Inhibition of Insulin-like Growth Factor I Receptor Increases the Antitumor Activity of Doxorubicin and Vincristine against Ewing's Sarcoma Cells[1]

Stefania Benini, Maria Cristina Manara, Nicola Baldini, Vanessa Cerisano, Massimo Serra, Mario Mercuri, Pier-Luigi Lollini, Patrizia Nanni, Piero Picci, and Katia Scotlandi[2]

Laboratorio di Ricerca Oncologica, Istituti Ortopedici Rizzoli, 40136 Bologna, Italy [S. B., M. C. M., N. B., V. C., M. S., P. P., K. S.]; and Dipartimento di Scienze Anatomiche Umane, Sezione di Fisiopatologia dell'Apparato Locomotore, ex Clinica Ortopedica [M. M.] and Dipartimento di Patologia Sperimentale, Sezione de Cancerologia [P-L. L., P. N.], Università degli Studi di Bologna, 40126 Bologna, Italy

ABSTRACT

Innovative treatment modalities are needed for Ewing's sarcoma (ES), a neoplasm with a disappointingly low survival rate despite the use of aggressive multimodal therapeutic approaches. We and others (D. Yee *et al.*, J. Clin. Investig., *86:* 1806–1814, 1990; K. Scotlandi *et al.*, Cancer Res., *56:* 4570–4574, 1996) have previously shown the existence and the pathogenetic relevance of an autocrine loop, mediated by the insulin-like growth factor-I receptor (IGF-IR), which is crucial for survival and proliferation of ES cells *in vitro*. Moreover, we reported that the IGF-IR-blocking monoclonal antibody (MAb), αIR3, as well as suramin, a drug that can interfere with growth factor by binding to the receptors, inhibited both the tumorigenic and the metastatic ability of ES cells in athymic mice. In this study, we analyzed whether agents that can block the IGF-IR-mediated loop are of value in association with conventional cytotoxic drugs for the design of more effective therapeutic regimens. Both αIR3 MAb and suramin treatment significantly increased the antitumor *in vitro* effects of doxorubicin and vincristine, two drugs with a leader action on ES. These findings were obtained by both simultaneous and sequential treatments. Analysis of the proliferation rate and of apoptosis revealed that αIR3 MAb and suramin significantly enhanced the G_1-phase rate induced by doxorubicin, without substantially affecting doxorubicin-G_2-M-blockage of cell cycle, and significantly increased the induction of apoptosis, which confirmed that the specific blockage of IGF-IR deprives ES cells of an important tool for the prevention of drug-induced apoptosis. Moreover, combination treatments of doxorubicin plus αIR3 MAb significantly increase the doxorubicin-induced impairment of the ability of ES cells to form colonies in soft agar. In conclusion, we showed that, in ES, the blockage of IGF-IR by a neutralizing MAb or by suramin may greatly potentiate the antitumor activity of conventional chemotherapeutic drugs.

INTRODUCTION

ES,[3] which includes conventional ES, Askin's tumor, and PNET, ranks second in frequency among primary bone tumors. It is an extremely aggressive, poorly differentiated neoplasm of uncertain histogenesis, usually arising in children and young adults. Combination chemotherapy associated with local control with surgery or radiation therapy have become standard practice in the treatment of patients with ES (1–3). However, despite the use of multimodal treatments and of very aggressive chemotherapeutic regimens, the long-term disease free survival of ES patients is still disappointingly low, particularly in high-risk groups (2, 4, 5). The identification of valuable new therapeutic targets for the design of innovative, more effective strategies is, therefore, urgently needed. In recent years, by analyzing the role of growth factors in the pathogenesis of ES, we and others have reported on the autocrine production of IGF-I and the constant presence of its corresponding receptor, the IGF-IR, in ES cells (6, 7). IGF-IR, a membrane-bound heterotetramer receptor with intrinsic tyrosine kinase activity, is emerging as an important factor in the regulation of cell growth in different tumor types, both *in vitro* and *in vivo*. In particular, IGF-IR, activated by its ligands, appears to regulate cell growth in at least three different ways: it is mitogenic; it is mandatory for the establishment and the maintenance of the transformed phenotype; and it protects cells from apoptosis. Targeting IGF-IR by using different approaches, including antisense technologies (8–10), antibodies against IGF-IR (11, 12), and dominant negative mutants of IGF-IR (13–15), resulted in the reversal of the transformed phenotype in several types of tumor cells. In ES, the blockage of IGF-IR-mediated circuit by αIR3 MAb, which specifically neutralizes IGF-IR greatly inhibits the growth and the migration ability of ES cells *in vitro* (6), as well as their tumorigenic and

Received 11/1/00; revised 2/21/01; accepted 3/22/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by grants from the Italian Association for Cancer Research, the Italian Ministry for University and Research, and the Rizzoli Institute. V. C. is the recipient of a Fellowship from the Italian Fondation for Cancer Research.
[2] To whom requests for reprints should be addressed, at Laboratorio di Ricerca Oncologica, Istituti Ortopedici Rizzoli, Via di Barbiano, 1/10, 40136 Bologna, Italy. Fax: 39-051-6366761; E-mail: katia.scotlandi@ior.it.

