

- Experimental  
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## Effect of Ibandronate on Disseminated Tumor Cells in the Bone Marrow of Patients with Primary Breast Cancer: A Pilot Study

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**Abstract.** *Background:* Breast cancer patients who experience disease relapse even 10-20 years after primary diagnosis. Recurrence is caused by dormant disseminated tumor cells (DTCs) in the bone marrow (BM). Whereas chemotherapy is unable to eradicate these non-proliferating cells, bisphosphonates are currently being discussed as eliminating DTCs. It was the purpose of our study to i) analyze the presence of DTCs in the BM of breast cancer patients 2-10 years after first diagnosis of cancer, and ii) to study the effect of ibandronate on DTCs in those patients with DTC persistence. *Patients and Methods:* Bilateral BM aspirates of 54 individuals diagnosed 2-10 years ago with breast cancer, but currently disease free, were analyzed for DTCs by immunocytochemistry using pan-cytokeratin antibody A45-B/B3. Patients with DTC persistence received oral ibandronate treatment (50 mg per day) for six months and bilateral BM aspirates were analyzed for DTCs again after therapy. *Results:* DTCs were found in 18/54 (33%) of the patients, with a median number of 3 disseminated tumor cells (range 1-6 cells). These 18 patients received ibandronate orally for 6 months and 17/18 patients were analyzed for DTCs again after therapy. Only 3/17 (18%) patients remained DTC-positive, with the detection of 1 (n=2 patients) and 3 DTCs, respectively. These three DTC-positive patients continued their ibandronate intake for a further six months and re-examination of the BM resulted in no detection of DTCs in any of the three patients. *Conclusion:* Our pilot study indicates the potential effect of ibandronate on DTCs and further studies are needed to demonstrate these findings in a larger patient cohort.

To reduce the risk of systemic metastasis for patients with operable breast cancer, adjuvant chemo-, antibody and endocrine therapy are commonly applied. Therapy decisions are based on the primary tumor size, the presence of axillary lymph node metastasis, grading of tumor differentiation, receptor and HER2 status, and age, as well as the hormonal status of the patient. However, a substantial number of patients will develop recurrent carcinoma (1) even 10-20 years after the first diagnosis of breast cancer. This recurrence rate is explained by early tumor cell dissemination to distant organs, preferentially bone marrow (BM), where disseminated tumor cells (DTCs) were shown to persist in a state of dormancy (2).

Although the consequences of secondary adjuvant therapy is under discussion, a therapy for targeting these cells has not been defined. The time-limited proliferation potential of DTCs might explain why adjuvant chemotherapy fails to prevent relapse in some patients with early breast cancer (3). Antibody therapy could be one option since it has been demonstrated that these cells express carcinoma-associated cell-surface molecules, including HER-2, Epithelial Cell Adhesion Molecule (EpCAM), Mucin 1 (MUC-1) and Lewis<sup>Y</sup> (4). One approach applied the murine monoclonal antibody 17-1A (Edrecolomab) directed against EpCAM. Ten patients were treated with a single dose of 500 mg of Edrecolomab and monitored by BM analyses. Although a marked reduction in the mean numbers of DTCs was found, a complete elimination of EpCAM positive cells was only possible in four patients (5).

One approach targeting these cells might be the use of bisphosphonates which reduce bone resorption and inhibit the activity of osteoclasts. The bisphosphonate clodronate (clodronic acid) has been shown to increase overall survival after a median follow-up of 8.5 years and reduce the frequency of skeletal complications and the incidence and number of new bony and visceral metastases in women with breast cancer who were at high risk for distant metastases (6, 7).

It was the purpose of our study to i) analyze the presence of DTC in the BM of breast cancer patients 2-10 years after

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first diagnosis of breast cancer and ii) to study the effect of ibandronate on DTC in those patients with DTC persistence.

## Patients and Methods

**Study design.** We conducted a retro- and prospective single institution trial to determine the role of bisphosphonates on DTC in BM for patients with non metastatic-breast cancer. Patients with diagnosed breast cancer and DTCs at the time of primary diagnosis who presented at our clinic from 1998-2007 were invited to have their BM analyzed again for DTCs. In cases of persistence of DTCs in the BM, patients received a 6-month oral bisphosphonate therapy with ibandronate (50 mg per day). After 6 months, the BM was re-analyzed for DTCs.

