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*Original Article*

# Systematic screen with kinases inhibitors reveals kinases play distinct roles in growth of osteoprogenitor cells

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**Abstract:** Cancer treatment-related bone loss has become growing problematic, especially in breast and prostate cancer treated with hormone/endocrine therapy, chemotherapy and radiotherapy. However, bone loss caused by targeted therapy in cancer patients is largely unknown yet. In present study, a kinase inhibitors screen was applied for MC3T3-E1, a murine osteoprogenitor cell line, and seven kinase inhibitors (GSK1838705A, PF-04691502, Dasatinib, Masitinib, GDC-0941, XL880 and Everolimus) were found to suppress the cell viability with dose- and time-dependent manner. The most interesting is that many kinase inhibitors (such as lapatinib, erlotinib and sunitinib) can promote MC3T3-E1 cell proliferation at 0.01 μM. 4 out of 7 inhibitors were selected to perform the functional study and found that they lead to cell cycle dysregulation, treatments of PF-04691502 (AKT inhibitor), Dasatinib (Src inhibitor) and Everolimus (mTOR inhibitor) lead to G1 arrest of MC3T3-E1 cells via downregulation of cyclin D1 and p-AKT, whereas XL880 (MET and VEGFR inhibitor) treatment results in increase of sub-G1 and G2/M phase by upregulation of p53 protein. Our work provides important indications for the comprehensive care of cancer patients treated with some targeted drugs.

**Keywords:** Cancer treatment-related bone loss, kinases inhibitors screening, osteoprogenitor cells

## Instruction

Over 400,000 individuals in the United States annually, including significant proportions of patients with breast, prostate, lung and other solid tumors, are affected by tumor metastasis to the skeleton, more than any other site of metastasis [1]. One the other hand, cancer and its treatment can comprise bone health, particularly in women with breast cancer and men with prostate cancer, leading to fracture, pain, loss of mobility, and hypercalcemia of malignancy [2, 3]. These suggest that bone microenvironment plays crucial roles in cancer metastasis and that cancer and cancer treatment aggravate the imbalance of bone hemostasis and eventually lead to bone loss-related phenotype. The long-term side effects associated with cancer therapies with hormone therapy (or endocrine therapy), chemotherapy or radiotherapy has become increasingly problematic [4], while bone loss caused by cancer treatment with targeted therapy has few clinical reports. In regard to the anti-proliferation effect of some targeted drugs for tumor cells as well as bone cells, the main reason for this difference may be in that the malignant progression of cancer and the high cost of targeted drugs hinder the long-term use of targeted drugs. With the advances in early diagnosis and wide use of targeted drugs in future, it is of great interest to uncover the possibility that targeted therapy results in bone loss.

In the healthy adult skeleton, bone maintenance is a coordinated, dynamic balance between bone resorption and bone formation. The resorption of old bone is as important to skeletal homeostasis as the formation of new bone. Resorption involves the osteoclasts, large cells originating in the bone marrow. Formation involves osteoblasts, differentiated cells of mesenchymal origin that produce the calcified bony matrix, and osteocalcin. However,

in aging people and cancer patients, the balance is broken. In treatment with estrogen-depleting therapies for breast cancer, such as aromatase inhibitors (AIs), accelerating bone resorption and bone loss then leads to osteopenia and osteoporosis [3, 5, 6]. In prostate cancer, therapeutic androgen deprivation leads to increased osteoclastic bone resorption and a progressive decrease in bone mineral density (BMD) [7-9]. In the cancer treatment for these two types of cancer, drug use makes the bone hemostasis both bias to bone resorption. While for the cancer targeted therapy, the situation may be different to some extent. Although there are no clinical reports, preclinical data give tips. Pinski et al reported in 2002 that Trk receptor inhibition induces apoptosis of proliferating but not quiescent human osteoblasts [10]. Singha et al reported in 2007 that rapamycin, a specific inhibitor of the mammalian target of rapamycin (mTOR), inhibits osteoblast proliferation and differentiation in MC3T3-E1 cells and primary mouse bone marrow stromal cells [11]. Duan et al reported in 2009 that insulin-like growth factor-I receptor (IGF1R) tyrosine kinase inhibitor cyclolignan picropodophyllin inhibits proliferation and induces apoptosis in multidrug resistant osteosarcoma cell lines, osteoblast-like cells [12]. O'Sullivan et al reported in 2011 that tyrosine kinase inhibitor nilotinib potently inhibited osteoblast proliferation at relative lower dose (0.01-1 μM) through inhibition of the platelet-derived growth factor (PDGFR) and have important effects on bone metabolism [13]. Chandra et al reported that epidermal growth factor receptor (EGFR) signaling promotes proliferation and survival in osteoprogenitors by increasing early growth response 2 (EGR2) expression [14], suggesting that EGFR inhibition can lead to osteoprogenitor cell death. Taken together, all these studies propose that many targeted drugs may inhibit the proliferation of osteoprogenitors and osteoblasts and hence interrupt bone formation. This hypothesis is worth of further study and may be implicated in the comprehensive care of cancer patients treated with some targeted drugs.

