Targeting Inflammatory Kinase as an Adjuvant Treatment for Osteosarcomas

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Background: A subset of patients with aggressive osteosarcomas responds poorly to conventional cytotoxic chemotherapy. Recent evidence from studies involving the liver, skin, stomach, and colon suggests that carcinogenesis is associated with inflammation. Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) has diverse roles in cancer and inflammation. The hypothesis of the present study is that targeted ERK1/2 inhibition will demonstrate anti-cancer effects in osteosarcoma both in vitro and in vivo.

Methods: The therapeutic effect of PD98059, a MAPK/ERK pathway inhibitor, was examined with respect to cell death, survival, and anti-apoptotic protein expression by means of flow cytometry and immunoblotting in vitro. Additionally, we transplanted green fluorescent protein and luciferase-tagged 143B osteosarcoma cells into the proximal part of the tibia of nude mice. Mice were randomly assigned to treatment with doxorubicin, PD98059, or both. Vehicle-treated mice served as controls. Treatment outcome was assessed by measuring bioluminescence and by monitoring survival.

Results: In vitro, ERK1/2 blockage increased the expression of pro-apoptotic proteins and increased cell death in 143B osteosarcoma cells. Doxorubicin treatment increased the expression of Bcl-2, an anti-apoptotic protein, but this upregulation was blocked by combined treatment with PD98059, suggesting a role for ERK1/2 in conferring drug resistance. In osteosarcoma-bearing mice, targeting ERK1/2 with PD98059 resulted in prolonged survival in comparison with vehicle-treated control mice (median survival time, sixty-seven days compared with seventy-four days; $p = 0.0272$; survival ratio = 0.9122; 95% confidence interval = 0.4354 to 1.389). Standalone doxorubicin treatment yielded similar animal morbidity (median survival time, sixty-seven days compared with seventy-six days; $p = 0.0170$; survival ratio = 0.8882; 95% confidence interval = 0.4181 to 1.358). Combined PD98059 and doxorubicin treatment further prolonged survival (median survival time, sixty-seven days compared with eighty-two days; $p = 0.0023$; survival ratio = 0.8232; 95% confidence interval = 0.3606 to 1.286).

Conclusions: Inhibiting ERK1/2 signaling resulted in osteosarcoma cell death by upregulating pro-apoptotic genes and inhibiting the Bcl-2-mediated resistance to doxorubicin. In osteosarcoma-bearing mice, ERK1/2 targeting alone or in combination with doxorubicin prolonged survival as compared with untreated mice.

Clinical Relevance: Our study highlights the anti-cancer effect of the inflammatory kinase inhibitor PD98059 on osteosarcoma cells by inducing cell death and by inhibiting a potential drug-resistance mechanism. Taken together, these results suggest that ERK signaling blockade (targeted therapy) may be considered as a new targeted adjuvant therapy for osteosarcoma.

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A commentary by John H. Healey, MD, is available at www.jbjs.org/commentary and is linked to the online version of this article.

Osteosarcoma is the most common primary malignant neoplasm of bone. While the five-year survival rate for patients with nonmetastatic osteosarcoma has increased over the past forty years, from between 10% and 20% to between 70% and 80%[1], there has been little improvement in the survival time of patients with metastatic or multifocal osteosarcoma[2]. Therefore, there is an unmet medical need for a new generation of pharmacologic treatments that target pathways involved in the pathogenesis of osteosarcoma.

Virchow postulated that cancer arises at sites of inflammation[3,4]. Recent evidence from studies involving the liver, skin, colon and stomach supports the notion that carcinogenesis can be associated with inflammation[3-5]. On the basis of these historical and epidemiologic observations, inflammatory pathways have been extensively examined in malignant tumors with respect to pathogenesis, and several targeted pharmacologic treatments have been developed[6]. Inflammation is a cascade of cellular events that are mediated by signal transducers. Kinases are among such transducers and participate in the regulation of the inflammatory process. Mitogen-activated protein kinase (MAPK) 1/2, also known as extracellular signal-regulated kinase 1/2 (ERK1/2), acts downstream of the RAS oncogene and has been implicated in the regulation of a wide range of cellular processes such as proliferation, differentiation, survival, and motility[6-11]. The MAPK pathway has also been identified as a key mechanism for aggressive behavior of malignant tumors with respect to metastasis, survival, and drug resistance[11], all of which are logical targets of cancer treatment. Inhibitors of the RAS/MAPK/ERK pathway have been developed for use in patients with melanoma[12]. With respect to osteosarcoma, previous in vivo animal experiments have shown that 143B cells, a high-grade human osteosarcoma cell line with elevated RAS activity, are associated with higher rates of orthotopic sarcoma growth and lung metastasis when compared with non-RAS-activated osteosarcoma cells[11].

In the present study, we hypothesized that targeted ERK1/2 inhibition would demonstrate anti-cancer effects in osteosarcoma both in vitro and in vivo. We treated human osteosarcoma cells with PD98059, a high-specificity pharmacologic inhibitor of the ERK1/2 kinase, MEK[13]. We compared the anti-cancer effect of PD98059 with that of doxorubicin, one of the most commonly used cytotoxic agents for osteosarcoma[14-17]. Doxorubicin intercalates with DNA[18] and also causes the formation of reactive oxygen species[19,20], both of which contribute to its cytotoxicity toward dividing cells. The underlying mechanism of action of PD98059 was also explored.

Materials and Methods

Cell Cultures and siRNA Transfections

Experiments were performed with use of two related human osteosarcoma cell lines. 143B cells were provided by Dr. Hue H. Luu, University of Chicago Medical Center. SaOS-2 cells were purchased from the American Type Culture Collection (Manassas, Virginia). 143B cells were grown in Dulbecco modified Eagle medium supplemented with 1% antibiotics, including penicillin and streptomycin, and 10% heat-inactivated fetal bovine serum (FBS) in 5% CO_2 at 37°C. SaOS-2 osteosarcoma cells were cultured in McCoy 5a medium with 1% antibiotics and 15% FBS. The culture medium was changed every two days. All experiments were performed on cells harvested at the mid-log phase of growth. Small interfering RNAs (siRNAs) decrease or knock down target mRNA expression. SiRNAs are necessary tools for loss-of-function experiments. The cells were transiently transfected with ERK1 and ERK2 siRNAs (Santa Cruz Biotechnology, Santa Cruz, California) with use of PrimeFect siRNA Transfection Reagent (Lonza, Walkersville, Maryland). Transfection Reagent and siRNA were mixed with OptiMEM medium (Invitrogen, Carlsbad, California). Fifteen minutes after incubation with the mixture, 1 μg/mL of siRNA was added[21].