**Eligibility criteria.** The eligibility criteria were as follows: histologically proven breast cancer, evidence of DTCs at time of primary diagnosis, completion of adjuvant treatment with operation and systemic chemotherapy, no local or distant metastasis, no severe uncontrolled co-morbidities or medical conditions, no second malignancies.

Prior adjuvant chemotherapy and radiation were permitted, as was therapy with clodronate. For those undergoing adjuvant endocrine therapy with tamoxifen or an aromatase inhibitor, continuing during the study was allowed. Before starting the study, all patients underwent an evaluation for local relapse and distant metastasis by clinical examination, ultrasound and mammography of the breast.

**Patient population and patient characteristics.** The study was conducted at the Department of Obstetrics and Gynecology in Essen. In total, 54 breast cancer patients with first diagnosis between 1998-2003 (n=21), or 2004-2007 (n=33), and who had DTCs in their BM aspirates at the time of primary diagnosis gave their written consent to participate in the study. Patients' characteristics at the time of diagnosis are shown in Table I. The patients ranged in age from 39 to 85 years. Most patients had a ductal carcinoma (n=40) of the breast; moderately (G2) differentiated tumors were predominant. Axillary lymph node dissection had been carried out in cases of clinically involved lymph nodes or after a positive sentinel lymph node biopsy (n=17 patients). Thirty-one patients had small tumors (pT1) and breast-conserving therapy was performed in 34 patients. Forty primary tumors were ER + and PR +, respectively, and 9 primary tumors had overexpression of HER2 (DAKO, Score 3+).

**Therapy.** Thirty-four patients received adjuvant chemotherapeutic treatment consisting of anthracyclines and taxanes. All patients with breast-conserving surgery received an adjuvant radiation. Patients with hormone receptor-positive tumors were treated with tamoxifen or aromatase inhibitors. Most of the patients (43/54) followed our recommendation and received oral clodronate therapy (2 x 520 mg per day) for at least 2 years. Only 11 patients did not receive bisphosphonate treatment at the time of primary diagnosis.

**Collection and analysis of BM.** Between 10 and 20 ml BM were aspirated under local anesthesia with mepivacain from the anterior iliac crests of 54 patients and processed within 24 hours. All specimens were obtained after written informed consent and collected using protocols approved by the Institutional Review

Table I. Patient characteristics at the time of primary diagnosis.

Patient characteristics	n (Patients)
Total number of patients	54
Median age (years)	61 (range 39-85)
Tumor size	
pT1	31
pT2-4	23
Nodal status (axilla)	
pT0	37
pTN1-3	17
Histology	
Ductal carcinoma	41
Lobular carcinoma	9
Other	4
Grading	
G 1	5
G 2	32
G 3	17
Receptor status	
ER positive	41
ER negative	13
PR positive	41
PR negative	13
HER2 overexpression	
Dako 0, +1 (negative)	44
Dako 3+ or 2+ and FISH positive	9
Unknown	1
Adjuvant chemotherapy	
Received	35
Not Received	19
Surgery	
Breast-conserving therapy	34
Mastectomy	120
Bisphosphonate at primary diagnosis	
Clodronate	43
No	11

Board (05/2856). Tumor cell isolation and detection was performed based on the recommendations for standardized tumor cell detection recently published by the German Consensus group of Senology (8). BM cells were isolated from heparinized BM (5000 U/ml BM) by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/mol; Pharmacia, Freiburg, Germany) at 400xg for 30 min. Interface cells were washed (400xg for 15 min) and resuspended in phosphate-buffered saline (PBS). A total of 1x10<sup>6</sup> mononuclear cells per 240/mm<sup>2</sup> from each aspiration side were directly spun onto glass slides (400xg for 5 min) coated with poly-L-lysine (Sigma, Deisenhofen, Germany) using a Hettich cytocentrifuge (Tuttlingen, Germany) for the detection of CK-positive cells. The slides were air-dried overnight at room temperature.