In present study, a kinase inhibitors screening was applied to MC3T3-E1, a mouse osteoprogenitor cell line, and seven kinase inhibitors were found to suppress the cell viability with dose- and time-dependent manner. 4 out of 7 inhibitors were selected to perform the functional study and found that they lead to cell cycle dysregulation, the underling mechanisms were examined by western blotting.

## Materials and methods

### *Cell culture*

MC3T3-E1 cell line (Subclone 14) was purchased from CellBank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies) in a humidified atmosphere containing 5% $CO_2$ at 37°C. Cells in the exponential growth phase were used for all the experiments.

### *Kinase inhibitors screen*

44 kinase inhibitors were purchased from Selleck Chemicals (USA) and list in **Table 1**. 300 MC3T3-E1 cells were seeded in each well of 384-well plate. 24 h later four concentrations (final concentrations were 0.01, 0.1, 1.0 and 10 μM, respectively) of all the 44 inhibitors were subjected to MC3T3-E1 cells. 6 days later, 5 μl of AlamaraBlue (CellTiter-Blue® Cell Viability Assay, Promega) was added to each well and incubated at 37°C for 2 h and the cell viability was assayed according to the manufacturer's instruction. Every treatment was triplicate in the experiment and the cell viability was calculated relative to the cells left untreated. The inhibitors that repress the cell viability by higher than 20% at relative lower concentration (0.1 μM) and that exert anti-proliferation effect by a dose-dependent manner were selected as positive hits which have critical effects on MC3T3-E1 survival.

### *Validation of anti-proliferation effects of positive hits on MC3T3-E1 cells*

7 positive hits were applied to MC3T3-E1 cells (1500 cells in each well of 96-well plate) at 3 different concentrations for 72 h. The lowest doses were the average cellular IC50 dose of that inhibitor to suppress the targeted enzyme activity, as described before [15-21]. The other two doses were 5-fold and 25-fold of the lowest doses, respectively. The final concentration for each inhibitor was shown in **Figure 2A**. And then MC3T3-E1 cells were treated with the 7 drugs at the corresponding highest doses used above, respectively, for 24 or 48 h to examine

