Abstract

**Purpose:** Signaling through insulin-like growth factor I receptor (IGF-IR) is important for growth and survival of many tumor types. Neuroblastoma is sensitive to IGF.

**Experimental Design:** We assessed the ability of NVP-AEW541, a recently developed small molecule that selectively inhibits IGF-IR activity, for neuroblastoma growth effects in vitro and in vivo. Our data showed that, in a panel of 10 neuroblastoma cell lines positive for IGF-IR expression, NVP-AEW541 inhibited in vitro proliferation in a submicromolar/micromolar (0.4–6.8) range of concentrations.

**Results:** As expected, NVP-AEW541 inhibited IGF-II–mediated stimulation of IGF-IR and Akt. In addition to growth inhibition, the drug also induced apoptosis in vitro. Oral administration of NVP-AEW541 (50 mg/kg twice daily) inhibited tumor growth of neuroblastoma xenografts in nude mice. Analysis of tumors from the drug-treated animals revealed a marked apoptotic pattern and a decrease in microvascularization compared with controls. Interestingly, quantitative real-time PCR detected both in vitro and in vivo a significant down-regulation of mRNA for vascular endothelial growth factor (VEGF) caused by NVP-AEW541. In addition, in Matrigel-coated chambers and in severe combined immunodeficient mice tail vein injected with neuroblastoma cells, tumor invasiveness was significantly reduced by this agent. Analysis of IGF-IR expression in a series of 43 neuroblastoma primary tumors revealed IGF-IR positivity in 86% of cases.

**Conclusions:** Taken together, these data indicate that NVP-AEW541 can be considered as a novel promising candidate for treatment of neuroblastoma patients.

Neuroblastoma, an embryonal tumor that originates from neural crest precursors, is the most common extracranial solid tumor in childhood, accounting for 8% to 10% of all pediatric malignancies (1). Despite the increasing understanding of its biological features, from a clinical point of view, neuroblastoma is probably one of the most puzzling among human tumors due to the remarkable heterogeneity of its clinical behavior ranging from those that regress spontaneously, some that are responsive to conventional treatment, and to those that are very aggressive and nonresponsive to intensified treatment that rapidly progress. Unfortunately, ~50% of patients with newly diagnosed neuroblastoma belong to the high-risk subset for which no substantial improvement in response rate and/or long-term survival has occurred over the last two decades (2, 3). In this subset of patients, the identification and evaluation of novel therapeutic approaches is particularly needed.

One such novel therapeutic approach is represented by the selective targeting of receptor tyrosine kinases. In normal cells, the activity of these molecules is strictly regulated, whereas in tumor cells their constitutive activation caused by gain-of-function mutation or fusion with a partner protein, or their abnormal autocrine or paracrine stimulation, can result in dysregulated cell growth (4, 5). In neuroblastoma, autocrine loops involving TrkB (6), c-Kit (7, 8), and epidermal growth factor receptor (9) have been described, and their selective inhibition has been shown to decrease tumor growth both in vitro and in vivo (9–13). An additional autocrine/paracrine loop involving the insulin-like growth factor I receptor (IGF-IR) and its ligand IGF-II has also been described in neuroblastoma and has been shown to promote tumor cell proliferation, survival, and motility (14–16). Accordingly, the suppression of the IGF-II/IGF-IR signaling through the use of IGF-IR antisense expression vectors (17) or blocking antibodies (18) has been

**Authors' Affiliations:** 1Section of Toxicology and Biomedical Sciences, Ente per le Nuove tecnologie l'Energia e l' Ambiente, Research Center Casaccia; 2La Sapienza University, Department of Pediatrics; 3Laboratory of Oncology, Bambino Gesù Children's Hospital, Rome, Italy; 4Royal Liverpool Children's NHS Trust Alder Hey, Department of Oncology, Liverpool, United Kingdom; and 5School of Reproductive and Developmental Medicine, Division of Child Health, Liverpool University, Liverpool, United Kingdom

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**Note:** B. Tanno and C. Mancini equally contributed to this work. B. Tanno was a fellow of Bambino Gesù Children's Hospital, Rome, Italy. R. Vitali is a fellow of “Fondazione Italiana per la Lotta al Neuroblastoma.”

**Requests for reprints:** Giuseppe Raschella, Section of Toxicology and Biomedical Sciences, ENEA Research Center Casaccia, Via Anguillarese, 301, 00060 Rome, Italy. Phone: 39-0630483172; Fax: 39-0630486559; E-mail: raschella@casaccia.enea.it.

