Antitumor Activity of the Insulin-Like Growth Factor-I Receptor Kinase Inhibitor NVP-AEW541 in Musculoskeletal Tumors

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Abstract

Identification of new drugs is strongly needed for sarcomas. Insulin-like growth factor-I receptor (IGF-IR) was found to provide a major contribution to the malignant behavior of these tumors, therefore representing a very promising therapeutic target. In this study, we analyzed the therapeutic potential of a novel kinase inhibitor of IGF-IR, NVP-AEW541, in Ewing’s sarcoma, osteosarcoma, and rhabdomyosarcoma, the three most frequent solid tumors in children and adolescents. NVP-AEW541 inhibits IGF-I-mediated receptor activation and downstream signaling. Ewing’s sarcoma cells were generally found to be more sensitive to the effects of this drug compared with rhabdomyosarcoma and osteosarcoma, in agreement with the high dependency of this neoplasm to IGF-IR signaling. NVP-AEW541 induced a G1 cell cycle block in all cells tested, whereas apoptosis was observed only in those cells that show a high level of sensitivity. Concurrent exposure of cells to NVP-AEW541 and other chemotherapeutic agents resulted in positive interactions with vincristine, actinomycin D, and ifosfamide and subadditive effects with doxorubicin and cisplatin. Accordingly, combined treatment with NVP-AEW541 and vincristine significantly inhibited tumor growth of Ewing’s sarcoma xenografts in nude mice. Therefore, results encourage inclusion of this drug especially in the treatment of patients with Ewing’s sarcoma. For the broadest applicability and best efficacy in sarcomas, NVP-AEW541 may be combined with vincristine, actinomycin D, and ifosfamide, three major drugs in the treatment of sarcomas. (Cancer Res 2005; 65(9): 3868-76)

Introduction

Altered expression of insulin-like growth factors (IGF) I and II and their corresponding receptors, particularly IGF-I receptor (IGF-IR), is shown in a variety of tumors, including sarcomas (for a review, see refs. 1, 2). In detail, osteosarcoma and Ewing’s sarcoma, the two most frequent bone tumors (although the cell of origin of Ewing’s sarcoma has not been clearly established) usually arising in children and adolescents (3), as well as rhabdomyosarcoma, the most common soft tissue sarcoma of childhood (4), show the presence of both active IGF-IR and the autocrine production of its ligands IGF-I and/or IGF-II (5–7). Although several other growth factor circuits are involved in deregulated cell growth of these neoplasms (8–12), the contribution of IGF-I/IGF-IR circuit to the malignant behavior of these cells has been clearly identified as of major importance. IGF-IR is implicated in autocrine and paracrine control of sarcoma growth and seems to be particularly relevant in pathogenesis of these tumors (13–18). Indeed, impairment of IGF-IR by using different approaches, including antisense technologies, antibodies against IGF-IR, and dominant-negative mutants of IGF-IR, reduces growth, increases apoptosis of sarcoma cells both in vitro and in vivo, and significantly decreases migration, invasion, and metastatic spread to lungs and bones (5, 19–25) as well as their angiogenetic properties (26), thus representing a good therapeutic approach against these tumors. Moreover, targeting IGF-IR resulted in chemosensitization to conventional cytotoxic drugs, including doxorubicin and vincristine (27, 28), which are leader drugs in treatment of sarcoma patients. This is of relevant importance from a clinical point of view, because despite recent advances in therapy one third of patients with nonmetastatic disease and the great majority of patients with metastases at diagnosis do not survive regardless of therapy (29–34).

In this study, we therefore chose to investigate the effects of a novel small molecule IGF-IR kinase inhibitor, NVP-AEW541 (35), a pyrrolo[2,3-d]pyrimidine derivative highly selective against IGF-IR, compared with the insulin receptor and other tyrosine kinases, on the growth of musculoskeletal tumors, including Ewing’s sarcoma, osteosarcoma, and rhabdomyosarcoma. In vitro effects of the compound in association with conventional drugs currently used in the treatment of these tumors were also analyzed to identify best drug combinations.