[3] The abbreviations used are: ES, Ewing's sarcoma; PNET, peripheral neuroectodermal tumor; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; IMDM, Iscove's modified Dulbecco's medium; MAb, monoclonal antibody; BrdUrd, bromodeoxyuridine.

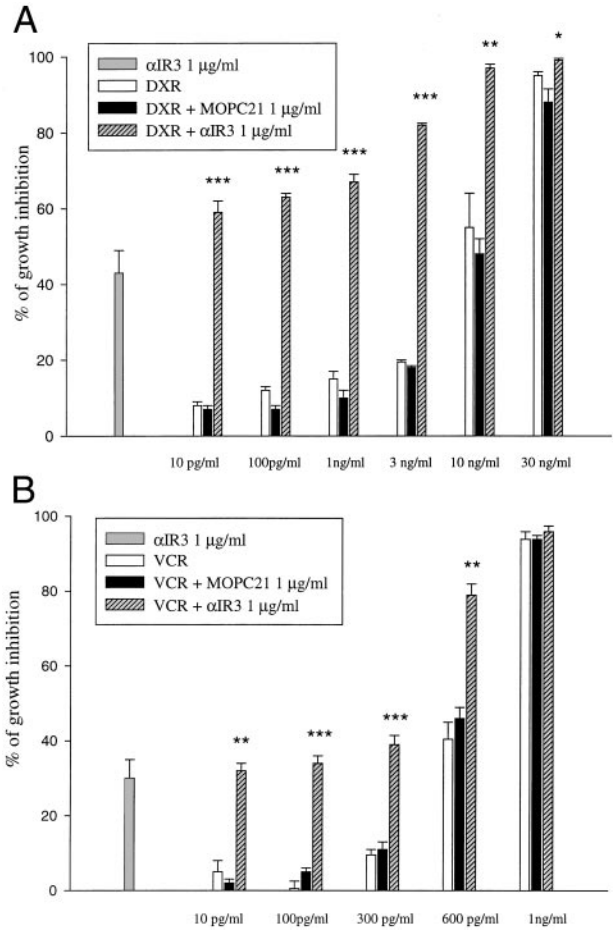

Fig. 1 Inhibitory effects of doxorubicin (*DXR*; *A*) or vincristine (*VCR*; *B*) in combination with the anti-IGF-IR αIR3 MAb on TC-71 cells after simultaneous and continuous treatments. TC-71 cells were treated with DXR or VCR at the indicated concentrations alone or associated with αIR3 MAb or control MOPC-21 MAb on the 1st day after cell seeding for a total of 96 h. Results represent the mean ± SE of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t* test, compared with the corresponding dose of DXR or VCR.