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**Cancer Therapy: Preclinical**

**Down-Regulation of Insulin-Like Growth Factor I Receptor Activity by NVP-AEW541 Has an Antitumor Effect on Neuroblastoma Cells In vitro and In vivo**

Barbara Tanno, Camillo Mancini, Roberta Vitali, Mariateresa Mancuso, Heather P. McDowell, Carlo Dominici, and Giuseppe Raschella
reported to inhibit tumor growth in in vitro (17, 18) and in vivo (17) neuroblastoma preclinical models. However, the difficulties in inhibiting selectively IGF-IR in a therapeutic setting without simultaneously interfering with the structurally closely related insulin receptor, have precluded any further exploitation of IGF-IR as a therapeutic target.

The recent availability of the novel IGF-IR inhibitor NVP-AEW541 (Novartis Pharma, Basel, Switzerland) capable of discriminating between IGF-IR and insulin receptor (19), prompted us to investigate the in vitro and in vivo antitumor activity of this compound in neuroblastoma preclinical models. We found that NVP-AEW541 effectively inhibits tumor growth, induces apoptosis, and reduces invasiveness in in vitro and in vivo neuroblastoma models.

Materials and Methods

Chemical compound. Pyrrole pyrimidine derivative NVP-AEW541 (19) was provided by Novartis Pharma. NVP-AEW541 was dissolved in DMSO (10 mmol/L) for in vitro studies and in 25 mmol/L l(+)-tartaric acid (5 mg/mL) for in vivo applications.

Cell lines. Human neuroblastoma cell lines HTLA-230, KCNR, SK-N-BE2c, SK-N-BE, LAN-5, GI-CA-N, SH-EP, SK-N-AS, and RN-GA were kept in culture as previously described (11). Human neuroblastoma cell line SY-5Y(N) was cultured in DMEM (Euroclone, Paignton, Devon, UK) supplemented by 10% FCS (Hyclone, Logan, UT), L-glutamine at 37°C, 5% CO2 (20).

Patients. Primary tumor samples were obtained by surgery from 43 children with newly diagnosed neuroblastoma admitted to the Department of Pediatrics at La Sapienza University and the Division of Oncology at Royal Liverpool Children’s NHS Trust Alder Hey. No selection criteria were applied except for the availability of frozen tumor tissue for molecular analysis. Patients were staged according to the International Neuroblastoma Staging System (21) as follows: 10 patients were at stage 1, 5 at stage 2, 6 at stage 3, and 22 at stage 4. Southern blot for the determination of MYCN copy number was done as previously described (22).

Cell proliferation and apoptosis. Cell growth was evaluated by seeding cells in triplicate in complete medium (medium supplemented with 10% FCS) in which cells were present at the concentration of 10³ cells/cm² was added in the same well. Base agar and top agar were prepared by including NVP-AEW541 (0.5, 2.0, and 8.0 µmol/L) in the formulation where needed. Plates were incubated at 37°C. 5% CO2 in a humidified incubator for 11 days and stained with 0.005% crystal violet for >1 hour. Single cells (defined as cells or aggregates of ≤20 cells) and clones (aggregates of >20 cells) were scored in the plates counting at least 200 elements in each plate. Each experimental point was carried out in duplicate. Experiments were repeated twice with overlapping results. Cell cycle analysis and evaluation of the hypodiploid peak were carried out using propidium iodide staining (50 µg/mL) followed by flow cytometric analysis (FACSCalibur, Becton Dickinson, Bedford, MA).

Caspase-3 and caspase-7 activities were measured using the luminescent Caspase-Glo 3/7 kit (Promega, Madison, WI) based on detection of a luminescent caspase-3/7 substrate following caspase cleavage. In each well, 1.5 × 10⁶ cells were seeded in RPMI 1640 supplemented with 10% FCS. NVP-AEW541 at concentrations 20% above the calculated IC50 of each cell line was added where needed.

