.7^{cf}	29.9 ± 2.5^c
10	35.5 ± 3.9^{cf}	31.2 ± 2.2^c

[^{3}H] thymidine incorporation After treatment with *L*-NMMA 0.25, 0.5, and 1.0 mmol·L^{-1} for 48 h, the [^{3}H] thymidine incorporation decreased by 32.3 %, 51.2 %, and 76.2 %, respectively, compared with control. At the condition of XW630 1.0 nmol·L^{-1}, the effect of *L*-NMMA was diminished (Tab 3).

Tab 3. Inhibitory effects of XW630 or E_2 (1.0 nmol·L^{-1}) on *L*-NMMA-induced depression in [^{3}H] thymidine incorporation (Bq/min) in human osteoblast-like cell line TE85. $n = 6$. $x \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* *L*-NMMA.

	L-NMMA	E_2 + *L*-NMMA	XW630 + *L*-NMMA
L-NMMA concentrations (mmol·L^{-1})			
Control	25.2 ± 2.9	26.1 ± 2.3	25.9 ± 2.2
0.25	23.4 ± 3.0^c	26.2 ± 1.0^f	23.6 ± 2.5
0.5	17.6 ± 1.7^c	21.4 ± 1.9^f	22.5 ± 2.5^f
1.0	14.6 ± 2.4^c	20.2 ± 0.8^f	18.2 ± 2.1^f

XW630 on iNOS activity and content of cGMP Treatment with XW630 for 30 min did not affect the iNOS activity and the intracellular cGMP content. However, treatment with XW630 for 12, 24, and 48 h enhanced the iNOS activities in a time-dependent manner. The cGMP content increased in a similar fashion. The effects of XW630 on iNOS and cGMP were stronger than those of E_2 (Tab 4).

DISCUSSION

In experimental osteoporosis of ovariectomized rat, XW630 not only increased bone mass but also improved trabecular spatial architecture and enhanced stability and strength of bone[8]. In this study, XW630 stimulated the proliferation of TE85 osteoblast-like cells as shown by increases in both [^{3}H] thymidine uptake and cell number. Turner, *et al*[13] have reported that E_2 promoted bone formation resulting from the increase in osteoblast number and high local concentration of E_2 stimulated osteoblast number and relative osteoid surfaces[14].

Experiments with NOS inhibitor *L*-NMMA suggest that endogenous NO may be an important regulatory factor for cell proliferation.

XW630 promoted iNOS activity in a time-dependent manner, which suggests that XW630 induced the expression of iNOS. The fact that cGMP content and iNOS activity increased simultaneously indicated that the increase of cGMP was mainly due to the elevation of NO production.

In conclusion XW630 stimulated cell proliferation, enhanced iNOS activity and cGMP content, which led to prevention of experimental osteoporosis in ovariectomized rats.

Tab 4. Time-dependent effect of XW630 or E_2 (1.0 nmol·L^{-1}) on iNOS activity [mmol·min^{-1}·g^{-1}(protein)] and cGMP content [mmol·g^{-1}(protein)] in human osteoblast-like cell line TE85. $n = 4$ experiments. $x \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* E_2.

Time/h	iNOS		cGMP	
	E_2	XW630	E_2	XW630
Control	12.0 ± 2.1	12.1 ± 2.1	0.123 ± 0.028	0.141 ± 0.022
0.5	12 ± 3^a	12.3 ± 1.4^a	0.126 ± 0.051^a	0.132 ± 0.013^a
12	35.1 ± 2.4^c	41 ± 3^c	0.214 ± 0.033^c	0.279 ± 0.023^c
24	52 ± 4^c	92 ± 4^{cf}	0.408 ± 0.021^c	0.684 ± 0.024^{cf}
48	73 ± 7^c	105 ± 7^{cf}	0.693 ± 0.034^c	0.982 ± 0.023^{cf}

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XW630 对人的类成骨细胞株 TE85 细胞增殖、iNOS 活性和 cGMP 含量的影响[1]

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关键词 XW630；雌激素类；四环素类；成骨细胞；胸苷；细胞计数；放射免疫测定；一氧化氮合酶；环鸟苷一磷酸

目的：研究 XW630 对人的类成骨细胞株 TE85 细胞功能的影响. **方法**：[^{3}H]胸腺嘧啶掺入法和细胞计数法测定细胞增殖、放射免疫法测定 cGMP 含量、精氨酸转化法测定 iNOS 活性. **结果**：在培养的人的类成骨细胞中，XW630 作用48 h后，细胞数增加 96.9 %，[^{3}H]胸腺嘧啶掺入量增加62.7 %. NOS 抑制剂 *L*-NMMA (0.25，0.5，1.0 mmol·L^{-1})作用48 h，可剂量依赖地抑制细胞增殖，这一作用可被 XW630 1.0 nmol·L^{-1}部分阻断. 作用时间在 30 min 内，XW630 对细胞 iNOS 活性及 cGMP 含量无影响. 延长作用时间，XW630 时间依赖地增加细胞 iNOS 活性及 cGMP 含量. **结论**：XW630 刺激人的类成骨细胞增殖，增加细胞 iNOS 活性和 cGMP 含量.

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