Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors

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Summary

Insulin-like growth factors and their receptor (IGF-1R) have been implicated in cancer pathophysiology. We demonstrate that IGF-1R is universally expressed in various hematologic (multiple myeloma, lymphoma, leukemia) and solid tumor (breast, prostate, lung, colon, thyroid, renal, adrenal cancer, retinoblastoma, and sarcoma) cells. Specific IGF-1R inhibition with neutralizing antibody, antagonistic peptide, or the selective kinase inhibitor NVP-ADW742 has in vitro activity against diverse tumor cell types (particularly multiple myeloma), even those resistant to conventional therapies, and triggers pleiotropic antiproliferative/proapoptotic molecular sequelae, delineated by global transcriptional and proteomic profiling. NVP-ADW742 monotherapy or its combination with cytotoxic chemotherapy had significant antitumor activity in an orthotopic xenograft MM model, providing in vivo proof of principle for therapeutic use of selective IGF-1R inhibitors in cancer.

Introduction

Insulin-like growth factors-1 (IGF-1) and -2 (IGF-2) have been implicated in the pathophysiology of a wide range of human neoplasias due to the mitogenic and antiapoptotic properties mediated by their type I receptor (IGF-1R or CD221) (reviewed in (LeRoith and Roberts, 2003). Expression of functional IGF-1R is required for neoplastic transformation in diverse tumorigenesis models (Werner and Le Roith, 1997). Until now, however, inhibition of the IGF-1R pathway had not been successfully applied as a major anticancer therapeutic strategy. This has been due not only to the lack of clinically applicable small molecule inhibitors of IGF-1R function, but also to the fact that the relative impact of inhibiting the IGF/IGF-1R pathway, compared to other cytokine/growth factor cascades, in tumor cell proliferation and survival have not been fully elucidated.

Herein, we confirm in a broad panel of tumor types that IGF-1R is universally expressed and that multiple strategies for specific inhibition of IGF-1R function, including a selective small molecule tyrosine kinase inhibitor, have potent antitumor effects in vitro. Of the diverse tumor types studied, multiple myeloma (MM) cell lines and primary tumor samples are particularly sensitive to IGF-1R inhibition, including cells with documented resistance to various conventional or investigational anticancer

SIGNIFICANCE

Epidemiological and experimental data have implicated the IGF/IGF-1R pathway in the establishment of human malignancies, but this cascade has not been previously viewed as a major therapeutic target due to widespread IGF-1R expression in normal tissues and a lack of clinically applicable small molecule inhibitors. Furthermore, while neutralizing monoclonal antibodies against human IGF-1R have shown efficacy against xenograft tumors, a lack of reactivity against the host rodent receptor confounds assessment of therapeutic index. This report validates IGF-1R as a therapeutic target for a broad spectrum of malignancies and provides in vivo proof of concept for use of selective IGF-1R kinase inhibitors as primary antitumor therapy or in synergistic combination as chemosensitizers. These results provide the rationale for clinical trials of small molecule IGF-1R inhibitors.

drugs. Comprehensive transcriptional and proteomic analyses indicate that IGF-1R inhibition confers a pleiotropic constellation of antiproliferative and proapoptotic molecular sequelae, which provide a mechanistic explanation for the ability of IGF-1R inhibitors to sensitize tumor cells to other anticancer drugs. IGF-1R inhibition also blunts tumor cell response to other growth factors, overcomes the drug resistance phenotype conferred by the bone microenvironment, and abrogates the production of proangiogenic cytokines. Importantly, in a clinically relevant mouse model of diffuse MM, systemic administration of the selective IGF-1R tyrosine kinase inhibitor NVP-ADW742 suppresses tumor growth, prolongs survival, and enhances the antitumor effect of cytotoxic chemotherapy. These studies provide proof of principle for IGF-1R kinase inhibition as a therapeutic strategy for a broad spectrum of malignancies, and elucidate previously unidentified aspects of the pathophysiology of IGF-1R and its downstream molecular mediators. These results also provide the rationale for the development of clinical therapeutic strategies targeting IGF-1R.

Results

Universal expression of IGF-1R in hematologic malignancies and solid tumors

We used flow cytometry to study a panel of 75 hematologic and solid tumor cell lines (Supplemental Table S1 at http://www.cancercell.org/cgi/content/full/5/3/221/DC1) for expression of IGF-1R using 2 different anti-human IGF-1R monoclonal antibodies. We found universal cell surface expression of IGF-1R in all cell lines tested (representative examples shown in Supplemental Figure S1), including hematologic malignancies (MM, various subtypes of leukemias and lymphomas), as well as solid tumors (prostate, breast, lung, colon, thyroid, ovarian, renal, adrenal cancer, sarcomas, and retinoblastoma). To preclude the possibility that IGF-1R expression is an artifact induced by in vitro propagation, we also examined 35 freshly isolated primary MM tumor specimens, including samples from patients with resistance to conventional or high-dose chemotherapy, as well as classes of antitumor agents recently added to the therapeutic armamentarium against MM, including thalidomide, its immunomodulatory analogs (CC-5013), and the proteasome inhibitor bortezomib (PS-341). Cell surface expression of IGF-1R was also universally present in these primary patient samples (representative examples shown in Supplemental Figure S1). There was no discernible pattern of association between the degree of surface IGF-1R expression and tumor type, histologic subtype, or resistance to anticancer drugs (e.g., alkylating agents, anthracyclines, dexamethasone, thalidomide, CC-5013, TRAIL/Apo2L, or PS-341).

In vitro antitumor activity of IGF-1R inhibitors

The functional impact of specific inhibition of IGF-1R activation in tumor cells was quantified in vitro by MTT colorimetric survival assays, to evaluate the degree to which inhibition of IGF-1R function can suppress the ability of serum (which contains IGFs) to stimulate increase of the population of viable tumor cells. These assays involved use of 10% or 20% fetal bovine serum and/or pooled sera from healthy donors or sera from MM patients, in comparison with serum-free conditions. Specific inhibition of IGF-1R function was achieved through use of a neutralizing monoclonal antibody α-IR3 (Flier et al., 1986), the IGF-1-like competitive peptide antagonist JB-1 (Hayry et al., 1995; Pietrzkowski et al., 1993), or the selective IGF-1R kinase inhibitor NVP-ADW742 (Novartis Pharma AG, Basel, Switzerland). Cellular kinase activity assays have demonstrated that NVP-ADW742 has >16-fold more potent inhibitory effect against IGF-1R than insulin receptor (InsR), the kinase with the highest homology to IGF-1R (IC_{50} 0.17 and 2.8 μM, respectively, in cellular autophosphorylation assays; Supplemental Figure S2A). The selectivity observed at the cellular level indicates that, despite the high identity of the kinase domains of IGF-IR and InsR, there are differences between these receptors which can allow the selective inhibition of IGF-1R, but not InsR. Furthermore, NVP-ADW742 has much higher IC_{50} values for other kinases (e.g., $IC_{50} > 10$ μM for HER2, PDGFR, VEGFR-2, or Bcr-Abl p210; and $IC_{50} > 5$ μM for c-Kit; Supplemental Figure S2A). Concordant with these results, immunoblot analysis verified that NVP-ADW742 blocks IGF-1-induced phosphorylation of IGF-1R and its known downstream target Akt (Mitsiades et al., 2002b) at submicromolar concentrations (Supplemental Figure S2B).