Protein analysis. Cellular proteins were extracted, separated on SDS-polyacrylamide gels, and Western blot analyses were carried out as previously described (23). Antibodies used were as follows: anti-IGF-IR, anti-phospho-IGF-IR (Tyre1131)/insulin receptor (Tyre1146), anti-Akt, anti-phospho-Akt (Ser473), anti-p44/42 mitogen-activated protein kinase, anti-phospho-p44/42 mitogen-activated protein kinase (Thr389/Tyr421), anti-cleaved-caspase-9 (Asp10; human specific) from Cell Signaling Technology, Inc. (Beverly, MA); anti-Bim from ProSci, Inc. (Poway, CA); anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-β-actin from Sigma-Aldrich (St. Louis, MO).

In vivo studies. Four-week-old male athymic nude mice (HSd: Athymic Nude-neu from Charles River Laboratories, Lecco, Italy) were fed ad libitum and kept in optimal hygienic conditions in a 12 hours light/12 hours dark cycle. Upon arrival, animals were kept in the animal facility for 1 week before starting the experiments. Twenty million cells (HTLA-230 or SK-NB2c) per animal were injected s.c. into the right flank of eight mice per group (control and NVP-AEW541-treated groups). Animals were kept under daily observation until the tumor mass reached a volume of ~100 mm³ (7-10 days). Tumor volumes (length × width × height × π/6) and body weights were determined thrice weekly. At the first day of treatment (day 0), the treated group (NVP-AEW541) and the control group (carrier only) were selected by stratification (eight animals per group, average tumor volume of ~100 mm³ per group). Animals were treated by oral gavage twice daily, 7 d/wk either with NVP-AEW541 (50 mg/kg; dissolved in 0.2 mL of 25 mmol/L l(+)-tartaric acid, treated group) or with 0.2 mL of 25 mmol/L l(+)-tartaric acid (control group). The experiments were concluded 14 days after day 0. Animals were sacrificed by CO2 inhalation, tumor masses were collected, and formalin-fixed for histologic and immunohistochemical analyses.

Histology and immunohistochemistry. Paraffin-embedded tissues were cut in 7 µm sections and processed for H&E staining according to standard techniques. For immunohistochemistry, 4 µm sections were cut and phospho-IGF-IR antigen was retrieved by microwave exposure (3 minutes, 790 W). Slides were then treated with 1% H2O2 for 10 minutes to eliminate endogenous peroxidase activity. Immunohistochemical analysis to detect the phosphorylated (active) form of IGF-IR was carried out using the phospho-IGF-IR–specific antibody (Cell Signaling) diluted 1:50 according to the specifications of the manufacturer. Control reactions were carried out by substituting the phospho-IGF-IR–specific antibody with normal rabbit serum. Staining was carried out using Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA).

PCR and quantitative real-time PCR. Total RNA was prepared by RNaize (Ambion, Austin, TX). RNA was cleaned from DNA contamination by means of DNA-free kit (Ambion) and was reverse transcribed with RETROscript kit (Ambion) according to the instructions of the manufacturer. An input of 500 ng was used for each reaction. Subsequent PCR to detect IGF-IRα was carried out for 30 cycles at the appropriate annealing temperature for the following pairs of primers: IGF-IR, forward: 5’-ACAACTACGCCCTGTGACATC-3’; IGF-IR, reverse: 5’-TGGCAACGACTCTTTGTCTTC-3’; Real-time PCR was carried out using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Taqman technology and the Assays-On-Demand kit (Hs00173626_m1) for human vascular endothelial growth factor

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(VEGF) were used. TAQMAN predeveloped kit part #4326315E for the human β-actin was used to normalize. Reactions were run in triplicate in two independent experiments.

**Statistical analysis.** Wilcoxon-Mann-Whitney exact test was applied to compare tumor growth between treated and control groups using GraphPad Prism 4 for Windows (GraphPad Software, Inc., San Diego, CA). Results are presented as mean ± SE. The level of significance was set at \( P < 0.05 \).

