Abstract

Tumor cells in the bone microenvironment are able to initiate a vicious cycle of bone degradation by mobilizing osteoclasts, multinucleated cells specialized in bone degradation. c-Src is highly expressed both in tumors and in osteoclasts. Therefore, drugs like AZD0530, designed to inhibit Src activity, could selectively interfere with both tumor and osteoclast activity. Here we explored the effects of AZD0530 on human osteoclast differentiation and activity. The effect on osteoclasts formed in vivo was assessed in mouse fetal calvarial explants and in isolated rabbit osteoclasts, where it dose-dependently inhibited osteoclast activity. Its effect on formation and activity of human osteoclasts in vitro was determined in cocultures of human osteoblasts and peripheral blood mononuclear cells. AZD0530 was most effective in inhibiting osteoclast-like cell formation when present at the onset of osteoclastogenesis, suggesting that Src activity is important during the initial phase of osteoclast formation. Formation of active phosphorylated c-Src, which was highly present in osteoclast-like cells in cocultures and in peripheral blood mononuclear cell monocultures, was significantly reduced by AZD0530. Furthermore, it reversibly prevented osteoclast precursor migration from the osteoblast layer to the bone surface and subsequent formation of actin rings and resorption pits. These data suggest that Src is pivotal for the formation and activity of human osteoclasts, probably through its effect on the distribution of the actin microfilament system. The reversible effect of AZD0530 on osteoclast formation and activity makes it a promising candidate to temper osteoclastic bone degradation in bone diseases with enhanced osteoclast activity such as osteolytic metastatic bone disease. (Mol Cancer Res 2009;7(4):476–88)

Introduction

The presence of bone metastasis is a frequent phenomenon in patients with common tumors such as breast (frequency of bone metastases, 65-75%), prostate (65-75%), and lung (30-40%) carcinomas (1). Tumor cells in close vicinity to the bone surface are able to contribute to the formation of osteoclasts (multinucleated cells specialized in bone degradation) and thus cause bone lysis, which often results in severe skeletal complications (1). Knowledge about proteins specifically involved in the activity of osteoclasts is a prerequisite for developing therapies targeting the osteoclast in pathologies such as cancer-related bone lysis and may also be beneficial in rheumatoid arthritis, osteoporosis, and periodontitis, where excessive and unwanted resorption occurs.

One of the characteristic manifestations of the osteoclast is its multinuclearity. In vivo, cells of the osteoblastic lineage play an orchestrating role in attracting osteoclast precursors and in delivering the proper imprint thought to be pivotal for osteoclast differentiation, such as macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL; reviewed in ref. 2). Ultimately, osteoclast precursor cells migrate to the bone surface and fuse (3, 4). At the bone surface, mature multinucleated osteoclasts form a specialized adhesive structure, the sealing zone. This zone contains a high level of filamentous actin (F-actin), which is characteristically distributed in a ring-like structure (reviewed in ref. 5). In conjunction with this structure, adhesion molecules, such as αvβ3 integrins (6-8), provide the means for the cells to strongly adhere to the bone surface. This unique structure is essential for bone resorbing osteoclasts. Osteoclasts lacking proteins critical for the assembly of the sealing zone cannot form a ruffled border, which is the extracellular secondary lysosome necessary for dissolving minerals and degrading bone matrix proteins (6-8).

One such protein critical for osteoclast activity is c-Src kinase, which is highly expressed in osteoclasts (9, 10). c-Src is indispensable for the formation of a sealing zone and subsequent ruffled border because osteoclasts from c-Src knockout mice lack the capacity to redistribute F-actin as ring-like structures (8, 11). Consequently, ruffled borders are not formed (12), leading to osteopetrosis and early death (13). The osteoclastic function of c-Src null mutants can be rescued with transplantation of bone marrow cells from wild-type mice (10, 14). Surprisingly, the only detectable defect in c-Src knockout mice is the malfunctioning osteoclasts, despite high expression of c-Src.
in brain and in platelets (13). This makes c-Src an attractive candidate target protein for therapeutic intervention in bone diseases with excessive bone resorption (15). It can be conceived that combating osteoclastic activity based on c-Src inhibition can be a valuable alternative for interfering with osteoclastic activity in patients with bone-lytic tumors because the present treatment with frequent use of bisphosphonates can lead to unwanted side effects like osteonecrosis of the jaw (16). Previous in vivo and in vitro studies indeed showed that Src inhibitors, such as CPG77675, can partially prevent ovariectomy-induced bone loss in rats (17). Treatment of rats with the Src inhibitors CPG77675 and CPG76030 caused an increase in bone mineral density, possibly by inducing osteoclast apoptosis and by reducing osteoclast formation (18). The Src inhibitor PP2 prevented transmigration of mouse osteoclasts underneath a layer of osteoblasts (4). However, it is unknown whether Src inhibitors inhibit the formation and activity of human osteoclasts.

Interestingly, high expression and/or activity of Src family kinases (including c-Src) is observed in many cancer cell lines and tissues. In a mouse model using breast cancer cells that metastasize to bone, the frequency of bone metastases decreases and the survival of mice increases when treated with the Src inhibitor CPG76030 (19). Furthermore, using a genetic approach, these mice survive on injection of c-Src–defective MDA-MB-231 breast cancer cells, whereas the same mice die on injection of MDA-MB-231 cells containing wild-type c-Src (19). Src inhibitor CPG76030 affected not only the osteoclasts, as indicated above, but also tumor cells (18, 19). This makes c-Src kinase an eligible molecule to design drugs against, which may combat both tumor load and osteoclastic activity.

