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TAK-778 Enhances Osteoblast Differentiation of Human Bone Marrow Cells via an Estrogen-Receptor-Dependent Pathway

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Abstract TAK-778, a derivative of ipriflavone, has been shown to induce bone growth in in vitro and in vivo models. However, there are no studies evaluating by which mechanism TAK-778 exerts its effect. Considering the evidences that its precursors act via classical estrogen-receptor (ER)-mediated signaling, in the present study, we tested the hypothesis that TAK-778 induces osteogenesis in human bone marrow cell culture via an ER-dependent pathway. Cells were cultured in 24-well culture plates at a cell density of 2×10^4 cells/well in culture medium containing: TAK-778 (10^{-5} M), Tamoxifen (10^{-5} M), TAK-778 (10^{-5} M) + Tamoxifen (10^{-5} M), and vehicle. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At 7, 14, and 21 days, cell proliferation, cell viability, total protein content, alkaline phosphatase (ALP) activity, and bone-like formation were evaluated. Data were compared by two-way ANOVA and Duncan's multiple range test. TAK-778 did not affect cell viability. Cell number was reduced by TAK-778. Total protein content, ALP activity, and bone-like formation were increased by TAK-778. In general, Tamoxifen did not have any effect on cell behavior. However, when cells were cultured in medium containing both TAK-778 and Tamoxifen, the effect of TAK-778 on osteoblast differentiation was inhibited. The present results show that TAK-778 enhances osteoblast differentiation in human bone marrow cell culture, at least in part, via an ER-dependent pathway, since its effect was inhibited by Tamoxifen, a well-known estrogen receptor antagonist. J. Cell. Biochem. 91: 749–755, 2004.

Key words: cell culture; cellular differentiation; drug therapy; estrogen receptor; osteogenesis

Although bone tissue has a conspicuous capacity for regenerative growth, there are clinical situations that require enhancement of the healing to ensure the rapid restoration of physiologic function, such as orthopedic and maxillofacial surgery.

Bone repair involves a complex cascade of several cell events including cell growth and differentiation. Thus, there is a great interest in studying the factors involved in these cell events to enhance osteogenesis. Notoya et al. [1994] previously reported that ipriflavone (7-isopropoxy-isoflavone), a derivative of natural isoflavone isolated from alfafa (*Medicago setiva L.*), enhances the formation of bone-like nodule in rat bone marrow cell culture due to stimulation of osteoblast differentiation. This finding and structure-activity relationship studies have led to the discovery of more potent 3-benzothiepin-2-carboxyamides derivatives from ipriflavone [Oda et al., 1999]. Of these, TAK-778 [(2R, 4S)-(−)-*N*(4-diethoxyphosphorylmethylphenyl)-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide] was selected for further investigation as a new drug to stimulate osteogenesis.

TAK-778 has been shown to induce bone regeneration and stimulate fracture healing in animal models [Hoshino et al., 2000, 2001]. In vitro studies have shown that TAK-778 increases both alkaline phosphatase (ALP) activity, one of the markers characteristic of the osteoblast phenotype, in rat bone marrow cell culture, and enhances the action of bone

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morphogenetic proteins in mouse osteoblastic cell line MC3T3-E1 [Oda et al., 1999]. TAK-778 also stimulates proliferation of uncommitted mesenchymal C3H10T1/2 cells without inducting of differentiation, whereas it inhibits proliferation of osteoblast-enriched rat calvarial cells accompanied by stimulation of ALP activity [Notoya et al., 1999]. Recently, we showed that TAK-778 enhances osteoblast differentiation in human bone marrow cell culture in a dose-dependent way [Rosa and Beloti, 2003a]. Also, it was observed that TAK-778 maintains its effect on osteoblast differentiation in presence of titanium, indicating that TAK-778 could be a useful drug to be used as an adjunct therapy to improve the osseointegration of titanium implants [Rosa and Beloti, 2003b]. In spite of these, the mechanism by which TAK-778 exerts its effect on osteoblast differentiation remains unclear.