Cell Survival Assay

The MTT assay measures the activity of enzymes that reduce yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to purple formazan. These reductions take place only when mitochondrial reductases are active, and therefore conversion is often used to identify living cells. Cells were seeded on ninety-six-well plates at a density of 7×10^3 cells/well. The cells were given 100 μL of fresh medium the next day, and 10 μL of a 12-mM MTT solution (Invitrogen) was added to each well. A negative control, containing 10 μL of the MTT solution added to 100 μL of medium alone and incubated at 37°C for four hours, was also included. After incubation, 100 μL of a solution containing 0.347-mM SDS-HCl was added to each well and mixed. The ninety-six-well plates were incubated at 37°C for four hours and then were read for absorbance at 570 nm.

Flow Cytometric Detection of Dying Cells

Cells were seeded at a concentration of 1×10^5 cells per well on a twelve-well plate. Twenty-four hours after seeding, the cells were changed to serum-free medium for sixteen hours. They were then treated with doxorubicin (0.1 μM), PD98059 (20 μM), or both for thirty-six hours. The cells were washed once with cold phosphate-buffed saline solution (PBS) and were collected with conditioned medium. The collected cells were placed into tubes and centrifuged at 1200 rpm for five minutes and were fixed with 70% ethanol in a –20°C freezer. The cells were resuspended in RNase buffer. Cells were then stained with annexin V (BD Pharmingen, San Diego, California) and propidium iodide (PI). Phosphatidylserine-positive cells and PI-permeant cells were counted with use of a flow cytometer (FACSCalibur; Becton Dickinson Biosciences, San Jose, California).

Immunocytochemical Detection of pERK1/2

Cells were seeded on four-well chamber slides at a density of 1×10^3 cells/well. The slides were treated with PD98059 and/or doxorubicin, as described previously. Cells were fixed with 4% formalin in PBS for fifteen minutes at room temperature. The fixed cells were washed with pure PBS and then were blocked and permeabilized for one hour at room temperature in PBS containing 0.25% Triton X-100, 0.5% Tween-20, and 1% bovine serum albumin. After blocking, the cells were stained with phosphorylated-ERK1/2 antibody (1:50 dilution) (Cell Signaling Technologies, Beverly, Massachusetts). Alexa Fluor 488 goat anti-rabbit-IgG (1:1000 dilution) (Invitrogen, Carlsbad, California) was used as a secondary antibody. The level of phosphorylated (activated) ERK1/2 (pERK1/2) was then visualized by means of fluorescence microscopy.

Detection of pERK1/2, Pro-Apoptotic, and Anti-Apoptotic Proteins with Western Blot Analysis

Cells were seeded on 10-cm dishes at a density of 1×10^6 cells/well. The cells were stimulated with doxorubicin and/or PD98059, as described, and were rinsed once with cold PBS. Cells were treated with lysis buffer and were sonicated. Protein lysate (20 μg) was applied to a 10% SDS (sodium dodecyl sulfate) gel. The proteins were separated by means of SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and were transferred to a PVDF (polyvinylidene fluoride) membrane. The membrane was incubated with 5% nonfat milk in TBST (Tris-buffered saline and Tween) (25 mM Tris/

HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20) for one hour at room temperature, was washed three times in TBST, and then was incubated overnight with antibodies raised against a variety of targets of interest, including pERK1/2, Bcl-2, Bcl-xL, Bim, and Bax. All antibodies were purchased from Cell Signaling Technologies and were diluted according to the manufacturer's recommendations. The membrane was washed three times with TBST and was incubated with HRP (horseradish peroxidase)-conjugated secondary antibody for thirty minutes. After incubation, the membrane was again washed three times with TBST, and proteins were visualized with use of a commercial HRP-detection reagent (Amersham ECL Plus; GE Healthcare, Piscataway, New Jersey).

Osteosarcoma Animal Model and Immunohistochemistry

All animal procedures were performed in accordance with approved Institutional Animal Care and Use Committee protocols. Six to eight-week-old female athymic nude mice were purchased from Jackson Laboratory (Bar Harbor, Maine). 1×10^6 GFP-Firefly luciferase-tagged 143B cells (kindly provided by Dr. Hue Luu, University of Chicago) in 50 μL of PBS were injected orthotopically into the proximal part of the left tibia with the animal under general anesthesia. 143B cells were chosen for xenografting because of their known aggressive growth in mouse models[11]. The first set of experiments (n = 17 mice) was performed to confirm the relationship between tumor size and bioluminescence signal intensity. Bioluminescence signal intensity and tumor volume were measured in a longitudinal cohort of mice every three days from transplantation until death. Tumor width and length were determined with use of electric calipers, and tumor volume was calculated according to the modified ellipsoid formula ½(length × width^2)[22,23].

The second set of experiments was performed to confirm that the PD98059 treatment regimen was sufficient to attain pharmacologic inhibition of pERK1/2 activity in vivo. Osteosarcoma-bearing mice were treated with intraperitoneal injections of PD98059 (5 mg/kg body weight), doxorubicin (5 mg/kg body weight), or both for three consecutive days (n = 5 for each of the three experimental groups and a control group; total = 20). The animals were killed in a carbon dioxide chamber, and the left leg (with tumor) was excised, fixed, decalcified, embedded, sectioned, and immunostained for pERK with use of monoclonal antibodies (Cell Signaling Technologies), as previously described[21].