All three anti-IGF-1R inhibitory strategies similarly suppressed serum-stimulated increase of the total population of viable tumor cells in all cell lines tested (Figures 1A and 1B). These results indicate that IGFs are key mediators of the ability of serum to stimulate the proliferation of tumor cells in vitro, and that specific inhibition of IGF-1R function supercedes the ability of other serum growth factors to stimulate proliferation/growth. To determine if there were any cell type-specific differences in response to IGF-1R inhibition, we assayed the effect of NVP-ADW742 on the viability of 58 hematologic and solid tumor cell lines. Dose-dependent inhibition of serum-induced cell growth was observed in all cell lines (Figure 1D). As a group, MM cell lines appeared to be more sensitive to the effects of NVP-ADW742, with IC_{50} values generally in the 0.1–0.5 μM range, in comparison to the relatively higher IC_{50} values observed in most other hematologic and solid tumor cell lines.

Focusing on MM, we evaluated the effect of IGF-1R inhibition on the viability of MM cells purified from primary patient samples in short-term culture assays immediately after isolation. In all primary patient samples tested, inhibition of IGF-1R with a neutralizing antibody or NVP-ADW742 completely suppressed serum-induced growth (Figure 1C and Supplemental Table S2). As a specificity control, an anti-IL-6 receptor (IL-6R) neutralizing antibody had no discernable effects on the viability of serum-cultured MM cells (Figures 1A and 1C and Supplemental Table S2). This finding may appear counterintuitive to the widely accepted role of IL-6 in tumor cell proliferation, survival, and drug resistance in MM (Anderson and Lust, 1999; Burger et al., 2001; Hallek et al., 1998) and other diseases, e.g., prostate cancer (Culig et al., 2002; Giri et al., 2001), raising the possibility that serum concentrations of IL-6 are not sufficient to stimulate tumor cells. However, we further found that the response of MM and prostate cancer cells to exogenous administration of IL-6 at levels in the 1–10 ng/ml range, which are 2–3 logs higher than serum levels (Nakashima et al., 2000), was also abrogated by inhibition of IGF-1R (Figure 2). These findings underscore an apparent pivotal role for IGF-1R function in the hierarchy of growth factor receptor systems in tumor cells.

Importantly, NVP-ADW742, α-IR3 or JB-1 were active even against MM cell lines with known resistance to conventional (cytotoxic chemotherapy, dexamethasone) or investigational (thalidomide, CC-5013, TRAIL/Apo2L, PS-341) anticancer agents (Figure 1), as well as primary tumor cells from MM patients with

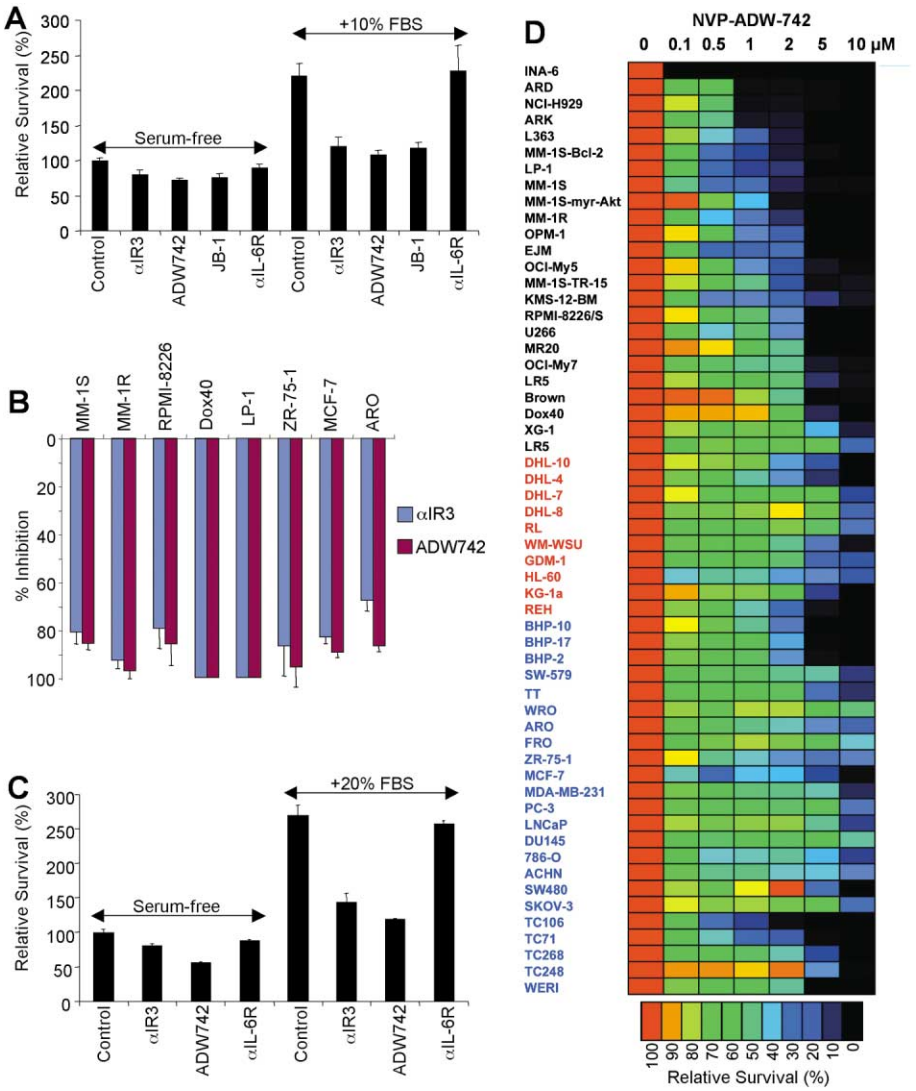

Figure 1. In vitro antitumor activity of selective IGF-1R inhibitions

A: In vitro antitumor activity of the IGF-1R inhibitors NVP-ADW742 (0.5 μM), α-IR3 (2 μg/ml), and JB-1 (2 μg/ml) against the dexamethasone-resistant MM-1R cell line. Cells were incubated with the indicated IGF-1R inhibitors or an IL-6R neutralizing antibody (10 μg/ml) for 2 days in the presence or absence of serum. Results (mean ± SD) are expressed relative to the respective control (DMSO, mouse IgG1 antibody, or scrambled peptide) and are representative of three independent experiments performed in triplicate for each experimental condition.

B: Comparative results of the effect of α-IR3 2 μg/ml or NVP-ADW742 0.5 μM for 72 hr on a panel of drug-sensitive and resistant MM cell lines, as well as prostate, breast, and thyroid carcinoma cell lines (see Supplemental Table S1 at http://www.cancercell.org/cgi/content/full/5/3/221/DC1). Results (mean ± SD) are presented as the % suppression of serum-induced increase in cell number and are representative of three independent experiments performed in triplicate for each experimental condition.