Materials and Methods

Cell lines. A panel of 8 osteosarcoma, 10 Ewing’s sarcoma, and 5 rhabdomyosarcoma cell lines were analyzed. Osteosarcoma cell lines Saos-2 and U-2OS, Ewing’s sarcoma cell lines SK-ES-1, SK-N-MC, and RD-ES, and alveolar rhabdomyosarcoma cell lines SJ-Rh 30 and SJ-Rh 4 were all obtained from the American Type Culture Collection (Rockville, MD). Ewing’s sarcoma cell lines TC-71 and 6647 were kindly provided by T.J. Triche (Children’s Hospital, Los Angeles, CA). All other osteosarcoma cell lines (SARG, MOS, IOR/OS7, IOR/OS9, IOR/OS10, and IOR/OS14) as well as Ewing’s sarcoma cell lines here considered (LAP35, IOR/BRZ; IOR/CAR; IOR/NGR; and IOR/RCH) were obtained from the Laboratory of Oncologic Research, Orthopaedic Rizzoli Institute (Bologna, Italy) and were characterized previously (11). The CCA cell line was obtained from a human embryonal rhabdomyosarcoma (36). The RMZ-RC2 cell line was obtained from an alveolar rhabdomyosarcoma (37). The RD/18 cell line is a clone of the commercially available human embryonal rhabdomyosarcoma cell line RD (Flow Laboratories, McLean, VA). Cells were routinely cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 20 units/mL penicillin, 100 μg/mL streptomycin (Sigma, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker Europe, Verviers, Belgium).

Drugs. NVP-AEW541 was kindly provided by Novartis Pharma (Basel, Switzerland). Stock solution of this drug was prepared in DMSO and stored...
at -20°C. Doxorubicin, cisplatin, methotrexate, vincristine, and actinomycin D were purchased from Sigma. D-18851, an ifosfamide analogue not requiring metabolic activation (22), was kindly provided by Baxter Oncology Research. (Frankfurt, Germany). Working dilutions of all drugs were prepared immediately before use.

In vitro cytotoxicity. To study the effects of NVP-AEW541 in standard or low-serum conditions, 20,000 to 100,000 cells were plated into 24-well plates in IMDM plus 10% FBS. After 24 hours, medium was replaced by IMDM plus 10% FBS or 1% FBS with or without (control) various concentrations of the compound (30 nmol/L-3 μmol/L, Novartis Pharma) up to 6 days. Effects of the neutralizing antibody anti-IGF-IR c1R3 (1 μg/ml, Calbiochem, San Diego, CA) were also evaluated in the same conditions as a comparison. Cell growth inhibition by daily administration of NVP-AEW541 was also considered. IC50 (drug concentration resulting in 50% inhibition of growth) values were determined by seeding 20,000 cells/cm2 in standard medium (IMDM + 10% FBS). After 24 hours, increasing doses of the drug were added. To evaluate the ability of IGF-I to induce cell recovery from the cytotoxic effects of NVP-AEW541, TC-71 cells were exposed to 300 nmol/L and 1 μmol/L NVP-AEW541 (corresponding to IC50 value) and 50 ng/ml IGF-I (Upstate Biotechnology Inc., Lake Placid, NY) for 48 hours. In all experiments, cell growth was evaluated on harvested cells by trypan blue vital cell count to estimate the percentage of growth inhibition compared with cells treated with DMSO-containing medium. Final concentration of DMSO in the medium was <0.001%, and in the present study, it had no effect on cell growth inhibition.

Combined in vitro treatments with NVP-AEW541 and conventional chemotherapeutics. 3 × 104 cells/cm2 of TC-71 Ewing’s sarcoma cell line were seeded in IMDM plus 10% FBS. After 24 hours, cells were treated with varying concentrations of doxorubicin (range. 0.3-10 ng/ml), cisplatin (range. 3-300 ng/ml), vincristine (0.01-1 ng/ml), actinomycin D (range. 0.01-1 ng/ml) and ifosfamide analogue D-18851 (range. 10 ng/ml-1 μg/ml) without (control) or with NVP-AEW541 (100 nmol/L, corresponding to the dose that gives ~20% to 25% growth inhibition in TC-71 cell line). After 72 hours of treatment, cell growth was evaluated as previously described.

