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Multiparameter Analysis of the “Off-Target Effects” of Dasatinib on Bone Homeostasis in Patients with Newly Diagnosed Chronic Myelogenous Leukemia

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Abstract

We assessed patients with chronic myelogenous leukemia for serum Ca, PO₄, bone alkaline phosphatase, N-telopeptide, osteoprotegerin levels and trabecular bone (TBA) in bone marrow (BM) specimens before and after treatment with dasatinib. We identified a significant increase in TBA % in post-dasatinib BM (p=0.022). This suggests that dasatinib therapy can increase TBA, without significant changes in bone and mineral metabolism.

Introduction—Interferences with bone homeostasis and mineral metabolism have been described in patients taking imatinib for chronic myelogenous leukemia (CML) or gastrointestinal stromal tumors. Dasatinib is a potent second generation tyrosine kinase inhibitor (TKI), designed to inhibit ABL and SRC kinases while also interfering with the c-KIT, PDGF-R and STAT5 pathways.

Patients and Methods—In this study, we use a multiparameter approach to examine the “off target effects” of dasatinib in 30 patients with CML treated between 2009 and 2012. We recorded serum calcium (Ca) and phosphate (PO₄) levels, analyzed markers of bone formation (bone alkaline phosphatase/ BAP) and bone resorption (N-telopeptide/NTX), measured osteoprotegerin (OPG) levels and digitally analyzed changes in trabecular bone area (TBA) in paired bone marrow biopsy specimens (BM) before and after treatment. We correlated all findings with each other and with results of conventional cytogenetic and molecular analysis.

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Disclosure

The authors declare they have no competing financial interests.

Results—We identified a significant increase in TBA% in post-dasatinib BM biopsies ($p=0.022$), and noted a decrease in serum OPG levels in 75% of patients. Ca, PO₄, BAP and NTX levels remained steady without significant changes. There was no correlation between biomarker levels, TBA%, and/or cytogenetic/ molecular response.

Conclusion—These findings suggest that dasatinib therapy (within the therapeutic range) can increase trabecular bone, without causing significant changes in bone and mineral metabolism. Nonetheless monitoring of bone health and skeletal integrity should be included into the long term management of patients treated with dasatinib to further enhance our understanding of its safety profile and potential role as a treatment modality for other bone diseases.

Keywords

Dasatinib; Bone homeostasis; Digital image analysis; Bone marrow; Trabecular bone

1. Introduction

With the introduction of targeted oral tyrosine kinase inhibitor (TKI) therapy, long-term/life-long therapy for patients with chronic myelogenous leukemia (CML) is now the reality. Imatinib (Novartis, Basel, Switzerland) was the first TKI approved for the treatment of CML, and was considered the standard of care for more than a decade. Notably, many *in vitro* studies demonstrated that imatinib can interfere with bone hemostasis by affecting the morphology and function of osteoclasts and osteoblasts through the c-fms (*Susan McDonough strain of feline sarcoma virus*), c-abl (*Abelson murine leukemia viral oncogene homolog 1*), and PDGFR (*platelet-derived growth factor receptor*) pathways. These result in alterations of the OPG/ RANK/ RANKL (*Osteoprotegerin/Receptor Activator of Nuclear Factor κ /Receptor activator of nuclear factor kappa-B ligand*) system, thus increasing the expression of osteogenic markers, such as osteocalcin, RUNX2 (*Runt-related transcription factor 2*), and BMP2 (*bone morphogenetic protein 2*).^{1–6} Simultaneously, several *in vivo* studies corroborated these findings, noting that CML patients undergoing treatment with first generation TKIs had indicators of altered bone metabolism in their peripheral blood as well as changes in bone mineral density as identified on serial dual energy X-ray absorptiometry (DEXA) scans shortly after commencing therapy.^{7–10}