There are evidences that isoflavones such as ipriflavone act via classical estrogen-receptor (ER)-mediated signaling [Petilli et al., 1995; Anderson et al., 1999; Chen et al., 2002]. In addition, previous studies have shown that osteoblasts contain both functional estrogen receptors α and β (ERα and ERβ) [Arts et al., 1997; Bord et al., 2001]. Thus, considering that TAK-778 is derivative from ipriflavone, in the present study, we tested the hypothesis that TAK-778 induces osteogenesis via an ER-dependent pathway. Human bone marrow cells were cultured in presence of TAK-778 and/or Tamoxifen, a well-known ER antagonist, and were evaluated the following parameters: cell proliferation, cell viability, total protein content, ALP activity, and bone-like formation.

MATERIALS AND METHODS

TAK-778 and Tamoxifen

TAK-778 was kindly supplied by Takeda Chemical Industries (Osaka, Japan). Tamoxifen was purchased from Zeneca Pharmaceuticals (Macclesfield, UK). Both compounds were dissolved in a solution of ethanol-dimethyl sulfoxide (1:1, v/v) at a concentration of 10^{-3} M before use, and diluted with culture medium to the designated concentration (10^{-5} M).

Culture of Human Bone Marrow Cells

Human bone marrow cells were obtained from healthy male donors, 23 and 28-year-old, under approved research protocols of the Brazilian National Committee of Ethics in Research (CONEP) for human tissue specimens, and cultured in α-MEM (Gibco—Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 50 μg/ml gentamicin (Gibco), 0.3 μg/ml fungizone (Gibco), 10^{-7} M dexamethasone (Sigma, St. Louis, MO), 5 μg/L ascorbic acid (Gibco), and 7 mM β-glycerophosphate (Sigma). Subconfluent cells in primary culture were harvested after treatment with 1 mM EDTA (Gibco) and 0.25% trypsin (Gibco) and the first passage was subcultured in 24-well culture plates (Falcon, Franklin Lakes, NJ) at a cell density of 2×10^4 cells/well in culture medium containing the same volume of TAK-778 (10^{-5} M), Tamoxifen (10^{-5} M), and TAK-778 (10^{-5} M) + Tamoxifen (10^{-5} M). Cells subcultured in medium supplemented with vehicle were used as a control. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 3 or 4 days.

Cell Proliferation

For proliferation evaluation, cells were cultured for 7, 14, and 21 days. The culture medium was removed and the wells were washed three times with PBS at 37°C. Cells were then enzymatically (1 mM EDTA and 1.3 mg/ml collagenase and 0.25% trypsin, Gibco) released from the well, and counted using a hemacytometer.

Cell Viability

For cell viability, aliquots of the same solutions used for calculating number of cells were assayed. These aliquots were incubated, for 5 min, with the same volume of 1% trypan blue (Sigma), that stains non-viable cells, and cells were counted using a hemacytometer. Cell viability was expressed as percentage of viable cells counted at 7, 14, and 21 days, respectively.

Total Protein Content

Total protein content was calculated, at 7, 14, and 21 days, according to a Lowry et al. [1951] modified method. The culture medium was removed, the wells were washed three times with PBS at 37°C and were filled with 2 ml of 0.1% sodium lauryl sulfate (Sigma). After 30 min, 1 ml of this solution from each well was mixed with 1 ml of Lowry solution (Sigma) and left for 20 min at room temperature. After

this period, it was added to 0.5 ml of the solution of phenol reagent of Folin and Ciocalteau (Sigma). This stood for 30 min at room temperature to allow color development and the absorbance was then spectrophotometrically measured (CE3021, Cecil, Cambridge, UK) at 680 nm and the total protein content was calculated from a standard curve and expressed as μg/ml. These data were normalized by the number of cells counted at 7, 14, and 21 days, respectively.

