Abstract

**Purpose:** Ewing tumor cell survival and proliferation depend on several autocrine loops. Targeting these loops is a promising therapeutic approach. We recently showed the cytostatic role of imatinib, an inhibitor of the SCF-KIT loop, on Ewing tumor cells, and in this study, we intend to analyze the inhibition of the insulin-like growth factor I receptor (IGF1R) loop.

**Experimental Design:** We analyzed IGF1R blockade by ADW742, a small molecule specific for this receptor, alone and in combination with imatinib, vincristine, and doxorubicin on Ewing tumor cell lines. We studied the effect on proliferation, apoptosis, cell cycle, pathway phosphorylation, soft-agar growth, motility, and vascular endothelial growth factor expression levels.

**Results:** Treatment with ADW742 induced down-regulation of IGF1R/AKT/mammalian target of rapamycin (mTOR) phosphorylation, which was deeper in cell lines having higher IGF1R activation levels. Treatment also induced dose-dependent inhibition of cell proliferation (IC50 = 0.55-1.4 μmol/L), inducing a G1 phase blockage and apoptosis. Addition of imatinib to ADW742 synergistically augmented these effects and was especially effective in inhibiting AKT/mTOR phosphorylation and reducing vascular endothelial growth factor expression in cell lines having high IGF1R activation levels. Combination with usual chemotherapeutic agents vincristine and doxorubicin showed synergistic interactions.

**Conclusions:** Inhibition of Ewing tumor cell proliferation by ADW742 is mediated through blockade of IGF1R signaling. Combination of ADW742 with imatinib, vincristine, and doxorubicin induces a significant reduction of tumor cell growth, mainly by the increase in apoptosis with a pattern depending on IGF1R activation levels. This study supports a potential role for ADW742 in the treatment of Ewing tumor and AKT/mTOR as a possible surrogate marker of response to therapy.
extracellular signal-regulated kinase 1/2, or mTOR, are of major interest in sarcoma treatment. From a clinical point of view, the activation levels of these molecules could also be very useful to predict the response to therapy, acting as surrogate markers of response (26).

Previous studies have shown that the blockage of IGF1R circuit reduces the tumorigenic and metastatic properties of Ewing tumor cells (24, 27–33). In our study, we intend to analyze the effects of the inhibition of this circuit on Ewing tumor cells by treatment with ADW742, an inhibitor of the kinase activity of this receptor. We previously reported that imatinib partially sensitizes Ewing tumor cells to treatment with the conventional therapy (34), is able to inhibit the MAPK pathway, but unable to inhibit phosphorylation of AKT. We, therefore, decided to study the combination of ADW742 with imatinib, doxorubicin, or vincristine and their effect on Ewing tumor cell phenotype. The results obtained raise the possibility of new combined treatments.

Materials and Methods

Cell lines. A673 cell line (obtained from the European Collection of Animal Culture Cells, Porton Down, United Kingdom) was cultured in DMEM medium (Invitrogen/Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen/Life Technologies). TC-71 (kindly given by Dr. Iranzu González, Universidad de Navarra, Pamplona, Spain) was cultured in Iscove’s modified medium (Invitrogen/Life Technologies) supplemented with 20% fetal bovine serum. SK-ES-1 (purchased from the American Type Culture Collection, Teddington, United Kingdom) were cultured in McCoy’s medium (Invitrogen/Life Technologies) with 15% fetal bovine serum. A4573 was cultured in RPMI medium (Invitrogen/Life Technologies) supplemented with 15% fetal bovine serum. Besides fetal bovine serum, all media were supplemented with 1% penicillin/streptomycin (Invitrogen/Life Technologies).

Drugs. ADW742 and imatinib (Gleevec-Glivec) were kindly provided by Novartis Pharma AG (Bazel, Switzerland). These compounds were resuspended in DMSO (10 mmol/L) and aliquoted in the desired working concentrations. Vincristine and doxorubicin, purchased from Sigma Chemical Co. (St. Louis, MO), were resuspended in distilled water.