**Results**

Previous studies showed that neuroblastoma cell lines are sensitive to IGFs (14). We analyzed the expression of IGF-IR, phospho-IGF-IR, Akt, phospho-Akt, Erk1/2, and phospho-Erk1/2 in 10 human neuroblastoma cell lines, either MYCN-amplified (HTLA-230, KCNR, SK-N-BE, SK-N-BE2c, LAN-5) or nonamplified [SH-EP, GI-CA-N, SY-5Y(N), RN-GA]. Detectable levels of IGF-IR were found in all cell lines tested (Fig. 1A). The presence of more than one band in most samples analyzed for IGF-IR expression is due to posttranslational modifications that affect electrophoretic mobility of IGF-IR (24). We determined the inhibitory activity on neuroblastoma cell growth of a recently developed chemical compound (NVP-AEW541) that inhibits IGF-IR activity with a high specificity (19). Each cell line was treated for 72 hours with scalar concentrations of NVP-AEW541 (0.5, 2.0, and 8.0 \( \mu \text{mol/L} \)) in normal culture conditions (medium supplemented by 10% FCS). Given the persistence of its effect (25), the drug was added at time 0 and the medium remained unchanged till the completion of the experiment. We found that NVP-AEW541 was able to inhibit cell growth in a dose-dependent manner within the range of concentrations used for the experiment (Fig. 1B). The IC\(_{50}\) values calculated by regression analysis were in the submicromolar/micromolar range (minimum IC\(_{50}\) 0.4; maximum IC\(_{50}\) 6.8 \( \mu \text{mol/L} \); Table 1). Notably, the effectiveness of NVP-AEW541 was not always proportional to the activation of IGF-IR. This is likely to be due to the different contribution of the IGF axis in stimulating proliferation in individual cell lines in which other signal transduction pathways may simultaneously be activated. Because the expected activity of NVP-AEW541 is on the activation of IGF-IR, we tested whether the drug was able to inhibit the IGF-II–mediated activation of IGF-IR. Cells were serum-starved for 18 hours, then left untreated or treated for 10 minutes with 100 ng/mL of recombinant IGF-II with or without a 2-hour pretreatment with NVP-AEW541 at concentrations 20% greater than the IC\(_{50}\) of each cell line. Pretreatment with NVP-AEW541 dramatically reduced phosphorylation of IGF-IR in all cell lines tested (Fig. 2). Signal transduction from IGF-IR involves the activation of phosphatidylinositol 3-kinase/Akt and/or MEK/Erk1/Erk2 pathways (26). We showed that in neuroblastoma cells, the NVP-AEW541–driven inhibition of IGF-IR causes a reduction of phosphorylation of Akt (Fig. 2), but not of Erk1 and Erk2 (data not shown). However, these results do not rule out the possibility that Erk1 and Erk2 inhibition could be detectable following longer exposures to IGF-II.

Because anchorage-independent growth is a hallmark of tumorigenicity, we assessed the ability of neuroblastoma cells to growth in semisolid agar in presence of scalar concentrations (0.5, 2.0, and 8.0 \( \mu \text{mol/L} \)) of NVP-AEW541. After 11 to 13 days from plating, single cells (groups of <20 cells) and clones (groups of >20 cells) were counted. NVP-AEW541 inhibited the ability of neuroblastoma cells to form clones in semisolid agar in the range of concentrations used (Table 2). Of interest, in GI-CA-N cells, the concentration of the drug that reduced clonogenicity by >50% (<2.0 \( \mu \text{mol/L} \)) was much lower than the IC\(_{50}\) calculated in bidimensional cultures (6.8 \( \mu \text{mol/L} \); Table 1).

Neuroblastoma cells treated with NVP-AEW541 were pyknotic and showed membrane blebbing that is a morphologic

![Fig. 1. A, IGF-IR, phospho-IGF-IR, Akt, phospho-Akt, Erk1/2, and phospho-Erk1/2 expression was detected in 10 human neuroblastoma cell lines by Western blotting. B, NVP-AEW541 inhibits growth of neuroblastoma cell lines (bidimensional cultures). NVP-AEW541 treatment at the indicated concentrations was prolonged for 72 hours.](image-url)

**Table 1. IC\(_{50}\) values of NVP-AEW541 in human neuroblastoma cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC(_{50}) (( \mu \text{mol/L} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI-CA-N</td>
<td>6.80</td>
</tr>
<tr>
<td>SH-EP</td>
<td>3.00</td>
</tr>
<tr>
<td>HTLA-230</td>
<td>0.50</td>
</tr>
<tr>
<td>SK-N-BE2c</td>
<td>1.10</td>
</tr>
<tr>
<td>SK-N-BE2</td>
<td>3.00</td>
</tr>
<tr>
<td>SY-5Y (N)</td>
<td>2.40</td>
</tr>
<tr>
<td>LAN-5</td>
<td>0.40</td>
</tr>
<tr>
<td>KCNR</td>
<td>0.40</td>
</tr>
<tr>
<td>RN-GA</td>
<td>1.30</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>2.80</td>
</tr>
</tbody>
</table>