Dasatinib (Bristol-Myers Squibb, New York, NY, USA) is a multitargeted, second-generation kinase inhibitor which was initially used in patients who were resistant or intolerant to Imatinib front-line therapy.^{11–14} Originally the drug was developed as an inhibitor of Abl and SRC- family kinases (*Rous sarcoma virus oncogene protein pp60 (V-SRC)*) including c-Src, Lck, Hck, Yes, Fgr, Lyn, and Fyn.¹¹ Dasatinib also inhibits platelet-derived growth factor (PDGF) family members, particularly c-Kit and PDGF receptor alpha/ beta (PDGFR-a, PDGFR-b) at therapeutic levels.¹⁵ In addition, it can bind to MAPK (mitogen-activated protein kinases), a tyrosine kinase discoidin domain receptor and can block downstream signaling of STAT5 (signal transducer and activator of transcription 5) which down regulates expression of STAT5 target genes including *Bcl-x*, *Mcl-1*, and *cyclin D1*.¹⁶ Dasatinib has an enhanced affinity for the BCR-ABL1 fusion transcript, can block BCR-ABL1 phosphorylation and is less sensitive to the common mutations in the *ABL* kinase domain.^{14,17} Thus, the drug is more potent through its multitargeted profile.

Dasatinib achieved significantly higher and faster rates of complete cytogenetic and major molecular responses in CML patients compared to imatinib and is FDA approved as a first line therapy.^{18–21}

Considering the effects of the first generation TKIs on bone homeostasis, it is no surprise that multiple *in vitro* studies were able to demonstrate that the second generation reagent with its higher potency and its broader effect on different kinase families can also influence bone metabolism.^{22–26} Dasatinib can disturb the balance between osteoblast-osteoclast interactions by producing a convergent effect comprised of increased bone anabolism and reduced bone resorption.²⁷ Interestingly these results subsequently led several authors to propose a potential (“off target-off label”) application for dasatinib in the treatment of other bone specific disorders^{28–30} which mandates the necessity to conduct comprehensive studies analyzing bone health in long term users.

Our goal in this study was to assess the “off target” effects of dasatinib monotherapy on bone homeostasis in chronic phase CML patients in order to better understand its effects on bone health in patients in general. We recorded calcium (Ca) and phosphate (PO₄) levels and measured levels of four additional selected biomarkers of bone health, namely serum and urinary N-Telopeptide (NTx) of collagen type 1, a marker of bone resorption; serum alkaline phosphatase isoenzyme/bone-specific alkaline phosphatase (BAP), a marker of osteoblast activity and bone matrix calcification; and serum osteoprotegerin (OPG) an osteoclastogenesis inhibitory factor established to have bone protective effects through the OPG/RANK/RANKL system. We also quantified trabecular bone areas (TBA) in BM biopsy specimens via digital image analysis as described previously³¹, tabulated cytogenetic and molecular treatment responses and correlated all findings with each other.

2. Patients and Methods

2.1 Patient selection

We prospectively identified adult patients with newly diagnosed Philadelphia chromosome (Ph) positive chronic phase CML who were being enrolled in a preexisting clinical trial which was approved by the relevant institutional review boards and ethics committees. All patients provided informed written consent in accordance with the Declaration of Helsinki. Inclusion criteria encompassed adequate organ function and no other serious medical conditions. Each patient was scheduled to undergo periodical bone marrow (BM) aspirations and biopsy, cytogenetic and molecular analysis, and serum and urine biomarker testing as part of the protocol. The first BM examination was performed at the time of study enrollment; a second BM specimen was obtained after 6 months of therapy and a third BM specimen was obtained 1 year after commencing Dasatinib monotherapy.

2.2 Cytogenetic and Molecular analysis

Conventional cytogenetic analysis and fluorescence *in situ* hybridization analysis (FISH) were performed on metaphase cells prepared from BM aspirate smears using standard techniques. Cytogenetic responses were classified according to degree of decreased in the Philadelphia chromosome (Ph+) metaphases compared to the pretreatment value. The

categories included: no cytogenetic response (continued presence of 100% Ph+ metaphases), minor cytogenetic response (35–90% Ph+ metaphases), partial cytogenetic response (5 to 34% Ph+ metaphases) and complete cytogenetic response (CCyR) with 0% Ph+ metaphases.

A quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assay was used for assessment of *BCR-ABL1* transcript levels as described previously.³² The assay was designed to detect residual leukemia with up to 4- to 5-log reduction from the baseline levels at diagnosis. A major molecular response (MMR) was defined as a *BCR-ABL1/ABL1* ratio < 0.1% and complete molecular response (CMR) was defined as undetectable *BCR-ABL1* levels. Missing samples were counted as no response.