Until recently, an important caveat of existing Src kinase inhibitors, however, was the applicability in humans. The recently described orally available Src inhibitor AZD0530 selectively inhibits Src and is well tolerated in man (20). AZD0530, which binds to the kinase domain of c-Src and its Src kinase family members (20), has antitumor properties because it inhibits tumor growth of human pancreatic cancer in a mouse model (20) and suppresses motility and invasiveness of MCF7 breast cancer cells (21). In addition, AZD0530 is the first Src inhibitor to show inhibition of the Src pathway in human tumor tissue (22). Preliminary data suggest that AZD0530 could specifically affect osteoclastic bone resorption because resorption markers present in serum and urine were significantly reduced after single (volunteers) or multiple daily doses of AZD0530 (volunteers and patients with cancer), whereas markers for bone formation were not affected (23-25). AZD0530 is now being tested specifically in cancer patients with metastatic bone disease to determine its effect on bone turnover markers in this population (22). These initial findings could be of paramount importance for future clinical applications; however, it is unknown whether and how AZD0530 affects the stages of human osteoclast formation and activity.

In the present study, we aimed to elucidate whether AZD0530 affects the different processes required for osteoclast formation and activity. First, we tested the effect of AZD0530 on mouse and rabbit osteoclasts formed in vivo. Second, we used a coculture system of human osteoblasts in conjunction with human peripheral blood mononuclear cells (PBMC; refs. 26, 27) because osteoclasts play a key role in the attraction of osteoclast precursor cells and in the formation of osteoclasts in vivo (3). Using these approaches, we thus assessed the effect of AZD0530 on all stages of (human) osteoclast formation and activity: adhesion of osteoclast precursor cells, formation of multinucleated cells, attachment of the cells to the bone surface, and resorptive activity.

**Results**

Effect of AZD0530 on Osteoclast Activity in Cultures of Mouse Calvarial Bone and Isolated Rabbit Osteoclasts

AZD0530 Inhibits Osteoclast Activity in Calvarial Bone Cultures. The Src inhibitor AZD0530 at concentrations of 5.0 and 10.0 μmol/L significantly inhibited 45Ca release by 30% and 50% from cultured fetal calvarial mouse bone explants (Fig. 1).

AZD0530 Inhibits Resorption by Isolated Rabbit Osteoclasts. Rabbit osteoclasts were seeded on cortical bone slices in the presence of different concentrations of AZD0530. After 24 hours, resorption pits could be visualized under all conditions (Fig. 2A). However, the percentage of resorbed bone (Fig. 2B), the number of pits per square millimeter (Fig. 2C), and the pit size (Fig. 2D) were significantly reduced at higher concentrations of AZD0530 (1.0 and 5.0 μmol/L).

Effects of AZD0530 on the Formation and Activity of Human Osteoclasts

AZD0530 Does Not Interfere with Initial Adhesion of PBMCs to Osteoblasts. Adhesion of osteoclast precursor cells to cells of the osteoblastic lineage represents one of the first steps in osteoclast formation in vivo. AZD0530 did not affect the initial adhesion of PBMCs to the osteoblasts. The number of adhered PBMCs ranged from 325 ± 152 for control cultures to 189 ± 97 in cultures incubated with 10 μmol/L AZD0530 (data are mean ± SD, n = 5; not significant).
AZD0530 Dose-Dependently Inhibits Osteoclast Formation in Osteoblast/PBMC Cocultures. We next assessed the effect of AZD0530 on the formation of tartrate-resistant acid phosphatase (TRACP)–positive multinucleated cells, representing osteoclast-like cells. In the presence of AZD0530 at 0.1 to 10 μmol/L, no effects were observed on osteoblast morphology when cultured in monoculture (Fig. 3A, top row). However, AZD0530 prevented spreading of PBMCs when cultured without (Fig. 3A, middle row) or with (Fig. 3A, bottom row) osteoblasts.

TRACP-positive multinucleated cells were formed after 21 days in the absence of AZD0530 (Fig. 3B). Treatment with AZD0530 caused an arrest of osteoclast differentiation (i.e., mononuclear cells were attracted to osteoblasts), but these cells failed to form a syncytium (Fig. 3C and D). The inhibitory effect of AZD0530 on the formation of osteoclast-like cells was dose dependent; maximal inhibition was reached at 5 μmol/L (Fig. 3E). Strikingly, only a certain percentage of osteoblasts attracted PBMCs (Fig. 3C and D), indicating that the osteoblast population is heterogeneous in its ability to bind PBMCs.

AZD0530 was not toxic in the 0.1 to 10 μmol/L range because adenylate kinase could not be measured in cocultures (Fig. 3F) or in monocultures of osteoblasts or PBMCs either cultured in the absence or presence of M-CSF and RANKL (data monocultures not shown).

Effect of Src Inhibition by AZD0530 on Gene Expression in Osteoblast/PBMC Cocultures. To gain insight into possible mechanistic aspects of Src inhibition, we assessed whether AZD0530 could alter the expression of genes important for osteoclast formation. Osteoblast/PBMC cocultures were incubated for 24 hours with AZD0530 and analyzed at two time points: 1 week and 3 weeks. These time points respectively represent the adhesion stage and the end stage where multinucleated TRACP-positive cells are present in the coculture. Adhesion molecule intercellular adhesion molecule-1 (ICAM-1), which is crucial for osteoblast-osteoclast precursor interaction (26), parathyroid hormone–related protein (PTHrP), which is highly expressed in metastatic clones of the c-Src expressing MDA-MB-231 cell line (28), osteoclastogenesis factor RANKL and its regulator osteoprotegerin (OPG), osteoblast marker tissue nonspecific alkaline phosphatase, and osteoclast
marker cathepsin K were assessed in osteoblast and osteoblast/PBMC cocultures (Fig. 4).