Because anchorage-independent growth is a hallmark of tumorigenicity, we assessed the ability of neuroblastoma cells to growth in semisolid agar in presence of scalar concentrations (0.5, 2.0, and 8.0 \( \mu \text{mol/L} \)) of NVP-AEW541. After 11 to 13 days from plating, single cells (groups of <20 cells) and clones (groups of >20 cells) were counted. NVP-AEW541 inhibited the ability of neuroblastoma cells to form clones in semisolid agar in the range of concentrations used (Table 2). Of interest, in GI-CA-N cells, the concentration of the drug that reduced clonogenicity by >50% (<2.0 \( \mu \text{mol/L} \)) was much lower than the IC\(_{50}\) calculated in bidimensional cultures (6.8 \( \mu \text{mol/L} \); Table 1).
hallmark of apoptosis. Thus, we measured by flow cytometry the hypodiploid peak of neuroblastoma cells treated for different times (24, 48, and 72 hours) with NVP-AEW541 at a concentration 20% greater than the calculated IC50 of each cell line. An increase in the hypodiploid fraction was detected in all cell lines tested that proceeded in parallel with the depletion of the S and G2-M compartments (Table 3). In some cases (GI-CA-N, HTLA-230, and KCNR), a block in G0-G1 was also detected. To further characterize the apoptotic event following exposure to NVP-AEW541 for 72 hours at a concentration 20% greater than the calculated IC50 of each cell line, a luminometric assay was carried out to assess the activation of caspase-3/7. In all cell lines, we detected caspase-3/7 activation that ranged from 1.44-fold (SK-N-BE2c) to 200-fold (GI-CA-N) in NVP-AEW541–treated cells compared with untreated controls (Table 4). The analysis of proapoptotic proteins belonging to Bcl-2 superfamily (27) showed up-regulation of Bim in most cases but not in HTLA230 that presented up-regulation of Bax instead (data not shown). Also, caspase-9 (28) was activated by treatment with NVP-AEW541 (data not shown). Collectively, these data show that NVP-AEW541 treatment induces apoptosis in neuroblastoma cells in vitro.

To evaluate the antitumor activity of NVP-AEW541 in vivo, we carried out xenotransplantation experiments by injecting s.c. HTLA-230 and SK-N-BE2c cells in nude mice. These cell lines were chosen for the following reasons: (a) they show submicromolar (HTLA-230, IC50 0.50 μmol/L) or micromolar (SK-N-BE2c, IC50 1.10 μmol/L) sensitivity to NVP-AEW541 in vitro; (b) both are MYCN amplified, thus representing the most aggressive neuroblastoma phenotype that still awaits a more effective therapeutic treatment. Twenty million cells were injected in the flank of mice that were divided in two groups (eight mice per group). Tumors were grown until the mean volume reached ~100 mm3 (8-10 days). NVP-AEW541 was administered by oral gavage [50 mg/kg in 0.2 mL of 25 mmol/L L-(+)-tartaric acid] twice a day for 14 consecutive days. The control group was similarly treated with 0.2 mL carrier [25 mmol/L L-(+)-tartaric acid] twice a day. Tumor volume and

Table 2. NVP-AEW541 inhibits anchorage-independent growth (semisolid agar) of neuroblastoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>NVP-AEW541 (μmol/L)</th>
<th>Inhibition of clonogenicity (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNR</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>42.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>53.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>GI-CA-N</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>58.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>96.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>97.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HTLA-230</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>38.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>61.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>97.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SK-N-BE2c</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>27.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>57.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>70.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SY-SY (N)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>100.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>100.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE: Semisolid cultures were carried out in duplicate; a total of at least 500 single cells (groups of ≤20 cells) or clones (groups of >20 cells) were counted in each culture. The experiment was repeated twice. Abbreviation: NS, not significant.

*Statistical significance determined by χ² test.

1In SY-SY(N) cells, the experiment did not reach statistical significance because of the low ability of this cell line to form clones in semisolid agar.
animal weight were measured thrice a week till the end of the treatment. At that time, animals were sacrificed and tumors were collected and formalin fixed for histologic and immunohistochemical analyses. In both cases, NVP-AEW541 treatment caused tumor shrinkage (Fig. 3A and C) that reached the statistical significance (P = 0.0156 and P = 0.0111 for HTLA-230 and SK-N-BE2c, respectively). Signs of systemic toxicity (lethargy, disturbances in feeding behavior) were not observed by daily monitoring during treatment. Animal weight was not significantly different in treated and untreated animals (Fig. 3B and D).