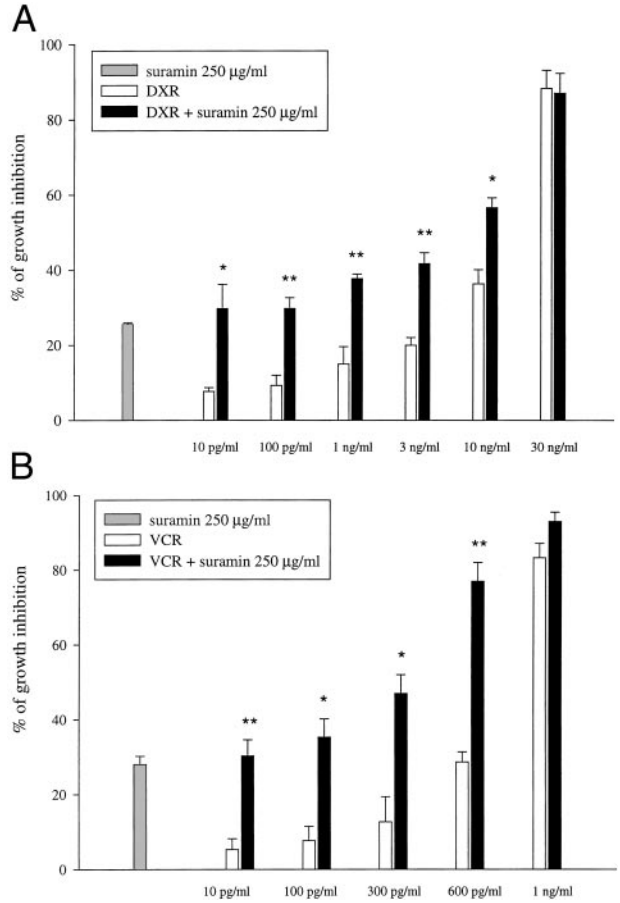

Fig. 2 Inihibitory effects of doxorubicin (*DXR*; *A*) or vincristine (*VCR*; *B*) in combination with suramin on TC-71 cells after simultaneous and continuous treatments. TC-71 cells were treated with DXR or VCR at indicated concentrations alone or associated with suramin on the 1st day after cell seeding for a total of 96 h. Results represent the mean ± SE of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$; Student's *t* test, compared with the corresponding dose of DXR or VCR.

metastatic ability *in vivo* (16). A significant inhibition of ES growth has also been successfully achieved *in vivo* by using suramin, a nonspecific growth factor antagonist that inhibits a number of autocrine circuits, including the IGF-IR-mediated loop (17). Therefore, impairment of IGF-IR appears to be a valuable therapeutic approach against ES. Moreover, Toretsky *et al.* recently reported that ES cells use an IGF-IR initiated signaling pathway through phosphoinositide 3-OH kinase and Akt for survival when treated with doxorubicin (18), indicating in IGF-IR a possible mechanism of ES resistance to chemotherapy. In this study, we investigated whether impairment of IGF-IR by αIR3 neutralizing MAb and/or suramin may be advantageously combined with conventional cytotoxic drugs for the design of more effective therapeutic regimens. Although suramin does not exert a specific action against IGF-IR, in ES/PNET its effects substantially overlap those obtained with the specific neutralizing antibody anti-IGF-IR. This is probably attributable to the peculiar and unique presence, in this neoplasm, of a autocrine IGF-IR-mediated loop (7). Suramin ability to specifically block IGF-IR function in ES/PNET was demonstrated previously (16).

MATERIALS AND METHODS

Cell Lines. The ES cell line TC-71 was a generous gift from T. J. Triche (Childrens Hospital, Los Angeles, CA). The Askin's tumor cell line SK-N-MC was obtained from the American Type Culture Collection (Rockville, MD). The PNET cell line IOR/LAP35 was previously established and characterized at the Istituti Ortopedici Rizzoli. Cells were routinely cultured in IMDM (Life Technologies, Inc., Paisley, Scotland), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% inactivated FCS (Biological Industries, Kibbutz Beth Haemek, Israel). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cell Culture and Growth Assay. Cells (200,000) of TC-71 and SK-N-MC or 500,000 cells of IOR/LAP-35 were

Table 1 Sensitivity of ES/PNET cells to chemotherapeutic agents after combined simultaneous treatments

ES/PNET cells were treated with progressive doses of doxorubicin (DXR) or vincristine (VCR) either alone or associated with αIR3 MAb (1 μg/ml), control MOPC-21 MAb (1 μg/ml) or suramin (250 μg/ml for TC-71 and LAP-35 cells; 100 μg/ml for SK-N-MC). Results represent the mean ± SE of triplicate experiments.

Cell lines	Treatment	IC_{50} values[a] (ng/ml)	Fold-increase in DXR and VCR sensitivity
TC-71	DXR	7.9 ± 0.65	
	DXR+MOPC-21 MAb	11.5 ± 0.45	
	DXR+αIR3 MAb	0.094 ± 0.012[b]	84
	DXR+suramin	0.721 ± 0.055[b]	11
	VCR	0.673 ± 0.077	
	VCR+MOPC-21 MAb	0.513 ± 0.053	
	VCR+αIR3 MAb	0.097 ± 0.008[c]	7
	VCR+suramin	0.101 ± 0.012[c]	7
SK-N-MC	DXR	4.5 ± 0.5	
	DXR+αIR3 MAb	0.26 ± 0.02[b]	17
	DXR+suramin	0.93 ± 0.04[c]	5
	VCR	0.133 ± 0.02	
	VCR+αIR3 MAb	0.052 ± 0.01[d]	3
	VCR+suramin	0.048 ± 0.005[d]	3
LAP-35	DXR	3.55 ± 0.15	
	DXR+αIR3 MAb	0.062 ± 0.01[b]	57
	DXR+suramin	0.27 ± 0.002[b]	13
	VCR	1.19 ± 0.21	
	VCR+αIR3 MAb	0.009 ± 0.002[c]	132
	VCR+suramin	0.27 ± 0.09[d]	4

[a] Drug concentration resulting in 50% inhibition of cell growth.
[b] $P < 0.001$; Student's *t* test.
[c] $P < 0.01$; Student's *t* test.
[d] $P < 0.05$; Student's *t* test.

