Specific Tyrosine Kinase Inhibitors Regulate Human Osteosarcoma Cells In vitro

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Abstract Inhibitors of specific tyrosine kinases are attractive lead compounds for development of targeted chemotherapies for many tumors, including osteosarcoma. We asked whether inhibition of specific tyrosine kinases would decrease the motility, colony formation, and/or invasiveness by human osteosarcoma cell lines (TE85, MNNG, 143B, SAOS-2, LM-7). An EGF-R inhibitor reduced motility of all five cell lines by 50% to 80%. In contrast, an IGF-1R inhibitor preferentially reduced motility by 42% in LM-7 cells and a *met* inhibitor preferentially reduced motility by 80% in MNNG cells. The inhibitors of EGF-R, IGF-1R, and *met* reduced colony formation by more than 80% in all tested cell lines (TE85, MNNG, 143B). The EGF-R inhibitor reduced invasiveness

by 62% in 143B cells. The JAK inhibitor increased motility of SAOS-2 and LM7 cells without affecting colony formation or invasiveness. Inhibitors of HER-2, NGF-R, and PDGF-Rs did not affect motility, invasiveness, or colony formation. These results support the hypothesis that specific tyrosine kinases regulate tumorigenesis and/or metastasis in osteosarcoma.

Introduction

Osteosarcoma, the most common bone sarcoma, predominantly affects rapidly growing bones in adolescents [25]. Although only approximately 400 cases occur in the United States per year, osteosarcoma is the fifth most frequent malignancy in 15 to 19 year olds [63]. Before the development of chemotherapy regimens, long-term survival rates were 10% to 20% with surgical resection, usually amputation, as the only treatment available [25, 39, 63]. During the 1970s, initiation of chemotherapy protocols in combination with aggressive surgical resection resulted in long-term survival rates of 60% to 70% in patients with localized disease [7, 38, 39]. However, patients with metastatic disease still face 20% to 30% survivorship 10 years after diagnosis [7, 39]. Thus, a greater understanding of the basic biology of osteosarcoma is needed to allow development of novel approaches to increase survival rates [25, 62].

Reduced dependence on growth factors is a common mechanism in many cancers, usually as a result of autocrine production of the growth factors themselves or overexpression or mutation of either growth factor receptors or downstream signaling molecules [18, 22]. Because many of the receptors and downstream signaling molecules are tyrosine kinases [18, 22], inhibitors of these kinases are a majority of the most promising anticancer drugs

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[4, 10, 21, 27]. Although osteosarcoma has not been as well studied as other types of cancer, overexpression in osteosarcoma has been reported for both growth factors and their tyrosine kinase receptors, and overexpression of some of these molecules correlates with metastasis and poor survival in patients with osteosarcoma [5, 8, 9, 15, 17, 20, 23, 28, 33, 36, 47, 49, 60, 65].

However, the value of tyrosine kinases to predict outcomes or responses to treatment in osteosarcoma has yet to be finalized. Several reports established an association between HER-2 expression and decreased overall patient survival [20, 45, 49], whereas others failed to confirm any association [1, 43]. However, this does not undermine the potential benefit that inhibitors of tyrosine kinases may play in future treatment of patients with osteosarcoma. Additionally, the vast majority of human tyrosine kinases have yet to be tested for correlation with long-term survival.

Current antiproliferative chemotherapies used to treat patients with osteosarcoma may induce debilitating side effects, including hematologic, liver, renal, cardiac, neurologic, and/or gonadal toxicity [39]. These agents are also mutagenic and can cause secondary malignancies, most commonly leukemia, brain cancer, soft tissue sarcomas, and breast cancer [39]. In contrast, therapies against specific targets such as tyrosine kinases would likely produce fewer side effects [4, 10]. Thus, such targeted therapies offer the hope of an improved quality of life as well as increased survival.

We asked whether inhibitors of specific tyrosine kinases alter the motility, colony formation, and invasiveness of osteosarcoma cell lines.