C: Representative in vitro activity of α-IR3, NVP-ADW742, and anti-IL-6R (concentrations as specified in **A**) against primary MM tumor cells isolated from a patient resistant to multiple conventional and novel anti-MM therapies (thalidomide, CC-5013, and PS-341). Results (mean ± SD) are expressed relative to serum-free control and are representative of two separate experiments performed in duplicate for each experimental condition.

D: Dose-response matrix of a panel of cell lines from MM (black letters), other hematologic malignancies (red letters), and solid tumors (blue letters) to NVP-ADW742 at 0–10 μM for 72 hr. Relative survival is visualized in an assigned color scale. Results represent the mean for at least 2 independent experiments for each cell line, performed in triplicate for each experimental condition.

multi-drug-resistant disease (Supplemental Table S2). Furthermore, the degree of sensitivity of MM or other neoplasias to IGF-1R inhibitors was not associated with the level of surface expression of IGF-1R (Supplemental Figure S1 and data not shown). Because the IGF/IGF-1R cascade can be counteracted by the expression of IGF-receptor 2 (IGF-2R, IGF-IIR, CD222, or CIMPR; reviewed in LeRoith, 1996), we also studied the expression of IGF-2R in our panel of tumor cells. We found that IGF-2R is expressed on the surface of a minority of tumor cell lines, but is found intracellularly in the entire tumor cell panel tested (Supplemental Figure S3). Again, we observed no discernible association between the degree of either surface or intracellular IGF-2R expression and tumor type, histologic subtype, or resistance to the aforementioned panel of conventional and investigational anticancer drugs. Furthermore, there was no apparent association between IGF-2R levels and sensitivity to IGF-1R inhibition.

In vivo antitumor activity of the selective IGF-1R kinase inhibitor NVP-ADW742

Focusing on MM as a model of IGF-1R-dependent neoplasias, we evaluated the in vivo antitumor activity of IGF-1R inhibition in a mouse xenograft model of MM. Given the importance of the tumor microenvironment in the biology of MM, we wished to establish an orthotopic MM model of bone and bone marrow disease. The MM cell line MM-1S was engineered to stably express firefly luciferase fused to a selectable marker (neomycin phosphotransferase or enhanced GFP). Sublethally irradiated NOD/SCID mice were injected intravenously with MM-1S-Luc cells and tumor distribution was followed by serial whole-body noninvasive imaging of visible light emitted by luciferase-expressing tumor cells upon injection of mice with luciferin (Armstrong et al., 2003). Pilot experiments demonstrated that i.v. injections of MM-1S-Luc cells led to their engraftment to bone marrow and bone and consistent establishment of multifocal bone lesions in the axial skeleton (vertebrae, skull, pelvis) and long bones (Figure 3A and data not shown). The anatomic distribution of these MM lesions was consistent with the presentation of disease in human MM patients (Healy and Armstrong, 1998).

We evaluated the in vivo antitumor efficacy of IGF-1R inhibition with NVP-ADW742 in our orthotopic MM model. Cohorts of mice with similarly increasing tumor burden were divided into control and treatment groups 3 weeks after tumor cell injection.

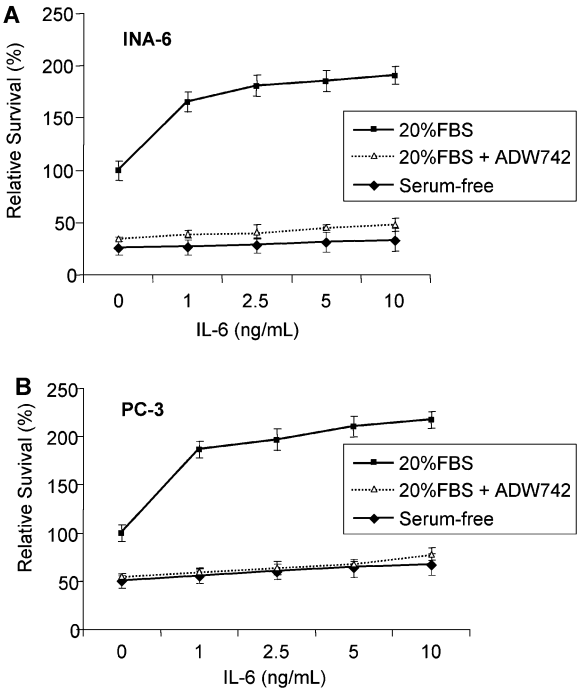

Figure 2. IGF-1R inhibition abrogates the responsiveness of tumor cells to IL-6

In the presence of serum, the INA-6 MM cells (**A**) and PC-3 prostate cancer cells (**B**) are responsive to IL-6 at the indicated concentration as shown by MTT assays after 72 hr of incubation (solid symbols and lines). However, this responsiveness is abrogated in the absence of serum (gray symbols and line) or upon cotreatment with 0.5 μM NVP-ADW742 (open symbols, dashed lines). Results are presented relative to control cultures in serum only, and represent the mean ± SD of triplicate samples.

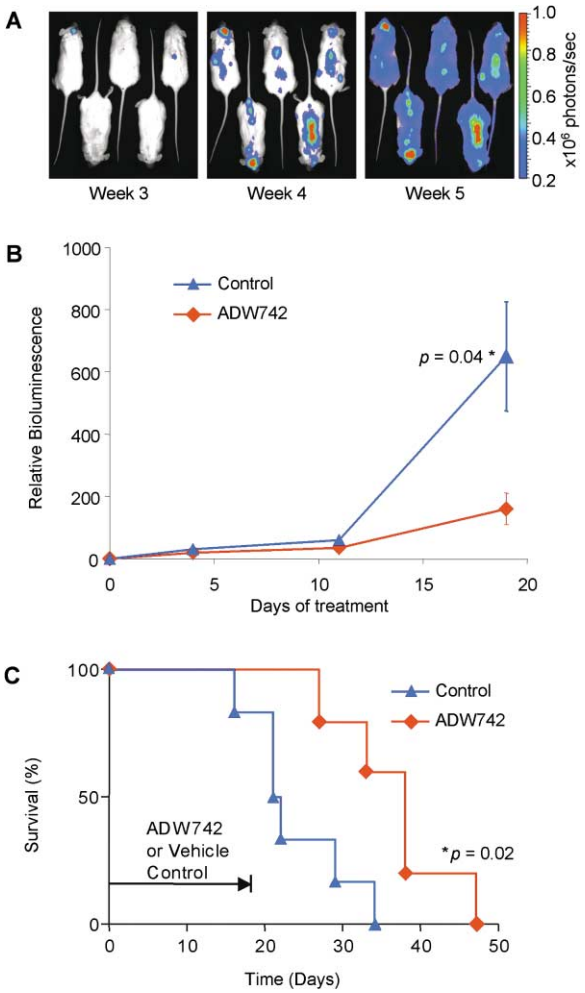

Figure 3. In vivo antitumor activity of the selective IGF-1R kinase inhibitor NVP-ADW742

A: Representative in vivo bioluminescence imaging of SCID/NOD mice injected i.v. with 5×10^6 MM-1S-Luc$^+$ human MM cells. Imaging 3 weeks after injection of cells reveals lesions in the spine, skull, and pelvis, with eventual progression to diffuse disease. Quantification of total tumor burden (**B**) and Kaplan-Meier survival curve (**C**) of MM-1S-Luc$^+$ orthografted mice receiving NVP-ADW742 (10 mg/kg i.p. twice daily) versus vehicle-treated control mice. Homogeneous cohorts of mice with established tumor lesions were divided into treatment groups, 3 weeks after injection of MM-1S-Luc$^+$ cells. Treatments were administered daily for 19 days. Relative bioluminescence is represented as mean ± SD, n = 6 per treatment group. Median survival of mice treated 19 days with NVP-ADW742 was 38 days, in comparison to 21 days for control mice ($p = 0.02$, log rank test).