The expression of ICAM-1 was higher in cocultures compared with osteoblast monocultures, but it was not changed by AZD0530 (Fig. 4, first row). Similarly, PTHrP expression was not changed by Src inhibitor AZD0530 (Fig. 4, second row). RANKL expression, however, was consistently lower in AZD0530-treated cultures, in osteoblast and in osteoblast/PBMC cultures, both at 1 and 3 weeks (Fig. 4, third row). The osteoclast marker cathepsin K was present in cocultures and not in monocultures of osteoblasts. In line with the time needed for osteoclast differentiation, cathepsin K expression was markedly higher at 3 weeks. Inhibition of Src did not change the expression levels of cathepsin K (Fig. 4, bottom row). Src inhibition did not alter the expression of OPG or of the osteoblast marker tissue nonspecific alkaline phosphatase or the macrophage colony forming factor M-CSF (data not shown).

AZD0530 Inhibits All Stages of Osteoclast Formation in Osteoblast/PBMC Cocultures. The data presented above indicate that Src kinase is essential for osteoclast formation, as shown by the inhibition of the formation of TRACP-positive multinucleated cells by AZD0530 when continuously present during the 3-week period needed for the formation of TRACP-positive multinucleated cells. We next assessed whether certain stages of osteoclast formation are more susceptible than others to inhibition of Src kinase activity. AZD0530 (1 μmol/L) was administered for 1 to 2 weeks, either at the beginning or at the end of the 3-week culture period, or continuously for 3 weeks (see Fig. 5A, black bars), and the effect on the formation of TRACP-positive multinucleated cells was investigated. Addition of AZD0530 to all cultures resulted in a significantly lower number of TRACP-positive multinucleated cells (Fig. 5B). Moreover, the inhibitory effect was strongest when AZD0530 was added during the initial phase of coculture. Equally low numbers of TRACP-positive multinucleated cells were found when cells were cultured in the presence of the inhibitory compound for the first week only or during the whole 3-week period.

To study the effect of AZD0530 on osteoclast formation by PBMCs in more detail, we made use of *in vitro* osteoclastogenesis assays without osteoblasts. The PBMCs were cultured at higher densities than in the cocultures with or without the cytokines M-CSF and RANKL (Fig. 5C-E). When PBMCs were cultured without cytokines, AZD0530 elicited its inhibitory effect when added in the first week (Fig. 5C), analogous to cocultures of osteoblasts and PBMCs. Formation of TRACP-positive multinucleated cells was completely abolished and resulted in the presence of exclusively mononuclear TRACP-positive cells, which were present at a very low density (Fig. 5E, top and middle). Inhibition of Src at later time points had no effect on osteoclast formation. Remarkably, formation of TRACP-positive multinucleated cells was not inhibited by AZD0530 in PBMC cultures, which were cultured in the presence of M-CSF and RANKL (Fig. 5D).

We next assessed the extent of c-Src expression and the effect of AZD0530 on the expression of the active phosphorylated form in the three types of osteoclastogenesis cultures (i.e., cocultures of osteoblasts and PBMCs, and PBMCs cultured at higher densities in the absence or presence of M-CSF and RANKL) by Western blotting (Fig. 6A) and quantitative real-time PCR (Fig. 6B). Typically, TRACP-positive multinucleated cells were present at the 3-week time point in all three assays. Compared with the positive control, the bone metastatic breast cancer cell line MDA-MB-231 expressed higher levels of c-Src (19, 28). c-Src was expressed at much higher levels in all osteoclastogenesis cultures and also in osteoblasts. Addition of 1 μmol/L AZD0530 for the last 24 hours consistently and considerably reduced the presence of the active phosphorylated form of c-Src (Fig. 6A, top). The expression of total c-Src at the protein level (Fig. 6A, middle) or at the mRNA level (Fig. 6B) was not affected by AZD0530.

AZD0530 Reversibly Inhibits the Formation of Actin Rings. For osteoclast activity analyses on bone slices, cocultures of osteoblasts and PBMCs in culture medium were supplemented with M-CSF and RANKL. In a previous coculture study and also in the present study (data not shown), we found that these molecules were pivotal for the newly formed osteoclasts to exert their osteoclastic activity (29). The cocultures in the present study were done for 3 weeks on bone slices, either in the absence or presence of 1 μmol/L AZD0530. We analyzed whether osteoclast activity recovered in the 2-week culture period after administration of 1 μmol/L AZD0530 during the first week. Actin rings were formed in control cultures (Fig. 7A), but the number of actin rings was reduced in cultures that received AZD0530 for the total 3-week culture period (Fig. 7B). More specifically, osteoblasts retracted from the bone surface at sites where multinucleated cells containing actin rings were formed in control cultures (Fig. 7D), whereas osteoclast precursors were arrested on top of the osteoblast layer in cultures treated with AZD0530 for 3 weeks (Fig. 7E). The effect of AZD0530 proved to be reversible because actin rings were present again in cultures that had received the Src inhibitor during the first week of the 3-week coculture period (Fig. 7C). The proportion of osteoclastic cells containing actin rings was significantly reduced in cultures treated for 3 weeks with the Src inhibitor compared with both untreated control cultures and cultures treated with AZD0530 during the first week only. The percentage of osteoclast-like cells with rings in the cultures that received AZD0530 in the first week only was similar to the control cultures, indicating that recovery of osteoclastogenesis had taken place (Fig. 7F). The actin ring perimeter was largest in untreated control cultures and smallest in cultures treated with the Src inhibitor for 3 weeks (Fig. 7G).