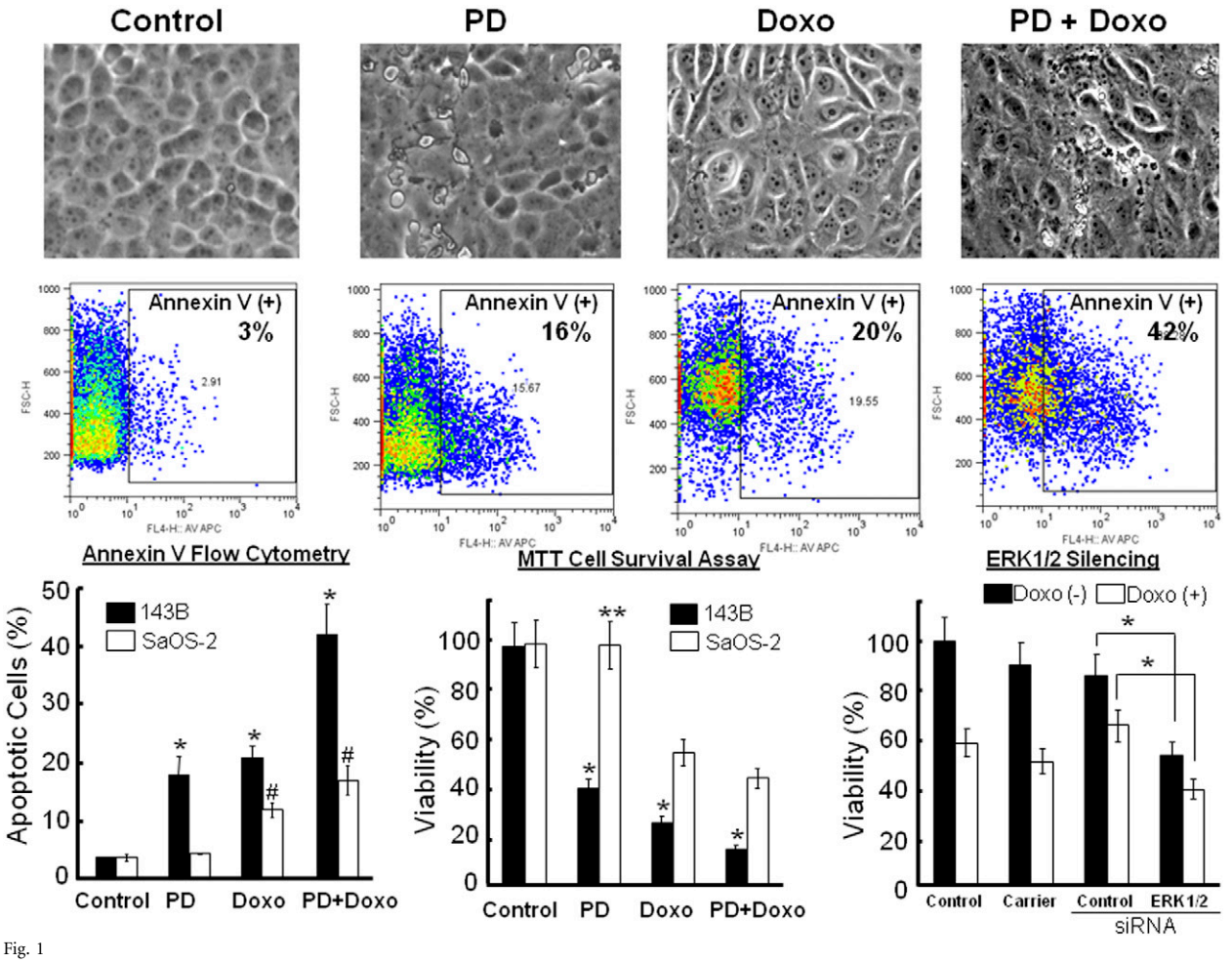


Fig. 1

Assessment of cell death and survival after inhibition of ERK1/2 by PD98059 and siRNA. PD98059 (PD) increased apoptosis in 143B osteosarcoma cells, which have higher basal pERK1/2 activity compared with SaOS-2 cells. Combined treatment with doxorubicin (Doxo) and PD98059 resulted in higher cell death compared with doxorubicin alone. Statistical analysis was performed with use of the Student t test (*$p < 0.005$, n = 3; **$p < 0.01$, n = 8; #$p < 0.05$, n = 3). The error bars denote the standard deviation.