In this model of MM, NVP-ADW742 monotherapy (10 mg/kg i.p. twice daily) significantly suppressed tumor growth (Figure 3B) and prolonged the survival of mice (Figure 3C). There was no difference in body weight between treatment groups ($p = 0.82$) and no other significant treatment-related toxicity discernable by necropsy and histopathological evaluation (data not shown). Consistent results were obtained with a second study with the same i.p. dosing, as well as a third study utilizing NVP-ADW742 dosed orally at 50 mg/kg twice daily (data not shown).

Molecular sequelae of IGF-1R inhibition

To investigate the molecular pathways implicated in IGF-1R activation and, conversely, its inhibition, we characterized the molecular sequelae triggered in MM cells upon their exposure to IGF-1R inhibitors (in the presence or absence of serum), using gene expression profiling and signal transduction proteomic profiling using multiplex immunoblotting analyses, as previously described (Mitsiades et al., 2003a, 2003c). The functional significance of findings detected in the global molecular profiling assays was subsequently confirmed by specific mechanistic assays.

Global expression profiling revealed distinct transcriptional profiles distinguishing serum-free from serum-stimulated cells (Figure 4A). Cells treated solely with physiological levels of IGF-1 (but otherwise serum-free) had expression profiles consistent with those of cells exposed to serum. Conversely, when serum-exposed cells were treated with either α-IR3 or NVP-ADW742, the expression profiles clustered with those of cells grown in serum-free conditions as shown by unsupervised hierarchical clustering (Figure 4A). Taken together, these results demonstrate that activation of IGF-1R by the IGFs contained in serum constitute a major part of the transcriptional effects of serum. Furthermore, the global effects of inhibiting IGF-1R by NVP-ADW742 or a specific neutralizing antibody are concordant, thus further supporting the specificity of NVP-ADW742 activity.

Of note, unsupervised hierarchical clustering analyses showed that the gene expression profiles of MM cells treated with IGF-1R inhibitor (NVP-ADW742 and α-IR3) corresponded

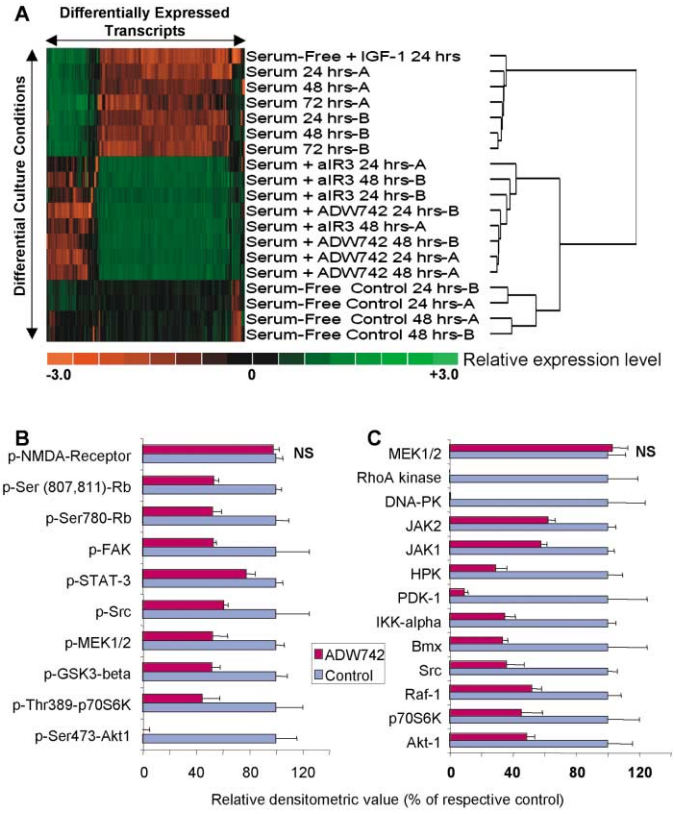

Figure 4. Molecular profiling of IGF-1R inhibition in myeloma cells

A: Unsupervised hierarchical clustering of transcriptional profiles of MM-1S cells cultured for the indicated times in the presence or absence of 10% fetal bovine serum, NVP-ADW742 0.5 μM, α-IR3 2 μg/ml, or IGF-1 200 ng/ml. In this diagram, rows represent individual samples grown under the indicated conditions, while columns represent transcripts which are differentially expressed in the various samples of this analysis. The expression of transcripts for each sample is visually represented as red for upregulated genes and green for downregulated genes, according to a logarithmic color scale (−3.0 corresponds to 3-log reduction and +3.0 to a 3-log increase in gene expression) shown at the bottom of the gene expression profile matrix. The branches of the dendrogram which appears on the right hand side of the graph indicates that, based on their gene expression profiles, the samples of this analysis can be separated in 2 main clusters, one corresponding to IGF-1- or serum-treated samples and the other representing samples cultured with IGF-1R inhibitors (NVP-ADW742 or αIR-3) or cultured in serum-free conditions.

B–C: Densitometric results of proteomic analyses of the signaling state of MM-1S cells cultured in serum-containing medium in the presence or absence of NVP-ADW742 at 0.5 μM. Results of phosphorylation state (**B**) and total levels (**C**) of signaling mediators in the setting of NVP-ADW742 treatment are represented as % of the respective controls (mean of 2 independent replicates ± SD). All differences were significant with $p < 0.05$, except where indicated (NS = not significant).

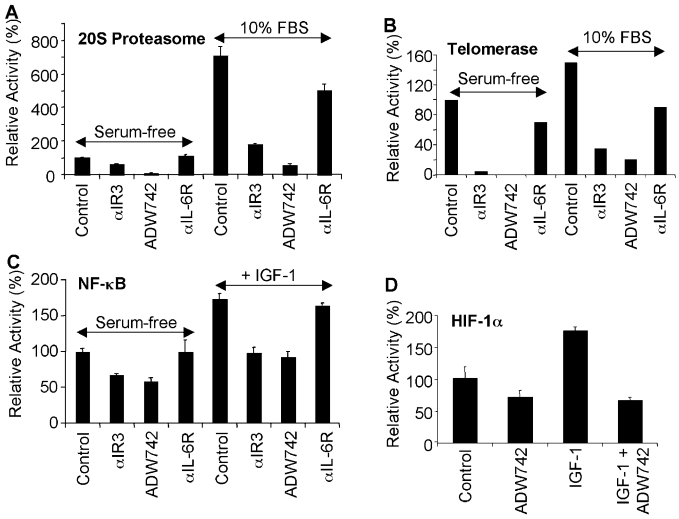

Figure 5. Effects of IGF-1R inhibition on proteasome, telomerase, NF-κB, and HIF-1α activities

MM-1S cells were grown for 24 hr in the absence (serum-free) or presence of 10% fetal bovine serum (10% FBS), IGF-1 (200 ng/ml), αIR3 (2 μg/ml), NVP-ADW742 (0.5 μM), or anti-IL-6R antibody (2 μg/ml).