Proliferation studies. First, dose response proliferation of the cell lines under the influence of ADW742 was analyzed to determine the IC50 of proliferation. Second, the percentage of proliferation inhibition was evaluated in cell lines treated with ADW742 combined with imatinib, vincristine, or doxorubicin. To determine the rate of proliferation, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Briefly, cells were seeded on 24-well culture plates and incubated with ADW742 (alone or with imatinib, doxorubicin, or vincristine) for 72 hours when 30% to 50% of confluence was reached. Then, medium was removed, and cells were incubated with a 1:10 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution [a total of 30 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in the specific medium for each cell line] for 75 minutes. The medium was removed and replaced by 500 µL DMSO. Absorbance was read in a plate reader at 570 nm.

Isobolographic analysis. The effects of the combination of ADW with imatinib, doxorubicin, or vincristine were analyzed by the Loewe’s isobolographic analysis (35), revised by Steel and Peckman (36, 37), which distinguishes three types of interactions: pure additivity, synergy, and antagonism. If synergy exists, then a lower concentration of DA and/or dB would be required to achieve the same effects of the theoretical dosages for additivity (38). For isoeffective dosages of a two-drug combination (DA + DB) and the individual drugs alone (DA and DB), combinations having a combination index > 1 are considered as antagonistic, those with combination index = 1 are additive, and those with combination index < 1 are synergistic, being the combination index = DA / DA + DB / DB. Combination index values for each condition were calculated using the IC50 of proliferation as the isoeffective point, and these values were determined by plotting the results obtained in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in a Hill Curve (using Origin 6.0 to plot the data). Isobolograms were created by plotting the IC50 of ADW742 on the Y-axis and the IC50 of imatinib, doxorubicin, or vincristine on the X-axis. The line that connects these two points is the line of additivity.

Western blot. Expression of IGF1R and its signaling pathway was determined by Western blotting. Preliminary studies were done to determine optimal doses and timing of all drugs and ligand (data not shown). Based on that [and in studies from González et al. (35), Mitsiades et al. (25), and García-Echeverría C (27)], all cell lines were pretreated with ADW742 and imatinib for 20 minutes and doxorubicin/vincristine for 1 hour and then with IGF1 for 15 minutes. The optimal concentrations of imatinib, doxorubicin, and vincristine were, respectively, 10 µmol/L, 100 to 250 ng/mL, and 2.5 to 5 ng/mL. Cell lines were treated with increasing concentrations of ADW742 between 0.1 and 0.25 µmol/L (depending on the cell line) to 5 µmol/L. Initially, cell lysates were obtained by scraping the treated cells on ice with 500 µL of lysis buffer [1% NP40, 150 mmol/L NaCl, 50 mmol/L EDTA, 10% glycerol, 20 mmol/L Tris-HCl (pH 7), protease inhibitor cocktail tablet (Roche), 50 mmol/L NaF, and 2 mmol/L Na3VO4] and sheared through a 25-gauge needle to reduce viscosity. Protein concentration was determined using the Bio-Rad Protein Assay Reagen (Pierce, Rockford, IL). Proteins (50 µg per lane) were resolved on 6% to 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were blocked with 5% bovine serum albumin in TBS-T, probed with the specific antibodies, and visualized by enhanced chemiluminescence detection reagents (Amersham, Barcelona, Spain). To quantitatively assess the changes of phosphorylation, the intensity of each “phospho-band” was analyzed by densitometry with Quantity One 4.3.1 software for GelDoc 2000 (Bio-Rad, Hercules, CA) and normalized against the total protein band. The antibodies used were anti-IGF1R, anti-phospho-IGF1R (anti-p-IGF1R; Tyr1158/1161), anti-phospho-mTOR (anti-p-mTOR; Ser2448), and anti-mTOR, all cell from Cell Signaling (Danvers, MA) with the exception of anti-IGF1R, which is from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-p-IGF1R [Tyr1138/1139], which is from Cellbiochem (La Jolla, CA)/Merck (Darmstadt, Germany), reference nos. sc-713,407707, 9101, 9102, 9271, 9270, 9271, and 9272, respectively. Western blot analysis was done in a FACSort Cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest and Paint-a-Gate software. For each analysis, 20,000 events were acquired.