Materials and Methods

We tested two families of osteosarcoma of genetically related osteosarcoma cell lines to determine if in vitro

differences in phenotypes correlated with their tumorigenic and metastatic potentials. The selected in vitro assays of motility, invasiveness, and colony-forming generally reflect the in vivo tumorigenic/metastatic potential of the osteosarcoma cell lines. TE85, MNNG, and 143B cell lines were obtained from the American Type Culture Collection (Manassas, VA); SAOS-2 and LM-7 cell lines were obtained from E. Kleinerman, MD (Anderson Cancer Center, Houston, TX). Each family includes a parental cell line (TE85 and SAOS-2) isolated from human osteosarcoma tissue that exhibits little tumorigenesis or metastasis when implanted in immunodeficient mice and a highly tumorigenic/metastatic cell line (143B and LM-7, respectively) derived from the parental cell line [12, 30, 40]. The TE85 family also includes a tumorigenic but only weakly metastatic cell line (MNNG) [40].

Unless otherwise specified, all cell cultures contained minimal essential medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone), nonessential amino acids (Mediatech, Herndon, VA), sodium pyruvate (Invitrogen, Carlsbad, CA), L-glutamine (Mediatech), and penicillin-streptomycin (Hyclone) and were maintained at 37°C in a humidified 5% CO₂ atmosphere. All experiments were performed on cells harvested at the mid-log phase of growth.

The effects of small molecule inhibitors that are relatively specific for individual tyrosine kinases were used in tests of motility, invasiveness, or nonadherent colony formation. Small molecule inhibitors were obtained from Calbiochem (San Diego, CA) and the concentrations of small molecule inhibitors used were based on publications demonstrating the effective concentrations in intact cell assays (Table 1). Inhibitors were dissolved in dimethyl sulphoxide (DMSO; Sigma, St Louis, MO) and aliquots were stored at −20°C.

Scrape motility assays were performed similarly to a previously described method [6, 14]. Cells (1.0×10^5 per 9.6-cm² well) were cultured overnight to allow a confluent

Table 1. Small molecule tyrosine kinase inhibitors

	Target	Inhibitor (Calbiochem catalog number)	Concentration used in this study	Reference number
Receptor tyrosine kinases	EGF-R*	EGFR inhibitor (324674)	10 μM	[67]
	HER-2 [†]	AG825 (121765)	20 μM	[50]
	IGF-1R [‡]	Picropodophyllin (407247)	0.5 μM	[19]
	<i>met</i> /HGF-R [§] and <i>tpo</i> - <i>met</i>	SU11274 (448101)	5 μM	[41, 58]
	NGF-R [¶]	AG879 (658460)	10 μM	[34, 48]
	PDGF-R [#]	AG1296 (658551)	5 μM	[35]
Nonreceptor tyrosine kinase	JAK**	Pyridone 6 (420099)	1 μM	[53, 61]

* EGF-R = epidermal growth factor receptor, ErbB-1; [†]HER-2 = ErbB-2/*neu*; [‡]IGF-1R = insulin-like growth factor receptor 1; [§]*met*/HGF-R = hepatocyte growth factor receptor; ^{||}*tpo*-*met* = *met*-related oncogene; [¶]NGF-R = nerve growth factor receptor; [#]PDGF-R = platelet-derived growth factor receptors; **JAK = Janus family protein kinases.

monolayer to form. Scrapes, approximately 0.6 mm in width, were made using a 1-mL pipette tip. Media was changed to remove cellular debris and the cultures were incubated with tyrosine kinase inhibitors or 1% DMSO as a vehicle control. Each scrape was photographed immediately and at the indicated time points using the 10x objective on a Leica DM IRB inverted, phase contrast microscope (Leica Microsystems, Deerfield, IL). Scrape widths were measured with ImageJ (National Institutes of Health, Bethesda, MD). Motility was calculated by subtracting the scrape width at the indicated time points from the initial scrape width and dividing by two.