Cell cycle analysis. After 24 to 72 hours of treatment, cell cultures were incubated with 10 μmol/L bromodeoxyuridine (Sigma) for 1 hour in CO2 atmosphere at 37°C. Harvested cells were fixed in 70% ethanol for 30 minutes. After DNA denaturation with 2 N HCL, 1 × 106 cells were processed for indirect immunofluorescence staining using α-bromodeoxyuridine monoclonal antibody diluted 1:4 as a primary antibody (Becton Dickinson, San Jose, CA) and analyzed by flow cytometry (FACS Calibur, Becton Dickinson). For analysis of DNA content, cells were fixed with cold 70% ethanol, treated with 0.5 μg/ml RNase, and stained with 20 μg/ml propidium iodide.

Analysis of apoptosis. For morphologic assessment of apoptotic nuclei, sarcoma cells were seeded in 60 mm dishes in IMDM plus 10% FBS. The following day, medium was changed in IMDM plus 10% FBS without (control) or with NVP-AEW541 (100 nmol/L-1 μmol/L). Twenty-four to 72 hours from treatment, cells were fixed in methanol/acetic acid (3:1) for 15 minutes and stained with 50 ng/ml Hoechst 33258 (Sigma). Detection and quantification of apoptotic cells was also obtained by flow cytometric analysis of Annexin V-FITC-labeled cells. This test was done according to the manifacturer's instructions.

Soft agar assay. Anchorage-independent growth was determined in 0.3% agarose (SeaPlaque, FMC Bioproducts, Rockland, ME) with a 0.5% agarose underlay. Cell suspensions (cells per 60 mm dish: 3,000-10,000 for TC-71, SK-N-MC, and U-205S; 33,000-100,000 for the other cell lines) were plated in a semisolid medium (IMDM + 10% or 1% FBS containing 0.33% agarose) with or without NVP-AEW541 (100 nmol/L-1 μmol/L). Dishes were incubated at 37°C in a humidified atmosphere containing 5% CO2, and colonies were counted after 7 to 15 days. Colonies with >50 cells were considered. Percentage of growth inhibition was calculated with respect to cells treated with vehicle alone.

Western blotting. Constitutive activation of IGF-IR was evaluated on a panel of Ewing’s sarcoma, osteosarcoma, and rhabdomyosarcoma cell lines. To analyze the effects of NVP-AEW541 compound on IGF-IR signaling pathway of TC-71, starved cells were pretreated for 2 hours with 300 nmol/L to 1 μmol/L NVP-AEW541 and then exposed to IGF-I (50 ng/ml, 5-60 minutes). In a second experiment, we followed NVP-AEW541 inhibitory effects on IGF-IR-related signaling pathways by exposing TC-71 to 300 nmol/L and 1 μmol/L of compound for 1 to 48 hours in standard medium. To determine phosphorylation status of Erk and Akt, two downstream mediators of mitogen-activated protein kinase (MAPK) kinase/MAPK and phosphatidylinositol 3-kinase (PI3K) pathways, cells lysates were prepared with a buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA, 1% deoxycholate, and protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate). Protein concentration was determined by Bio-Rad protein assay (Hercules, CA) and equivalent amounts of total cell lysate (50 μg) were separated by 7.5% or 10% SDS-PAGE under denaturing conditions and transferred onto nitrocellulose membrane. Membranes were incubated overnight with primary antibodies [anti-phospho-IGF-IR (Ty313)] dilution 1:200, anti-phospho-Akt (Ser473) dilution 1:1,000, anti-phospho-p44/p42 MAPK (Thr202/Tyr204) dilution 1:1,000, anti-IGF-IR dilution 1:1,000, and anti-Akt dilution 1:1,000 (New England Biolabs, Cell Signaling Technology, Beverly, MA) and then incubated with secondary anti-rabbit antibody conjugated to horseradish peroxidase (Amersham, Buckinghamshire, United Kingdom; dilution 1:1,500). Membranes were revealed by enhanced chemiluminescence Western blotting detection reagents (Amersham). For analysis of IGF-IR tyrosine phosphorylation status by immunoprecipitation, total cell lysate (50 μg) was incubated with 1.5 μg specific anti-IGF-IR β-subunit monoclonal antibody (clone C-20, Santa Cruz, San Diego, CA) overnight. Protein G-Sepharose (40 μL, Calbiochem) were then added and incubation continued for 2 hours. Protein G-Sepharose was collected, washed thrice with lysis buffer, and resuspended in SDS-gel sample buffer. Western blotting was done using anti-phospho-tyrosine antibody (clone 42, BD Biosciences, San Diego, CA) followed by a secondary horseradish peroxidase-linked anti-mouse Ig antibody. Membranes were reprobed with anti-IGF-IR β-subunit antibody.