AZD0530 Inhibits Osteoclast Activity. Finally, we analyzed the effect of AZD0530 on osteoclast resorptive activity. No bone resorption was observed in osteoblast/PBMC cocultures in the absence of M-CSF and RANKL. Resorption pits were formed in all cocultures where M-CSF and RANKL were added (Fig. 8A). However, bone resorption was completely blocked in cultures treated with AZD0530 for 3 weeks (Fig. 8B). Resorption pits were detectable in one of five osteoblast/PBMC cocultures when AZD0530 was present during the first week only (Fig. 8C). Under these conditions, not only was the number of pits very low but also the size of the pits was much smaller (Fig. 8D).
Src Inhibitor AZD0530 Inhibits Osteoclast Formation

Discussion

In an aging society, it can be foreseen that the incidence of diseases associated with undesired bone loss, such as metastatic bone disease, rheumatoid arthritis, osteoporosis, and periodontitis, will increase considerably. Thus, drugs specifically targeting osteoclasts and their activity are desirable, possibly as supplementary agents to existing therapies. Orally applicable Src inhibitors could be advantageous compared with the widely used bisphosphonates because the latter can cause complications in the upper gastrointestinal tract (15) or can lead to osteonecrosis of the jaw (16). Indeed, the Src inhibitor CGP76030 was shown to reduce the number of osteoclasts in rat tibiae more efficiently than the bisphosphonate alendronate (18). Little is known, however, of the effect of Src inhibitors on human osteoclasts. Here, we describe the effect of Src inhibitor AZD0530 on osteoclast formation and activity in the different models used (osteoblast/PBMC cocultures, PBMCs cultured at high density without and with M-CSF + RANKL). AZD0530 did not only inhibit osteoclast activity in a mouse ex vivo model as well as in a rabbit in vitro model but it also inhibited the formation and activity of human osteoclasts, where c-Src was very highly expressed. AZD0530 grossly reduced the levels of the active phosphorylated form of c-Src in all human osteoclast models used. The inhibitory effect on osteoclast formation and activity proved to be reversible because withdrawal of AZD0530 reversed the inhibition of actin ring formation as well as bone resorption. In search of an alternative for bisphosphonates for the treatment of patients who are at risk of developing osteonecrosis of the jaw, this reversibility of action of AZD0530 (23, 24) is an important consideration because cessation of bisphosphonates does not seem to stop the development of osteonecrosis of the jaw (16).

Dosages for maximal effect of AZD0530 to inhibit osteoclast formation and activity varied between the model systems. For example, AZD0530 was effective at 0.1 μmol/L on isolated rabbit osteoclasts, 1 μmol/L was sufficient in human osteoclastogenesis assays, whereas 10 μmol/L inhibited only 50% osteoclastic activity in the mouse calvaria assays. The range of dosages used is consistent with concentrations found in human target tissues such as tumors, where AZD0530 accumulates at concentrations in the micromolar range.5

Considering the effect of AZD0530 on the sequence of events in osteoclast formation, it became apparent that AZD0530 did not interfere with the initial binding of PBMCs to osteoblasts, but did so with stages of osteoclast differentiation. In line with the lack of an effect on binding is the unaltered mRNA expression of ICAM-1, a cell-cell adhesion molecule important in osteoclastogenesis (26). An intriguing finding was the difference in binding of PBMCs to osteoblasts. To some osteoblasts, high numbers of PBMCs were attached whereas other osteoblasts were devoid of PBMCs. Possibly, cultured osteoclasts are heterogeneous in their expression of cell adhesion molecules. Such heterogeneity has previously been described for osteoblasts expressing ICAM-1, where ICAM-1-positive osteoclasts were particularly involved in the attachment and subsequent differentiation of osteoclast precursor cells (26). Interestingly, we found that AZD0530 caused an arrest at the prefusion stage of osteoclast precursor cells (i.e., they did not attach to osteoclasts). Migration of PBMCs from the osteoblasts to the bone surface and subsequent fusion were blocked, possibly due to hampered actin redistribution in both osteoblasts and osteoclast precursors, which both express high levels of c-Src (Fig. 6). In cancer cells, c-Src activity promotes dissemination by weakening intercellular adhesions (30). AZD0530 restores cell-cell contacts of breast carcinoma cells (21). Analogously, in our study osteoclast precursors stayed firmly attached to osteoblasts when c-Src activity was inhibited by AZD0530. Osteoclast formation from RAW264 cells, in which c-Src was knocked out, was also completely inhibited, possibly due to hampered cell migration (31). In c-Src knockout mice, however, osteoclast formation was enhanced rather than inhibited (12). As a reconciliation of this apparent discrepancy between our findings and the findings in knockout mice, it should be noted that AZD0530 is not a specific c-Src inhibitor but rather a selective inhibitor of the whole family of Src kinases. Thus, one may assume that other Src family kinases possibly involved in osteoclastogenesis are also affected. A combined blockade of cellular activities of both osteoblasts and osteoclast precursors