Colony formation assays were performed similarly to a previously described method [40]. Cells at $8.0 \times 10^3/\text{mL}$ were suspended in 160 μL of collagen gel, 1.69 mg/mL of rat tail Type I collagen (BD Biosciences, Bedford, MA), $0.8 \times \text{EMEM}$ with 5% FCS (Hyclone), and 0.75% NaHCO_3 (Invitrogen) overlying a lower layer of collagen gel (185 μL) in a 2-cm² culture well. Gels were covered with 1 mL of media containing 10% FBS plus sufficient levels of the tyrosine kinase inhibitors or DMSO as a vehicle control to obtain the indicated concentrations after complete equilibration with both layers of collagen gel. Media and inhibitors were changed every 48 hours. Colonies (five or more intact cells) were counted after 4 days (TE85, MNNG, 143B) or 7 days (SAOS, LM7) using phase contrast microscopy.

Basement membrane invasion assays were performed similarly to a previously described method [2] using 96-well CultrexTM chambers (Trevigen, Gaithersburg, MD) as recommended by the manufacturer. Basement membrane layers (CultrexTM) were composed mainly of laminin, Type IV collagen, entactin, and heparin sulfate proteoglycan. Cells at $5.0 \times 10^5/\text{mL}$ were placed in the upper chamber in serum-free media containing 0.1% bovine serum albumin (Proliant Biologicals, Ankeny, IA). The lower chamber contained media with 1.0% FBS as a chemoattractant. Tyrosine kinase inhibitors or 1% DMSO as a vehicle control were added to both the upper and lower chambers. Cells that transversed the basement membrane layer within 24 hours were measured by fluorescence (GENios Pro, Multimode microplate reader; Tecan, Durham, NC) after incubation with calcein AM (Trevigen).

We determined differences in motility distance, quantification of colony formation, and quantification of invasiveness between the experimental groups with tyrosine kinase inhibitors and the control groups with vehicle using analysis of variance with Fisher's least significant difference post hoc tests (SigmaStat, San Jose, CA). All groups of data demonstrated a normal distribution as assessed by the Kolmogorov-Smirnov normality test (SigmaStat). All figures illustrate mean $\pm$ standard error of the mean. Asterisks in each figure represent $p < 0.001$.

Results

The metastatic 143B cell line migrated 65% faster ($p = 1.0 \times 10^{-10}$, except at 4 hours, where $p = 9.9 \times 10^{-8}$ for the comparison between the 143B and TE85 cell lines) than the genetically related nonmetastatic TE85 and MNNG cell lines (supplemental Figs. 1, 2). Migration rates of the LM-7 cell line were similar ($p = 0.18$) to those in its genetically related nonmetastatic parental cell line SAOS-2 (supplemental Figs. 1, 2). The migration rates reflect motility rather than proliferation because they were not affected by aphidicolin, a DNA polymerase inhibitor that blocked cell growth (supplemental Fig. 3). The metastatic 143B cell line formed 47% to 50% more colonies than the nonmetastatic TE85 and MNNG cell lines ($p = 0.016$ and $p = 0.022$, respectively) (supplemental Figs. 4, 5). The 143B cell line formed numerous large colonies (supplemental Fig. 5). The MNNG cell line formed smaller colonies that were more spread out (supplemental Fig. 5). The parental TE85 cell line formed smaller and fewer colonies compared with the genetically related metastatic line (supplemental Fig. 5). The tumorigenic MNNG ($p = 0.013$) and 143B ($p = 0.030$) cell lines were five- to sevenfold more invasive than the parental TE85 cell line (supplemental Fig. 6).