In vivo treatments with NVP-AEW541 alone or in combination with vincristine. Female athymic 4- to 5-week-old Crl:C-1-nu/nu BR mice (Charles River Italia, Lecco, Italy) were used. Mice were treated according to institutional and European Union guidelines. Tumor growth was determined after s.c. injection of 5 × 106 TC-71 cells. Mice were randomized into controls and three treated groups when tumors started to be measurable (7 days after cell inoculation, day 0 of treatment). In the group treated with NVP-AEW541 alone, each mouse received 50 mg/kg dissolved in 255 nmol/L 1,2-tartaric acid p.o. twice daily, 7 days a week. The second group received vincristine alone (1 mg/kg/d) on days 0 and 1 of treatment. The third group received either NVP-AEW541 p.o. or two i.p. injections of vincristine following the time schedule mentioned above. The control group was treated p.o. with 25 mmol/L 1,2-tartaric acid only.

Tumor growth was assessed thrice weekly by measuring tumor volume, calculated as π/6 × (a2 × b), where a is maximal tumor diameter and b is tumor diameter perpendicular to a. For ethical reasons, mice with tumor were sacrificed when they reached a tumor volume of 2.5 mL. Otherwise, mice were sacrificed 5 months after cell inoculation by CO2 inhalation and necropsied.

Statistical analysis. Differences among means were analyzed using a two-sided Student’s t test. IC50 for each particular drug was defined as concentration of drug that reduces growth by 50% compared with untreated control cells and was calculated from linear transformation of dose-response curves. Analysis of drug combination effects was done by using the fractional product method.

Results

NVP-AEW541 selectively inhibits insulin-like growth factor-1–mediated growth and signaling. To confirm the inhibitory activity of NVP-AEW541 toward IGF-IR kinase and signaling, starved TC-71 cells were treated with doses of 300 nmol/L and 1 μmol/L for 2 hours followed by stimulation with IGF-I for 5 to
30 minutes. Figure 1A shows that both IGF-IR autophosphorylation and the two major IGF-IR-related intracellular signaling pathways, MAPK and PI3K pathways, are completely inhibited by NVP-AEW541. Selective effects of NVP-AEW541 were also confirmed on IGF-I-stimulated Ewing’s sarcoma proliferation. Despite the presence of the autocrine loop, Ewing’s sarcoma cells maintained the ability to respond to exogenous IGF-I by moderately increasing their proliferation (38). Inhibitory effects of NVP-AEW541 were maintained and IGF-I could not rescue cells from growth inhibition induced by the compound (Fig. 1B).

**Figure 1.** Effects of NVP-AEW541 on IGF-I-mediated receptor activation and cell growth. A, starved cells were treated with NVP-AEW541 for 2 hours followed by stimulation with 50 ng/mL IGF-I for 5 to 30 minutes (5 minutes for evaluation of MAPK family members Erk1/2 and IGF-IR phosphorylation and 30 minutes for PI3K mediator Akt activation). IGF-IR was immunoprecipitated and blotted with an anti-phospho-tyrosine antibody to visualize its level of tyrosine phosphorylation. Erk1/2 and Akt phosphorylation was immunodetected with specific antibodies on whole cell lysates. Anti-Akt, anti-Erk, or anti-IGF-IR antibodies were used to detect total proteins as controls. B, cells were grown in low-serum medium and exposed to NVP-AEW541 and/or IGF-I (50 ng/mL) for 48 hours before being counted. Columns, mean of three independent experiments; bars, SE. *, P < 0.05 with respect to control (IMDM + 1% FBS). Student’s t test. C, in vitro growth curves of TC-71 cells after exposure to NVP-AEW541 in low-serum medium (IMDM + 1% FBS) or in standard medium (IMDM + 10% FBS). Cells were seeded in IMDM + 10% FBS. After 24 hours, medium was replaced with or without (medium) different doses of the inhibitor. The number of cells was estimated at indicated times. Points, mean of two independent experiments; bars, SE.