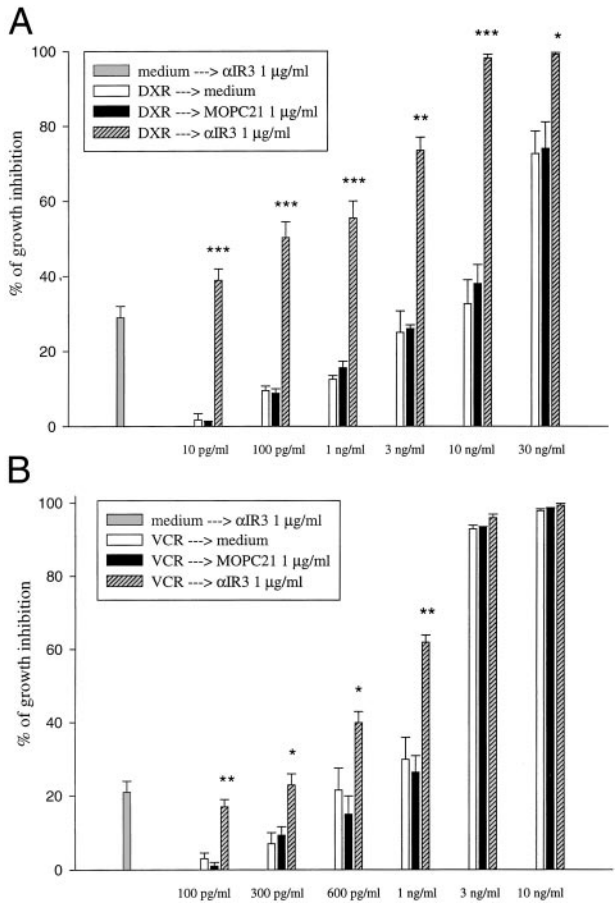

Fig. 3 Additive cytotoxicity of doxorubicin (*DXR*; *A*) or vincristine (*VCR*; *B*) in combination with anti-IGF-IR αIR3 MAb on TC-71 cells after sequential treatments. TC-71 cells were treated with DXR or VCR at indicated concentrations on the 1st day after cell seeding for a total of 12 h. Cells were then continuously exposed to αIR3 MAb or control MOPC-21 MAb for another 96 h. Results represent the mean ± SE of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t* test, compared with the corresponding dose of DXR or VCR.

seeded in 6-well plates in IMDM plus 10% FCS. After 24 h, cells were treated with varying concentrations of doxorubicin (range 10 pg/ml–100 ng/ml) or vincristine (range 10 pg/ml–1ng/ml) without (control) or with the blocking MAb αIR3 (1 μg/ml; Calbiochem-Novabiochem Co., San Diego, CA) or suramin (250 μg/ml; kindly provided by Bayer AG, Leverkusen, Germany; 100 μg/ml was used for the SK-N-MC cell line because these cells are particularly sensitive to suramin). As an additional control, the isotype-matched control MAb MOPC-21 (1 μg/ml; Sigma Chemical Co., St. Louis, MO) was also used. After 96 h of treatment, cell growth was evaluated on harvested cultures by trypan blue vital cell count. For sequential treatments, cells were distributed into 6-well plates, and treatment started the following day. Doxorubicin or vincristine were added to appropriate wells, at the concentrations indicated in the figure legends. After 12–24 h, depending on the doubling time of the cell line, the drugs were removed by washing the cells twice, followed by the addition of cell culture medium plus αIR3 MAb (1 μg/ml) or suramin (250 μg/ml; 100 μg/ml was used for the SK-N-MC cell line, because these cells are particularly sensitive to suramin). After 96 h of continuous presence of αIR3 MAb or suramin, cell growth was evaluated on harvested cells by trypan blue vital cell count.

BrdUrd Labeling Index. Five thousand to 10,000 TC-71 cells/cm^2 were seeded in IMDM plus 10% FCS. After 24 h, cells were treated with varying concentrations of doxorubicin (range, 13–100 ng/ml) either without (control) or with αIR3 MAb (1 μg/ml), or suramin (250 μg/ml), respectively. After 36 h from seeding, cell cultures were incubated with 10 μM BrdUrd (Sigma Chemical Co.) for 1 h in a CO_2 atmosphere at 37°C. Harvested cells were fixed in 70% ethanol for 30 min. After DNA denaturation with 2 N HCl for 30 min at room temperature, cells were washed with 0.1 M $Na_2B_4O_7$ (pH 8.5). Cells (10^6) were then processed for indirect immunofluorescence staining, using α-BrdUrd (Euro-Diagnostics, Milan, Italy) diluted 1:4 as a primary MAb, and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Milan, Italy). For the cell cycle analysis, 70% ethanol-fixed cells were pretreated with 100 μg/ml RNase for 30 min at 37°C and stained with 20 μg/ml propidium iodide before flow cytometric analysis.