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5 T.P. Green, unpublished results.
FIGURE 5. Temporal inhibition of Src reduces formation of TRACP-positive multinucleated cells in osteoblast/PBMC cocultures. A. AZD0530 at 1 μmol/L was added for 1, 2, or 3 wk during a total of 3 wk of osteoblast/PBMC coculture at the indicated time frame. Black columns, exposure period with AZD0530. B and D. Formation of TRACP-positive multinucleated cells after exposure to the Src inhibitor AZD0530 for the indicated time periods. B. Osteoblast/PBMC cocultures. C. PBMCs cultured without cytokines. **, P < 0.01, compared with controls and to conditions 1+2A and 2+1A. D. PBMCs cultured in the presence of M-CSF and RANKL. E. Micrographs of TRACP-positive multinucleated cells (white arrows) formed in PBMC cultures without cytokines (−) or cultured with M-CSF and RANKL (MR). Examples are shown of cultures not exposed to AZD0530 (left) or exposed in the first week (1A+2; middle) or in the last 2 wk (1+2A; right). Columns, mean number of TRACP-positive multinucleated cells; bars, SD. Cocultures with osteoblasts were from five patients; PBMC cultures are results of a triplicate plating. *, P < 0.05; **, P < 0.01; ***, P < 0.001, significant effect of AZD0530. Culture conditions are labeled as follows: −, 3 wk without AZD0530; 1A+2, 1 wk with AZD0530 followed by 2 wk without AZD0530; 2A+1, 2 wk with AZD0530 followed by 1 wk without AZD0530; 3A, 3 wk with AZD0530; 1+2A, 1 wk without AZD0530 followed by 2 wk with AZD0530; 2+1A, 2 wk without AZD0530 followed by 1 wk with AZD0530.
could also cause a lack of osteoclast formation. AZD0530 also inhibits family members of c-Src such as c-Yes (20), but osteoclasts were not affected in c-Yes knockout mice (32).

Our study revealed that AZD0530 elicits its inhibitory effect on osteoclast formation primarily when present early during the 3-week culture period. The formation of multinucleated cells was inhibited to the same extent when AZD0530 was added for the first 1 or 2 weeks or was present continuously during 3 weeks of culture. Significantly more osteoclast-like cells were formed in cultures that did not receive the Src inhibitor for the first 1 or 2 weeks, followed by 2 weeks or 1 week of culture in the presence of AZD0530. Our results seem to imply that inhibition of c-Src–dependent signaling events is critical especially during the early stages of osteoclast differentiation. When M-CSF and RANKL were added, rescue of osteoclast activity was seen after withdrawal of Src inhibitor on bone slices. It could be that addition of M-CSF (and RANKL) may attenuate this reversible effect, which was similarly reported for mice deficient in DAP12, which, like Src, is a molecule pivotal in actin remodeling (33). Compared with PBMCs, which were cultured without cytokines, addition of M-CSF and RANKL to PBMCs, which were cultured in the absence of osteoblasts, abolishes the effect of AZD0530 on osteoclast formation when added during the first week of culture. Previously, Nakamura et al. (34) described that prefusion osteoclast precursor cells from c-Src−/− mice are severely impaired in adhesion to and spreading on vitronectin-coated and noncoated surfaces, as a consequence of a lack of phosphorylation of proteins downstream of c-Src. Interestingly, defective adhesion and spreading could be overcome when adding M-CSF, which activates phospholipase Cγ also in the absence of c-Src (34). Analogous in our study, spreading of prefusion osteoclast precursors without or with reduced c-Src activity due to AZD0530 was possibly rescued by M-CSF (and RANKL), resulting in normal osteoclastogenesis.

Even when administered at high concentrations, AZD0530 seemed to have no effect on the morphology of osteoblasts (Fig. 3A) and proved to be nontoxic (Fig. 3F). Furthermore, expression of alkaline phosphatase was not altered by AZD0530 (data not shown). In line with these observation are the findings that AZD0530 treatment does not alter serum levels of osteoblast-related proteins like bone alkaline phosphatase in healthy volunteers (23) or in cancer patients with advanced disease (24). Like AZD0530, Src inhibitor CGP97675 seemed to be nontoxic for osteoblasts (17). Our findings further suggest that AZD0530 could also contribute to a reduced osteoclast formation by decreasing RANKL expression in osteoblasts.

Actin rings were not formed in cultures where AZD0530 was supplemented continuously. However, we show that human osteoclasts containing F-actin rings are present not only in osteoblast/PBMC cocultures that were cultured without AZD0530 but also in cultures that had received AZD0530 only in the first week. This finding is in agreement with the observation that bone resorption was present in one of the five cocultures with the same treatment. Hitherto, this aspect of reversibility has not been addressed in any study using Src inhibitors. In line with the reversibility of the action of the compound is the observed increase toward baseline in serum and urine levels of osteoclast markers on cessation of intake of AZD0530 (23). This feature makes AZD0530 a potential drug for applications where temporal inhibition of osteoclast activity is desired, albeit one can expect that physiologic bone turnover will also be affected.