In vitro activity of NVP-AEW541 on musculoskeletal sarcoma cells. To determine effects of NVP-AEW541 on cell proliferation in standard medium as well as in low-serum medium, TC-71 Ewing’s sarcoma cells were treated with different concentrations of the inhibitor (Fig. 1C). A remarkable, dose-dependent, growth inhibitory effect of NVP-AEW541 was observed in low-serum medium as well as in 10% FBS–containing medium. Therefore, to closely mimic in vivo settings in which tumor cells are exposed to a complex mix of growth factors, all experiments testing effectiveness of NVP-AEW541 were done in standard medium.
A time course evaluation of inhibitory effects of 300 nmol/L NVP-AEW541 on MAPK and PI3K signaling pathways in standard medium, however, revealed transient effects on MAPK pathway, particularly for the dose of 300 nmol/L, whereas PI3K pathway appeared to be blocked up to 48 hours (Fig. 2A). Consequently, we determined whether a daily in vivo administration of NVP-AEW541 gave a benefit in terms of growth inhibition. Figure 2B shows similar inhibitory effects were obtained in TC-71 cells with single or a repeated treatment using NVP-AEW541. This indicates that the stable inhibition of PI3K pathway is sufficient to guarantee remarkable growth inhibitory effects of NVP-AEW541. Growth inhibitory activity of the compound was maintained for at least 72 hours after its removal (33% of growth inhibition with the dose of 300 nmol/L and 50.3% of growth inhibition with the dose of 1 μmol/L; *P < 0.05*).

In vitro cytotoxic effects of NVP-AEW541 were examined on a panel of 10 Ewing’s sarcoma, 8 osteosarcoma, and 5 rhabdomyosarcoma cell lines. A comparison between effectiveness of the neutralizing antibody anti-IGF-IR eIR3 (1 μg/mL) and NVP-AEW541 (300 nmol/L) indicate a similar activity for the two agents in all cell lines here considered, further confirming the specificity of action of the kinase inhibitor (data not shown). Figure 3A shows IC_{50} values obtained for these cells. Ewing’s sarcoma cells were more sensitive to NVP-AEW541, showing IC_{50} values at submicromolar doses. Rhabdomyosarcoma cell lines rank second with respect to drug sensitivity, whereas osteosarcoma cells confirm that they are more refractory to strategies targeting IGF-IR (11). Interestingly, among rhabdomyosarcoma, the three cell lines of alveolar origin (RH4, RH30, and RC2) show a level of sensitivity comparable with that of Ewing’s sarcoma cells, whereas cell lines of embryonal origin are definitely less sensitive. The level of sensitivity does not correlate with the level of expression of IGF-IR, because all cell lines here considered express the receptor at similar level. However, when activation status of IGF-IR was examined, we found a correlation between constitutive phosphorylation of IGF-IR and level of sensitivity to NVP-AEW541 (Fig. 3B).

The same spectrum of activity was also observed in anchorage-independent conditions, with Ewing’s sarcoma and osteosarcoma cells at opposite ends of sensitivity (Fig. 4A and B). Interestingly, when cells were prevented to adhere, the general effectiveness of NVP-AEW541 seemed to be higher in soft agar, which is an accepted criterion for transformation rather than for growth in monolayer conditions.

**Effects of NVP-AEW541 on cell cycle and apoptosis.** Two major mechanisms may explain the inhibitory effects of NVP-AEW541 on cell proliferation: inhibition of cell cycle progression and/or induction of apoptosis, both of these cellular processes being modulated by IGF-IR signaling. NVP-AEW541 inhibited cell cycle progression in a dose-dependent manner, inducing specific G_{1} arrest in all six sarcoma cell lines here examined (Fig. 5A). In contrast, with respect to apoptosis, a significant induction of apoptotic rate after treatment with NVP-AEW541 was observed only in Ewing’s sarcoma cells and, among osteosarcoma and rhabdomyosarcoma, only in those cell lines that showed a high level of sensitivity toward the drug (Fig. 5B and C). Therefore, although the effect on the cell cycle appeared as a general action of the NVP-AEW541, the induction of apoptosis appeared to be dependent on the cellular context and determined the level of sensitivity of the cells to the compound.