Specific tyrosine kinase inhibitors (Table 1) reduced motility of the osteosarcoma cell lines tested as assessed by scrape motility assays. Motility was reduced (TE85 [$p = 9.4 \times 10^{-9}$], MNNG [$p = 1.0 \times 10^{-10}$], 143B [$p = 1.0 \times 10^{-10}$], SAOS-2 [$p = 6.0 \times 10^{-9}$], LM7 [$p = 1.0 \times 10^{-10}$]) 50% to 80% in all five cell lines by an inhibitor that targets EGF-R (Fig. 1). This inhibitor also induced a less adherent, more rounded phenotype (Fig. 2). None of the other inhibitors induced this change in morphology (Fig. 2) and (data not shown). The IGF-1R inhibitor preferentially reduced ($p = 1.6 \times 10^{-4}$) motility by 42% in the LM-7 cell line and the *met* inhibitor preferentially reduced ($p = 1.0 \times 10^{-10}$) motility by 80% in the MNNG cell line (Fig. 1). The JAK inhibitor increased the motility by 61% ($p = 3.6 \times 10^{-8}$) and 46% ($p = 2.8 \times 10^{-5}$) in the SAOS-2 and LM-7 cell lines, respectively (Fig. 1). The inhibitors of HER-2, NGF-R, and PDGF-R did not detectably affect motility (Fig. 1).

Specific tyrosine kinase inhibitors (Table 1) reduced colony formation of the osteosarcoma cell lines tested as assessed by the nonadherent collagen assays. Colony formation was reduced over 80% by the EGF-R ($p = 9.2 \times 10^{-9}$), IGF-1R ($p = 9.2 \times 10^{-9}$), and *met* inhibitors (TE85 cell line [$p = 9.2 \times 10^{-9}$], MNNG cell line [$p = 9.9 \times 10^{-7}$], 143B cell line [$p = 1.04 \times 10^{-8}$]) in all three cell lines (Figs. 3, 4). The inhibitors of HER-2, JAK, NGF-R, and PDGF-R did not detectably affect colony formation (Figs. 3, 4).

Fig. 1 Specific tyrosine kinase inhibitors reduced motility by the osteosarcoma cell lines. Motility was measured 4 hours after scraping in the presence of the tyrosine kinase inhibitors listed in Table 1 or 1% DMSO as a vehicle control. Bars represent the means $\pm$ standard error of mean of four to six independent experiments, each with four to six scrapes per group. Asterisks denote differences in motility

compared with the control group without inhibitor (EGF-R inhibitor: TE85 [$p = 9.4 \times 10^{-9}$], MNNG [$p = 1.0 \times 10^{-10}$], 143B [$p = 1.0 \times 10^{-10}$], SAOS-2 [$p = 6.0 \times 10^{-9}$], LM7 [$p = 1.0 \times 10^{-10}$], IGF-1R inhibitor: LM7 [$p = 1.6 \times 10^{-4}$]; *met* inhibitor: MNNG [$p = 1.0 \times 10^{-10}$]; JAK inhibitor: SAOS-2 [$p = 3.6 \times 10^{-8}$], LM7 [$p = 2.8 \times 10^{-5}$]).

Fig. 2A–J Photomicrographs show the effects of specific tyrosine kinase inhibitors on motility. Images of the same microscopic field of the 143B cell line at the time of initial scraping (A–E) and after

culture for 4 hours in the presence of 1% DMSO as a vehicle control (F), an inhibitor of EGF-R (G), an inhibitor of HER-2 (H), an inhibitor of IGF-1R (I), and an inhibitor of *met* (J).

Specific tyrosine kinase inhibitors (Table 1) reduced invasiveness of the osteosarcoma cell lines tested as assessed by basement membrane invasion assays. Invasiveness was reduced 62% ($p = 6.1 \times 10^{-5}$) by the EGF-R inhibitor in 143B cells (Fig. 5). The inhibitors of HER-2, JAK, NGF-R, and PDGF-R did not detectably affect invasiveness (Fig. 5).