Bone metastases occur frequently in the most common tumors (1). Tumor cells in close vicinity to the bone surface contribute to the formation of osteoclasts, which then cause bone

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**FIGURE 6.** Reduced levels of active phosphorylated c-Src protein in osteoclast cultures and in osteoblasts after AZD0530 exposure. PBMCs were cultured in the presence of osteoblasts (OB+PBMC), without (PBMC) or with M-CSF and RANKL (PBMC+MR) for 3 wk, either followed (+) or not (−) by 24-h treatment with 1 μmol/L AZD0530. Western blots (A) show that AZD0530 reduces (arrows) the presence of the phosphorylated form (Y418) of c-Src (top) and not total c-Src (middle). Loading control, vinculin (bottom). Total protein (10 μg) was loaded per lane. Quantitative PCR (B) revealed that AZD0530 did not effect the expression of c-Src. For mRNA analysis, cocultures from three osteoblasts and triplicates from PBMC cultures and MDA-MB-231 are shown.
lysis often resulting in severe skeletal complications (1). These patients typically receive bisphosphonates to combat osteoclast function (35-37). Interference with Src activity could be considered for the treatment of bone lysis. Inhibition of Src may temper not only osteoclast activity but also tumor cell properties such as motility and invasiveness. The Src inhibitor AZD0530 has been shown to inhibit cell migration and invasion as well as the formation of focal adhesions in human breast cancer cells (21, 30). We report here for the first time on the inhibitory effect of AZD0530 on the formation and activity of human osteoclasts. These findings, together with its tolerability and effectiveness at inhibiting serum bone resorption peptides (β-CTX) in both human volunteer subjects (23) and phase I cancer patients (24), make AZD0530 an important candidate drug (22) to evaluate inhibition of bone metastatic disease at both the tumor and osteoclast levels.

Materials and Methods
Reagents
Culture medium for all coculture experiments consisted of DMEM (Life Technologies, Inc.) supplemented with 10% FCS (HyClone), 1% antibiotics [100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (antibiotic antymycotic solution, Sigma)], 10⁻⁸ mol/L dexamethasone (Sigma), and 10⁻⁷ mol/L 1,25-dihydroxyvitamin D₃ (Sigma). A 10 mmol/L solution of AZD0530 (salt: Mw 774.0 g/mol) in DMSO was diluted 1:10 in culture medium to obtain a 100 μmol/L stock solution, which was used for preparing all dilutions of AZD0530. All cultures (i.e., with or without AZD0530) contained 0.01% DMSO. AZD0530 was supplied by AstraZeneca. The concentrations of AZD0530 used in vitro were potentially clinically relevant based on currently available pharmacokinetic data (20).

FIGURE 7. Reversible inhibition of actin ring formation in osteoclasts by AZD0530 on cortical bone slices. Low magnification of actin staining (green) in osteoblast/PBMC cocultures in the absence (A) and presence (B) of AZD0530 (1 μmol/L) or in cultures where AZD0530 (1 μmol/L) was added during the first week of the 3-wk culture period (C). Actin rings (arrows) were present in A and C. In more detail (D and E; nuclei in red, actin in green), syncitia were present on the bone surface at sites where osteoblasts retracted from the bone surface when cultured without AZD0530 (D). PBMCs remained on top of the layer of osteoblasts when cultured for the total 3-wk culture period in the presence of AZD0530 (E). Percentage of osteoclasts (OC) and osteoclast precursors (OCp) with actin rings (F) and actin ring size (G) were significantly reduced when cells were cultured in the presence of AZD0530 for 3 wk (3A). The number of actin rings and actin ring size were not affected or less affected when cells were cultured for 1 wk with AZD0530 followed by 2 wk without the inhibitor (1A+2). Columns, mean percentages (F) and mean ring size (G); bars, SD. Cocultures with osteoblasts were from five patients. *, P < 0.05; **, P < 0.01, significant effect of AZD0530. Bar, 150 μm (A-C); 60 and 100 μm (D and E).
Osteoclast Activity Assay Using Mouse Calvarial Explants

Permission for the use of animals to perform the experiments described in this study was obtained from the Animal Welfare committee of the VU University Amsterdam. Bone organ cultures were used to investigate the effects of AZD0530 on calcium release, which is a measure of osteoclast resorption activity.

Bones were labeled in utero by injection of the mother with 100 μCi 45CaCl2 (Amersham Biosciences) at 16 d of gestation. Seven-day-old neonatal mice were sacrificed and calvarial bones were dissected as described previously (38). Each calvaria was divided into halves. Calvarial bones were cultured overnight in α-MEM (Life Technologies) supplemented with 1 mmol/L β-glycerophosphate (Sigma), 0.6 mmol/L ascorbic acid (Sigma), and 10% FCS to exchange the free 45Ca. Paired calvarial bones were cultured for 3 d, one with and one without the addition of 0.1 to 10 μmol/L AZD0530. All cultures received the same amount of the vehicle DMSO. The amount of 45Ca released into the medium was measured with a liquid scintillation counter (Wallac). 45Ca present in the calvariae was extracted with 5% trichloroacetic acid. The amount of 45Ca released into the medium is given as a percentage of the total amount (38).

Osteoclast Activity Assay Using Rabbit Osteoclasts

Osteoclasts were isolated as described before (25). Briefly, long bones were dissected from 5-d-old Chinchilla rabbits, and the periosteum were removed. The bones were minced with scissors in α-MEM containing 5% FCS. The minced bone fragments were sedimented, and the supernatant containing osteoclasts was centrifuged for 1 min at 200 × g. The cell pellet was reconstituted, and 2 × 10⁶ cells were seeded in a 50 μL volume on 650-μm-thick bovine cortical bone slices in 24-well culture plates and allowed to adhere for 1 h. The bone slices were incubated for 48 h in 300 μL α-MEM containing 5% FCS and supplemented with AZD0530 at different concentrations (0.1-5 μmol/L) or with vehicle (DMSO). All cultures received the same amount of the vehicle DMSO. Medium was changed after 24 h. After 48 h, the bone sections were cleaned carefully in 0.25 mol/L NH₄OH, thereby removing all cells, washed in distilled water, and stained with Coomassie brilliant blue. The percentage of bone resorption and the individual pit sizes were assessed using a computerized X-Y tablet (QWin, Leica). The percentage of bone resorption was expressed as the percentage of resorbed bone surface per total bone surface area.