**Cytotoxicity of combined in vitro treatments.** Experiments were carried out to determine effects of conventional chemotherapeutic drugs (doxorubicin, vincristine, cisplatin, actinomycin D, and ifosfamide) in combination with NVP-AEW541 on the growth of musculoskeletal sarcoma cells. TC-71 cells were simultaneously exposed to increasing concentrations of conventional agents and to a concentration of NVP-AEW541 that gave ~20% growth inhibition after 72 hours. Combined treatment with NVP-AEW541 and vincristine, actinomycin D, or the ifosfamide analogue D-18851 resulted in a significantly enhanced inhibition of cell growth with respect to the therapeutic efficacy of these drugs alone (Fig. 6A). In contrast, when cells were exposed to NVP-AEW541 and doxorubicin or cisplatin concomitantly, a subadditive cytotoxic effect was generally observed. Fractional product method confirmed antagonism when cells were treated with NVP-AEW541 and doxorubicin or cisplatin concomitantly and additive effects when NVP-AEW541 were combined with vincristine, actinomycin D, and ifosfamide.
Antitumorigenic effects of NVP-AEW541 treatment alone or in combination with vincristine. To analyze inhibitory effects of NVP-AEW541 alone or in association with vincristine on tumor growth, TC-71 cells were s.c. inoculated in athymic mice. Drug treatment started 7 days after cell injection, when all mice had measurable tumors (day 0 of treatment). NVP-AEW541 alone produced a slight reduction in tumor growth when compared with controls. Vincristine was able to inhibit tumor growth for some days, but later tumor size became similar to control. NVP-AEW541 in association with vincristine significantly inhibited TC-71 growth compared with untreated mice after 11 days until the end of the treatment period (Fig. 6B).

Discussion

The identification of novel therapeutic strategies and new potent drugs effective against sarcomas is a high priority goal. In fact, since the identification of ifosfamide, no new agents for sarcoma therapy have proven to be effective. Recent clinical studies have indicated that the first improvements in the cure rate of patients with localized disease have been achieved by dose intensification of conventional therapeutics, therefore paying the price of severe toxicity and high rate of life-threatening late events, such as secondary malignancies. Moreover, treatments for high-risk Ewing’s sarcoma family of tumors patients are still completely inadequate (29–34). In this study, we investigated the therapeutic potential of the novel, selective inhibitor of IGF-IR kinase, NVP-AEW541 (35), against a panel of representative musculoskeletal sarcoma cell lines.

IGF-IR signaling is an attractive target for new therapeutic strategies in sarcoma based on its role in the pathogenesis and progression of these tumors (4–7, 13–18). Indeed, IGFs promote tumor growth, survival, and migration of these cells and, by inducing vascular endothelial growth factor-A production, may favor their blood supply essential for the progressive growth of primary malignancies and for the development of metastases (19–26). Impairment of IGF-IR functions was therefore found to substantially contribute to control of sarcoma malignancy, especially of Ewing’s sarcoma. Indeed, very promising and convincing preclinical results were obtained using a variety of approaches targeting IGF-IR, which include neutralizing antibodies, antisense IGF-IR RNAs, and competitive inhibitors, such as dominant-negative mutants. However, none of these approaches may be promptly applied in clinical research. In this respect, the recent availability of a selective small molecule, inhibiting IGF-IR signaling, raises new prospective for clinical studies. The compound, a pyrrolo[2,3-d]pyrimidine derivative, was recently presented as an optimized IGF-IR kinase inhibitor that selectively distinguishes at the cellular level between the native IGF-IR and the closely related insulin receptor. Its effects on other protein tyrosine kinases have been excluded, confirming the specificity of action (35). NVP-AEW541 shows a 27-fold inhibitory selectivity for the IGF-IR versus the insulin receptor (35) and is slightly more active (IC_{50} = 0.086 mol/L, toward IGF-IR kinase) and more selective than another pyrrolo[2,3-d]pyrimidine derivative that was recently studied in a series of tumor cell lines (39, 40). In addition, NVP-AEW541 has the advantage of being an orally bioavailable tyrosine kinase small molecule inhibitor. Considering the high degree of sequence identity between the IGF-IR and the insulin receptor, the high selectivity toward the IGF-IR is a major point of attractiveness.