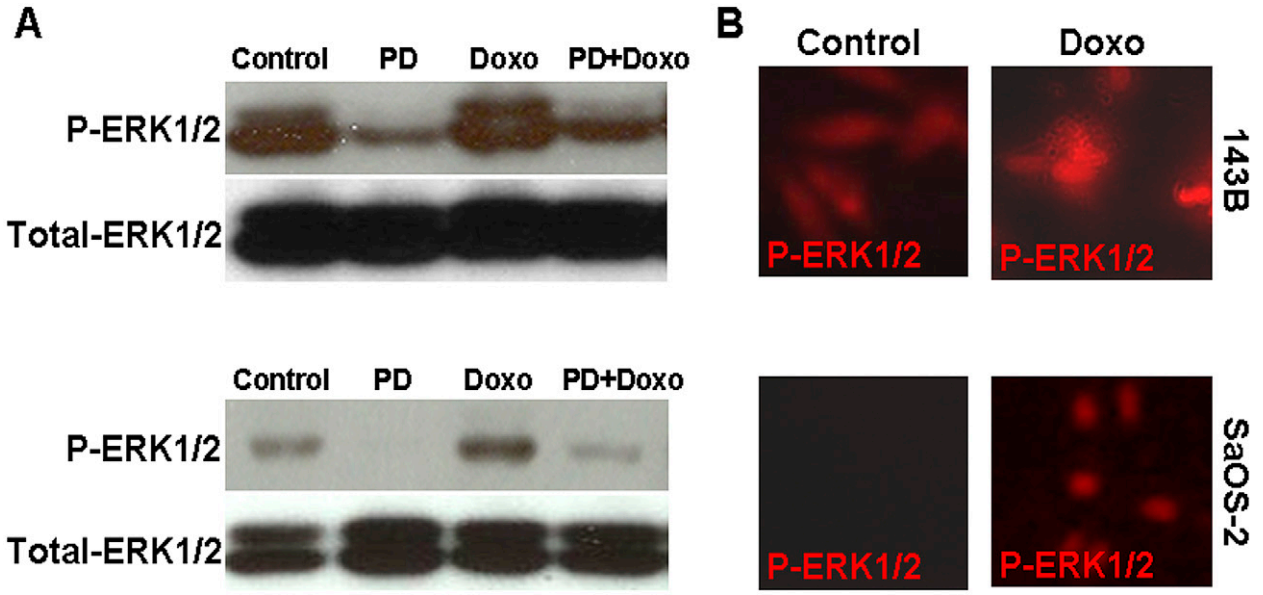


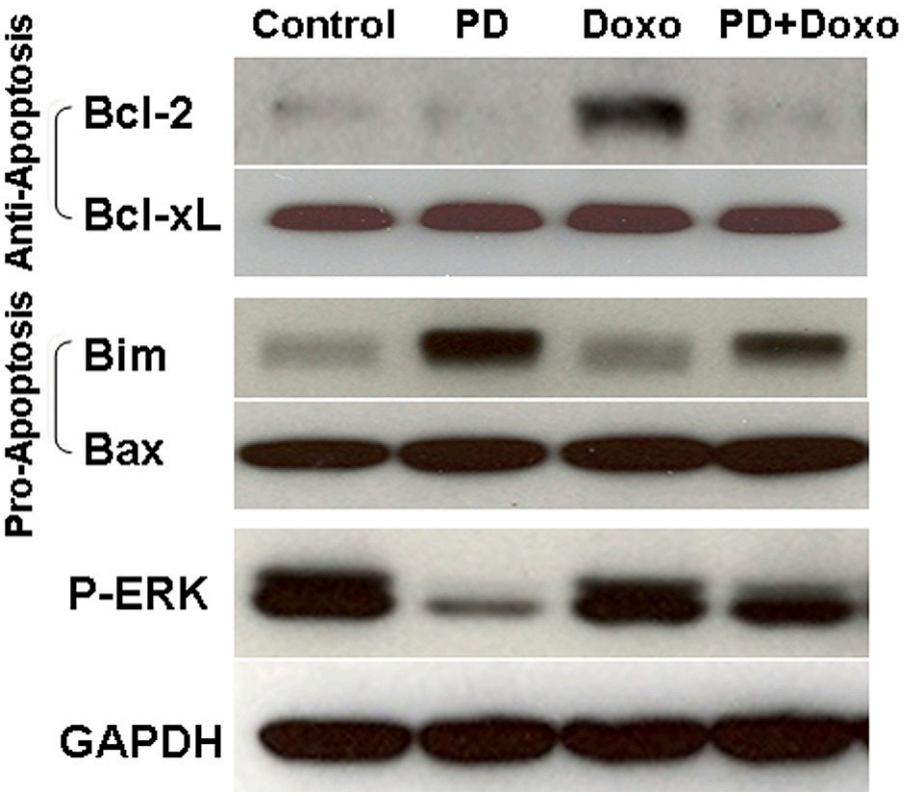
Fig. 2

Western blotting and immunocytochemistry with anti-pERK antibody, demonstrating increased pERK activity in 143B and SaOS-2 cells following doxorubicin treatment. 143B cells, which are known to demonstrate more aggressive tumor growth in vivo, exhibit higher pERK activity at basal level. PD = PD98059, Doxo = doxorubicin.

The third experiment was performed to compare the survival of untreated sarcoma-bearing mice (control) with that of mice treated with PD98059 and/or doxorubicin regimens (n = 15 for each group; total = 60).

Statistical Analysis

The Student t test and Kaplan-Meier survival analysis were performed. The level of significance was set at $p < 0.05$. The sample size was estimated



Immunoblotting of 143B whole cell lysates for pro-apoptotic and anti-apoptotic proteins following treatment with PD98059 (PD) and/or doxorubicin (Doxo). pERK inhibition by PD98059 increased Bim, a pro-apoptotic regulator. Doxorubicin treatment increased expression of Bcl-2, an anti-apoptotic regulator that promotes cell survival under stress. Combined treatment with PD98059 and doxorubicin blocked this effect, returning Bcl-2 to control levels. Bax and Bcl-xL did not change after PD98059 treatment, suggesting specific effects of PD98059 on Bcl-2 and Bim.

Fig. 3

on the basis of the results of previously published studies[24,25]. A study power analysis was performed to confirm the resulting power of the study.

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This research was funded by the Orthopaedic Science and Research Foundation. The funds were used for materials and supplies.

Results

In Vitro Anti-Cancer Effects of ERK1/2 Targeting

Annexin V flow cytometry demonstrated that 15.67% of PD98059-treated cells and 19.55% of doxorubicin-treated cells underwent apoptosis, compared with 2.91% in the control group ($p < 0.05$) (Fig. 1). Furthermore, when cells were treated with both PD98059 and doxorubicin, 42% of osteosarcoma cells underwent apoptosis (Fig. 1). These results support earlier findings that MEK inhibitors have an independent anti-cancer effect[26]. Combined treatment with PD98059 and doxorubicin showed a summative effect compared with treatment with either therapy individually.

Functional Validation of ERK1/2 Targeting

To study the effects of PD98059 on osteosarcoma cells in vitro, 143B and SaOS-2 cells were treated with PD98059, doxorubicin, or both. Western blot analysis confirmed that PD98059 treatment of 143B and SaOS-2 cell cultures diminished the levels of activated (phosphorylated) ERK1/2 protein while leaving total ERK1/2 levels unchanged (Fig. 2, *A*). This result was expected, given that PD98059 is a known MAPK/ERK pathway inhibitor. Furthermore, both 143B and SaOS-2 cells showed increased ERK1/2 activation in response to doxorubicin treatment (Fig. 2, *A*), which was confirmed with fluorescence immunocytochemistry (Fig. 2, *B*). When 143B cells were treated with both PD98059 and doxorubicin, PD98059 reversed the doxorubicin-induced activation of ERK1/2 and restored pERK1/2 expression to control levels (Fig. 2, *A*).