2.3 Digital image analysis of bone marrow biopsy specimens

We used a digital image analysis algorithm to examine BM specimens similar to a method described previously.³¹ Limited quality or suboptimal quality biopsy specimens (substantial crush or aspiration artifact) as well as subcortical biopsy specimens were excluded from the analysis. Routine hematoxylin and eosin (H&E) stained slides from formalin-fixed, paraffin embedded BMx were digitally scanned at 2×, 10×, 20× and 40× using a whole slide digital imaging (Philips, beta tester, Sweden). A bounding box was manually aligned around the scanned area/area of interest in order to compute the total biopsy specimen area. Spurious fragments, shattered areas, cortical bone and areas with crush artifact were excluded. Trabecular bone was identified, encircled and labeled with a numerical value. Each numerical value is representative of a specifically encircled pixel area which allows objects to be counted as a percentage of the total number of measured objects and as a percentage of total measured areas. Measurements were taken through an Image-Pro Plus software area pixel count algorithm (Image Pro Plus system Version 6.3, MediaCybernetics, Bethesda, MD, USA). Areas of trabecular bone (TBA) and non-trabecular bone area (n-TBA) were calculated as a percentage of total biopsy specimen area. Percentage of total TBA (TBA% = sum of trabecular area/total biopsy area) and percentage change (TBA%) between two different biopsy specimens (TBA% = (2nd BX TBA% – 1st BX TBA%)/1st BX TBA% × 100) were computed and recorded.

2.4 Ca, PO4, BAP, NTX and OPG levels

All whole blood, serum and urine samples were taken in the morning with patients being in a fasting state. Calcium (total) and phosphate levels were recorded as part of a routine metabolic panel. Measurements for urine and serum N-Telopeptide (UNTx, SNTx) and bone specific alkaline phosphatase (BAP) were taken every 3 to 6 months for the first 12 months, beginning at enrollment into the study and every 6 months thereafter as per the protocol. BAP was measured with a solid-phase biotin-labeled, BAP-specific monoclonal antibody EIA micro plate system (Immunodiagnostic Systems). After the formation of a solid phase/capture antibody/BAP complex, we removed unbound BAP by washing the micro plates, and then incubated the plates with an enzyme substrate. The rate of substrate turnover is thereby determined calorimetrically by measuring the absorbance of the quenched reaction at 405 nm in a micro plate reader. The absorbance is proportional to the concentration of BAP in the test sample. The calculation of BAP concentration in the sample is based on concurrent testing of BAP calibrators and a Zero Calibrator/Diluent. We used a competitive-

inhibition enzyme-linked immunosorbent assay (EIA) (Osteomark) to UNTx and SNTx levels for collagen type 1. NTx from the collected specimen samples binds to a monoclonal antibody while competing with the solid-phase NTx. The antibody amount bound to the solid-phase NTx is inversely proportional to the amount of patient NTx in the specimen. Absorbance was determined by spectrophotometry and the NTx concentration was calculated using a standard calibration curve. The final values were reported in nanomoles per liter (nM/L) of bone collagen equivalent (BCE) per millimole per liter (mM/L) of creatinine. Serum levels of OPG were measured at baseline and after 12 or 18 months with a competitive inhibition EIA assay with human OPG specific-specific polyclonal antibodies (Human ELISA kit abcam). Test samples were added to precoated 96-well plates. Samples were washed and biotinylated human specific polyclonal detection antibodies were added. Samples were washed again and HRP-conjugated streptavidin was pipetted to the wells. To visualize the HRP enzymatic reaction a substrate TMB was used. HRP catalyzes TMB which produces a blue color product that changes into yellow after adding acidic stop solution. The density of yellow is proportional to the human OPG amount captured in the plate. The values were reported as pg/ml.

2.5 Statistical analysis

The paired t-test was used to compare analysis results between different visits. Fisher's exact test was used to compare result changes (increase vs. decrease) among different dose groups and Kruskal-Wallis test was applied to compare the percent changes for each patient among different dose groups. Wilcoxon signed rank test was applied for comparison of biomarker levels in each of the 2nd, 3rd and 4th visits with that of the 1st visit. Spearman correlation coefficients were computed to evaluate the association between dose and changes in TBA and biomarker levels as well as between TBA changes and cytogenetic response and best overall PCR response rate.