Coculture Experiments Using Human Osteoblasts and PBMCs

Cells

Osteoblasts. Bony waste material was obtained with informed consent from osteoarthritic patients who underwent knee or hip surgery at the Department of Orthopaedic Surgery of the VU Medical Centre (VUmc) in Amsterdam. Osteoblast cultures were established as described previously (39). The osteoblastic character of the cells was consistently shown (26). Outgrowth of osteoblasts from bone chips of ∼1 mm occurred after ∼1 to 2 wk. Third or fourth passage cells were used for all experiments.

PBMCs. PBMCs from buffy coats (Sanquin) were isolated as described previously (29). Buffy coats were diluted 1:1 in HBSS containing 2% FCS. Twenty-five milliliters of diluted blood were carefully layered on 15-mL lymphoprep (Axis-Shield Po CAS) and centrifuged for 30 min at 1,200 × g without brake. The interphase containing PBMCs was washed and then recovered in DMEM containing 10% FCS and 1% antibiotics.

FIGURE 8. Inhibition of bone resorption by AZD0530. Bone resorption was assessed on cortical bone slices after a 3-wk culture period. Osteoblast/PBMC cocultures were established in the presence of M-CSF+ RANKL, either in the absence (A) or presence of 1 μmol/L AZD0530 (B), or 1 wk with 1 μmol/L AZD0530 followed by 2 wk without the inhibitor (C). Bone resorption was present in all (A), none (B), or in 1 of 5 specimens (C). Resorption pits (arrows) were visualized with Coomassie brilliant blue (resorption assay was done with cocultures with osteoblasts from five patients). Bar, 100 μm. D. Pit size was smaller in cultures that received 1 wk of 1 μmol/L AZD0530 followed by 2 wk without the inhibitor (1A+2); *** P < 0.001. No pits were observed in cultures that had received AZD0530 for 3 wk (3A). Columns, mean size of 48 (control) and 84 (1A+2) pits; bars, SE.
Cell Adhesion

Preliminary experiments showed that proper adhesion of human osteoblasts and PBMCs in cocultures was achieved after 3 d of culture (data not shown). Seeding of $1.5 \times 10^4$ osteoblasts in 48 wells followed by overnight incubation resulted in a confluent cell layer. The next day, $5 \times 10^4$ PBMCs were seeded in 400 μL medium per well without or with 0.1 to 10 μmol/L of AZD0530 in 0.01% DMSO. All cultures received the same amount of the vehicle DMSO. After 3 d, the supernatants were removed and cells were washed thrice with 0.5 mL of culture medium. Adherent cells were counted on five micrographs per well.

Osteoclastogenesis Assays

Coculture experiments were done in 48-well plates as described (29). Osteoblasts and PBMCs were cultured in the presence of $10^{-8}$ mol/L dexamethasone and $10^{-7}$ mol/L 1,25-dihydroxy vitamin D$_3$ (dex + vit D$_3$). Osteoblasts from five patients were used for each coculture experiment. Culture medium (1 mL/well) was replaced twice a week. In experiments testing the effect of AZD0530 on osteoclast activity, the PBMCs were cocultured with osteoblasts on cortical bone slices in the absence or presence of M-CSF and RANKL. The cells were cultured for 10 d followed by 24 h of treatment with AZD0530 (range, 0.1-10 μmol/L). Release of adenylate kinase was measured with ToxiLight Nondestructive Cytotoxicity BioAssay kit (Cambrex). As a positive control, cells were incubated for 10 min in culture medium containing 0.5% Triton X-100.

mRNA Analysis and Real-time Quantitative PCR

RNA isolation and real-time quantitative PCR were done as described in detail in ref. 29. Real-time PCR primers were designed using the Primer Express software, version 2.0 (Applied Biosystems; Table 1). To avoid amplification of genomic DNA, each ampiclon spanned at least one intron. The standard curve used in the PCR reactions, cDNA was used from bone marrow that was cultured for 4 d with M-CSF + RANKL. The PCR reactions of the different ampiclons had equal efficiencies.

Porphobilinogen deaminase (PBGD) was used as the housekeeping gene (43). The expression of this gene was not affected by the experimental conditions. Samples were normalized for the expression of PBGD by calculating the ΔCt (CtPBGD − Ct gene of interest) and expression of the different genes was expressed as $2^{-\Delta \Delta Ct}$.

Western Blot Analysis

The expression of c-Src and the effect of AZD0530 on activated/phosphorylated c-Src were established in the various osteoclastogenesis assays. Osteoblasts, cocultures of osteoblasts with PBMCs, and PBMCs in the presence or absence of M-CSF and RANKL were cultured for 21 d followed by a 24-h culture in the presence of 1 μmol/L AZD0530. Cells were minced. Multinucleated cells were analyzed using Image-Pro Plus software (Media Cybernetics).