20S proteasome activity (**A**), telomerase activity (**B**), NF-κB DNA binding activity (**C**), and HIF-1α DNA binding activity (**D**) were assayed and represented as mean ± SD of duplicate samples.

to a distinct cluster, separate from other clusters corresponding to profiles of MM cells treated with other potent anti-MM agents (including PS-341, hsp90 inhibitors and histone deacetylase inhibitor; Mitsiades et al., 2002a, 2002c, 2003b). This suggests that the distinct molecular sequelae of IGF-1R inhibition do not reflect a nonspecific cytotoxic or antiproliferative effect (data not shown).

Expression profiling studies, proteomic analyses, and subsequent confirmatory assays showed that tumor cell exposure to IGF-1 or serum was associated with a highly pleiotropic constellation of proliferative/antiapoptotic molecular events, which were suppressed by IGF-1R inhibition with NVP-ADW742 or α-IR3 (Figures 4–6)., The mechanistic implications of IGF-1R inhibition-induced transcriptional changes are comprehensively analyzed in a separate study (C.S.M. et al., unpublished data).

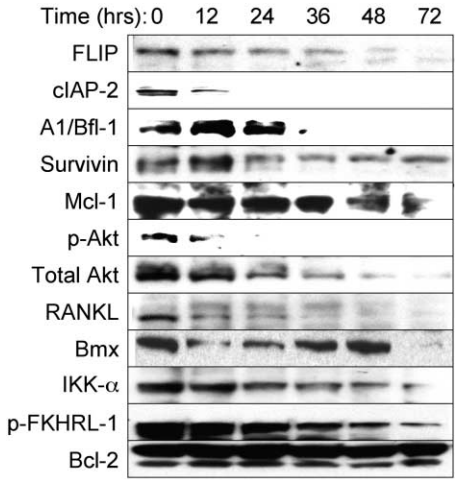

Figure 6. Effects of IGF-1R inhibition on antiapoptotic signaling cascades

Confirmatory immunoblotting analyses indicate that upon NVP-ADW742 treatment (0.5 μM) of MM-1S cells in 10% FBS-containing medium, IGF-1R inhibitor-induced cell death is preceded by pleiotropic antiproliferative/proapoptotic molecular events, such as early suppression of Akt, MEK1/2, and FKRHL-1 phosphorylation, as well as decrease in intracellular levels of Akt, Bmx, IKK-α, and the osteoclastogenic stimulator (and mediator of bone resorption in osteolytic MM lesions) RANKL. No change in Bcl-2 levels is apparent over the course of treatment.

Briefly summarized, IGF-1R inhibition led to decreased expression of genes implicated in cell cycle progression/proliferation and decreased Rb phosphorylation; decreased levels of caspase inhibitors (e.g., FLIP, XIAP, cIAP-2, survivin) and other antiapoptotic regulators (e.g., A1/Bfl-1); suppression of multiple genes involved in DNA synthesis and DNA damage repair; significant decrease in constitutive and serum-stimulation of telomerase activity; suppression of genes implicated in oncogenic transformation (e.g., c-myb, DEK) and decreased phosphorylation (and total levels) of the Aurora family of kinases; decreased expression of transcripts encoding the 26S proteasome subunits, modulation of other genes regulating proteasome function (e.g., ubiquitin-specific proteases and ubiquitin conjugating enzymes), and suppression of constitutive and serum-induced activation of proteasome activity; decreased expression of genes for nucleocytoplasmic transport and other solute carrier proteins regulating uptake of glucose and other metabolites; suppression of heat shock proteins (e.g., hsp90); decreased phosphorylation of critical kinases and kinase targets in the PI-3K/Akt pathway (including Akt, p70S6K, GSK3β, FKHRL-1), Raf/ERK1/2 pathway (MEK1/2), Src, STAT3, and FAK (focal adhesion kinase); suppression of total intracellular levels of kinases implicated in proliferative/antiapoptotic responses, including Akt, p70S6K, Raf, Src, Bmx, IKK, and PDK1 (PI-3K-dependent kinase); and suppression of transcriptional activity of NF-κB and HIF-1α, 2 downstream targets of IKK and Akt, respectively.

The highly pleiotropic constellation of proliferative/antiapoptotic pathways which are stimulated by serum and conversely inhibited by NVP-ADW742 may explain not only the potent growth/survival signal mediated by IGF-1R in this study and in previous ones (Mitsiades et al., 2002b, 2002c; Poulaki et al., 2002, 2003), but also the major impact of IGF-1R inhibitors on a wide range of tumor types in this study. Several molecular sequelae of IGF-1R inhibition elucidated by these studies are consistent with previously reported findings, e.g., those pertaining to Akt, FKHRL-1, NF-κB, and HIF-1α function or caspase inhibitor expression (Mitsiades et al., 2002b, 2002c; Poulaki et al., 2002, 2003). Other previously unidentified findings also emerged. For example, IGF-1R inhibition appears to modulate the signaling state of tumor cells not only by changes in phosphorylation of the components of PI-3K/Akt, Ras/Raf/ERK1/2, IKK/NF-κB, or other signaling cascades, but also by modulating the intracellular concentration of components of these pathways, including key kinases such as Akt, Raf, and IKK. Our findings that IGF/IGF-1R signaling activates/upregulates several signaling effectors (e.g., Akt, Raf, IKK) participating in signal transduction cascades triggered by other cytokines/growth factors and their receptors, and that the transcriptional profiles of IGF-1R inhibitor treatment of serum-cultured MM cells are comparable to the profiles in serum-free conditions (Figure 4A), highlight a critical role of IGF-1R function for the transcriptional responses of tumor cells to the entire spectrum of growth factors present in serum. These results provide putative mechanistic explanation for our prior finding that the biologic activity of IGFs/IGF-1R may be required for priming of tumor cells to respond to other cytokines, which may not be sufficient per se to trigger cell proliferation.

Several pathways abrogated by IGF-1R inhibition are critical for tumor cell resistance to proapoptotic therapies and/or constitute known targets for anticancer therapies. For example, Akt, NF-κB, and caspase inhibitors confer resistance to multiple caspase-dependent proapoptotic anticancer therapies including dexamethasone, TRAIL/Apo2L, proteasome inhibitors, and thalidomide analogs (Chauhan et al., 2001; Mitsiades et al., 2002c, 2002d, 2002e, 2002f). Furthermore, NF-κB activity and DNA damage repair genes play critical roles in tumor cell resistance to cytotoxic chemotherapy (Mitsiades et al., 2003c; Wang et al., 1999). In addition, proteasome function is a target of the recently emerging small molecule inhibitor PS-341, also known as bortezomib (Hideshima et al., 2001b; Mitsiades et al., 2002c, 2003c). Because inhibition of IGF-1R function attenuated all of these pathways associated with cytoprotection and drug resistance, we decided to evaluate whether IGF-1R inhibition can enhance the antitumor activity of existing therapeutic strategies, e.g., cytotoxic chemotherapy, dexamethasone, or proteasome inhibition. Furthermore, because the aforementioned antiapoptotic pathways (which are suppressed by IGF-1R inhibition), e.g., PI-3K/Akt, Ras/Raf/ERK1/2, IKK/NF-κB, are also important mediators of the protective effects of bone marrow stromal elements on MM and other tumor cells from osteotropic neoplasias (Hideshima and Anderson, 2002; Mitsiades and Koutsilieris, 2001), we also decided to characterize the ability of IGF-1R inhibitors to abrogate these tumor-stromal interactions.