Soft agar assay. To test the effect of ADW742 and imatinib on anchorage-independent colony formation, 1 × 103 cells/mL were suspended in complete medium containing 0.3% agar and ADW742, imatinib, vincristine, or doxorubicin and then plated in six-well plates coated with a basal layer of complete medium containing 0.6% agar. Colonies were counted after 14 to 21 days using a Zeiss AxioVert 135 microscope, with a ×10 ocular.

In vitro motility assay. The in vitro influence of ADW742 and imatinib on the motility of Ewing tumor cell lines was done by the “wounding assay,” as described previously (17). Briefly, cells were grown to confluence on a glass microscopy slide. Then, a wound was created by scratching the slide with a razor blade, creating a portion of slide without cells. Pictures were taken at 0, 24, and 72 hours. Cell motility from the cutting zone to the area of wound was assessed using...
a Zeiss AxioVert 135 microscope, with a ×10 ocular. The number of cells migrating >5 mm and <5 mm for each condition was counted.

ELISA assay. All subconfluent cell lines were treated with ADW742 and/or imatinib for 72 hours, and then the levels of VEGF released to the cellular medium were assessed by a commercial ELISA (Quantikine Human VEGF Immunoassay, R&D Systems, Minneapolis, MN), following manufacturer’s instructions.

Results

Ubiquitous expression of IGF1R in Ewing tumor cell lines. We first checked the constitutive expression and activation of this receptor in a panel of four Ewing tumor cell lines. We found constitutive and consistent expression in all cell lines (Fig. 1A), with plasma membrane reactivity (Supplementary Fig. S1). A673 had the lowest expression of IGF1R and almost null IGF1R basal phosphorylation; A4573 and TC-71 cell lines had the highest levels of phosphorylated receptor.

Blocking effects of ADW742 on the activation of IGF1R and its pathway. We studied the influence of ADW742 on IGF1R activation by Western blotting. We observed that Ewing tumor cell line A673 was less sensitive to ADW742. Dose-dependent blockade of basal and IGF1-induced phosphorylation was seen with ADW742 in all cell lines (IC50 of phosphorylation = 171-508 nmol/L). We observed that, as shown in Fig. 1 and Supplementary Figs. S2-S4, the effects of this compound depended on the levels of IGF1R protein expression and basal phosphorylation of each cell line. Furthermore, AKT pathway activation induced by IGF1 was markedly blocked with the pretreatment with ADW742. Phospho-AKT and p-mTOR (Fig. 1C-D; Supplementary Figs. S2-4) were inhibited in a dose-dependent manner. Sensitivity to the drug showed a positive correlation with the levels of expression and basal phosphorylation of IGF1R. In contrast, MAPK42/44 remained active even when pretreated with high concentrations of ADW742, in all cell lines except A4573.

Effects of ADW742 on cell proliferation, apoptosis, and cell cycle progression. To determine whether the inhibition of IGF1R could affect the proliferation rate of Ewing tumor cells, we treated all cell lines with different concentrations of ADW742 and quantified proliferation by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. As shown in Fig. 2A and in Table 1, growth inhibition after treatment with ADW742 was strong and dose dependent. All cell lines had IC50 for proliferation around 600 nmol/L, except...
A673, the cell line showing lower activation of IGF1R, in which IC\textsubscript{50} for proliferation was the only one above 1,000 nmol/L.