Cytotoxicity

The cytotoxicity of AZD0530 was assessed for the following cultures: monoculture of osteoblasts, cocultures of osteoblasts with PBMCs, and monoculture of PBMCs in the absence or presence of M-CSF and RANKL. The cytotoxicity of AZD0530 was assessed for the effect of AZD0530 on activated/phosphorylated c-Src were established in the various osteoclastogenesis assays. Osteoblasts, cocultures of osteoblasts with PBMCs, and PBMCs in the presence or absence of M-CSF and RANKL were cultured for 21 d followed by a 24-h culture in the presence of 1 μmol/L AZD0530. Cells were

### Table 1. Quantitative PCR Primers Used

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sequence 5′→3′</th>
<th>Amplicon Length (bp)</th>
<th>Ensembl Gene ID</th>
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<tr>
<td>PBGD</td>
<td>TgCAGTTTTGAAATCATCTgTGCTTCGTC</td>
<td>84</td>
<td>ENSG00000113721</td>
</tr>
<tr>
<td>c-Src</td>
<td>GACATCAAGAGTCCGCAAGCT</td>
<td>106</td>
<td>ENSG00000197122</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>TgATCTGTTCGTTGACATACTG</td>
<td>104</td>
<td>ENSG00000090339</td>
</tr>
<tr>
<td>PTHrP</td>
<td>AGCGTCGCGGTGTTCCT</td>
<td>98</td>
<td>ENSG0000007494</td>
</tr>
<tr>
<td>RANKL</td>
<td>CAGCTCAAATCTTGGATGGACTT</td>
<td>60</td>
<td>ENSG00000120659</td>
</tr>
<tr>
<td>OPG</td>
<td>CATCGGGCTGGTTCGTCCAT</td>
<td>100</td>
<td>ENSG00000164761</td>
</tr>
<tr>
<td>M-CSF</td>
<td>CCAGCGAGGCTCTCGAGTGAC</td>
<td>100</td>
<td>ENSG00000184371</td>
</tr>
<tr>
<td>Tissue nonspecific alkaline phosphatase</td>
<td>gCTCAAAACgAgATCACAgACgAg</td>
<td>149</td>
<td>ENSG00000184371</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>TgCATCAATgTTCCAgAgAg</td>
<td>100</td>
<td>ENSG00000156387</td>
</tr>
</tbody>
</table>

NOTE: For each gene, the first oligonucleotide sequence represents the forward primer, and the second sequence the reverse primer.
subsequently washed in DMEM containing or not containing 1 μmol/L AZD0530 and lysed in lysis buffer [0.5% Triton X-100, 20 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA] containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride and 1 μg/mL leupeptine) and phosphatase inhibitors (1 mmol/L NaF and 2 mmol/L sodium vanadate). The breast cancer cell line MDA-MB-231 (generous gift from Prof. J. Borst), which metastasizes to bone in nude mice (18), and human c-Src–transfected 3T3 cells were used as positive controls for c-Src expression. Lysates were freeze-dried and resuspended in small volumes. Samples were prepared for Western blotting by boiling in sample buffer for 5 min. Samples were diluted 1:1 in sample buffer [125 mmol/L Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.02% bromophenol blue, 58 mmol/L DTT] and boiled for 5 min. Samples were prepared so that 50 μg of sample were loaded onto 8% Tris-glycine gels (Invitrogen) in a constant volume of 35 μL. The gels were then run at 125 V for 2 h, after which they were transferred onto nitrocellulose membranes (Invitrogen) at 30 V for 1 h. Subsequently, the membranes were blocked in 5% powdered milk (Marvel, Premier International Foods)/0.05% TBST for 1 h, after which they were incubated in primary anti-p-Src (pY418) antibody CST #2101 (New England Biolabs), anti–total Src #05-184 (Millipore/Upstate, Co.), or anti-vinculin V9131 (Sigma Aldrich) overnight at 4°C. The following morning, the membranes were washed in TBS/0.05% Tween for 4 × 15 min and then the secondary antibody (CST HRP-linked antirabbit IgG #7074 or antimouse CST #7076) was added for 1 h at room temperature. The membranes were again washed for 4 × 15 min in TBS/ 0.05% Tween, after which they were exposed to West Pico SuperSignal (Pierce 34080) and then exposed to chemiluminescence film (GE Healthcare) developed on The KODAK XOMAT2000 developer.

**Bone Resorption**

Maximal numbers of multinucleated cells were usually seen after 3 wk in a PBMCs/osteoblasts coculture system (29). To allow for significant bone resorption, cocultures of PBMCs and osteoblasts were assessed for bone resorption after a culture period of 4 wk. The cells present on the bovine cortical bone slices were removed with 0.25 mol/L NH4OH. The slices were period of 4 wk. The cells present on the bovine cortical bone and osteoblasts were assessed for bone resorption after a culture allowing for significant bone resorption, cocultures of PBMCs

**Actin Staining**

F-actin was stained using Alexa 488–conjugated phalloidin (Molecular Probes) as described previously (3). Nuclei were stained with propidium iodide (Sigma). Image stacks were taken using confocal laser scanning microscopy (Leica). F-actin ring surface area was analyzed on compressed stacks using QWin-software (QWin, Leica).

**Statistical Analysis**

The Kruskal-Wallis nonparametric ANOVA test followed by Tukey-Kramer’s multiple comparison test was used when multiple comparisons were made. Two-sided t test was used when data obtained from paired samples were compared. P ≤ 0.05 was considered significant.

**Disclosure of Potential Conflicts of Interest**

A grant was received from AstraZeneca Pharmaceuticals, and Drs. Tim P. Green and Neil James are employees of AstraZeneca Pharmaceuticals.

**Acknowledgments**

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**References**


The Src Inhibitor AZD0530 Reversibly Inhibits the Formation and Activity of Human Osteoclasts

Teun J. de Vries, Margriet G. Mullender, Marion A. van Duin, et al.