Morphological Assessment of Apoptotic Nuclei. TC-71 Cells were seeded and treated with doxorubicin with or without αIR3 MAb as reported for BrdUrd labeling index. After a 24–96 h of *in vitro* treatment, cells were fixed in methanol/acetic acid (3:1) for 15 min, and stained with 50 ng/ml HOECHST

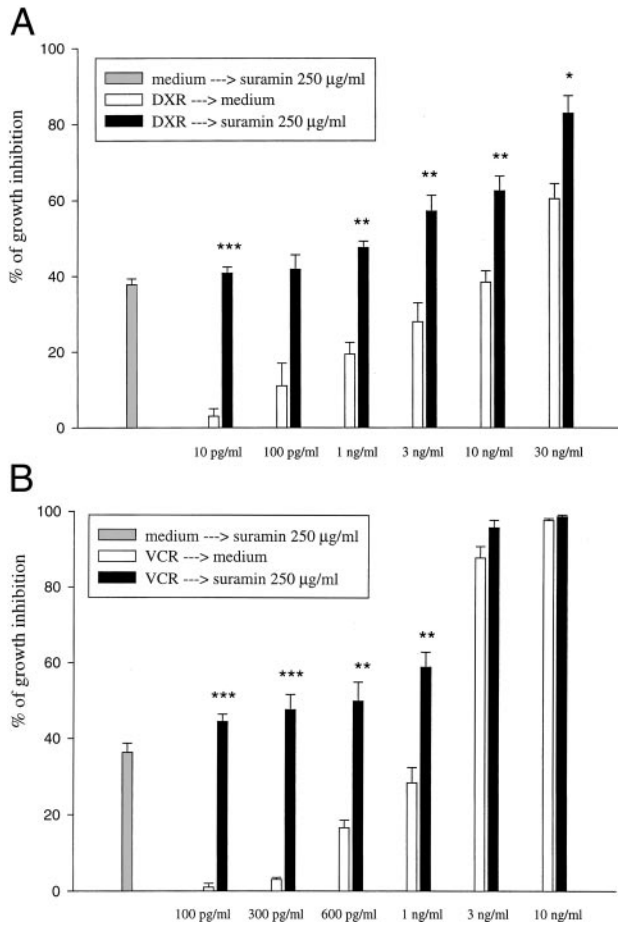

Fig. 4 Additive cytotoxicity of doxorubicin (*DXR*; *A*) or vincristine (*VCR*; *B*) in combination with suramin on TC-71 cells after sequential treatments. TC-71 cells were treated with DXR or VCR at indicated concentrations on the 1st day after cell seeding for a total of 12 h. Cells were then continuously exposed to suramin for another 96 h. Results represent the mean ± SE of triplicate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's *t* test, compared with the corresponding dose of DXR or VCR.

33258 (Sigma Chemical Co.). Cells with three or more chromatin fragments were considered as apoptotic. The percentage of apoptotic nuclei was evaluated based on 1000–2000 nuclei.

Soft-Agar Assay. Anchorage-independent growth was determined in 0.33% agarose (SeaPlaque; FMC BioProducts, Rockland, ME) with a 0.5% agarose underlay. TC-71 cell suspensions (1000–3300 cells/dish) were plated in a semisolid medium (IMDM plus 10% FCS containing 0.33% agarose) with or without varying concentrations of doxorubicin (range 3–100 ng/ml) or vincristine (300 pg/ml–1 ng/ml) plus anti-IGF-IR αIR3 MAb (1 μg/ml). The MAb MOPC-21 (1 μg/ml) was also used as an additional control. Dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2, and colonies were counted after 7 days.

Statistical Analysis. Differences among means were analyzed using a two-sided Student's *t* test. The analysis of drug combination effects was performed by using the fractional product method.

Table 2 Sensitivity of ES/PNET cells to chemotherapeutic agents after combined sequential treatments

ES/PNET cells were treated with progressive doses of doxorubicin (DXR) or vincristine (VCR) for 12–24 h and then were exposed to αIR3 MAb (1 μg/ml), or control MOPC-21 MAb (1 μg/ml), or suramin (250 μg/ml for TC-71 and LAP-35 cells; 100 μg/ml for SK-N-MC cells). Results represent the mean ± SE of triplicate experiments.