Functional Relationship Between ERK1/2 and Cancer Cell Dynamics

To investigate the possibility that ERK inhibition realized its anti-cancer effect through suppression of a drug-resistance/cell-survival mechanism, 143B cells were treated with PD98059 and/or doxorubicin, and Western blot testing was performed for several well-known apoptotic regulators of the Bcl-2 family, including Bcl-2, Bcl-xL, Bim, and Bax[27]. PD98059 treatment alone was found to increase the expression of pro-apoptotic Bim (Fig. 3). This finding correlates well with the increase in apoptosis seen with annexin V staining (Fig. 1). Notably, when

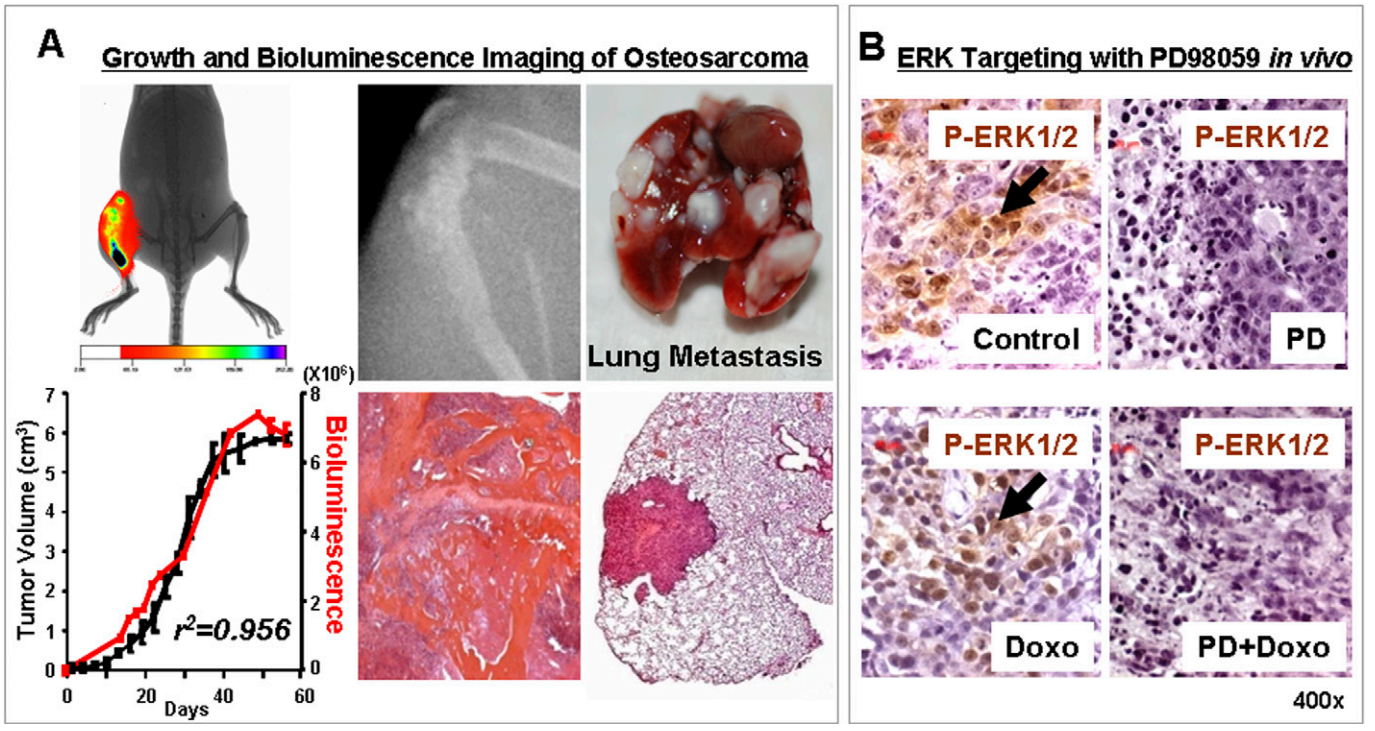

Fig. 4

Transplantation of luciferase-tagged 143B cells in the proximal part of the tibiae of nude mice. *A:* 143B cells grew aggressively in the tibia and readily metastasized to the lung. Bioluminescence intensity correlated well with tumor volume (n = 17). Tumor volume and bioluminescence imaging demonstrated a three-phase growth pattern consisting of an initial toe region, a linear growth period between three and seven weeks, and a plateau at seven weeks. PD = PD98059, Doxo = doxorubicin. *B:* Immunohistochemical staining of necropsy specimens with pERK1/2 antibodies demonstrated that a three-day PD98059 treatment regimen successfully blocked pERK expression in vivo. The arrows indicate pERK1/2 expression.

treated with doxorubicin alone, 143B cells were found to up-regulate production of anti-apoptotic Bcl-2 as compared with the control (Fig. 3). This doxorubicin-induced Bcl-2 upregulation was blocked by combined treatment with doxorubicin and PD98059 (Fig. 3). Bcl-xL and Bax expression levels were not affected by PD98059 and/or doxorubicin.

Anti-Cancer Effect of PD98059 in Vivo

The anti-cancer effect of ERK1/2 inhibition in vivo was examined via bioluminescence imaging of tumor-bearing mice. Mice were given intra-tibial injections of 143B cells, which were allowed to grow for four weeks (Fig. 4). The mice were then treated with PD98059, doxorubicin, or both. Bioluminescence imaging intensity was found to correlate with caliper-measured tumor size ($R^2 = 0.95$) (Fig. 4, *A*). Therefore, we used bioluminescence imaging intensity as an indirect indicator of drug response. Bioluminescence imaging intensity diminished significantly over the first six days in the PD98059-treated group when compared with the control (Fig. 5). The duration of PD98059 effect was less than one week. Interestingly, in the doxorubicin-alone treatment group, no significant difference in bioluminescence imaging intensity when compared with the control was demonstrated until day nine (Fig. 5). The combined treatment group showed more pronounced anti-tumor effects on Day 9 (Fig. 5). These results indicate that PD98059 may have early anti-cancer effects that doxorubicin alone lacks.