**Table 1.** 44 small-molecule inhibitors of kinases

| No | Name | Target | Cellular IC50 of target enzyme activity |
|---|---|---|---|
| S2672 | PF-562271 | FAK \| PYK2 | 5 nM |
| S1028 | LapatinibDitosylate | EGFR \| HER2 | 200 nM \| 80 nM |
| S1181 | ENMD-2076 | Flt \| Aurora Kinase \| Src \| VEGFR | 130 nM |
| S2205 | OSI-420 (erlotinib) | EGFR | 20 nM |
| S1042 | Sunitinib Malate | FLT3 \| PDGFR \| VEGFR \| CSF-1R | 10-50 nM |
| S2248 | CX-4945 | CK2 | 100 nM |
| S1032 | MotesanibDiphosphate | VEGFR \| PDGFR \| c-Kit \| Ret | 207 nM \| 37 nM |
| S1249 | JNJ-7706621 | CDK \| Aurora Kinase | 100 nM |
| S1207 | AV-951 (Tivozanib) | VEGFR \| c-Kit \| PDGFR | 100-200 nM |
| S1361 | MGCD-265 | c-Met \| VEGFR \| Tie-2 | 20-30 nM |
| S1078 | MK-2206 | Akt | 20~120 nM |
| S1226 | KU-0063794 | mTOR | 200 nM |
| S1101 | Vatalanib | VEGFR \| c-Kit \| Flk \| Flt \| PDGFR | 17-34 nM |
| S2617 | TAK-733 | MEK | 1.9 nM |
| S1360 | GSK1059615 | PI3K \| mTOR | 40 nM |
| S1134 | AT9283 | Bcr-Abl \| JAK \| Aurora Kinase | 30 nM |
| S1108 | NVP-TAE684 | ALK | 4 nM |
| S1486 | AEE788 | EGFR \| ErbB2 | 11 nM |
| S5001 | CP-690550 (Tofacitinib) | JAK | 77 nM |
| S1065 | GDC-0941 | PI3K | 30-50 nM [19] |
| S1187 | PIK-90 | VEGFR \| c-Kit \| PDGFR | 100 nM |
| S1451 | Aurora A Inhibitor I | Aurora A | 0.19 μM |
| S2622 | PP121 | PDGFR \| Hck \| mTOR \| VEGFR2 \| Src \| Abl | 20 nM |
| S1111 | XL880 (GSK1363089) | MET\|VEGFR | 21~23 nM [20] |
| S1046 | Vandetanib | VEGFR-2 | 60 nM |
| S2680 | PCI-32765 | BTK | 11 nM |
| S1003 | ABT-869 (Linifanib) | RTK \| VEGFR \| PDGFR \| FGFR | 0.2 nM \| 2 nM \| 4nM \| 7 nM |
| S2216 | Mubritinib | HER2 \| CDK | 100 nM |
| S1171 | CYC116 | Aurora Kinase \| VEGFR | 60 nM |
| S2158 | KW 2449 | FLT3 \| ABL | 100 nM |
| S2201 | BMS 794833 | Met \| VEGFR-2 | 40 nM |
| S2743 | PF-04691502 | AKT | 32 nM [15] |
| S2703 | GSK1838705A | IGF-1R \| INSR \| ALK | 100 nM [21] |
| S1526 | AC-220 | FLT3 | 4 nM |
| S2730 | Crenolanib (CP-868569) | PDGFRα \| PDGFRβ | 10 nM |
| S1490 | AP24534 | RTK | 10-100 nM |
| S1040 | SorafenibTosylate | VEGFR \| PDGFR \| RAF | 40-80 nM |
| S2719 | AMG 900 | Aurora Kinase | 50 nM |
| S1021 | Dasatinib | SRC \| Abl | 15 nM [16] |
| S2231 | Telatinib | VEGFR-2 \| VEGFR-3 \| c-Kit \| PGFR-β | 19 nM |
| S1178 | BAY 73-4506 (Regorafenib) | c-Kit \| B-Raf \| VEGFR | 10-100 nM |
| S1005 | Axitinib | VEGFR | 0.2 nM |
| S1064 | Masitinib (AB1010) | KIT | 150 nM [17] |
| S1120 | Everolimus (RAD001) | mTOR | 20 nM [18] |