ALP Activity

ALP activity was assayed as the release of tymolphtaleine from tymolphtaleine monophosphate using a commercial kit (Labtest Diagnostica SA, MG, Brazil), and specific activity was calculated. Aliquots of the same solutions used for calculating total protein content were assayed for measuring ALP activity. Absorbance was spectrophotometrically measured at 590 nm and ALP activity was calculated from a standard measure. Results were calculated as μmol tymophtaleine/h and data were expressed as ALP activity normalized by the number of cells counted at 7, 14, and 21 days, respectively.

Bone-Like Formation

At 7, 14, and 21 days, the cells were washed three times with PBS at 37°C. The attached cells were fixed in 3% glutaraldehyde (Electron Microscopy Sciences, Washington, PA) in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) for 2 h at room temperature and rinsed once in the same buffer. After fixation, the specimens were dehydrated through a graded series of alcohol and processed for staining with Alizarin red S (Sigma), that stains areas rich in calcium. The specimens were evaluated using an image analyzer (Image Tool, University of Texas Health Science Center, San Antonio, TX) and the amount of bone-like formation was calculated as a percentage of total well area.

Statistical Analysis

Data presented in this study are the result of two separate experiments in cell cultures established from two different donors with $n = 5$, for each group, for each evaluated parameter. All data were submitted to two-way analysis of variance (ANOVA) and Duncan's multiple range test when appropriate. Differences at $P \leq 0.05$ were considered statistically significant.

RESULTS

Cell Proliferation

Cell number was affected by culture treatment (ANOVA: $F = 9.06$; $df = 3$; $P = 0.0001$) in the following order: TAK-778 < Control = Tamoxifen = TAK-778 + Tamoxifen. Cell proliferation was a time-dependent event (ANOVA: $F = 29.84$; $df = 2$; $P = 0.00001$) being greater after 21 days. Data are presented in Figure 1.

Cell Viability

Cell viability was affected neither by culture treatment (ANOVA: $F = 0.89$; $df = 3$; $P = 0.45$) nor by culture period (ANOVA: $F = 4.17$; $df = 2$; $P = 0.20$). Data are presented in Table I.

Total Protein Content

Total protein content was affected by culture treatment (ANOVA: $F = 91.68$; $df = 3$;

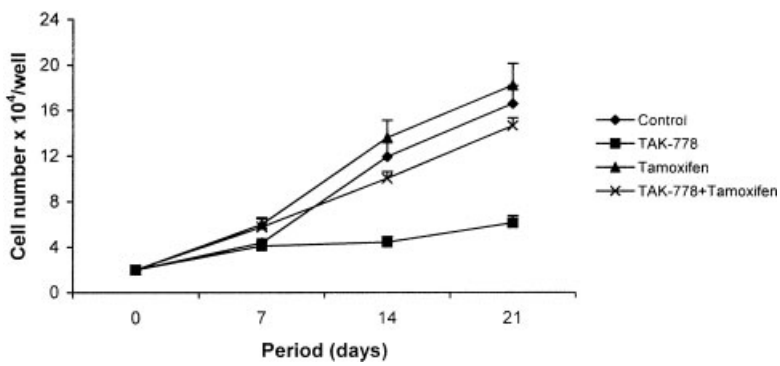

Fig. 1. Proliferation of human bone marrow cells at 7, 14, and 21 days in presence of vehicle, TAK-778, Tamoxifen, and an association of TAK-778 and Tamoxifen. Data are reported as mean ± standard deviation ($n = 5$).

TABLE I. Cell Viability Expressed as Percentage of the Total Number of Cells Counted at 7, 14, and 21 Days in Presence of Vehicle, TAK-778, Tamoxifen, and an Association of TAK-778 and Tamoxifen

Period (days)	Group			
	Control	TAK-778	Tamoxifen	TAK-778 + Tamoxifen
7	96.92 ± 2.41	95.75 ± 1.90	95.64 ± 2.66	97.84 ± 2.07
14	94.80 ± 2.33	96.24 ± 2.88	93.77 ± 2.12	94.84 ± 2.41
21	94.21 ± 2.24	95.71 ± 2.43	93.84 ± 1.75	93.95 ± 2.54

Data are reported as mean ± standard deviation (n = 5).