Histologic examination revealed stunning differences in the tumors of the control animals compared with those treated with NVP-AEW541. Tumors from controls were highly cellular and with a rich network of blood microvessels (Fig. 4A, B, E, and F); conversely, tumors from NVP-AEW541–treated animals showed many pyknotic cells with frequent presence of micronuclei and scant or no microvascularization (Fig. 4C, D, G, and H). The activation of IGF-IR in tumors of NVP-AEW541–treated and control animals was also analyzed by immunohistochemistry using a phosphospecific antibody that recognizes the active (phosphorylated) form of the receptor. A strong membrane-associated staining pattern was detectable in tumors from controls, whereas tumors from NVP-AEW541–treated animals were mostly negative (Fig. 5, compare A, B, E, and F with C, D, G, and H). Because IGF/IGF-IR pathway is involved in the control of VEGF expression (29), we carried out a quantitative real time reverse transcription-PCR from RNA of HTLA-230 and SK-N-BE2c cell lines exposed for 24 hours to a concentration of NVP-AEW541 20% greater than the calculated IC50, and from RNA of NVP-AEW541–treated tumors (SK-N-BE2c xenografts). In all cases, significantly lower amounts of VEGF mRNA were detected in tumor cells treated with NVP-AEW541 (Fig. 6).

Several experimental data indicate that IGF/IGF-IR is involved in invasion and metastatization in different tumor types (30). We investigated whether NVP-AEW541 was able to reduce the invasiveness of neuroblastoma cells in vitro by using Matrigel-coated invasion chambers. Cells were pretreated with scalar concentrations (0.5, 2.0, and 8.0 μmol/L) of NVP-AEW541 for 18 hours and replated in the Matrigel-coated invasion chambers. After 24 hours, the number of cells that migrated into the lower part of the chamber was counted.

### Table 4. Activation of caspase-3/7 in neuroblastoma cell lines untreated and treated with NVP-AEW541

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Untreated (RLU ± SE)</th>
<th>Treated (RLU ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNR</td>
<td>563 ± 23</td>
<td>1,347 ± 31</td>
</tr>
<tr>
<td>GI-CA-N</td>
<td>264 ± 13</td>
<td>53,266 ± 1,173</td>
</tr>
<tr>
<td>HTLA-230</td>
<td>363 ± 24</td>
<td>545 ± 16</td>
</tr>
<tr>
<td>SK-N-BE2c</td>
<td>698 ± 3</td>
<td>1,007 ± 29</td>
</tr>
<tr>
<td>SY-SY (N)</td>
<td>504 ± 30</td>
<td>19,152 ± 1,273</td>
</tr>
</tbody>
</table>

NOTE: NVP-AEW541 was used at a concentration 20% greater than the calculated IC50 for each cell line (see Table 1). Abbreviation: RLU, relative luminometric units.
NVP-AEW541 significantly decreased invasion in all cell lines tested within the used range of concentrations (Fig. 7). Interestingly, the ability of NVP-AEW541 to reduce invasion did not always parallel the inhibitory activity on cell proliferation as assessed in bidimensional cultures.

A pseudometastatic model of neuroblastoma (31) based on tail vein injection of HTLA-230 cells in severe combined immunodeficient mice was used to evaluate the effectiveness of NVP-AEW541 in reducing the spreading of tumor cells to target organs. As not all neuroblastoma cell lines are suitable for tail vein injection, HTLA-230 was selected being the most suitable model described thus far (31). The same day of the injection (day 0), administration of NVP-AEW541 (50 mg/kg twice daily) was started and continued for 14 days. Animals were sacrificed after the occurrence of the first death. Autopsy showed a substantial reduction of tumor cell burden in target organs (adrenals, kidneys, liver, and lungs) in animals treated with NVP-AEW541 compared with controls. Adrenals and kidneys were the most invaded organs. From each mouse of the NVP-AEW541–treated and control groups, histologic specimens were prepared and the largest sagittal section of each organ was evaluated by using an image analysis software to measure the extent of tumor involvement in adrenals and kidneys. A significant reduction of the involved areas was clearly detectable in NVP-AEW541–treated animals (Fig. 8A and B). Furthermore, the number and size of the metastatic foci were strongly diminished in treated animals (Fig. 8C).