To know the cause for this decrease in proliferation, we evaluated induction of apoptosis and/or inhibition of cell cycle progression by ADW742. Figure 3 and Supplementary Fig. S6 (the first three bars of each bar graph) show the data obtained; 500 nmol/L ADW742 alone (approximately the IC\textsubscript{50} for proliferation in SK-ES-1, A4573, and TC-71 cell lines) induced apoptosis ranging between 20% and 30%.

We also observed that ADW742 induced a blockade in G\textsubscript{1} phase, higher in SK-ES-1 and A4573 cell lines (see Supplementary Fig. S7). These results suggest that ADW742 is inhibiting proliferation not only by inducing apoptosis but also as a cytostatic by interfering with cell cycle progression.

Synergistic effects of ADW742 combined with imatinib. Because both drugs target two relevant signaling pathways in Ewing tumor, we studied the interaction of these two compounds as a possible new approach to the therapy of Ewing tumor patients.

The effect of combination of ADW742 with imatinib on IGF1R pathway activation is seen in Fig. 1, in which TC71 cell line is shown as an example. AKT and mTOR were the major targets of this combined therapy, their IC\textsubscript{50} of phosphorylation being reduced with respect to ADW742 alone (twice in p-AKT and three to seven times in p-mTOR; see Table 1). Although previous reports showed imatinib alone did not change the levels of p-AKT, our experiments show that combination of this drug with ADW742 inhibited p-AKT and p-mTOR in a synergistic manner, with a combination index of 0.70, whereas no changes were seen on p-MAPK42/44. Similar results were obtained in the other cell lines (Supplementary Figs. S2-S4).

As depicted in isobolograms of Fig. 2, combination of these two compounds synergistically inhibited cell proliferation in all cell lines. Synergy was high (combination index around 0.5) with concentrations of ADW742 as low as 50 nmol/L.

The results obtained in the studies of apoptosis and cell cycle (Fig. 3; Supplementary Figs. S6 and S7) showed that the major effect of the combined treatment in reducing cell growth is driven through induction of apoptosis because imatinib was unable to improve the effects of ADW742 alone on cell cycle progression.

Synergistic effects of ADW742 combined with doxorubicin and vincristine. We studied the combination of ADW742 with
vincristine and doxorubicin because these drugs are the most commonly used in Ewing tumor treatment.

As we did with imatinib, we also studied the effects of the combination of ADW742 with doxorubicin and vincristine on IGF1R pathway. As shown in cell line TC71 (Fig. 1) and Supplementary Figs. S2 to S4, p-AKT is again the major target of combined therapy. Its IC50 of phosphorylation was reduced to 180 to 900 nmol/L (Table 1). MAPK42/44 activation did not almost change with the combined therapy except for a mild inhibition in A4573 cell line, the one showing the highest levels of IGF1R/p-IGF1R.

Both combinations of ADW with doxorubicin or vincristine synergistically inhibited cell proliferation, using ADW742 in the range of 50 to 250 nmol/L (Fig. 2; Supplementary Fig. S5). As expected, the major effect of the combination of ADW742 with doxorubicin and vincristine was the induction of apoptosis (Fig. 3), up to 75% with the concentrations used, regardless the cell line studied. We only detected synergistic interactions between ADW742 and doxorubicin in A4573 cell line, with combination indexes ranging from 0.74 to 0.87.

Effects of ADW742 combined with imatinib, doxorubicin, and vincristine on motility and soft agar growth. To further characterize the interaction among these drugs, we also did motility and soft agar growth studies. As shown in Fig. 4A, ADW742 was particularly useful to induce a migration arrest and drug combination did not greatly improve these results. Conversely, the number of colonies formed was lower when a combined treatment was given with respect to single-agent administration (Fig. 4B), suggesting that, besides of inducing more apoptosis, the combined therapy may also have advantages in the inhibition of tumor growth.

Effects of ADW742 combined with imatinib on VEGF expression. It has been reported that an altered IGF1R autocrine loop has proangiogenic effects in Ewing tumor.