Survival Analysis

Mice were followed for the remainder of their lives to determine the effects of treatment on long-term survival. Untreated mice eventually died with extensive lung metastasis (Fig. 4, *A*). The median survival time for untreated mice was sixty-seven days (Fig. 6). Treatment groups that were given doxorubicin, PD98059, or both were found to have a significant increase in the median survival rate as compared with the control ($p = 0.017$, 0.0272, and 0.0023, respectively) (Fig. 6). These data indicate that PD98059 can be effective for prolonging median survival time when given alone or in combination. While the treatment group that received both doxorubicin and PD98059 did not demonstrate significant difference from the groups that were treated with either PD98059 alone or doxorubicin alone, the longest mean survival time (eighty-two days) and the longest single survival time (108 days) were both found in the combined doxorubicin and PD98059 treatment group.

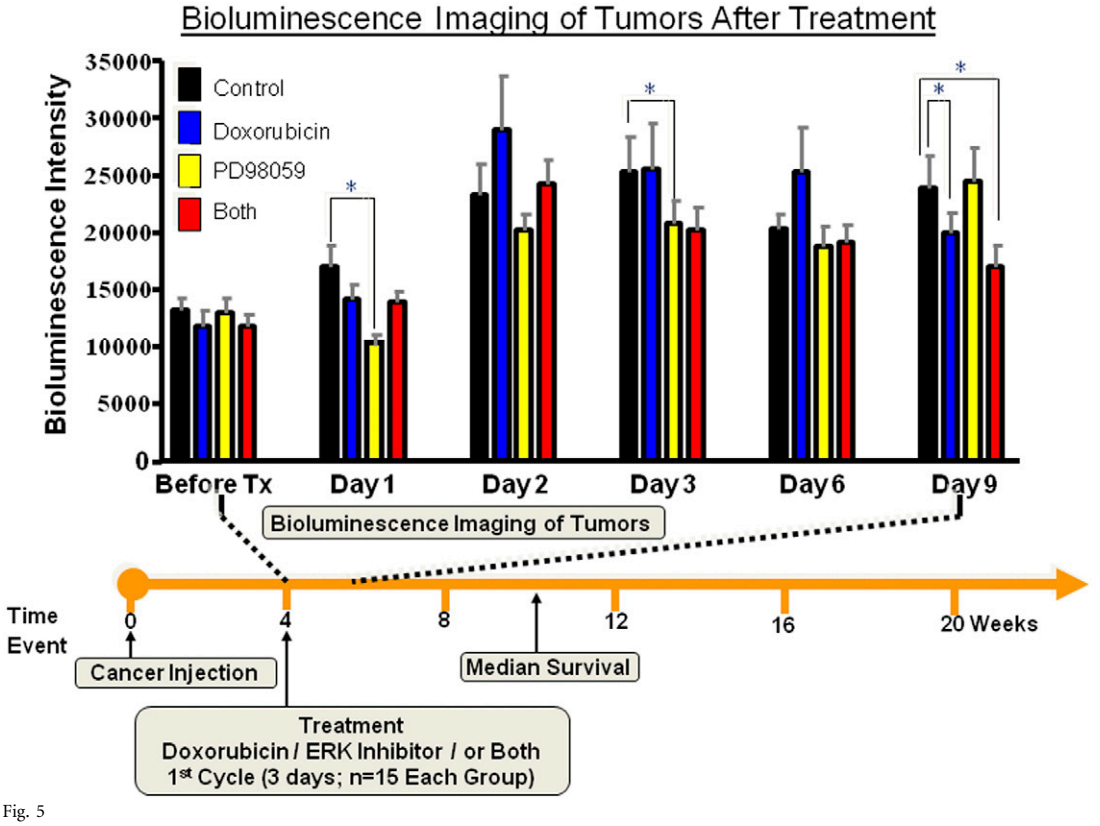

Fig. 5

Assessment of tumor growth with use of bioluminescence imaging. PD98059 treatment showed suppression of tumor growth in the first three days, whereas doxorubicin did not show significant effects until day nine, suggesting different mechanisms for the anti-cancer effects of these two drugs. *$p < 0.05$. The error bars denote the standard deviation.