Fig. 4. Histologic analysis of neuroblastoma xenografts. A to D, xenografts of HTLA-230 cells; E to H, xenografts of SK-N-BE2c. A, B, E, and F, tumors from untreated animals. C, D, G, and H, tumors from NVP-AEW541–treated animals. A, E, C, and G, ×40 magnification; B, F, D, and H, ×100 magnification. Several apoptotic cells and a strong decrease of blood microvessel network are present in tumors of NVP-AEW541–treated animals (C, D, G, and H).

Fig. 5. Immunohistochemical detection of the phospho-IGF-IR (active form) in neuroblastoma xenografts. A to D, xenografts of HTLA-230 cells; E to H, xenografts of SK-N-BE2c. A, B, E, and F, tumors from untreated animals. C, D, G, and H, tumors from NVP-AEW541–treated animals. A, E, C, and G, ×40 magnification; B, F, D, and H, ×100 magnification. Tumors from NVP-AEW541–treated animals show a strong inhibition of IGF-IR phosphorylation (C, D, G, and H). Control reactions were carried out by substituting the anti-phospho-IGF-IR antibody with normal rabbit serum (framed insets on the right of A and E).
Because the data reported above suggest a possible benefit of an IGF-IR–targeted therapy, a preliminary reverse transcription-PCR analysis was done in a series of 43 neuroblastoma primary tumors to evaluate the frequency of IGF-IR expression. Detectable levels of IGF-IR (defined as any signal visible by using ethidium bromide staining after 30 cycles of PCR amplification) were found in 37 of 43 (86%) cases analyzed (Table 5).

Discussion

IGF-IR is the most important individual component of the IGF axis that includes the ligands IGF-I and IGF-II, six high-affinity IGF-binding proteins, several proteases, and three receptors (32). After the engagement of the ligand to the receptor, many intracellular proteins take part to the process of signal transduction (33). In the nucleus, the activation of IGF-IR signaling may eventually modify the expression of several genes. It has been shown that IGF-IR signaling inhibits p53-dependent apoptosis by regulating MDM2 activity (34). In general, the activation of IGF/IGF-IR signaling promotes proliferative and survival mechanisms that can be usefully exploited by tumor cells. The role of the IGF axis in contributing to tumorigenesis is also supported by the evidence of an increased risk of colorectal, prostate, and breast carcinoma in subjects with elevated expression of IGF-I (reviewed in ref. 26).

Previous work has shown that IGFs are important for proliferation, survival, chemosensitivity, and motility of neuroblastoma tumor cells (15, 16, 35). In this article, we show that NVP-AEW541, a potent IGF-IR inhibitor, has a remarkable in vitro and in vivo antiproliferative activity in neuroblastoma. Growth inhibition caused by NVP-AEW541 is paralleled by apoptosis induction consequent to caspase-3 and caspase-7 and, in most cases, caspase-9 activation. Furthermore, the strong inhibition of anchorage-independent growth caused by the drug pointed to a loss of tumorigenicity that prompted us to evaluate the antitumor activity of NVP-AEW541 in vivo. The growth of tumor xenografts in nude mice was significantly inhibited by treatment with NVP-AEW541 at a dose (100 mg/kg/d) that was not associated with any major systemic toxicity as evaluated by substantial weight loss and variation of animal behavior. Interestingly, the activation of IGF-IR in NVP-AEW541–treated tumors was significantly reduced compared with controls, thus confirming in vivo the target-directed activity of the drug that had already been shown in our in vitro studies.

Further interesting information obtained from the histologic analysis was the evidence of inhibition of the microvasculature in the xenografts of NVP-AEW541–treated animals. Recently, it has been shown that IGFs up-regulate VEGF expression by activating HIF1α through Akt- and mitogen-activated protein kinase–dependent pathways (29). In this study, we detected a partial but yet significant reduction of VEGF mRNA in the NVP-AEW541–treated tumors as well as in the NVP-AEW541–treated cell lines, a finding that further strengthens the role of IGF-IR in controlling angiogenesis and that suggests that NVP-AEW541 acts also in vivo not only by inhibiting cell growth and stimulating apoptosis, but also by interfering with the proangiogenic signals released by neuroblastoma cells.