Cell lines	Treatment	IC_{50} values[a] (ng/ml)	Fold-increase in DXR and VCR sensitivity
TC-71	DXR→medium	21.2 ± 1.25	
	DXR→MOPC-21 MAb	33.8 ± 2.52	
	DXR→αIR3 MAb	0.016 ± 0.002[b]	1325
	DXR→suramin	0.301 ± 0.056[b]	70
	VCR→medium	1.38 ± 0.095	
	VCR→MOPC-21 MAb	1.17 ± 0.042	
	VCR→αIR3 MAb	0.34 ± 0.025[b]	4
	VCR→suramin	0.15 ± 0.02[b]	8
SK-N-MC	DXR→medium	5.9 ± 1.5	
	DXR→αIR3 MAb	0.843 ± 0.04[c]	7
	DXR→suramin	0.443 ± 0.02[c]	13
	VCR→medium	0.466 ± 0.09	
	VCR+αIR3 MAb	0.072 ± 0.01[c]	6
	VCR+suramin	0.086 ± 0.005[c]	5
LAP-35	DXR→medium	2.95 ± 0.15	
	DXR→αIR3 MAb	0.008 ± 0.0002[b]	369
	DXR→suramin	0.38 ± 0.04[b]	8
	VCR→medium	2.5 ± 0.2	
	VCR+αIR3 MAb	0.01 ± 0.008[b]	250
	VCR+suramin	0.008 ± 0.002[b]	312

[a] Drug concentration resulting in 50% inhibition of cell growth.
[b] $P < 0.001$; Student's *t* test.
[c] $P < 0.05$; Student's *t* test.

RESULTS

Increased Cytotoxicity of Combined *in Vitro* Treatments. Experiments were carried out to determine the effect on the growth of ES/PNET cells of two conventional chemotherapeutic drugs (doxorubicin or vincristine) in combination with anti-IGF-IR MAb αIR3 or suramin. Continuous treatments with either αIR3 MAb or suramin produced a dose-dependent inhibition of growth (7, 16). The combined treatment with increasing concentrations of doxorubicin or vincristine and 1 μg/ml of αIR3 MAb resulted in a synergistic or additive inhibition of TC-71 cell growth with respect to the therapeutic efficacy of doxorubicin or vincristine alone, respectively (Fig. 1, *A* and *B*). The increased antitumor activity of anticancer agents was not observed when cells were treated with chemotherapeutic drugs in combination with MOPC-21 MAb, an isotype-matched MAb, used as a control. An additive cytotoxic effect was also obtained when doxorubicin or vincristine was used in combination with suramin (Fig. 2*A* and *B*). Table 1 summarizes the increased sensitivity to doxorubicin or vincristine of three cell lines representative of the whole spectrum of ES/PNET tumors, including conventional ES (TC-71), PNET (LAP-35), and Askin's tumor (SK-N-MC), after combined simultaneous treatments. To closely mimic the *in vivo* treatment conditions, we performed sequential treatments. In particular, ES/PNET cells were exposed to doxorubicin or vincristine for only 12–24 h, a time that corresponds to the *in vitro* doubling time of these cell lines, and then were maintained in the presence of αIR3