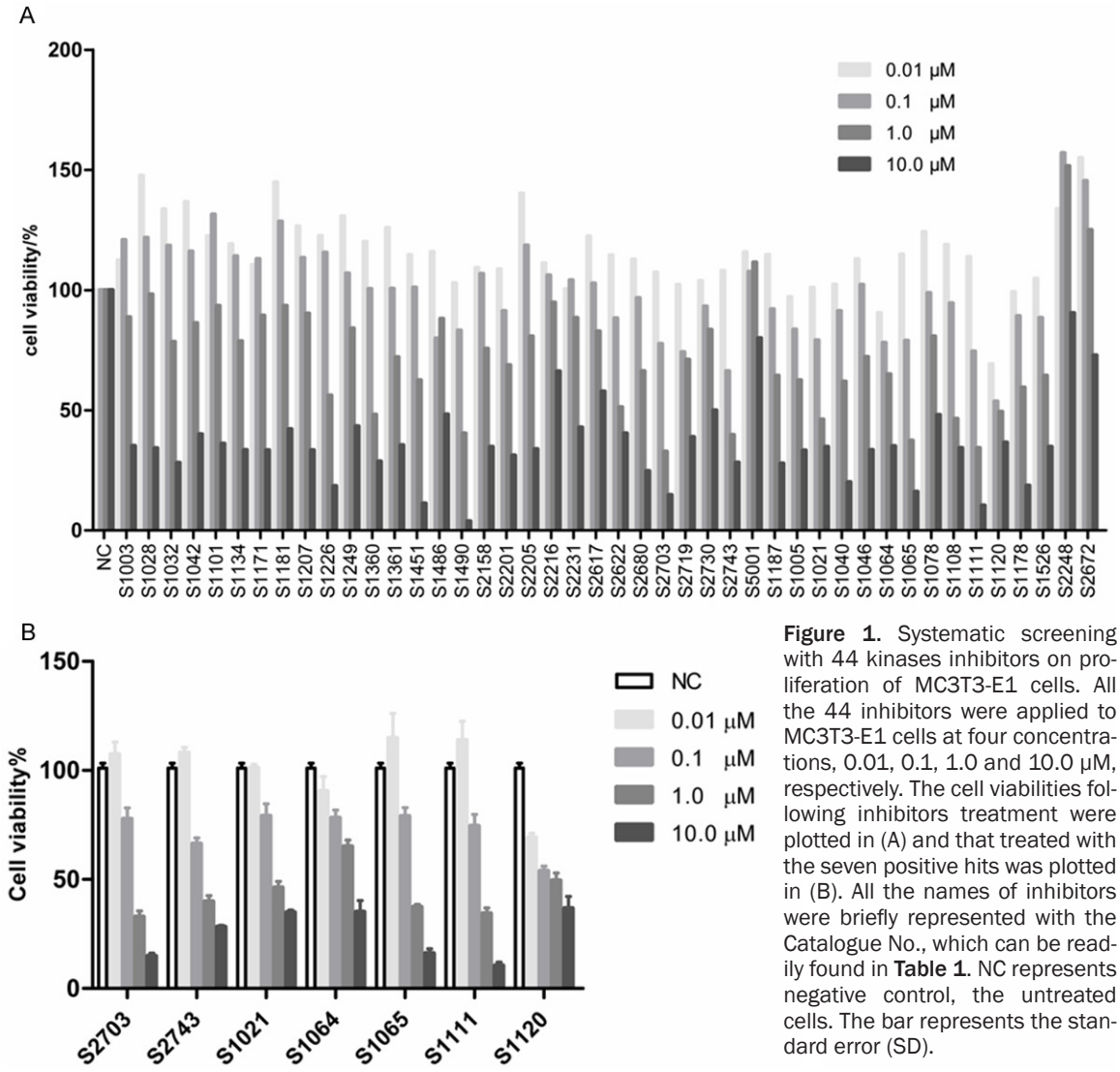


**Figure 1.** Systematic screening with 44 kinases inhibitors on proliferation of MC3T3-E1 cells. All the 44 inhibitors were applied to MC3T3-E1 cells at four concentrations, 0.01, 0.1, 1.0 and 10.0 μM, respectively. The cell viabilities following inhibitors treatment were plotted in (A) and that treated with the seven positive hits was plotted in (B). All the names of inhibitors were briefly represented with the Catalogue No., which can be readily found in **Table 1**. NC represents negative control, the untreated cells. The bar represents the standard error (SD).

the time effects of these drugs on MC3T3-E1 cell proliferation. Then 10 μl of AlamaraBlue was added to each well and incubated at 37°C for 2 h and the cell viability was assayed according to the manufacturer's instruction. Every treatment was triplicate in the experiment and the cell viability was calculated relative to the cells left untreated.

*Flowcytometry*

Cells left untreated or treated with 0.5 μM of 4 inhibitors (PF-04691502, Dasatinib, XL880 and Everolimus) respectively, for 48 h. Then cells were harvested with trypsin-EDTA, washed with PBS, and fixed with 70% ethanol at -20°C for a few days. The fixed cells were pelleted, resuspended in 100 μL of hypotonic citric buffer (192 mmol/L $Na_2HPO_4$ and 4 mmol/L citric acid), and incubated for 30 minutes at room temperature. The cells were pelleted and suspended in PI/RNase/PBS (100 μg/mL propidium iodide and 10 μg/mL RNase A) overnight at 4°C. Analysis of DNA content was done on a FACSCalibur system (BD Immunocytometry Systems, San Jose, CA).

*Protein isolation and western blotting*

Cell pellets were resuspended in 1 × SDS loading buffer (1 mmol/L $Na_3VO_4$, 10 mmol/L NaF, 1 mmol/L PMSF) containing protease inhibitors. Lysates (20 μg each lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for GAPDH (Abmart, 080922), CCND1 (Santa Cruz, SC-450), p53 (Abclonal, A0263), BAX (Abclonal,