**Immunocytochemistry.** Staining for CK-positive cells was performed using the murine monoclonal antibody Mab A45-B/B3 (Micromet, Germany), directed against a common epitope of CK polypeptides including the CK heterodimers 8/18 and 8/19. The protocol has been described in detail elsewhere (9). Briefly, the method includes a) permeabilization of the cells with a detergent (5 min), b) fixation with a formaldehyde-based solution (10 min), c) binding of the

conjugate Mab A45-B/B3-alkaline phosphatase to cytoskeletal CKs (45 min), and d) formation of an insoluble red reaction product at the site of binding of the specific conjugate (15 min) using the DAKO-APAAP detection kit (DakoCytomation, Denmark) according to the manufacturer's instructions. Subsequently, the cells were mounted with Kaiser's glycerol/gelatine (Merck, Darmstadt, Germany) in Tris EDTA buffer (Sigma, Deisenhofen, Germany). A control antibody (conjugate of Fab-fragment; Micromet) served as negative control. For each test, a positive control slide with the breast carcinoma cell line MCF-7 (ATCC, Rockville, MD, USA) was treated under the same conditions.

*Evaluation of CK-positive cells.* Microscopic evaluation of the slides was carried out using the ARIOL system (Applied Imaging International, Newcastle upon Tyne, UK) according to the ISHAGE evaluation criteria and the DTC consensus (8, 9). This automated scanning microscope and image analysis system consists of a slide loader, camera, computer and software for the detection and classification of cells of interest based on particular color, intensity, size, pattern, and shape.

*Immunohistochemical analysis of the primary tumor.* For each of the 54 patients, the tumor type, TNM staging and grading were assessed according to the WHO Classification of tumors of the breast (10) and the sixth edition of the TNM Classification System (11). The estrogen receptor (ER) and progesterone receptor (PR) status were determined by immunohistochemistry. Sections of 5- $\mu$ m-thickness were cut and mounted on SuperFrost<sup>®</sup> Plus slides (Menzel, Braunschweig, Germany). Following individually optimized heat-based antigen retrieval for each antibody, each glass slide was immunostained with commercially available antibodies. The following antibodies were used: anti-ER [clone SP1, DCS (Hamburg, Germany), dilution 1:300, antigen retrieval: 30 min 95°C], citrate buffer, pH 6.0 and anti-PR [clone 16, DCS (Hamburg, Germany), dilution 1:200, antigen retrieval: 30 min 95°C, citrate buffer, pH 6.0]. Automated immunohistochemistry was performed using a Dako Autostainer Plus System (DakoCytomation, Carpinteria, CA, USA) with the anti-mouse IgG EnVision Plus detection kit (DakoCytomation) for secondary and tertiary immunoreactions. Reaction products were developed with diaminobenzidine (DAB), according to general protocols. Positive and negative control sections were included in each run, which showed appropriate results.

The DAKO score for the expression of HER2 was determined by the pathologists using the HercepTest<sup>®</sup> and the results were interpreted as follows: 0=no membranous staining, 1+=faint, partial membranous staining, 2+=faint, complete membranous staining in more than 10% of invasive cancer cells, 3+=intense, complete membranous staining in more than 10% of invasive cancer cells. Fluorescence in situ hybridization (FISH) analyses in cases of 2+ staining were determined by the HercepTest and performed as described (12). For patients with first diagnosis between 1998-2003, the DAKO score was determined retrospectively by the pathologists.

## Results

*Specificity for immunocytochemistry.* The applied antibody A45-B/B3 for the detection of CK-positive cells is directed against a common epitope of CK polypeptides and is complexed with alkaline phosphatase anti-alkaline

phosphatase (APAAP) molecules so that the missing Fc part prevents unspecific binding of the antibody to Fc receptors on mononuclear cells. A BM analysis of 165 non-carcinoma control patients resulted in only two false positive results, indicating that the A45B/B-3 gives reliable results for the detection of single DTCs (13).

*Patient characteristics at study start.* The complete data set for every patient who participated in the study is documented in Table II. All patients had a good general and nutritional condition and no evidence of primary or distant metastasis of their breast cancer. During the study, no patient received any chemotherapy or radiotherapy. Patients with hormone receptor-positive tumors (n=10) continued their adjuvant hormonal treatment according to their menopausal status.

*Detection of DTCs before study start.* DTCs were analyzed in all 54 patients. The clinical data for these patients are shown in Table I and are described in the Patients and Methods section. Whereas no DTCs were found in 36/54 (66%) of these patients, 18/54 (33%) patients showed a persistence of DTCs 2-10 years after the first diagnosis of breast cancer. These 18 patients were included in our pilot study consisting of oral ibandronate therapy for 6 months.