Based on that assumption, we decided to study VEGF levels secreted to the cellular medium of all Ewing tumor cell lines treated with ADW742 and/or imatinib. Our ELISA assay (Fig. 5) showed a VEGF reduction after treatment of all Ewing tumor cell lines. Reduction ranged between 10% (A673) and 35% (A4573). Combination of ADW742 with imatinib was additive, showing 30% to 40% more inhibition than in the treatment with each drug alone.

### Discussion

Search for new therapeutic approaches is an urgent need in sarcomas. IGF1R is a receptor with high homology with the insulin receptor (>70 %) that belongs to an autocrine loop very relevant to cell proliferation and survival in Ewing tumor (10). IGF1R blockade is therefore a promising approach in the therapy of Ewing tumor. Several studies have shown experimental inhibition of this receptor using blocking monoclonal antibodies or several drugs, and a main requirement for such therapy is a selective and specific action against IGF1R, without inhibiting insulin receptors at therapeutic doses (24, 25, 27–33).

ADW742 is a powerful and specific inhibitor of IGF1R (16 times more specific for IGF1R than for insulin receptor; ref. 25), which in our hands is able to inhibit IGF1-mediated phosphorylation of IGF1R (IC50 = 0.171-0.508 μmol/L) and its signaling pathway, especially AKT. It is interesting that sensitivity of several members of IGF1R pathway in Ewing tumor cell lines seem to depend on the levels of expression of IGF1R and basal IGF1R activation that cell lines bear. For example, AKT inhibition is deeper in TC-71 or A4573 cell lines, which show very high levels of IGF1R activation. The opposite situation is seen in A673. On the other hand, AKT phosphorylation, a key event in cell survival signaling, is

### Table 1. IC50 of phosphorylation (μmol/L) of IGF1R, AKT, and MAPK after ADW742 treatment alone or combined with imatinib, doxorubicin, or vincristine and IC50 of proliferation of all cell lines after ADW742, imatinib, doxorubicin, or vincristine treatment at 72 hours of incubation