Discussion

Tyrosine kinases regulate several cellular processes such as proliferation, motility, differentiation, and apoptosis. In osteosarcoma, as well as many other cancer cells, these processes may go unregulated leading to tumorigenesis and/or metastasis. The goal of this study was to determine

Fig. 3 Specific tyrosine kinase inhibitors reduced colony formation by the osteosarcoma cell lines. Colony formation assays were performed in the presence of the tyrosine kinase inhibitors listed in Table 1 or 1% DMSO as a vehicle control. Bars represent the means $\pm$ standard error of mean of three individual experiments, each with three wells per group. Asterisks denote a decrease in the colony count compared with the control group without inhibitor ($p = 9.2 \times 10^{-9}$ for each asterisk except: 143B cells in the presence of the *met* inhibitor [$p = 1.04 \times 10^{-8}$] and MNNG cells in the presence of the *met* inhibitor [$p = 9.9 \times 10^{-7}$]).

if inhibitors of specific tyrosine kinases decreased the motility, colony formation, or invasiveness of human osteosarcoma cells in vitro.

A limitation of this study was the in vitro nature of motility, colony formation, and invasion assays. However, the in vitro behavior (motility, invasiveness, colony formation) of the human osteosarcoma cell lines used in this study corresponds to their in vivo tumorigenic and metastatic potential (supplemental Figs. 1–6). Nonetheless, future studies will be needed to determine whether the same tyrosine kinases also regulate tumorigenesis and metastasis in vivo. A second limitation of this study is the potential for nonspecific effects of the small molecule inhibitors [3, 31]. However, the tyrosine kinase inhibitors used in this study were selected based on their relatively specific effects (Table 1). Moreover, several of the currently available tyrosine kinase inhibitors are well tolerated

Fig. 5 Specific tyrosine kinase inhibitors reduced invasiveness by the 143B cell line. Basement membrane invasion assays were performed in the presence of the tyrosine kinase inhibitors listed in Table 1 or 1% DMSO as a vehicle control. Bars represent the means $\pm$ standard error of mean of three to five individual experiments, each with five wells per group. The asterisk denotes a decrease ($p = 6.1 \times 10^{-5}$) in invasiveness compared with the control group without inhibitor.

by patients in clinical trials [4, 37] despite their nonspecific effects [3, 31]. Nonetheless, future studies will be needed to determine whether more specific approaches such as siRNA or antisense demonstrate similar effects as the small molecule inhibitors used in this study.

Our in vitro assays of motility, colony formation, and invasiveness reflect the tumorigenic and metastatic capacity of the human osteosarcoma cell lines used in this study, similar to data published by other groups [30, 40]. These results provide rationale not only for testing the effects of tyrosine kinase inhibition, but also using these cell lines and in vitro assays for the initial screening of new therapeutic compounds.

Our data demonstrate specific tyrosine kinases regulate motility, colony formation, and invasiveness of osteosarcoma cells, all of which are critical components of tumorigenesis and/or metastasis. The EGF-R inhibitor substantially decreased motility, colony formation, and invasiveness of all tested cell lines. The receptor for EGF

Fig. 4A–E Photomicrographs show the effects of specific tyrosine kinase inhibitors on colony formation. Images of the 143B cell lines were collected after culture for 4 days in the presence of 1% DMSO

as a vehicle control (A), an inhibitor of EGF-R (B), an inhibitor of HER-2 (C), an inhibitor of IGF-1R (D), and an inhibitor of *met* (E). (D–E) reveal air bubbles, which became trapped in the collagen gel.

mediates growth of malignant cells and promotes cell survival [4], and uncontrolled activation of the EGF pathway causes many types of cancer [10]. Overexpression of EGF and its receptor is present in patients with osteosarcoma [5, 28, 65] and causes continuous growth and antiapoptosis signals in osteosarcoma cells [5, 28, 29]. Additionally, EGF stimulates motility [32, 64], invasion, and tumor progression of other types of cancer cells [44, 46]. Our results further support the development of EGF-R inhibitors as a novel osteosarcoma therapy. For example, gefitinib, a specific inhibitor of EGF-R, is currently in clinical trials for osteosarcoma and is well tolerated by children [16]. Also, CI-1033, which inhibits EGF-R and related receptor tyrosine kinases (HER-2, ErbB-3, ErbB-4), is currently in clinical trials for osteosarcoma [29].