Figure 3. A, in vitro sensitivity of 10 Ewing’s sarcoma, 8 osteosarcoma, and 5 rhabdomyosarcoma cell lines to NVP-AEW541. Cells were exposed for 72 hours to different doses of the compound and IC_{50} doses were calculated. Columns, mean of three independent experiments; bars, SE. B, constitutive phosphorylation of IGF-IR on a representative panel of Ewing’s sarcoma, osteosarcoma, and rhabdomyosarcoma cell lines.
and fulfills the need for agents that exhibit selectivity for IGF-IR versus the insulin receptor (2). Therefore, the compound has all the prerequisites for being considered a potential new drug for sarcomas, provided its effectiveness is proven and best treatment modalities are identified.

NVP-AEW541 was found to fulfill the key features expected from an IGF-IR inhibitor. It selectively inhibits IGF-I-mediated growth and signal transduction in Ewing’s sarcoma cells. Of note, the inhibitory effects of NVP-AEW541 were not reverted in the presence of exogenous IGF-I. We believe that this is a major point because sarcoma cells, which always express IGF-IR, are likely to be locally exposed to paracrine and autocrine stimulation by IGF-I. In fact, IGF-I is normally stored in high quantity in bone matrix (41) and may be easily released by osteolysis induced by growing sarcoma cells. NVP-AEW541 shows higher growth inhibitory effectiveness in soft agar than in monolayer conditions, in keeping with the notion that IGF-IR is the key for growth of tumor cells under anchorage-independent conditions, but less for their growth as adherent monolayer. Comparing the effectiveness of NVP-AEW541 among the three major pediatric sarcomas (i.e., Ewing’s sarcoma, rhabdomyosarcoma, and osteosarcoma), we found a generally higher activity toward Ewing’s sarcoma versus rhabdomyosarcoma and, above all, osteosarcoma. Analysis of the constitutive activation status of IGF-IR in sarcoma cells indicated a correlation between level of phosphorylation of the receptor and sensitivity to the compound, indicating that the evaluation

Figure 4. A, effects on colony formation in soft agar on a representative panel of Ewing’s sarcoma, osteosarcoma, and rhabdomyosarcoma cell lines. Cells were plated as described in Materials and Methods in 0.33% agarose with medium + FBS in the absence (control) or in the presence of increasing concentration of NVP-AEW541 and colonies with >50 cells were counted after 7 to 15 days. Percentages of growth inhibition were calculated with respect to control (IMDM + 10% FBS). *, P < 0.05; **, P < 0.01, Student’s t test. B, representative soft agar picture of TC-71 cells treated with NVP-AEW541 300 nmol/L.
Figure 5. A, effects of NVP-AEW541 on proliferative rate of a representative panel of Ewing’s sarcoma, osteosarcoma, and rhabdomyosarcoma cell lines after 24-hour treatments. Columns, mean percentage of cells in different cell cycle phases as determined by flow cytometry (two similar experiments). $P < 0.05$, statistically significant differences by Student’s $t$ test. B, apoptotic effects of NVP-AEW541 on musculoskeletal sarcoma cells by Annexin V test after 24 hours of NVP-AEW541 treatment. *, $P < 0.05$, Student’s $t$ test. C, morphologic evaluation of apoptotic nuclei with Hoechst 33258 after 48 hours of NVP-AEW541 treatment. *, $P < 0.05$, Student’s $t$ test.
of phosphorylated level of IGF-IR is a valuable predictor of response to NVP-AEW541. In addition, Ewing's sarcoma cells are more dependent on IGF-IR functions for growth, survival, and migration (5) than rhabdomyosarcoma and osteosarcoma, which show a redundancy of autocrine loops (11, 12). Analysis of the effects of NVP-AEW541 on cell cycle and apoptosis on a panel of cell lines with a spectrum of sensitivity toward the compound revealed that NVP-AEW541 determines blockage of cells in G1 phase in all cell lines, whereas apoptotic effects were observed only in those cells that show a high level of sensitivity. This different activity of NVP-AEW541 seems to be due to a differential effect of the compound on intracellular signaling pathways. In fact, in cells that are sensitive to NVP-AEW541, such as the TC-71 Ewing's sarcoma cell line, the compound efficiently inhibited Akt activation in standard medium (i.e., in the presence of serum), whereas in cell lines that are less sensitive NVP-AEW541 had minimal effect on Akt activity (data not shown). These data provide further evidence that cell lines that are particularly sensitive to NVP-AEW541 are highly dependent on IGF-1 for activation of critical signaling pathways. In addition, these findings suggest that efficient inhibition of PI3K-Akt signaling is a prerequisite for growth inhibition. Indeed, when a time course analysis of the effects of NVP-AEW541 on MAPK and PI3K signaling was done, we observed a transient inhibitory effect on Erk phosphorylation but a stable inhibition of Akt, in keeping with the inhibitory effects on cell growth.