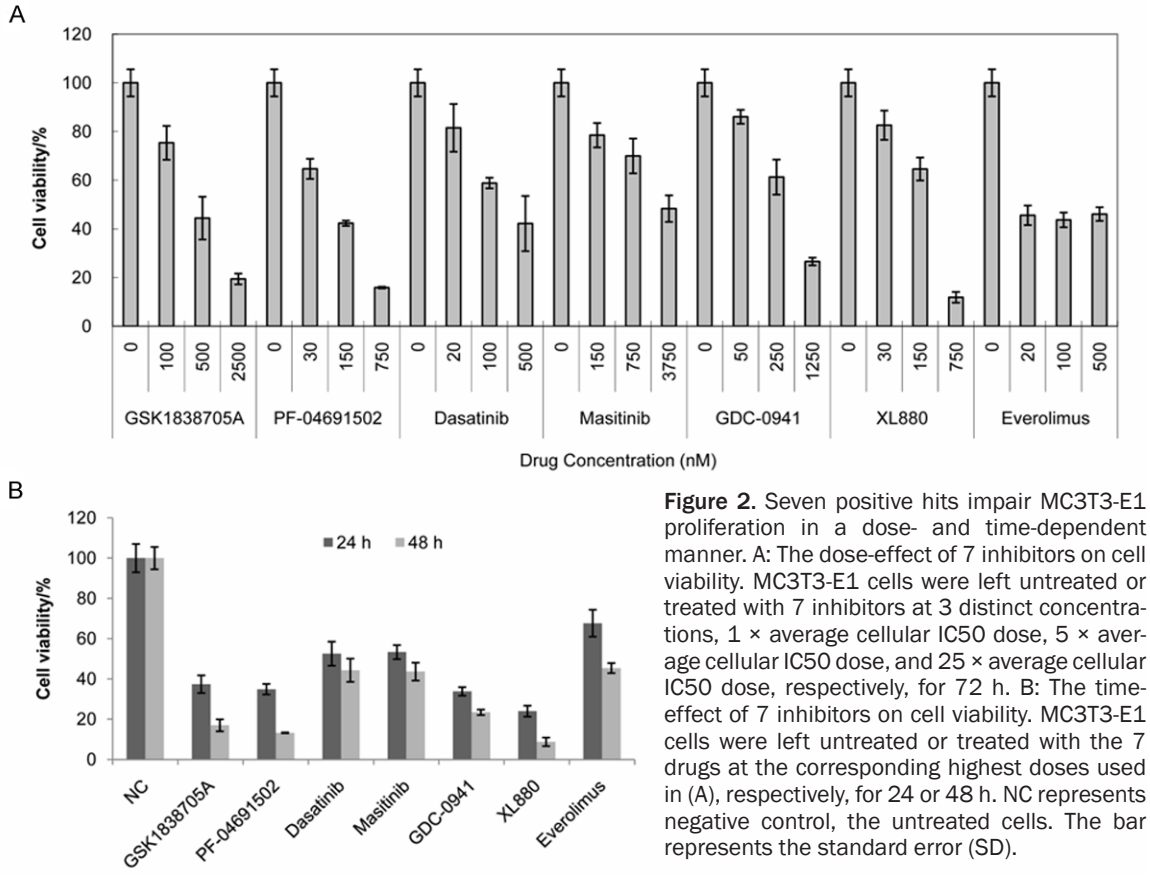


**Figure 2.** Seven positive hits impair MC3T3-E1 proliferation in a dose- and time-dependent manner. A: The dose-effect of 7 inhibitors on cell viability. MC3T3-E1 cells were left untreated or treated with 7 inhibitors at 3 distinct concentrations, 1 × average cellular IC50 dose, 5 × average cellular IC50 dose, and 25 × average cellular IC50 dose, respectively, for 72 h. B: The time-effect of 7 inhibitors on cell viability. MC3T3-E1 cells were left untreated or treated with the 7 drugs at the corresponding highest doses used in (A), respectively, for 24 or 48 h. NC represents negative control, the untreated cells. The bar represents the standard error (SD).

A0207), AKT (Santa Cruz, sc-8312) and p-AKT (Santa Cruz, SC-7985-R, pS473), were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and visualized by chemiluminescence detection system (Millipore, WBKLS0500).

## Results

*Kinase inhibitors screen reveals that osteoprogenitor cells have different sensitivities to kinase inhibitors*

To examine the influence of kinase on growth of osteoprogenitor cells, a kinase inhibitors screen was applied to MC3T3-E1 cells. Almost all the 44 inhibitors at final dose of 10 μM display potent inhibition to cell growth by higher than 50%, except for five inhibitors (**Figure 1A**). When cells were treated with 1 μM kinase inhibitors, 20 inhibitors repressed cell growth by > 30% and 4 inhibitors had no effect on cell growth (**Figure 1A**). There were 9 inhibitors suppressed cell growth by > 20% at 0.1 μM (**Figure 1A**), while only one inhibitor, Everolimus (RAD001, targeted to mTOR), had apparent anti-proliferation effect at 0.01 μM and inhibited cell growth by 31% (**Figure 1A**). As 0.1 μM is a dose near to the cellular IC50 dose of most kinase inhibitors, those inhibitors that repressed the cell growth by higher than 20% at 0.1 μM and that inhibited the cell growth with a significant dose-dependent manner were selected as positive hits in the screen. There were 7 positive hits in our screen, they were GSK1838705A (targeted to IGF-1R/INSR/ALK), PF-04691502 (targeted to AKT), Dasatinib (targeted to SRC/Abl), Masitinib (AB1010, targeted to KIT), GDC-0941 (targeted to PI3K), XL880 (GSK1363089, targeted to MET/VEGFR) and Everolimus (RAD001, targeted to mTOR), respectively.