3. Results

3.1 Patient characteristics

Thirty patients with early chronic phase CML without evidence of clonal evolution were evaluated. The median age was 51 years (range, 25–63) with a male to female ratio of 1.5: 1 and a median follow up of 24 months (range, 12–54). Of the 10 female patients, 6 women were post-menopausal, 2 were peri-menopausal and 2 were pre-menopausal. Two different dose groups were assessed (40 mg bid and 100 mg qd).

3.2 Cytogenetic and Molecular Responses

The percentage of patients who achieved CCyR at any time was 97%. The median time to CCyR was 3.1 months. The cumulative MMR rate by 24 months (calculated using the cumulative incidence approach–Kaplan-Meier method and allowing for competing events) was about 80%. There were no significant changes between the two dose groups.

3.3 CA, PO4, BAP, NTX and OPG levels

No significant changes in Ca, PO4, BAP or NTX (serum/urine) levels were observed in all 30 patients when evaluated by linear mixed regression analysis with Tukey adjusted pairwise

comparisons. There was a moderate correlation between first visit BAP and first visit NTX levels with a Spearman correlation coefficient of 0.35 (p value =0.003). Twelve of 16 patients (75%) showed a significant decrease in OPG levels in the evaluated time frame (p value = 0.014) (Table 1). There was no significant difference in findings between the two dose groups (Wilcoxon rank test).

3.4 Digital image analysis

Paired BM specimens of adequate quality were available in 23 of 30 patients. The paired samples were obtained at the time of diagnosis and after commencing dasatinib therapy. The median follow-up time between the two biopsy specimens was 24 months (range: 12–51 months). A significant increase in trabecular bone area (TBA) was noted on post dasatinib BM specimens compared to initial (p=0.022) (Figure 1; Table 2). Seventeen patients (74%) showed an increase in the TBA (mean absolute increase: 5.2%) following dasatinib therapy. Notably 6 patients (3 post-menopausal women, 1 peri-menopausal woman and 2 men) showed a decrease in TBA (mean absolute decrease: 3.1%). No significant association was detected between absolute (p-value = 0.08) or percent change (p-value = 0.12) in TBA and the different dose groups.

3.5 Association between TBA, different biomarkers, cytogenetic and molecular response

No significant association was detected between percent change in biomarker levels and percent change in TBA using Spearman correlation coefficients (correlation coefficient: 0.08, p-value = 0.78). When applying a linear regression model, there was no correlation between TBA change and OPG levels ($R^2=0.0005$, p= 0.9). However, we noted a trend between percent TBA change and OPG level change between visit 1 and 2 yet these numbers were too small to provide statistical power (Figure 3). There was no association between biomarker levels, CCyR, CMR or MMR. There was also no association between TBA change and CCyR, CMR or MMR or between degree of TBA change and decline in *BCR-ABL1* transcripts (p value =0.14) (Figure 4). In addition none of the comparisons at 6 months, 9 months, 12 months, 18 months or 24 months were statistically significant at the 5% level. No adjustment was made for the multiplicity of testing.

4. Discussion

Our results demonstrate that dasatinib can induce changes in trabecular bone remodeling within the therapeutic range in CML patients. Though the amount of bone sampled in a needle biopsy is very small, we more often observed an increase than a decrease in trabecular bone area. We did not identify any significant changes in total Ca, phosphate, serum BAP, serum NTX or urine NTX levels over the evaluated time period. In addition we did not detect any significant correlation between those biomarker levels when compared with each other.

We also did not find any association with cytogenetic and molecular response rates, which is in accordance with prior studies showing that TKIs can interfere with bone metabolism in CML patients independently of cytogenetic/molecular response by abrogating/revoking signal transduction through different independent pathways.^{1–10,31,33–36} This phenomenon,

first described with imatinib, is now known as a potential class effect throughout the majority of TKIs based on their effects on mesenchymal stem cells.³⁷ Dasatinib, as a potent, multitargeted kinase and non-kinase inhibitor and with its somewhat different spectrum of molecular targets from the older agents recapitulates this principle, and causes disturbances in the balance between osteoblast-osteoclast interactions independent of cytogenetic or molecular response rates.³⁸

In a small subset of patients we measured OPG levels and noted a decrease between initial analysis and after commencing therapy. Unfortunately OPG and particularly serum OPG levels are produced by a variety of tissues and cell types and experience great variations based on several regulatory and counter regulatory mechanisms^{39–43} making its use as a surrogate marker for a particular subset of tissue or disease process debatable. Nevertheless, although the sample size of our analysis was too small to provide any sufficient statistical power, we noted a trend where the decrease in OPG levels correlated in 90% of patients with an increase in TBA. The significance of this finding is unknown and further investigations are needed in order to be able to draw a causal relationship.