**Figure 6.** A, inhibitory effects of doxorubicin, cisplatin, vincristine, actinomycin D, or ifosfamide in combination with NVP-AEW541 (100 nmol/L) after simultaneous and continuous treatments. Cells were treated with the drugs at the indicated concentrations alone or in association with NVP-AEW541 the day after cell seeding for a total of 72 hours. Points, mean of duplicate or triplicate experiments compared with the corresponding dose of the single drug; bars, SE. *, P < 0.05, Student’s t test. B, inhibition of TC-71 tumor growth in nude mice by NVP-AEW541 and vincristine. In vivo growth curves of TC-71 tumors (5 x 10^5 s.c.) in groups of 5 (vehicle and vincristine), 10 (NVP-AEW541 + vincristine), or 20 (NVP-AEW541) athymic mice treated with vehicle (25 mmol/L L(+)-tartaric acid; DI p.o. twice daily; 7 days a week for 2 weeks; vincristine alone i.p. (1 mg/kg/d) on days 0 and 1 of treatment (●); NVP-AEW541 p.o. alone twice daily, 7 days a week for 2 weeks (○); or NVP-AEW541 p.o. twice daily, 7 days a week + vincristine i.p. (1 mg/kg/d) on days 0 and 1 of treatment (■). Treatments began when tumors started being measurable. *, P < 0.05, Student’s t test.
As a final step, we investigated in vitro growth effects of NVP-AEW541 in combination with conventional therapeutic agents that are currently used in the treatment of Ewing's sarcoma, osteosarcoma, or rhabdomyosarcoma. It is now generally accepted that IGF-I attenuates the response of cancer cells to several chemotherapeutic agents (1, 2). Thus, inhibition of IGF-I action could be a useful adjuvant to cytotoxic chemotherapy. Our in vitro findings illustrate that the compound may be advantageously used in combination with vincristine, actinomycin D, and ifosfamide but not with doxorubicin and cisplatin. The agonistic effect of NVP-AEW541 and vincristine was also confirmed in the in vivo study. Significant inhibition of Ewing's sarcoma tumor growth was indeed observed only with combined treatments. However, this observed synergistic in vivo effects of the IGF-IR inhibitor and vincristine in this s.c. model may not reflect the situation when the tumor localizes to the bone. Therefore, this drug merits further in vivo evaluation for what concerns distance metastases in a appropriate model prior to human studies.

In conclusion, we show that the availability of the selective IGF-IR kinase inhibitor NVP-AEW541 may be a promising approach in the treatment of Ewing's sarcoma. However, for the broadest applicability and best efficacy in sarcomas, NVP-AEW541 may be combined with vincristine, actinomycin D, and ifosfamide, three major drugs in the treatment of these tumors. In addition, NVP-AEW541 by showing a significant increase of the inhibitory effects of these drugs may reduce their toxicity allowing a decrease in drug dosage.

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