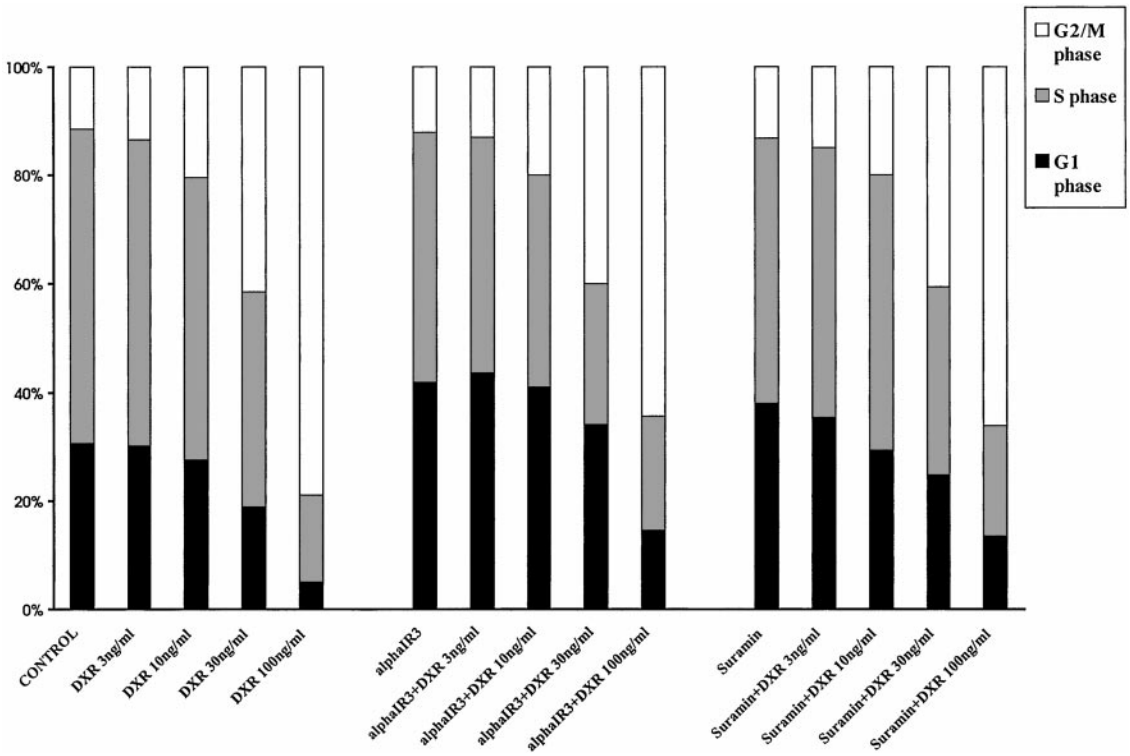

Fig. 5 Effects of treatments with doxorubicin (*DXR*) in combination with anti-IGF-IR αIR3 MAb or suramin on TC-71 cell cycle. Asynchronously growing TC-71 cells were cultured in the absence (*control*) or presence of increasing concentrations of doxorubicin ± αIR3 (1 μg/ml) or suramin (250 μg/ml) for 36 h. Data are from one experiment representative of three.

MAb or suramin for an additional 96 h. Again, a synergistic or additive inhibitory effect on TC-71 cell growth was observed (Fig. 3, *A* and *B*; Fig. 4, *A* and *B*) and the sensitivity of ES/PNET cells to doxorubicin or vincristine was significantly enhanced with combined sequential treatments (Table 2). These effects occurred at clinically relevant drug concentrations. The *in vitro* conditions closely simulated drug plasma levels that may persist for a prolonged period and are sufficient to kill tumor cells without severe toxic effects (17, 19, 20).

Analysis of Cell Cycle and Apoptosis after Combined Treatments. To ascertain whether the inhibitory effect on TC-71 cell growth *in vitro* was attributable to IGF-IR impairment on the cell cycle and/or induction of apoptosis, the incorporation of BrdUrd and the percentage of cells in the different phases of cell cycle, as well as the percentage of apoptotic nuclei, were analyzed after treatment with different doses of doxorubicin alone or in association with αIR3 MAb (1 μg/ml) or suramin (250 μg/ml). Doxorubicin progressively induced a consistent reduction in the G_1 and S-phase rate, as well as an enhancement in the percentage of cells in G_2-M phase of the cell cycle. In combined treatments, αIR3 MAb and suramin significantly increased the G_1-phase rate, only partly affected the doxorubicin-induced decrease of BrdUrd-positive cells, and failed to significantly modify the G_2-M blockage induced by doxorubicin (Fig. 5). With regard to the percentage of apoptotic nuclei after treatment with αIR3 MAb or with suramin and doxorubicin, a synergistic effect was observed in the combined treatment using αIR3, whereas the association of suramin with doxorubicin gave an additive increase in the apoptotic rate observed with doxorubicin alone (Fig. 6, *A* and *B*). These findings confirmed the hypothesis that the specific blockage of IGF-IR deprives ES cells of an important tool for preventing apoptosis that is induced by chemotherapeutic agents.

Effects on Colony Formation in Soft Agar. Treatment of TC-71 cells with increasing concentrations of doxorubicin induced a progressive inhibition in the number of colonies observed in semisolid medium (Table 3). Combined treatments of doxorubicin plus αIR3 MAb synergistically increased growth inhibition induced by doxorubicin alone, whereas the combined treatment with vincristine had an additive effect. These findings appeared to be specific for the blockage of IGF-IR, because MOPC-21 MAb did not affect the cytotoxic effect of doxorubicin.