The *met* inhibitor blocked colony formation by all tested cell lines and preferentially slowed the motility of the MNNG cell line. The preferential effect on motility reflects the known expression of the *tpo-met* oncogene by the MNNG cell line [51, 55]. Overexpression of HGF and *met*/HGF-R has been demonstrated in patients with osteosarcoma [15, 17] and causes malignant transformation of primary osteoblasts [52]. HGF increases motility, proliferation, and invasion of osteosarcoma cells [11, 42]. Our data suggest the *met* inhibitor affects not only the MNNG cell line, which expresses *tpo-met*, but also blocks colony formation by the TE85 and 143B cell lines, which do not express *tpo-met* [51, 55]. Inclusion of a *met* inhibitor in a chemotherapeutic regimen could therefore be potentially beneficial in patients with osteosarcoma, even in the absence of *tpo-met* expression.

The IGF-1R inhibitor blocked colony formation by all tested cell lines and preferentially slowed the motility of the SAOS-2 and LM-7 cell lines. IGF-1R and its ligands are overexpressed in human osteosarcoma cell lines [8]. IGF-1R overexpression mediates proliferation, provides apoptosis protection, and has been associated with increased invasiveness and metastasis [56]. Furthermore, IGF-1R activation induces motility [64] and protects tumor cells from apoptosis-inducing agents such as cytotoxic drugs, osmotic stress, and hypoxia [13, 54, 56]. Therefore, IGF-1R inhibition may provide tumor chemosensitization and enhance the effect of traditional chemotherapeutic agents when used in combination [56, 59]. Our results support the development and investigation of novel IGF-1R inhibitors.

The JAK family inhibitor preferentially increased motility of the SAOS-2 and LM-7 cell lines. This result is consistent with the finding that JAK-mediated activation of STAT1 suppresses metastasis of other types of tumors [24, 66]. In contrast, JAK-mediated activation of STAT3 or STAT5 increases metastasis of other tumors [24].

The inhibitors that target HER-2, NGF-R, and PDGF-R did not detectably affect motility, colony formation, or invasiveness of any of the tested cell lines. These results should not be interpreted as demonstrating these receptors are unimportant in patients with osteosarcoma. Rather, the data only show inhibitors of these receptors did not alter the malignant phenotype of the tested cell lines. The receptors may be overexpressed or activated in many patients with osteosarcoma but not in the cell lines used in this study.

Unlike many types of cancer that are the result of single genetic changes (translocations, mutations, deletions, and so on), osteosarcoma is characterized by a large number of genetic changes in each patient and a great diversity of genetic changes between patients [26, 57]. It is therefore unlikely a single “magic bullet” therapy will be uniformly successful for patients with osteosarcoma. Thus, development of a series of novel therapies is needed as well as methods to allow selection of the most appropriate therapy or therapies for each patient. Tyrosine kinases are a promising class of potential targets for development of such therapies [10, 21, 27]. However, the roles of most of the 90 tyrosine kinases in the human genome have not been studied in osteosarcoma or in other types of tumors. Ongoing studies in our laboratory are designed to identify novel tyrosine kinases that may contribute to tumorigenesis and/or metastasis in osteosarcoma. Discovery of new and interesting tyrosine kinases important in the malignant transformation of osteosarcoma will be the first step in developing new therapeutic agents. In vitro results will lead to experiments focusing on establishing cellular pathways and understanding mechanisms of action with the goal of transitioning to an animal model and eventually human clinical trials.

These data demonstrate specific tyrosine kinases regulate motility, colony formation, and invasiveness of osteosarcoma cell lines. Therefore, tyrosine kinase inhibition provides a promising avenue in the evolving treatment of osteosarcoma.

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