*Detection of DTCs before and after ibandronate treatment.* DTCs were detected in all 18 patients before ibandronate intake, with a median number of 3 cells (range 1-6 cells). BM aspiration was performed in 17/18 patients after ibandronate therapy and resulted in only 3/17 (18%) DTC-positive patients, with the detection of 1 (n=2 patients) and 3 DTCs, respectively. These three DTC-positive patients continued their ibandronate intake for a further six months and re-examination of the BM resulted in no detection of DTCs in either patients. The detailed analysis for the follow-up of DTC detection, including the detection at primary diagnosis, before and after ibandronate therapy is documented in Table II.

## Discussion

Bisphosphonates, such as clodronate, ibandronate and zoledronate, are potent inhibitors of bone resorption, they inhibit bone loss and reduce the risk of skeletal-related events in patients with bone metastases. The efficacy of oral clodronate and ibandronate in disease with bone resorption is very good and oral therapy has been shown to be safe with a low rate of side-effects (14,15). Zoledronic acid prevents bone loss associated with aromatase inhibitor use in postmenopausal women and premenopausal women with early breast cancer (16,17). In a small study, monthly oral ibandronate prevented anastrozole-induced bone loss during adjuvant aromatase inhibitor therapy for breast cancer (18).

Table II. Patient characteristics at study start.

Patient	PT	PN	M	G	Histology	ER	PR	Her2	Surgery	Radio-therapy	Chemo-therapy	Endo- crine therapy	Clodronate therapy at prim. diagnosis	Number of DTCs			
														at primary diagnosis	at study start	after ibandronate treatment for 6 months	after ibandronate treatment for 12 months
1	1	0	0	2	Ductal	Pos	Pos	Neg	BCT	Yes	No	Yes	No	3	6	n.d.	N.d.
2	2	0	0	3	Ductal	Neg	Pos	Neg	BCT	Yes	6x FEC	Yes	Yes	6	1	3	0
3	1	0	0	3	Medullar	Neg	Neg	Neg	BCT	Yes	6x TAC	No	No	5	4	0	N.d.
4	1	0	0	2	Ductal	Pos	Pos	Neg	BCT	Yes	No	Yes	Yes	1	1	0	N.d.
5	1	0	0	2	Ductal/lobular	Pos	Pos	Neg	BCT	Yes	No	Yes	Yes	1	1	0	N.d.
6	3	2	0	2	Neuroendocrine	Pos	Pos	Neg	Mastectomy	Yes	4x EC, 4x DOC	Yes	Yes	6	2	0	N.d.
7	2	0	0	2	Mucinous	Pos	Pos	Neg	Mastectomy	No	No	Yes	No	1	6	0	N.d.
8	2	0	0	2	Lobular	Neg	Pos	Neg	BCT	Yes	No	Yes	No	4	3	0	N.d.
9	2	1	0	2	Ductal	Pos	Pos	Pos	BCT	Yes	6x FEC	Yes	Yes	1	2	0	N.d.
10	3	1	0	3	Ductal	Neg	Neg	Neg	Mastectomy	No	6x TAC	No	Yes	27	2	0	N.d.
11	1	0	0	3	Ductal	Pos	Pos	Neg	BCT	Yes	4x FEC	Yes	Yes	36	3	0	N.d.
12	1	1	0	3	Ductal	Neg	Neg	Pos	BCT	Yes	6x FEC	No	Yes	375	4	1	0
13	2	3	0	2	Lobular	Neg	Neg	Neg	Mastectomy	Yes	6x TAC	No	No	4	2	0	N.d.
14	1	1	0	3	Lobular	Neg	Neg	Pos	Mastectomy	No	4x EC, 4x T	No	Yes	5	1	0	N.d.
15	1	0	0	2	Ductal	Pos	Pos	Neg	BCT	Yes	No	Yes	No	2	1	0	N.d.
16	1	0	0	3	Ductal	Pos	Neg	Neg	BCT	Yes	6x EC	Yes	No	56	2	0	N.d.
17	1	0	0	3	Ductal	Neg	Neg	Pos	BCT	Yes	6x TAC	No	Yes	11	1	0	N.d.
18	2	0	0	3	Ductal	Pos	Pos	Neg	Mastectomy	No	3x FEC, 3xDOC	Yes	Yes	1	7	1	0

Abbreviations: PT=Pathological tumor stage, PN=Pathological nodal status, M=Metastasis, G=Grading, ER=Estrogen receptor status, PR=Progesterone receptor status, Pos=Positive, Neg=Negative, BCT=Breast-conserving therapy, F=5-Fluorouracil, E=Epirubicin, C=Cyclophosphamide, DOC=Docetaxel, T=Taxol, A=Adriamycin, N.d.=Not done.