$P = 0.00001$) in the following order: Control = Tamoxifen < TAK-778 + Tamoxifen < TAK-778. However, the period of culture did not affect total protein content (ANOVA: F = 0,14; df = 2; $P = 0.87$). Data are presented in Figure 2.

ALP Activity

ALP activity was affected by culture treatment (ANOVA: F = 98.40; df = 3; $P = 0.00001$) in the following order: Control = Tamoxifen < TAK-778 + Tamoxifen < TAK-778. However, the period of culture did not affect ALP activity (ANOVA: F = 1.49; df = 2; $P = 0.23$). Data are presented in Figure 3.

Bone-Like Formation

After 7 days in culture there was no evident bone-like formation, so these data were not included in statistical analysis. Bone-like formation was affected by culture treatment (ANOVA: F = 4.12; df = 3; $P = 0.013$) in the following order: Control = Tamoxifen < TAK-778 + Tamoxifen < TAK-778. Process of bone-like formation increased in a time-dependent way (ANOVA: F = 79.36; df = 1; $P = 0.0001$) being greater after 21 days in culture. Data are presented in Figure 4.

DISCUSSION

In the present study, the response of human bone marrow cells cultured in presence of non-endogenous chemical compounds, TAK-778 and/or Tamoxifen was evaluated. The results showed that TAK-778 stimulates expression of markers characteristic of the osteoblast phenotype in human bone marrow cells, which are ALP activity and bone-like formation. In addition, it was observed that Tamoxifen inhibits the stimulant effect of TAK-778 on these markers of osteoblast phenotype. The in vitro concentration of TAK-778 used in this study

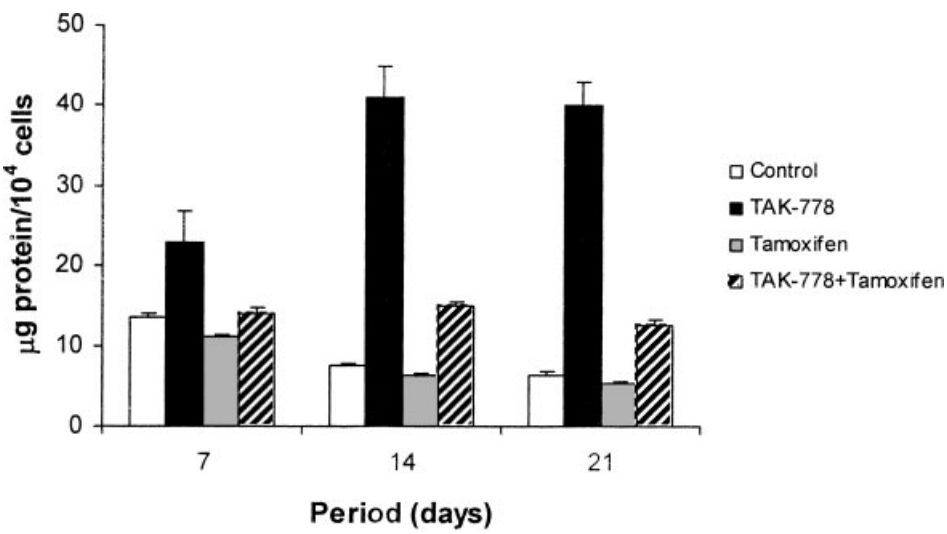

Fig. 2. Total protein content (µg/ml), normalized by the number of cells, at 7, 14, and 21 days, in presence of vehicle, TAK-778, Tamoxifen, and an association of TAK-778 and Tamoxifen. Data are reported as mean ± standard deviation (n = 5).