The IGF/IGF-IR signaling has been described to be involved in local invasiveness and metastatic spreading in several tumor types (30). IGF-I regulates adhesion and motility of breast cancer cells via the α5β1 integrin–mediated activation of the signaling molecule SHC (36). Furthermore, it has been reported that in human neuroblasts, IGF-I stimulates the development of a distinct mesh of actin filaments (37). The latter observation is very important because the acquisition of metastatic properties often requires the reorganization of the actin cytoskeleton. The IGF/IGF-IR signaling has also been identified as a positive regulator of the ECM-degrading enzyme matrix metalloproteinase-2 in lung carcinoma cells (38). Neuroblastoma rarely metastasizes at distant sites where...
a paracrine supply of IGF-II is not available (15), whereas aggressive neuroblastoma tumors and cell lines often express IGF-II (15, 39). Here, we show that NVP-AEW541 is effective in inhibiting the in vivo spreading of a pseudometastatic model of neuroblastoma in severe combined immunodeficient mice (31). This finding is further supported by the additional observation that NVP-AEW541 strongly inhibits the invasive potential of neuroblastoma cells in Matrigel-coated chambers in vitro. Taken together, these data strongly suggest that the inhibition of IGF-IR could be a viable therapeutic strategy to prevent neuroblastoma metastatic growth.

A direct causative role of epigenetic modifications in transformation has been shown less frequently. Nevertheless, many tumors show increased expression of signaling pathways that is associated with enhanced proliferation and aggressiveness. Neuroblastoma is an IGF-sensitive tumor for which previous studies have shown that IGF-II expression and, more generally, signaling through the IGF-IR are important for proliferation and survival (15, 39). Inhibition of the IGF/IGF-IR signal transduction pathway has recently been suggested as a novel promising strategy for tumor treatment (26). This suggestion is also based on the evidence that, in adults, the inhibition of IGF-IR is well tolerated (30). The safety of an intermittent selective inhibition of IGF-IR in children is presently unknown, as no comparable clinical syndrome has been identified thus far. However, it is reasonable to suppose that the overall side effects will be transient and no more severe than those secondary to cytotoxic chemotherapy.

Notwithstanding, the use of IGF-IR blockade for therapeutic purposes has been prevented by the high degree of structural homology between IGF-IR and insulin receptor. Over the last few years, a number of different approaches aimed at creating IGF-IR-specific inhibitors have been attempted to circumvent this problem. For instance, a humanized single chain anti-IGF-IR antibody has been shown to be able to render MCF-7 breast cancer cells refractory to the mitogenic effects of IGF-I. A dominant negative IGF-IR has been shown to induce apoptosis and inhibit cell growth, transformation, as well as metastasis. The recent availability of a small molecule that inhibits IGF-IR with high selectivity (19) has prompted its use in IGF-sensitive tumors (25, 40). In this study, we have shown that NVP-AEW541 inhibits neuroblastoma growth in vitro as well as in vivo by inducing apoptosis, decreasing tumor vascularization, and reducing metastasis formation. Although additional studies are warranted to investigate the toxicity profile of NVP-AEW541 in humans, this molecule represents a novel promising tool for neuroblastoma treatment.

**Table 5. IGF-IR expression in 43 primary neuroblastoma tumors**

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>No. patients</th>
<th>MYCN-amplified patients</th>
<th>IGF-IR-positive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2, 4-S</td>
<td>15</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>3, 4</td>
<td>28</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td></td>
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NOTE: Thirty-seven of 43 patients analyzed were positive for IGF-IR expression (86%).

Fig. 8. NVP-AEW541 inhibits tumor invasion in vivo. Severe combined immunodeficient mice were injected with $3 \times 10^6$ HTLA-230 cells in the tail vein. The same day (day 0), administration of NVP-AEW541 (50 mg/kg twice daily) was started and prolonged for 14 days. Animals were sacrificed after the occurrence of the first death (35th day). The largest sagittal section was evaluated for tumor involvement in adrenals (A) and kidneys (B). A, tumor involvement is expressed as the percentage of the invaded area compared with the total area of the section. Columns, mean; bars, SD. C, representative adrenals and kidneys from untreated (left) and NVP-AEW541-treated (right) animals.

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References


Down-Regulation of Insulin-Like Growth Factor I Receptor Activity by NVP-AEW541 Has an Antitumor Effect on Neuroblastoma Cells In vitro and In vivo

Barbara Tanno, Camillo Mancini, Roberta Vitali, et al.


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