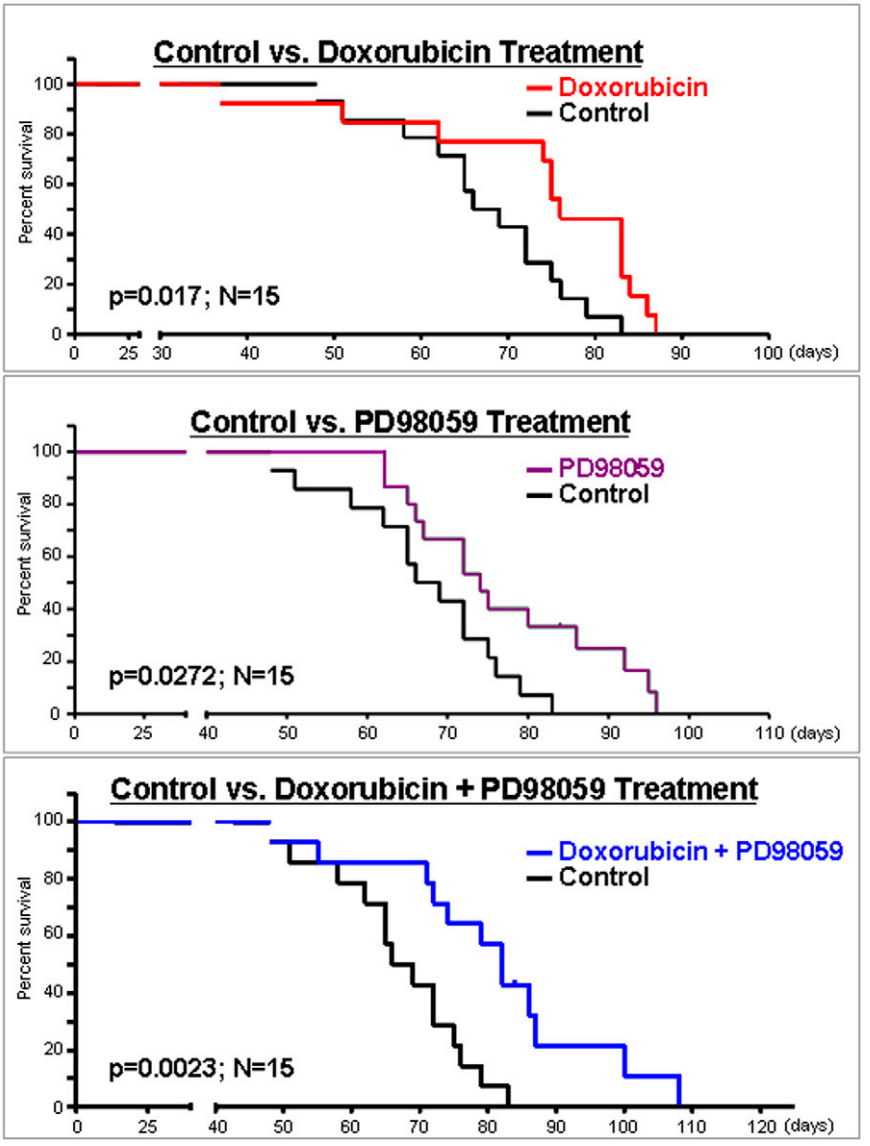

Fig. 6

Survival analyses demonstrating the effect of a single three-day course of treatment with pERK inhibitor and/or doxorubicin in osteosarcoma-bearing mice. Consistent with in vitro experiments, pERK inhibition alone prolonged the survival of osteosarcoma-bearing mice compared with untreated mice (median survival time, sixty-seven days compared with seventy-four days; $p = 0.0272$; survival ratio = 0.9122; 95% confidence interval = 0.4354 to 1.389). Doxorubicin treatment increased the median survival time (median survival time, sixty-seven days compared with seventy-six days; $p = 0.0170$; survival ratio = 0.8882; 95% confidence interval = 0.4181 to 1.358). Combined treatment with PD98059 and doxorubicin further prolonged the survival compared with control (median survival time, sixty-seven days compared with eighty-two days; $p = 0.0023$; survival ratio = 0.8232; 95% confidence interval = 0.3606 to 1.286).

A study power analysis was performed with use of PASS 2008, a power analysis software program from NCSS (Kaysville, Utah). With use of one-way analysis of variance (ANOVA) to compare the test groups to the control group, with $p < 0.05$, the study power of the experiment was 0.824. The average effect size was 0.643[24,25].

Discussion

Chronic inflammation may play a role in the pathologic basis for a majority of human malignancies, including osteosarcoma. From the late 1800s, when Virchow noticed leukocytes in neoplastic tissues, and continuing to the present day, our understanding of the relationship between cancer and

inflammation continues to expand[2-5]. Although inflammation provides a protective function to the host, chronic activation of an inflammatory response can be detrimental. Several lines of evidence suggest that the development of carcinomas of the stomach, liver, gallbladder, prostate, and pancreas can be attributed to multiple chronic states of inflammation, including *Helicobacter pylori*-induced gastric inflammation, chronic hepatitis, cirrhosis, cholecystitis, inflammatory atrophy of the prostate, and chronic pancreatitis[4].

Patients with osteosarcoma demonstrate multiple signs of inflammation such as redness (rubor), swelling (tumor), heat (calor), pain (dolor), and loss of function (functio laesa) throughout the course of the disease at both the gross and cellular levels. On a cellular level, various inflammatory pathways are activated. One such pathway, RAS/MAPK/ERK1/2, is implicated in inflammation as well as tumor formation in more aggressive forms of osteosarcoma.

PD98059, an ERK1/2 inhibitor, was developed to selectively inhibit the RAS/MAPK/ERK1/2 cascade[13,28]. Previous use of ERK1/2 inhibitors suppressed proliferation, limited invasiveness, and moderately induced apoptosis in several types of tumor cells. Furthermore, when ERK1/2 inhibitors were combined with conventional chemotherapy, they enhanced the effectiveness of the traditional treatment form. For example, ERK1/2 inhibitors enhanced the cytotoxic effects of doxorubicin in H460 human non-small-cell lung cancer cell lines in vitro[29]. In the present study, we found that PD98059, a pharmacological inhibitor of ERK1/2, produced anti-cancer effects in osteosarcoma cells by inducing cell death, inhibiting drug resistance mechanisms, demonstrating early anti-tumor activity, and prolonging survival time in an aggressive metastatic osteosarcoma experimental model.

ERK1/2 targeting with PD98059 exhibited anti-cancer effects on osteosarcomas both in vitro and in vivo. PD98059 treatment alone resulted in an increase in apoptosis of 143B human osteosarcoma cells in vitro. In vivo, PD98059 treatment significantly decreased tumor activity within the first seven days and prolonged the median survival of osteosarcoma-bearing mice as compared with the control. Cell death increases correlated with increased Bim expression and provide insight to the apoptotic signaling mechanism by which PD98059 inhibits tumor growth. These findings indicate that PD98059 has a therapeutic potential as an effective stand-alone anti-cancer treatment for osteosarcoma.

Doxorubicin, also known by the tradename Adriamycin, is an important anthracycline antibiotic used for the treatment of osteosarcomas. Multiple mechanisms have been proposed for the cytotoxic and cytostatic actions of doxorubicin, including intercalation into DNA, topoisomerase II inhibition, and free radical formation[14-17,19,20]. From our data, it appears that PD98059 exerts its anti-cancer effects by a different mechanism and through different timing than doxorubicin does. While only PD98059 and doxorubicin interactions were accessed in the current study, future studies will explore other commonly used chemotherapeutic agents such as cisplatin and methotrexate.

PD98059 played a role in reversing doxorubicin-resistance mechanisms in 143B cells and thus may function as an adjuvant therapy with doxorubicin. We found that doxorubicin treatment increased the expression of pERK1/2 and Bcl-2, a pro-survival protein, in 143B osteosarcoma cells. Combined treatment with doxorubicin and PD98059 blocked both responses, returning both pERK1/2 and Bcl-2 to control levels. This paradoxical upregulation of Bcl-2 by doxorubicin may represent a drug-resistance mechanism activated by osteosarcoma cells in response to cytotoxic stress. PD98059 may exert its anti-cancer effect through blockade of this survival machinery.