IGF-1R inhibition sensitizes tumor cells to other anticancer agents

In vitro, we found that NVP-ADW742 or α-IR3 could increase the sensitivity of MM cells to various cytotoxic chemotherapeutic agents (doxorubicin, melphalan), dexamethasone, TRAIL/Apo2L, or PS-341 (Figures 7A–7C and Supplemental Figure S4 at http://www.cancercell.org/cgi/content/full/5/3/221/DC1), the sensitivity of prostate cancer cells to doxorubicin, or the response of SK-N-MC sarcoma cells to Fas ligation (data not shown). These data indicated a broader role of IGFs/IGF-1R signaling in attenuating anticancer drug responsiveness in multiple neoplasias and the potential usefulness of IGF-1R inhibitors in enhancing the antitumor activity of a broad spectrum of anticancer therapeutic strategies.

Furthermore, the suppression of proteasome function by IGF-1R inhibition provides first evidence of growth factor-mediated regulation of proteasome function. This finding offers a mechanistic explanation for the demonstrated synergy between IGF-1R inhibition and PS-341 (Figure 7B), is consistent with our previous observation that IGF-1-induced signaling attenuates MM cell sensitivity to PS-341 (Mitsiades et al., 2002c), and bears major implications for the potential clinical applications of IGF-1R inhibitors to enhance the antitumor activity of proteasome inhibitors.

In vivo chemosensitizing effect of IGF-1R inhibition with NVP-ADW742

Based on the in vitro chemosensitizing effect of NVP-ADW742 on tumor cells, we evaluated the in vivo activity of a pulse of NVP-ADW742 (10 mg/kg i.p. twice daily, 4 days per week) administered after low dose melphalan (2.5 mg i.p once a week). In contrast to continuous administration of NVP-ADW742, pulsatile administration of NVP-ADW742 had no significant antitumor activity (Figure 7D, and data not shown). However, consistent with the in vitro data, the combination of subtherapeutic melphalan followed by subtherapeutic NVP-ADW742 had synergistic effect in reducing tumor burden measured by in vivo imaging (data not shown) as well as prolonging the survival of

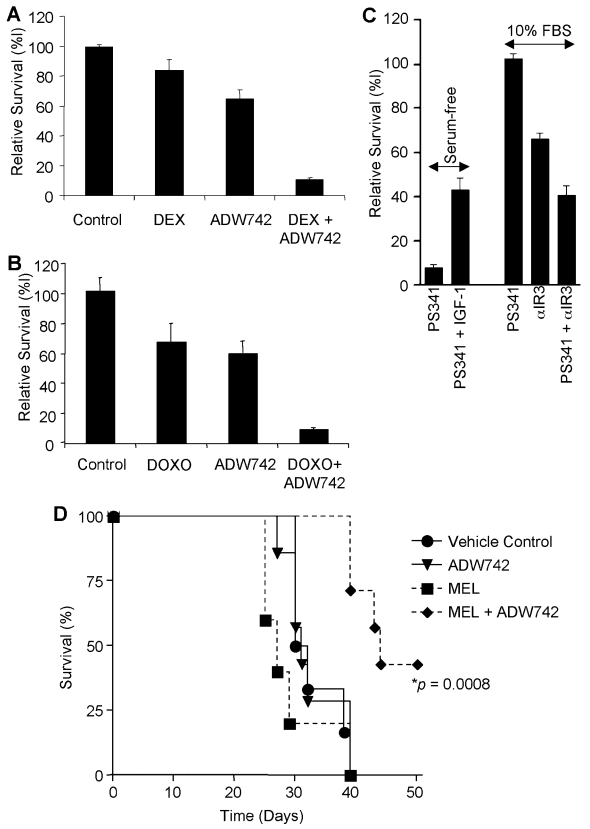

Figure 7. IGF-1R inhibition sensitizes tumor cells to other anticancer therapies in vitro and in vivo

The in vitro anti-MM activities of dexamethasone (Dex 0.1 μM, 72 hr, **A**) and doxorubicin (Doxo, 50 ng/ml, 48 hr, **B**) are enhanced by NVP-ADW742 treatment (0.75 μM for the final 24 hr of incubation) in MM-1S cells.
C: IGF-1R inhibition (by α-IR3 2 μg/ml) enhances the activity of PS-341 (2 nM, overnight incubation) against MM-1S cells. In all cases, cells were cultured in the presence of 10% FBS, and results are expressed as % of the viability of cells in the respective controls (cultures in serum-free conditions or supplemented with 10% serum, respectively) and correspond to the mean ± SD of 3 independent experiments.
D: In vivo combination therapy with NVP-ADW742. Two weeks after i.v. injection with MM-1S-Luc$^+$ cells, a group of NOD-SCID mice with established diffuse MM lesions was divided into 4 cohorts, with statistically equivalent tumor burden between cohorts (as assessed by bioluminescence imaging). Cohorts were treated with melphalan alone (2.5 mg/kg i.p. once a week), NVP-ADW742 alone (10 mg/kg i.p. twice daily, four days per week), melphalan followed 18 hr later by NVP-ADW742, or vehicle control. NVP-ADW742 enhanced the in vivo antitumor effect of melphalan, as shown by the prolongation of overall survival of mice receiving the combination of the 2 agents (log-rank test, p = 0.0008; n = 5 per treatment group).