<table>
<thead>
<tr>
<th>Drug</th>
<th>Molecule</th>
<th>Cell line</th>
<th>A673</th>
<th>TC-71</th>
<th>SK-ES-1</th>
<th>A4573</th>
</tr>
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<tr>
<td><strong>IC50 of phosphorylation (μmol/L)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>ADW</td>
<td>p-IGF1R</td>
<td></td>
<td>0.508 ± 0.12</td>
<td>0.244 ± 0.098</td>
<td>0.184 ± 0.154</td>
<td>0.171 ± 0.053</td>
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<td></td>
<td>p-AKT</td>
<td></td>
<td>1 ± 0.54</td>
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<td>0.35 ± 0.013</td>
<td>0.81 ± 0.028</td>
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<td></td>
<td>p-MAPK</td>
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<td>&gt;6</td>
<td>&gt;6</td>
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</tr>
<tr>
<td>ADW + imatinib</td>
<td>p-IGF1R</td>
<td></td>
<td>0.567 ± 0.09</td>
<td>0.176 ± 0.01</td>
<td>0.217 ± 0.08</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>p-AKT</td>
<td></td>
<td>4.1 ± 0.8</td>
<td>3.085 ± 1.29</td>
<td>1.498 ± 0.039</td>
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<td>p-MAPK</td>
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<td>&gt;6</td>
<td>&gt;6</td>
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<td>&gt;6</td>
</tr>
<tr>
<td>ADW + doxorubicin</td>
<td>p-IGF1R</td>
<td></td>
<td>0.55 ± 0.08</td>
<td>0.231 ± 0.078</td>
<td>0.186 ± 0.03</td>
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<tr>
<td>ADW + vincristine</td>
<td>p-IGF1R</td>
<td></td>
<td>0.97 ± 0.89</td>
<td>0.291 ± 0.093</td>
<td>0.23 ± 0.093</td>
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<td>p-AKT</td>
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<td>4.61 ± 0.52</td>
<td>3.656 ± 2.31</td>
<td>0.93 ± 0.028</td>
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<tr>
<td></td>
<td>p-MAPK</td>
<td>&gt;6</td>
<td>&gt;6</td>
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</tr>
<tr>
<td><strong>IC50 of proliferation</strong></td>
<td></td>
<td></td>
<td>1410 ± 280</td>
<td>670 ± 80</td>
<td>550 ± 50</td>
<td>640 ± 60</td>
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<tr>
<td>ADW (nmol/L)</td>
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<td>Imatinib (μmol/L)</td>
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<td>276 ± 35.17</td>
<td>82 ± 11.8</td>
<td>86 ± 8.24</td>
<td>65.5 ± 3.06</td>
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<tr>
<td>Doxorubicin (ng/mL)</td>
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<td>3.76 ± 0.43</td>
<td>3.5 ± 0.19</td>
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<tr>
<td>Vincristine (ng/mL)</td>
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<td>3.20 ± 0.35</td>
<td>3.76 ± 0.43</td>
<td>3.5 ± 0.19</td>
<td>3.7 ± 0.27</td>
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Fig. 3. Effects of ADW742 and imatinib (IMA), doxorubicin (DXR), or vincristine (VCR) on apoptosis of A673 and A4573 cell lines. Subconfluent cells were treated with ADW742 (0.1-1 μmol/L) and imatinib (2.5-10 μmol/L), doxorubicin (15-200 ng/mL), or vincristine (0.5-2.5 ng/mL) for 72 hours, and then apoptosis was analyzed by flow cytometry by Annexin V/propidium iodide detection. A, graphic representation obtained for several treatment conditions of A4573 cells with ADW742 and imatinib, doxorubicin, or vincristine. While imatinib alone (10 μmol/L) was unable to produce apoptosis, its combination with ADW742 was able to increase the apoptosis induced by the latter. The combination of ADW with doxorubicin and vincristine also induced more apoptosis than each drug alone. B, bar graph representation of all conditions. The higher the concentration of both drugs, the higher the ratio between apoptotic/necrotic cells and vital cells, indicating an increase in the induction of cell death. The effect of combining ADW742 with imatinib, doxorubicin, or vincristine resulted in an additional increase of 20% to 40% in apoptosis, depending on the cell line. We mainly observed additive effects, but in A4573, we saw synergistic effects when combining ADW + doxorubicin or ADW + imatinib, as described in Results. A, ADW742; I, imatinib; D, doxorubicin; E, apoptotic; N, necrotic (to see the results of SK-ES-1 and TC-71 cell lines, see Supplementary Fig. S6).
blocked in a dose-dependent manner by ADW742 and could then constitute a good surrogate marker of response to therapy using this drug. IGFR1-mediated expression of VEGF is also abolished by ADW742 in all Ewing tumor cell lines, being the VEGF levels secreted to the cellular medium lower in the treatment condition than in the controls. This suggests that AKT signaling pathway is the main mechanism mediating VEGF expression in Ewing tumor [in contrast to Stramiello et al. (22)]. Furthermore, we observed strong and dose-dependent inhibition of mTOR phosphorylation. In the combined treatment, p-mTOR inhibition was synergistic, showing even a deeper effect than the observed on p-AKT levels. We think that this fact indicates that we are detecting, together with the direct and widely reported effect of AKT over mTOR activation, another AKT-independent mTOR activation pathway that is being inhibited by imatinib but not by ADW742. We are currently performing detailed proteomic studies to evaluate this possibility.

All these data underline the central role of p-AKT/mTOR for cell signaling in Ewing tumor. In other words, whereas basal levels of IGFR1 and p-IGFR1 are predictive of AKT/mTOR response to ADW742 [in agreement with Scotlandi et al. (24)], levels of p-AKT/mTOR could be useful to monitor actual response to therapy by noninvasive techniques.