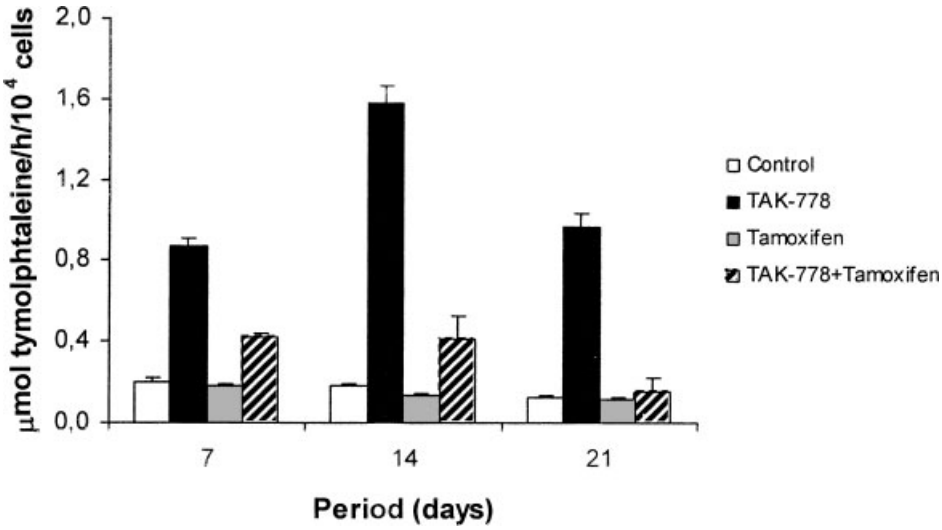

Fig. 3. Alkaline phosphatase (ALP) activity (µmol tymolphtaleine/h), normalized by the number of cells, at 7, 14, and 21 days, in presence of vehicle, TAK-778, Tamoxifen, and an association of TAK-778 and Tamoxifen. Data are reported as mean ± standard deviation (n = 5).

was based on our previous results [Rosa and Beloti, 2003a]. The concentration of Tamoxifen was selected based on our pilot study of the dose-dependent effect of Tamoxifen in presence or absence of TAK-778 (unpublished data).

Tamoxifen is a triphenylethylene-derivative, non-steroidal antagonist that is widely used for treatment of breast cancer [Heerdt and Borgen, 1999]. It competes with estrogen and estrogen-like molecules for binding to ERα and ERβ [Schwartz et al., 2002]. Recent in vitro experiments have suggested that Tamoxifen can stimulate both cell proliferation and differentiation [Fournier et al., 1996; Qu et al., 1999]. However, in our study, Tamoxifen did not present any effect on cell behavior. An explanation for such discrepancy could be the difference in the cell source used. For example, Fournier et al. [1996] used human osteoblast-like osteosarcoma cells. On the other hand, Qu et al. [1999] used mouse bone marrow cells. Besides cell origin, differences in the experimental methods make direct comparisons of results difficult or even questionable. Tamoxifen was

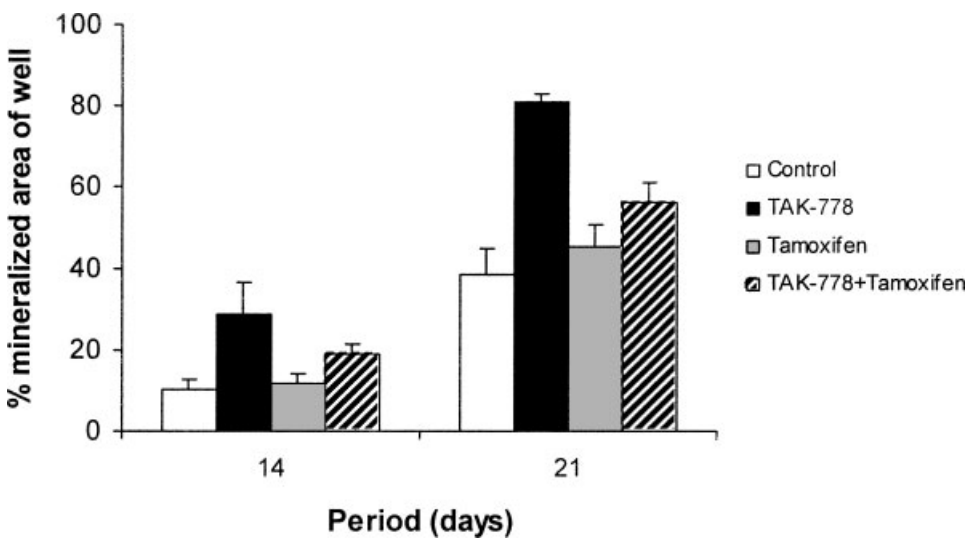

Fig. 4. Bone-like formation expressed as percentage of total well area at 14 and 21 days, in presence of vehicle, TAK-778, Tamoxifen, and an association of TAK-778 and Tamoxifen. Data are reported as mean ± standard deviation (n = 5).

selected because it competes with estrogen and estrogen-like molecules for binding to ER and also because human bone marrow cells were not affected by its presence.