There is growing evidence that bisphosphonates have antitumor and antimetastatic properties, including the inhibition of angiogenesis, tumor cell invasion, adhesion in bone, the induction of apoptosis, antitumor synergy with cytotoxic chemotherapy and immunomodulatory effects through induction of  $\gamma/\delta$  T-cells (19-21).

It is not fully understood how bisphosphonates effect tumor cells. It has been assumed that the release of growth factors, such as transforming growth factor and insulin-like growth factor, during degradation of bone matrix promotes the proliferation of tumor cells. Thus, by inhibition of the activity of osteoclasts, bisphosphonates can influence the interaction between tumor cells and bone cells at very early stages of the disease. Since chemotherapy seems to have only a limited effect on the elimination of dormant DTCs, bisphosphonates at early stages could be effective. Two studies using zoledronic acid demonstrated the effect on DTC elimination. As part of an interventional pilot study, 31 DTC-positive patients who had completed surgery leading to R0 resection of their tumor and adjuvant chemotherapy for at least 6 months were treated with zoledronate. BM was re-

examined after a median of 8 months, resulting in a persistence of DTCs in only 4 patients (22). Similar results were found in a multicenter trial treating DTC-positive patients with zoledronate and adjuvant chemotherapy (23). Our data show that neither the timespan since primary diagnosis of breast cancer, nor the adjuvant chemotherapy had any significant influence on the persistence of DTCs. The proportions of DTC-positive and DTC-negative patients were similar in the group of patients with primary diagnosis 1998-2003 (57% DTC-negative) and the group that had primary diagnosis 2004-2007 (75% DTC-negative). The ratio of DTC-positive (33%) and -negative patients (66%) is nearly the same with and without adjuvant chemotherapy.

A significant benefit of zoledronic acid with respect to disease-free survival has been demonstrated by Gnant *et al.* in a clinical trial (17). This effect may be explained by several antitumor mechanisms as demonstrated in preclinical models where zoledronic acid acted synergistically with many chemotherapy agents. Moreover, in the integrated analysis of the Zometa-Femara Adjuvant Synergy Trial, zoledronic acid significantly reduced disease recurrence among

postmenopausal women with early breast cancer when it was administered at the dose used in premenopausal women (17). There was a nonsignificant trend in overall survival in favour of adding zoledronic acid. Zoledronic acid not only reduced the rate of development of bone metastases, but there was also a marked reduction in locoregional, contralateral breast and distant recurrence (excluding bone metastases) (17).

Further data supporting the potential anticancer activity of zoledronic acid comes from the AZURE trial. This phase III randomized study of neo-adjuvant or adjuvant chemotherapy and/or hormonal therapy with or without zoledronic acid reported the results of a sub-group analysis at the San Antonio Breast Cancer Symposium in 2008. Pathological complete response in breast and axilla was higher in retrospective examination of pathological samples in those who received chemotherapy plus zoledronic acid than those who received chemotherapy alone (24).

As known from the data from Diel *et al.* (6, 7) oral clodronate can reduce the incidence and number of new bone and visceral metastases and increases the overall survival after a median follow-up of 8.5 years in women with breast cancer who are at a high risk for distant metastases. In our collective of 54 patients that had DTCs at the time of primary diagnosis, only one out of three patients showed a persistence of DTCs in their BM aspirates that we took for the screening for our study. We put this finding down to the application of bisphosphonates at the time of breast cancer diagnosis. At our centre we have recommended oral clodronate for patients with DTC-positive breast cancer since 1998. In conclusion our data support the results of Diel *et al.* (6, 7).

After treatment with ibandronate for 6 months, only three patients still had DTCs in their BM aspirates. After 12 months of treatment, no patient had DTCs. The much higher relative potency of ibandronate could be the reason for the elimination of DTCs in our population. Even after more than 8 relapse-free years since the diagnosis of breast cancer, ibandronate is effective in eliminating DTCs.

Taken together, our data, as previous data, indicate that bisphosphonates may exert antitumor effects both in and outside the bone. Currently large-scale clinical trials such as SO 307, GAIN and SUCCESS are investigating our hypothesis and will show if the antitumoral potential of ibandronate can reduce the morbidity of breast cancer patients or have further effects.

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