Accordingly, ADW742 inhibits cell proliferation in a dose-dependent manner, with IC$_{50}$ in the nanomolar range (550-1,410 nmol/L). Again, cell lines with high levels of IGFR1 activation were more sensitive to the drug. Our previous studies showed that blockade of MAPK pathway by imatinib was responsible for a mild decrease (20-30%) in cell proliferation in Ewing tumor (34). The effect of ADW742 through AKT/mTOR signaling seems to be much more pronounced and induces a deeper cell proliferation arrest (40-70%). Our data, therefore, suggest that AKT/mTOR pathway is a major determinant of cell proliferation/resistance to apoptosis in Ewing tumor.

Because the SCF/KIT pathway is also active in Ewing tumor (34), we decided to combine ADW742 with imatinib to inhibit MAPK pathway in a more efficient way. Blockade of MAPK phosphorylation did not change significantly by combining both drugs. Surprisingly, our results show that, using ADW742 combined with imatinib, phosphorylation of AKT/mTOR is inhibited at much lower concentrations of the former (IC$_{50}$ = 176-610 nmol/L). Combination studies of ADW742 and imatinib showed that the effects on cell proliferation and apoptosis are deeper than when both drugs are used alone. Importantly, the mathematical method employed [the Loewe (35) method described by Berenbaum blocked in a dose-dependent manner by ADW742 and could then constitute a good surrogate marker of response to therapy using this drug. IGFR1-mediated expression of VEGF is also abolished by ADW742 in all Ewing tumor cell lines, being the VEGF levels secreted to the cellular medium lower in the treatment condition than in the controls. This suggests that AKT signaling pathway is the main mechanism mediating VEGF expression in Ewing tumor [in contrast to Stramiello et al. (22)]. Furthermore, we observed strong and dose-dependent inhibition of mTOR phosphorylation. In the combined treatment, p-mTOR inhibition was synergistic, showing even a deeper effect than the observed on p-AKT levels. We think that this fact indicates that we are detecting, together with the direct and widely reported effect of AKT over mTOR activation, another AKT-independent mTOR activation pathway that is being inhibited by imatinib but not by ADW742. We are currently performing detailed proteomic studies to evaluate this possibility.

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Because the SCF/KIT pathway is also active in Ewing tumor (34), we decided to combine ADW742 with imatinib to inhibit MAPK pathway in a more efficient way. Blockade of MAPK phosphorylation did not change significantly by combining both drugs. Surprisingly, our results show that, using ADW742 combined with imatinib, phosphorylation of AKT/mTOR is inhibited at much lower concentrations of the former (IC$_{50}$ = 176-610 nmol/L). Combination studies of ADW742 and imatinib showed that the effects on cell proliferation and apoptosis are deeper than when both drugs are used alone. Importantly, the mathematical method employed [the Loewe (35) method described by Berenbaum...
(36) and later modified by Steel and Peckman (37)] showed that this particular combination is synergistic, especially at low concentrations of both drugs. In fact, whereas results were largely additive when concentrations of 1 to 5 μmol/L ADW742 were used in 24-hour incubations (data not shown), synergy was evident when much lower concentrations were used for 72-hour periods. Synergy was stronger in the cell lines showing a higher expression level of IGF1R, probably more dependent on this molecule for survival and proliferation. Besides these effects, the ADW742 + imatinib combination also seemed to be effective in reducing the levels of VEGF, suggesting again the benefits of the combined therapy. This is a clinically relevant finding because two recent articles pointed out that VEGF is the single most important regulator of angiogenesis in Ewing tumor and therefore a therapeutic target for this neoplasm (39, 40).