We did not identify any significant changes or trends in any of the other markers evaluated and we assume that the lack of interference with Ca, PO₄, BAP and NTX levels might suggest that there are probably many other factors, not yet identified, that also interact with bone metabolism and homeostasis.

We acknowledge that there are limitations to this study. A longer follow up in regards to blood, serum and urine biomarkers as well as measurements of PTH and vitamin D levels might have been beneficial to further enhance our understanding of bone homeostasis in CML patients on TKI. In addition we were unable to perform digital image analysis on sequential BM needle biopsy specimens, as the vast majority of patients achieved CCyR and/or MMR and further follow-up consisted only of BM aspiration with cytogenetic / molecular testing. Therefore we are unable to identify any potential fluctuations in TBA % over an extended period of time, in these small biopsy samples. Lastly, as our patient cohort was enrolled in a pre-existing clinical trial which had non-skeletal primary endpoints, we were not able to perform pre and post therapy radiologic assessments from bone densitometry studies or computed tomographic scans in order to allow for a more comprehensive understanding of their skeletal integrity. This became of increasing importance as six patients showed a decrease in TBA, and it is unclear if these patients should have been referred to an endocrinologist for further evaluation.

In summary, our multi-parameter approach takes us one step further in understanding the safety profile and potential interferences of dasatinib in regards to bone health. Our preliminary findings demonstrate that dasatinib can interfere with trabecular bone remodeling in chronic phase CML patients at least in part, as seen in small biopsy samples. Along these lines, several recent studies have noted the effect of dasatinib in the treatment of bone metastasis or its potential role for other endocrine or malignant bone/soft tissue related processes.^{29,30,44–47} While these findings, in conjunction with the most recent literature appear promising with regards to a potential off target drug use, further investigations are needed.

5. Conclusion

The interaction of dasatinib with agents of bone homeostasis is gaining increasing interest. This study represents the first multi-parameter investigation regarding some of the off target effects of dasatinib. However further investigations are needed to understand effects on bone homeostasis in patients on continuous therapy. Therefore we suggest that the ongoing management of CML patients on TKI therapy should include monitoring of bone health and skeletal integrity on a long term basis in order to further enhance our understanding of the safety profile of dasatinib and its efficacy as a potentially new treatment modality for other bone diseases.

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Figure 1. Percentage trabecular bone area (TBA%) at initial diagnosis (1st visit) and after commencing dasatinib therapy (2nd visit)

b

Figure 2.

a: Boxplot of serum BPA by visit

b Boxplot of serum NTX by visit

c: Boxplot of urine NTX by visit

Figure 3.
Correlation between delta OPG and delta TBA (linear regression model $R^2 = 0.0005$, $p = 0.9$)

Figure 4.
Association between percentage change trabecular bone area (Δ TBA) and the rate of change in *BCR-ABL1* transcript levels (Δ BCR-ABL1)

Table 1

Comparison of OPG levels (pg/ml) between the 1st and the 2nd visit

	OPG levels (pg/ml) Mean +/- std, median (range)	Mean of difference in (OPG 1st - OPG 2nd) (95% CI)	Paired t test p value
1 st visit	92.04 +/- 15.6, 97.2 (69.2 – 117.4)	8.13 (1.91 – 14.4)	0.014
2 nd visit	83.9 +/- 17.1, 86.3 (54.96 – 114.6)		

Table 2

Comparison of TBA% between the 1st and the 2nd visit

	N	Mean (std)	Median (range)	Paired t test P value
1st visit TBA %	23	18.67 (7.52)	18.15 (5.89, 37.68)	0.022
2nd visit TBA %	23	23.87 (9.13)	22.59 (6.07, 51.10)	