PD98059 provides early anti-cancer effects in osteosarcomas that are not seen with doxorubicin treatment alone in vivo. After osteosarcoma-bearing mice were treated with either PD98059, doxorubicin, or both, tumor growth was assessed with use of bioluminescence imaging. Treatment with PD98059 alone generated significant changes in tumor bioluminescence when compared with the control within six days after treatment. Doxorubicin, on the other hand, did not show anti-tumor effects until nine days after treatment. These data indicate that PD98059 may improve osteosarcoma treatment by decreasing tumor response time to therapy. This further affirms the potential therapeutic role of ERK1/2 targeting as an adjuvant with doxorubicin and perhaps other current cytotoxic agents such as methotrexate and cisplatin.

One strength of the present study is the longitudinal survival analysis. Targeting ERK1/2 with PD98059, doxorubicin, or both resulted in prolonged survival when compared with control. Based on our results, targeting ERK1/2 exhibited anti-cancer effects by upregulating Bim, a pro-apoptotic protein, and by inhibiting doxorubicin-induced expression of Bcl-2, a pro-survival protein (Fig. 7). This study of increased survival time provides a unique look at the effect of therapy with PD98059 that, to our knowledge, has never been assessed in osteosarcomas. Although tumor resection is commonly performed after preoperative neoadjuvant treatment, it was not technically feasible to conduct tumor resection or limb preservation in such small animals. Nevertheless, patients with multifocal osteosarcomas or unresectable osteosarcomas of the spine or pelvis are often treated with palliative chemotherapy. Thus, even without surgical resection our experiment remains clinically relevant.

In this proof-of-concept study, we limited the drug treatment regimen to a single three-day cycle. For future study, we would consider additional treatment cycles given that the median survival time was approximately nine weeks after tumor transplantation. Given that the treatment group that received both doxorubicin and PD98059 was not found to differ significantly from the groups that received either treatment alone, the treatment regimen may need to be intensified with respect to the dose and number of cycles in order to replicate the summative effect seen in vitro. We are aware of some limitations of using a small animal osteosarcoma-xenograft model. Nude (athymic) mice possess T-cell deficiency and are inclined to accept human cancer xenografts. However, these mice possess competent B-cells, macrophages, and natural killer cells.

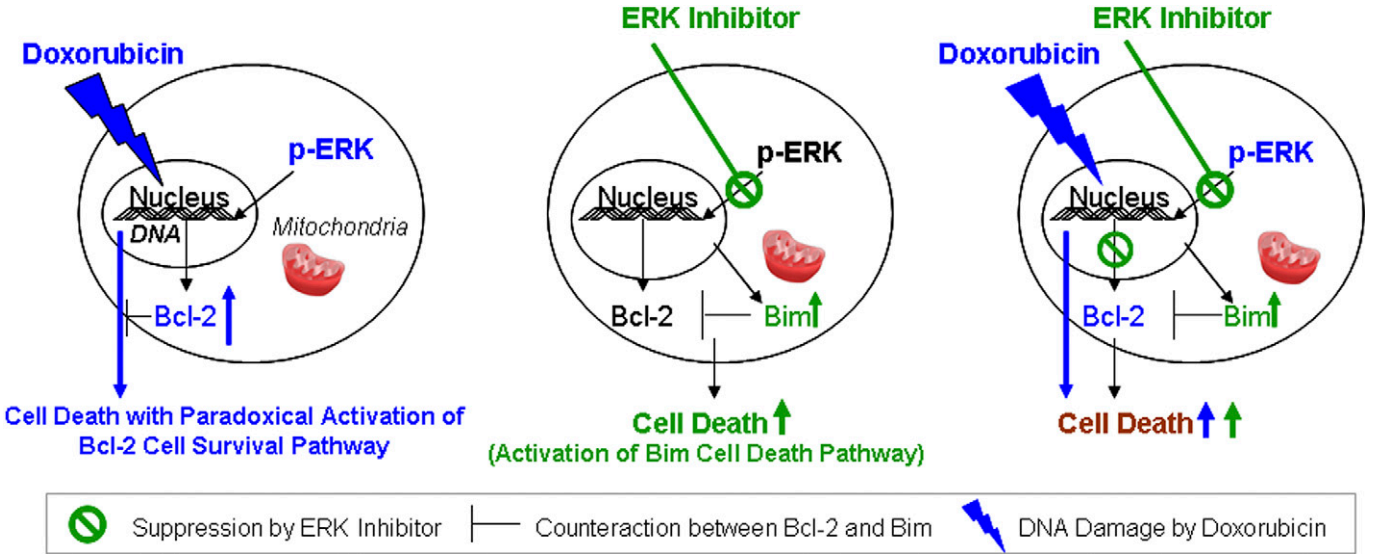

Fig. 7

Three models demonstrating mechanisms of anti-cancer effects. *Left:* Doxorubicin exhibits anti-cancer effects by intercalating DNA. However, doxorubicin concurrently upregulates expression of Bcl-2, an anti-apoptotic protein. *Middle:* ERK1/2 inhibition results in an increase of Bim expression, which promotes cell death by counteracting Bcl-2. *Right:* Combined treatment with ERK1/2 inhibitor and doxorubicin demonstrates enhanced anti-cancer effects by inducing Bim-mediated cell death, DNA targeting by doxorubicin, and blockage of doxorubicin-induced Bcl-2 expression.

Spontaneously occurring osteosarcomas have been identified in conditional mice lacking p53 and pRb[30-32]. Although immune cells are competent in syngeneic mice receiving murine osteosarcoma cells, tumor burdens and clonal variations may interfere with data interpretation. For preclinical study, naturally occurring osteosarcomas in large-animal models are more ideal than small-animal models. However, recruitment of osteosarcoma-bearing dogs and heterogeneous cancer biology entail high experimental cost and obstacles. Further collaborative studies with a veterinary oncologist would provide a new opportunity for preclinical experiments[33-35]. pERK is now being used in clinical trials in patients with advanced cancer[36]. Additional studies will be necessary to further explore pERK inhibitors in osteosarcoma. Last, we envision that customized or personalized treatment for osteosarcoma may be designed by screening inflammatory kinase activity in the biopsy specimens prior to chemotherapy. Taken together, the increased survival time demonstrated in the present study strongly encourages further investigation into the long-term benefits of the combination of ERK1/2 targeting with conventional cytotoxic agents. ■

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