Because IGF1 is able to attenuate the effect of conventional chemotherapeutic drugs (32, 33), we combined ADW742 with doxorubicin and vincristine, two of the most widely used drugs in Ewing tumor treatment, in an attempt to further sensitize Ewing tumor cell lines to doxorubicin or vincristine treatment. We observed that ADW742 actually sensitizes Ewing tumor cell lines to the treatment with these drugs, especially through the induction of apoptosis. Again, and in contrast to what was reported using NVP-AEW541, another pyrrolo[2,3-d]pyrimidine derivative highly selective against IGF-IR (24), combination of ADW742 with doxorubicin and vincristine was synergistic for cell proliferation and apoptosis, meaning that concentrations as low as 50 to 250 nmol/L ADW742 and 10 to 50 ng/mL doxorubicin or 0.5 to 2 ng/mL vincristine are able to induce significant levels of apoptosis in all cell lines tested. This is of potential clinical interest because doses of both drugs can be diminished when combined, saving Ewing tumor patients from drug toxicity.

As a closer approach to in vivo conditions, we also performed soft agar and mobility assays. ADW742 dramatically suppressed colony growth and motility even with very low concentrations of ADW742 alone (100 nmol/L). Drug combinations showed only additive results. These results, showing anchorage-independent growth, suggest the possible benefits of ADW742 treatment in vivo, probably inhibiting other processes involved in survival through cell-cell and cell-matrix contact.

Our results raise the possibility that AKT/mTOR could be a useful new surrogate marker of the response to ADW742 + imatinib treatment because it accomplishes, at least partially, the three criteria that need to be satisfied (26): (a) p-AKT is biologically associated with survival signals in Ewing tumor cells (41); (b) the treatment is directly associated with p-AKT levels; and (c) p-AKT mediates the effects of treatment on cell proliferation (this molecule seems to fully “capture” the IGF1R and c-Kit signals, and the reduction of his activation seems to be enough to reduce proliferation, with no MAPK change). In actual tumors, much more complex than cell lines, it is likely that a panel of surrogate markers would be required to predict the full effect of treatment, but we suggest that AKT should be included in this panel.

In summary, IGF1R expression and activation determines at least in part the response to ADW742 in Ewing tumor cell lines. For example, A4573 is more sensitive to ADW742 treatment, alone and especially when combined with imatinib. Their levels of proliferation, cell cycle arrest, and apoptosis induction are deeper, VEGF levels are lowered more effectively, and they can show p-MAPK inhibition. In contrast, A673 shows the opposite situation. On the other side, IGF1R expression is a determinant of p-AKT/p-mTOR inhibition. It is tempting to speculate that it would be important to stratify Ewing tumor patients at the time of diagnosis with respect to tumor expression and activation of IGF1R. Interestingly, we have previously reported using a well-characterized series of patients (42) that extensive IGF1R expression was seen in biopsies of a subgroup of Ewing tumor patients and was strongly associated to tumor samples with high proliferative activity and EWS-FLI1 no-type 1 fusions (P = 0.015). ADW742 alone or combined with imatinib, vincristine, or doxorubicin could be then especially useful in the treatment of patients with Ewing tumor having high IGF1R activation levels/EWS-FLI1 no-type 1 fusions, which is a subgroup of patients showing a dismal prognosis (8, 9) when treated with conventional agents (like vincristine or doxorubicin).

Clinical routine analysis of expression of a given receptor (i.e., by immunohistochemistry) could be not enough to ensure it constitutes a therapeutic target. To ensure the success of a tyrosine kinase inhibitor in cancer treatment, it is important that at least three other requirements are accomplished: this receptor should be constitutively active (either by ligand exposure or activating mutations); activity of this receptor should be important for cell survival; and a selective inhibitor orally bioavailable should be designed. Our results show that all these requirements are met with ADW742 in Ewing tumor, and that this drug, alone or combined with imatinib, vincristine, or doxorubicin, is a powerful therapeutic alternative in Ewing tumor cells.

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Insulin-Like Growth Factor I Receptor Pathway Inhibition by ADW742, Alone or in Combination with Imatinib, Doxorubicin, or Vincristine, Is a Novel Therapeutic Approach in Ewing Tumor

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