*Positive hits specifically inhibit MC3T3-E1 cells viability in a dose- and time-dependent manner*

Then the 7 positive hits were further validated by another cell viability experiments. First, we investigated the dose effects of the 7 positive hits on MC3T3-E1 cells viability. The cellular IC50 dose of each inhibitor, 5-fold and 25-fold

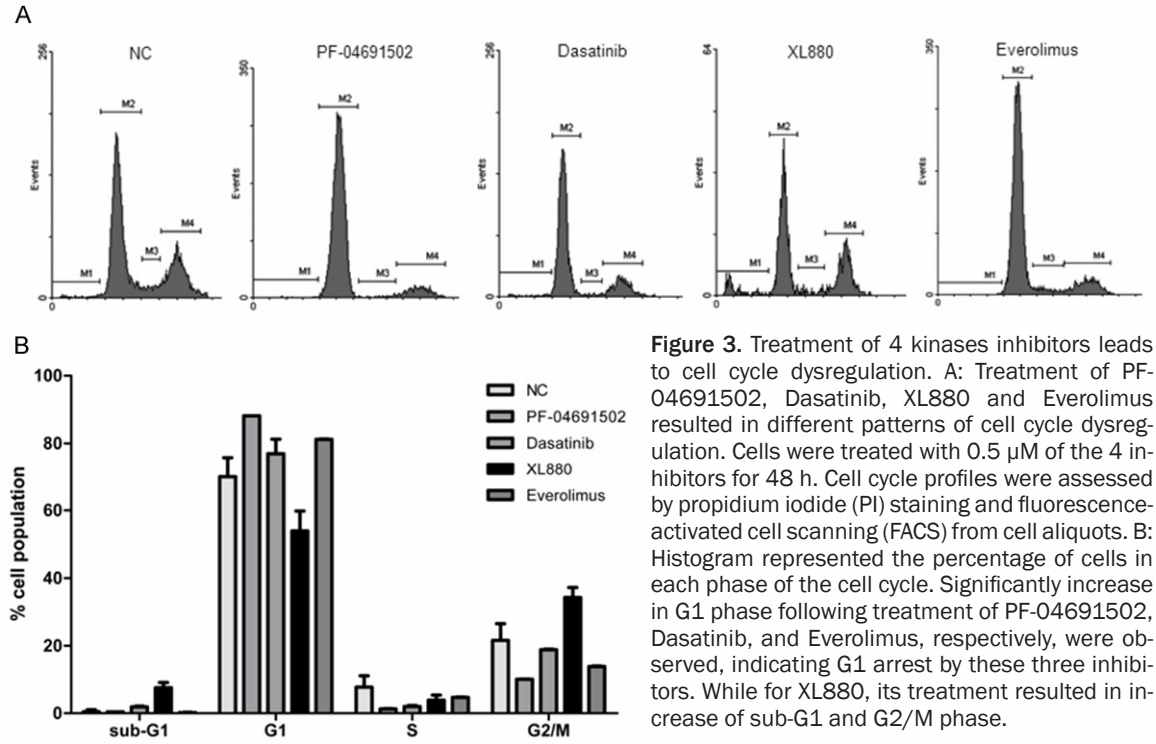


**Figure 3.** Treatment of 4 kinases inhibitors leads to cell cycle dysregulation. A: Treatment of PF-04691502, Dasatinib, XL880 and Everolimus resulted in different patterns of cell cycle dysregulation. Cells were treated with 0.5 μM of the 4 inhibitors for 48 h. Cell cycle profiles were assessed by propidium iodide (PI) staining and fluorescence-activated cell scanning (FACS) from cell aliquots. B: Histogram represented the percentage of cells in each phase of the cell cycle. Significantly increase in G1 phase following treatment of PF-04691502, Dasatinib, and Everolimus, respectively, were observed, indicating G1 arrest by these three inhibitors. While for XL880, its treatment resulted in increase of sub-G1 and G2/M phase.

of cellular IC50 dose were applied to MC3T3-E1 cells (**Figure 2A**). The results showed that 6 inhibitors exhibited apparent dose-dependent inhibition to cell growth, while Everolimus (RAD001, targeted to mTOR) displayed almost the same anti-proliferation effect on MC3T3-E1 cells at the three different concentrations, all inhibited cell growth by 60% or so. Second, the time effects of the 7 positive hits on MC3T3-E1 cells viability were examined. MC3T3-E1 cells were treated with the 7 drugs at the corresponding highest doses used in **Figure 2A**, respectively, for 24 or 48 h. And the results showed that all the 7 drugs exerted time-dependent inhibition effects on MC3T3-E1 cells growth. Collectively, the 7 drugs specifically inhibit MC3T3-E1 cells viability in a dose- and time-dependent manner.