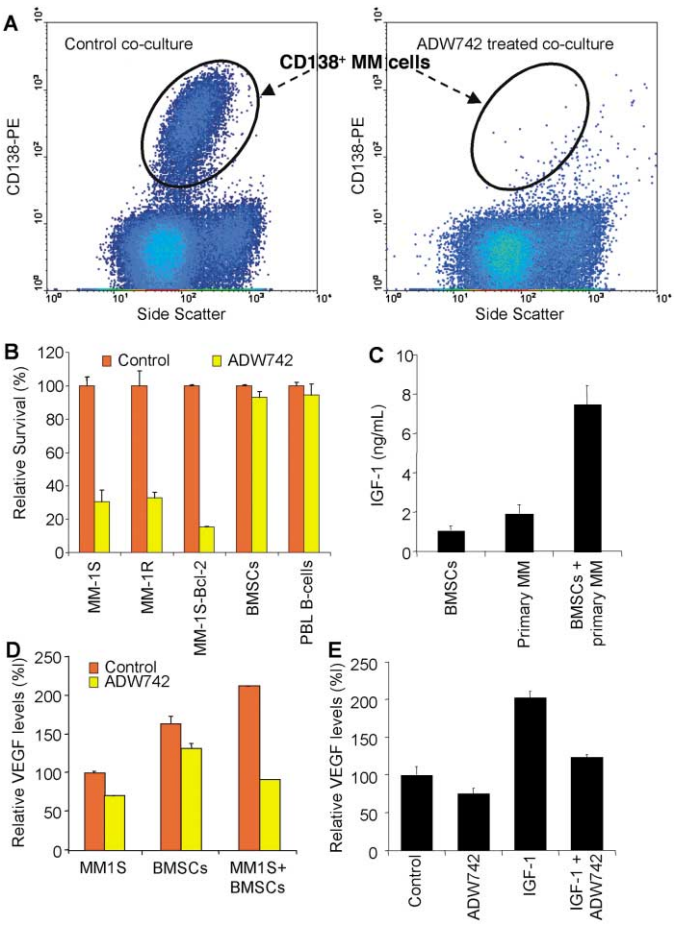

Figure 8. IGF-1R inhibition overcomes the protective effects conferred to MM tumor cells by their local bone microenvironment

A: Unpurified bone marrow (BM) mononuclear cells from BM aspirate of MM patient (which contain CD138$^+$ MM cells and CD138$^-$ normal cells of the bone microenvironment, such as stromal cells) were cultured in the presence of 20% serum for 72 hr. NVP-ADW742 treatment (0.5 μM) led to specific reduction in the percentage of the CD138$^+$ population of malignant cells, but not BM stromal cells.
B: NVP-ADW742 (0.5 μM for 72 hr) does not significantly affect the viability of BM stromal cells (BMSCs) or normal peripheral blood B cells (PBL B cells), in comparison to MM cell lines (MM-1S, MM-1R, MM-1S-Bcl-2).
C: Coculture of primary MM cells with BMSCs for 24 hr triggers an increase in IGF-1 levels in the coculture supernatant.
D: IGF-1R inhibition by NVP-ADW742 (0.5 μM for 24 hr) suppresses the constitutive and coculture-induced secretion of VEGF by myeloma cells and BMSCs.
E: NVP-ADW742 (0.75 μM) suppresses the IGF-1 (200 ng/ml in serum-free medium)-induced secretion of VEGF by the SW579 papillary thyroid carcinoma cells. All data are presented as the mean ± SD of triplicate samples.

mice (Figure 7D). Taken together, these results demonstrate that abrogation of IGF-1/IGF-1R signaling after cytotoxic therapy may enhance the efficacy of conventional therapies by removing key antiapoptotic signals.

Impact of IGF-1R inhibition on interactions of tumor cells with the bone marrow microenvironment and VEGF production

The in vivo antitumor effects of IGF-1R inhibition may reflect both tumor cell-autonomous and -nonautonomous effects. The importance of stromal elements in supporting tumor growth is becoming increasingly clear, particularly in osteotropic malignancies, such as MM, where stromal elements of the BM microenvironment confer protective effects to tumor cells against various antitumor therapies (Hideshima and Anderson, 2002; Mitsiades and Koutsilieris, 2001). This protective effect may be particularly relevant for strategies targeting IGF-1R, since IGFs are locally produced in the BM milieu by BM stromal cells (BMSCs) and osteoblasts, and because coculture of MM cells with BMSCs significantly enhances production of IGF-1 in vitro (Figure 8C). However, in coculture assays of MM cells and BMSCs, their interaction did not overcome the antitumor effects of NVP-ADW742 on MM cells (Figure 8A), even at concentrations

which did not affect the viability of BMSCs (Figure 8B). Furthermore, consistent with its ability to suppress IGF-1-induced HIF-1α activity, NVP-ADW742 decreased the production of VEGF by tumor cells and bone marrow stromal cells (Figure 8D), and suppressed the IGF-1-induced secretion of VEGF by various tumor types such as thyroid cancer cells (Figure 8E) or MM cells (data not shown), suggesting putative antiangiogenic effects of IGF-1R inhibition. These results not only suggest that IGF/IGF-1R signaling plays important roles in the interactions of tumor cells with their local microenvironment, including stromal protection of tumor cells or tumor-associated angiogenesis, but also propose an additional mechanistic rationale for use of IGF-1R kinase inhibitors in order to maximize the in vivo efficacy of other antitumor therapies.

Discussion

Prospective epidemiological studies have indicated that high IGF-1 levels confer increased risk for development of diverse solid tumor types (Chan et al., 1998; Hankinson et al., 1998), and prior studies have suggested a role for IGF-1R activation in malignant transformation of certain tumor types (reviewed in LeRoith and Roberts, 2003). However, less emphasis has been placed on understanding how IGFs/IGF-1R influence the clinical response of tumor cells to anticancer therapies, and only limited focus has been placed on targeting the IGFs/IGF-1R pathway in the clinical setting (Koutsilieris et al., 2002). This study highlights the potential utility of therapeutic targeting of IGF-1R function in cancer therapy. We demonstrate that IGFs in serum constitute critical promoters of tumor cell proliferation, survival, and drug resistance in multiple neoplasias, and inhibition of IGF-1R significantly suppresses these effects. Global transcriptional and proteomic profiling offers insight into the molecular sequelae of IGF-1R activation and inhibition, including modulation of a pleiotropic constellation of proliferative and antiapoptotic pathways. Finally, demonstration of in vivo efficacy of systemic administration of a small molecule IGF-1R kinase inhibitor establishes proof of principle that selective IGF-1R inhibition can achieve in vivo antitumor activity with a favorable therapeutic window.

Focusing on MM, the second most commonly diagnosed hematologic malignancy, as a disease critically dependent on IGF-1R function, we demonstrated significant in vitro and in vivo antitumor activity of the selective IGF-1R tyrosine kinase inhibitor NVP-ADW742 (both as a single agent, as well as in combination with cytotoxic chemotherapy), without significant treatment-related toxicity. NVP-ADW742 recapitulated the biologic effects of anti-IGF-1R-specific neutralizing antibody or inhibitory peptide, induced pleiotropic antiproliferative/proapoptotic biologic sequelae in tumor cells, and was active even against primary tumor cells from patients with multi-drug-resistant disease or tumor cell lines with known resistance to various conventional or investigational anticancer drugs. IGF-1R inhibition attenuates tumor cell responsiveness to other cytokines/growth factors, selectively increases tumor cell sensitivity to other (conventional or novel) anticancer therapies, overcomes the protective effect of bone marrow stromal cells, and abrogates the production of proangiogenic cytokines such as VEGF by tumor and stromal cells.