With regard to the relationship between cell growth and phenotypic expression during osteoblastic differentiation, Owen et al. [1999] proposed that a reciprocal relationship exists between the decrease in proliferation and the subsequent induction of cell differentiation in rat calvarial cell cultures. Therefore, the decrease in cell number caused by TAK-778, observed in this study, seems to be related to the progression of differentiation into mature osteoblasts. In agreement with these findings, Notoya et al. [1999] showed that TAK-778 at a concentration of 10^{-5} M significantly reduced the saturated cell density and increased the ALP activity of rat calvarial cells. In addition, Tamoxifen did not have any effect on cell proliferation since no statistical difference was observed between the control and the group cultured in medium containing Tamoxifen. When the cells were treated with TAK-778 and Tamoxifen, the effect of TAK-778 on cell proliferation was inhibited suggesting that TAK-778 and Tamoxifen could share the same signaling pathway. Despite reducing cell proliferation, TAK-778 did not affect cell viability. Also, the presence of Tamoxifen did not affect cell viability.

Previous studies show that TAK-778 and ipriflavone exposure result in an increase in the cell synthesis activity [Notoya et al., 1999; Hoshino et al., 2000]. Our results showed that, for human cells, TAK-778 increased the amount of total protein produced. Considering that the amount of protein was normalized by the number of cells, these results evaluated the cell secretory activities that were stimulated by TAK-778. The presence of only Tamoxifen did not affect the cell synthesis activity. However, Tamoxifen reduced the effect of TAK-778 on cell synthesis activity in approximately 40% on day 7, 63% on day 14, and 68% on day 21. This result also suggests that TAK-778 and Tamoxifen have their effects through the same signaling pathway.

Rat bone cells present an increase of ALP activity in presence of TAK-778, mainly at concentrations of 10^{-6} M or higher [Notoya et al., 1999]. In the present study, the increase of the ALP activity caused by TAK-778 could be detected in early period of culture, and peaked at day 14. A positive correlation between ALP activity and bone-like nodule formation has been shown by Perizzolo et al. [2001] and Lincks et al. [1998]. Our results are in agreement with these findings because it was possible to observe that treatments capable of sustaining a higher ALP activity result in more bone-like formation. Corroborating the results of cell proliferation and cell synthesis activity, ALP activity and bone-like formation was not affected by Tamoxifen. However, when cells were cultured in presence of both TAK-778 and Tamoxifen, the effect of TAK-778 on osteoblast differentiation, expressed by both ALP activity and bone-like formation was inhibited. Tamoxifen reduced the effect of TAK-778 on ALP activity in approximately 52% on day 7, 74% on day 14, and 83% on day 21. The effect of TAK-778 on bone-like formation was reduced by Tamoxifen in approximately 33% on day 14, and 30% on day 21. These results strongly suggest that TAK-778 act via classical ER-mediated signaling.

Although the bone stimulator effect of TAK-778 has been documented in different systems of cell culture, this is the first experimental evidence showing that TAK-778 enhances osteogenesis in human bone marrow cell culture, at least in part, via an ER-dependent pathway. However, the discovery by Kuiper et al. [1996] of β isoform of ER suggests that the molecular regulation of osteoblast activity by estrogens, or estrogen-like molecules, is more complex than that previously thought. New experiments are necessary to clarify which ER underlie the mechanism triggered by TAK-778 on osteogenesis, if ERα, ERβ or both. This investigation is being carried out in our laboratory.

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