*Flowcytometry showed that 4 inhibitors treatments lead to MC3T3-E1 cell cycle dysregulation*

To elucidate how the positive hits inhibit the cell growth, MC3T3-E1 cells treated with 4 kinase inhibitors (PF-04691502, Dasatinib, XL880 and Everolimus), respectively, were subjected to flowcytometry. The results showed that treatments of PF-04691502, Dasatinib and Everolimus resulted in increased fraction of G1 phase and subsequent decrease in S phase (**Figure 3**), suggesting that MC3T3-E1 cells were arrested at G1 phase following these three inhibitors treatments. While for XL880, its treatment caused significant increase in sub-G1 and G2/M phase, indicating its dual roles in promoting apoptosis and G2/M arrest.

*Immunoblotting showed 4 inhibitors repress MC3T3-E1 proliferation by different mechanisms*

Subsequently, cells untreated or treated with the 4 inhibitors at 0.5 μM concentrations, respectively, were applied for western blotting to figure out the underlying mechanisms by which these drugs dysregulate the cell cycle of MC3T3-E1. Cyclin D1 was found to be markedly downregulated following PF-04691502, Dasatinib and Everolimus treatment, respectively (**Figure 4A**). While for XL880, its treatment resulted in a moderate upregulation of Cyclin D1 protein (**Figure 4A**). And then, protein expression of p53, Bax, AKT and p-AKT was examined for cells treated with 0.5 μM of Dasatinib and XL880, respectively (**Figure 4B**). The results showed that Dasatinib treatment led to inactivation of AKT whereas p53 protein was upregulated following XL880 treatment.

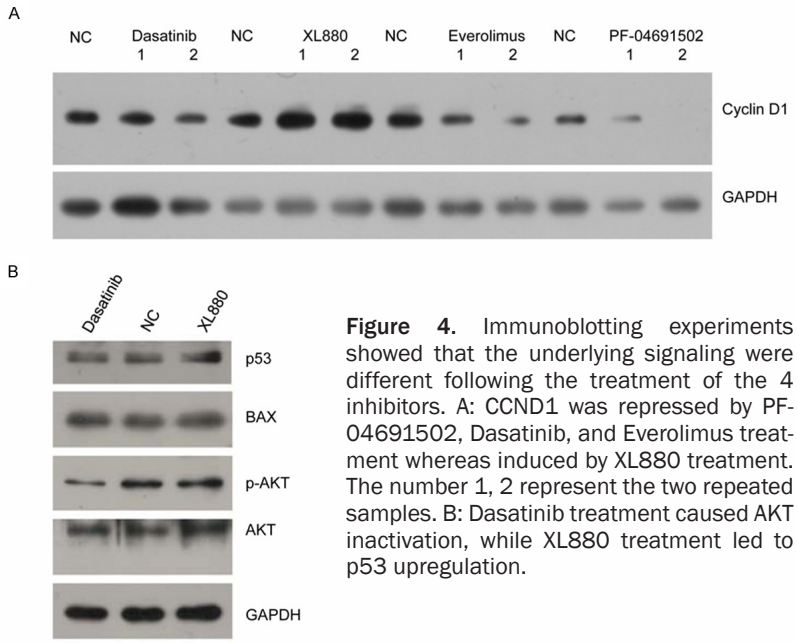


**Figure 4.** Immunoblotting experiments showed that the underlying signaling were different following the treatment of the 4 inhibitors. A: CCND1 was repressed by PF-04691502, Dasatinib, and Everolimus treatment whereas induced by XL880 treatment. The number 1, 2 represent the two repeated samples. B: Dasatinib treatment caused AKT inactivation, while XL880 treatment led to p53 upregulation.

## Discussion

Cancer treatment-related bone loss has become increasing problems, especially in breast and prostate cancer patients treated with hormone/endocrine therapy, chemotherapy or radiotherapy. Although there are few clinical reports on bone loss caused by targeted therapy for cancer patients, preclinical studies have given a lot tips on this topic. With the improvements of early diagnosis and wide use of targeted drugs for long-term, it is possible that bone loss resulted from targeted therapy comes into a new big trouble. So, it is worth of studying deeply to this field now.

In this work, a mouse osteoprogenitor cell line, MC3T3-E1, was subjected to a kinase inhibitors screen. We found that 7 inhibitors can repress MC3T3-E1 cell proliferation in a dose- and time-dependent manner. 4 out of 7 drugs exhibit this effect by different mechanisms.