The major clinical responses achieved by imatinib mesylate (STI-571, Gleevec) against neoplasias critically dependent on bcr-abl, c-kit, or PDGF receptor illustrated the concept of therapeutic intervention with small-molecule kinase inhibitors which neutralize single molecular targets that are mutationally activated, uniquely expressed, or overexpressed in tumor versus normal cells (Druker et al., 2001; Druker et al., 1996). Our current study presents a conceptually different therapeutic approach, because it focuses on a target which is expressed in a wide range of neoplasias, but is not a pathognomonic neoplastic alteration since it is also expressed in many, if not most, normal tissues (LeRoith et al., 1995). However, our study shows that IGF-1R inhibition not only has potent antitumor activity in vitro, but also a favorable in vivo therapeutic window. These results suggest that IGF-1R function is critically required for tumor cell survival, but dispensable for survival of normal cells in adult animals. This highlights the notion that development of new anticancer therapeutic strategies, which traditionally focuses on molecules with significant differential expression in tumor versus normal cells, should also address molecular targets present in both cell types, but with differential functional importance for tumor cell survival and proliferation. Selective targeting of such nonpathognomonic pathways may also be associated with a favorable therapeutic window. Furthermore, our finding that inhibition of IGF-1R modulates responsiveness to other growth factors highlights a potential therapeutic application of IGF-1R kinase inhibitors to enhance response to therapeutic strategies targeting other growth factor/receptor signaling systems in tumor cells.

Because of the highly pleiotropic nature of the molecular sequelae of IGF-1R and, conversely, its inhibition, the delineation of which one(s) of these effects is more important for the biologic activity of IGF-1R or for the antitumor effect of its inhibitors is difficult, particularly because many of the downstream effectors of IGF-1R or its inhibition constitute per se targets sufficient to trigger tumor cell death in vitro and in vivo, including the ubiquitin/proteasome pathway (as evidenced by the clinical activity of proteasome inhibitors; Richardson et al., 2003) and the PI-3K/Akt, Raf/ERK1/2, and IKK/NF-κB pathways (Hideshima et al., 2001a, 2002; Mitsiades et al., 2002b, 2002c, 2002e, 2003c; Wang et al., 1999). In fact, the pleiotropic molecular effects of IGF-1R inhibition can explain not only its significant in vitro and in vivo antitumor activity, but also its ability to target such a wide range of tumor types from diverse lineages and significant differences in their molecular profiles. This wide spectrum of activity of IGF-1R inhibitors and, importantly, their equally pleiotropic effects in selectively sensitizing tumor cells to a wide range of anticancer agents, not only highlight the major role of IGF/IGF-1R signaling for human malignant cells, but also provide the framework for future clinical trials of specific tyrosine kinase inhibitors of IGF-1R to improve the outcome of patients with IGF-responsive malignancies.

Experimental procedures

Cell lines and primary tumor specimens

Cell lines were obtained from the ATCC (Manassas, VA) or were kindly provided by sources indicated in the Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/5/3/221/DC1. Primary MM patient samples were freshly isolated from bone marrow aspirates from patients undergoing diagnostic or restaging bone marrow aspirates under an IRB-approved protocol.

Transfections and retroviral transductions
Stable transfections of MM-1S cells with vectors encoding myristoylated (constitutively active) Akt or Bcl-2 (Upstate Biotechnologies, Lake Placid, NY), or with empty (neo) vectors, were performed using Lipofectamine 2000 (Life Technologies) and G418-containing selection media, as previously described (Mitsiades et al., 2002c). Retroviral transduction of MM-1S and MM-1R cells with a pGC-*gfp/luc* vector (kind gift of C.G. Fathman, Stanford University) or pMMP-LucNeo was performed as previously described (Armstrong et al., 2003).

Ex vivo drug sensitivity assays
Cell survival of IGF-1R inhibitor-treated cells was examined using the 3- (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) colorimetric assay, as previously described (Mitsiades et al., 2001).

In vivo antitumor activity of IGF-1R inhibition
The in vivo anti-MM activity of NVP-ADW742 was evaluated in a model of diffuse skeletal lesions of luciferase-expressing MM cells in SCID/NOD mice, serially monitored with whole-body bioluminescence imaging, as previously described (Armstrong et al., 2003). Briefly, 6- to 8-week-old male SCID/NOD mice (Jackson Laboratories, Bar Harbor, ME) were sublethally irradiated (300 rads) using ^{137}Cs γ irradiator source. After 3–6 hr, 5×10^6 MM-1S-luc$^+$ cells in 100 μl of phosphate-buffered saline (PBS) was injected via the tail vein into each mouse. Mice were monitored daily for changes in their body weight, signs of infection, and paralysis, and weekly by whole-body bioluminescence imaging utilizing the In Vivo Imaging System (IVIS, Xenogen Corp, Alameda, CA) with total imaging time of 2 min, bin 2. Total body bioluminescence was quantified by integrating the photonic flux (photons/sec) through a standardized region of interest encompassing each mouse (Living Images, Xenogen). NVP-ADW742 (Novartis Pharma AG, Basel, Switzerland) was formulated in 25 mM tartaric acid and administered by i.p. injection or by oral gavage. Melphalan (Alkeran, GlaxoSmithKline, Research Triangle Park, NC) was formulated according to manufacturer's instructions, and was administered by i.p. injection. All animal studies were approved by the Dana-Farber Cancer Institute Animal Care and Use Committee.

Flow cytometry, immunoblotting, and functional assays
Flow cytometric analyses for IGF-1R and IGF-2R were performed as previously described (Mitsiades et al., 2001, 2002e, 2002f) on a EPICS-XL-MCL flow cytometer (Beckman Coulter). Previously described protocols were applied for immunoblotting analyses (Mitsiades et al., 2001, 2002c); TRAP telomerase activity assay (Akiyama et al., 2002; Mitsiades et al., 2004); 20S proteasome chymotryptic activity assay (Shringarpure et al., 2003); and DNA binding activity ELISAs for quantitative assessments of NF-κB and HIF-1a transcriptional activities (Mitsiades et al., 2002c, 2002e, 2002f, 2003c). VEGF and IGF-1 ELISAs were performed according to manufacturer's instructions (R&D Systems, Minneapolis, MN)

Molecular profiling of IGF-1R inhibition
The gene expression profile of MM cells treated with IGF-1R inhibitors (NVP-ADW742, α-IR3), in the presence or absence of serum, was analyzed using U133A olignucleotide microarrays (Affymetrix Inc, Santa Clara, CA), using previously described protocols for total RNA extraction and purification, synthesis of cDNA and biotinylated cRNA, hybridization with human U133A Affymetrix chips, and scanning in HP ChipScanner to detect hybridization signals (Mitsiades et al., 2002c, 2003c). Subsequent analysis of scanned image output files was performed with Affymetrix GeneChip Microarray Analysis Suite 5.0 software (Affymetrix), normalized, and analyzed by hierarchical clustering, functional clustering, and relevance network algorithms, as in previous studies (Mitsiades et al., 2002c, 2003c). High-throughput global proteomic analysis of the signaling state of IGF-1R-inhibitor-treated MM cells was performed by multiplex immunoblotting, as previously described (Mitsiades et al., 2003a, 2003c; Zhang et al., 2002).

Statistical analysis
Statistical significance for in vitro assay results was examined by 2-way analysis of variance, followed by Duncan's post-hoc test. Statistical significance for bioluminescence imaging studies was determined by two-tailed Student's t test. In all analyses, $p < 0.05$ was considered statistically significant. For assessment of in vivo antitumor activity, the overall survival of mice was defined as the time between i.v. injection of tumor cells and sacrifice, or death and was compared across different treatment groups with Kaplan-Meier survival analysis (Kaplan and Meier, 1958).

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