The phosphoinositide 3-kinase and AKT (protein kinase B) signaling pathway (PI3K/AKT) plays a central role in the control of cell survival, growth, and proliferation throughout the body. With regard to bone, and particularly in osteoblasts, there is an increasing amount of evidence that the many signaling molecules exert some of their bone-specific effects in part via selectively activating some of the generic effects of the PI3K/AKT pathway in osteoblasts [22-27]. Therefore, it is not surprising that two inhibitors, PF-04691502 (targeted to AKT) and GDC-0941 (targeted to PI3K), were screened to be positive hits for MC3T3-E1 growth. For the other two inhibitors, GSK1059615 (targeted to PI3K/mTOR) and MK-2206 (targeted to AKT), although their inhibition effects on MC3T3-E1 cell proliferation were not as potent as the former two drugs, they can impair osteoprogenitor cells growth at higher doses (when treated with 1 μM of MK-2206, the cell viability was 81%; while treated with 10 μM of MK-2206, the cell viability was 48%). In regard to that the cellular IC50 doses of these four drugs were roughly the same level, above results indicate that GSK1059615 and MK-2206 may be more potential therapeutic drugs for long-term cancer PI3K/AKT-targeted therapy with slight adverse effects on bone loss. mTOR is mechanistic target of rapamycin (serine/threonine kinase), belongs to PI3K pathway. For mTOR inhibitors, the situation is the same as the PI3K/AKT inhibitors. There mTOR inhibitors (KU-0063794, GSK1059615 and PP121) also exert inhibition effects at higher dose of 1 μM, while Everolimus inhibits MC3T3-E1 cell growth even at 0.01 μM, suggesting that the long-term use of Everolimus in cancer therapy may do great damage to bone formation.

Src is a proto-oncogene. SRC protein is a tyrosine-protein kinase and is implicated in the regulation of embryonic development, cell growth, adhesion and migration. Recently, SRC has been reported to cross-talk with PI3K/AKT pathway and to be involved in osteoblast and osteoblast progenitor proliferation, commitment, differentiation [28-31]. Our data showed that Src inhibition by Dasatinib results in p-AKT downregulation and hence suppress the growth of osteoblast progenitor cells. Dasatinib is a small inhibitors targeted to Src and Abl, now have been approved by FDA for treatment of Acute lymphoblastic leukemia and Chronic

myelogenous leukemia. Garcia-Gomez et al reported that low dasatinib concentrations (1-2 nM) show convergent bone anabolic and reduced bone resorption effects [32], while our screening data showed that MC3T3-E1 cell growth was not influenced following 10 nM of dasatinib treatment. This contradiction may be caused by the different cell lines used in the studies and may be worth of further study. Actually, there were many inhibitors can promote the proliferation when used at lower concentration. For example, viabilities of cells treated with 0.01 μM of lapatinib, erlotinib or sunitinib, respectively, were all increased by higher than 40%. It is an intriguing phenotype and this proposes that not all targeted drugs lead to osteoblast and osteoblast progenitor cells death at any event.

c-Met receptor tyrosine kinase is one of the most mentioned cancer therapeutic targets. This kinase and its ligand, hepatocyte growth factor (HGF), play a central role in cell proliferation and the survival of several human cancers [33] and are associated with increased invasion and progression of human cancer [34]. XL880 (Foretinib), a multitargeted receptor tyrosine kinase inhibitor of several receptors, including MET and vascular endothelial growth factor receptor 2 (VEGFR2), is in phase I studies as well as in phase II studies in solid tumors, including Metastatic Gastric Cancer, Recurrent or Metastatic Squamous Cell Cancer of the Head and Neck, and Papillary Renal-Cell Carcinoma. In our data, XL880 exerts its antiproliferation effect on MC3T3-E1 cells by markedly distinct mechanism with the other three kinase inhibitors. MC3T3-E1 cells were arrested at G2/M phase and induced to apoptosis following XL880 treatment, which is in consistent with previous studies in mouse [35] and pancreatic islet cancer cells [36], respectively. However, the concurrent occurrence of G2/M arrest and apoptosis may be worth of further study to investigate the possibility whether this phenotype is specific for osteoprogenitor cells when treated with XL880.

Collectively, our kinases inhibitors screen uncovered 7 inhibitors that impair MC3T3-E1 cell proliferation in a dose- and time-dependent manner. Treatments of PF-04691502, Dasatinib and Everolimus lead to G1 arrest of MC3T3-E1 cells via downregulation of cyclin D1 and p-AKT, whereas XL880 treatment results in increase of sub-G1 and G2/M phase by upregulation of p53 protein.

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## Disclosure of conflict of interest

None.

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