DISCUSSION

In recent years, treatment modalities based on growth factor-receptor interactions or on interference with their signal transduction pathways seemed to be promising therapeutic possibilities, especially for those neoplasms such as ES in which conventional chemotherapy, although successful in many cases, has clearly shown an impasse (2). In previous studies, we found that IGF-IR signal transduction plays a critical role in the regulation of *in vitro* and *in vivo* ES cell growth (7, 16). In particular, impairment of IGF-IR functions by using a specific neutralizing MAb or suramin, a drug that is able to disrupt IGF-I

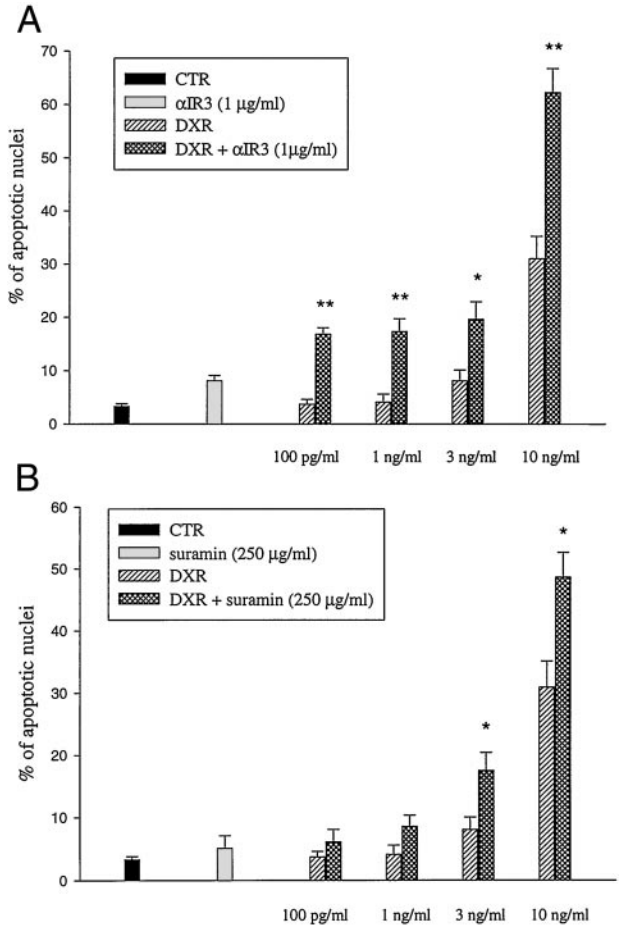

Fig. 6 Effects on apoptosis of TC-71 cells by treatments with doxorubicin (*DXR*) in combination with anti-IGF-IR αIR3 MAb (*A*) or suramin (*B*). The percentage of apoptotic nuclei was evaluated by morphological assessment after 96 h of simultaneous and continuous *in vitro* treatments. Results represent the mean ± SE of triplicate experiments. ∗, $P < 0.05$; ∗∗, $P < 0.01$; Student's *t* test, compared with the corresponding dose of DXR.

binding from its receptor (17), resulted in a loss of tumorigenic and metastatic ability of ES cells in nude mice (16). Targeting IGF-IR seems, therefore, to be of potential therapeutic help in the treatment of ES patients. However, from a clinical point of view, to be of significant therapeutic value, MAbs against IGF-IR and suramin should be effectively combined with anticancer drugs in mediating their antitumor activity. The present study showed that combined treatments using IGF-IR inhibiting agents (both αIR3 MAb or suramin) in association with conventional cytotoxic agents, such as doxorubicin and vincristine, currently included in all ES/PNET chemotherapeutic regimens, significantly enhance the *in vitro* cytotoxic effects of chemotherapeutic drugs.

Most anticancer agents, including doxorubicin and vincristine, kill cancer cells by inhibiting cell cycle and/or inducing apoptosis (21–23). The apoptotic action of chemotherapeutic drugs has been extensively studied in the last few years, and it has been claimed to be the main mechanism of action. On the other hand, IGF-IR, activated by its ligands, is emerging as a powerful inhibitor of apoptosis induced by a variety of agents, including anticancer drugs and ionizing and nonionizing radiation (24–27). In particular, Dunn *et al.* (27) first showed that IGF-I could induce a 20–40% increase in cell survival of breast cancer cells treated with clinically relevant and functionally diverse anticancer drugs, which supported the idea that IGF-I may significantly contribute to decreasing the effectiveness of chemotherapy. In ES, a recent study clearly indicates that IGF-I can act as a survival factor and that the activation of IGF-IR makes cells more resistant to doxorubicin-induced apoptosis (18). Therefore, blockage of IGF-IR functions by using a neutralizing MAb or suramin might sensitize ES cells to apoptosis that is induced by chemotherapeutic drugs, potentiating in this way their cytotoxic action. Indeed, by targeting IGF-IR, we observed an additive or, in some cases, a synergistic effect of growth inhibition induced by doxorubicin or vincristine. The percentage of growth inhibition from simultaneous or sequential treatments with chemotherapeutic drugs plus αIR3 MAb or suramin was enhanced by 30–35% compared with the effects induced by anticancer agents alone. This percentage corresponds to the percentage of growth inhibition induced by αIR3 MAb or suramin as single agents. As may be expected, αIR3 MAb, which specifically blocks IGF-IR, generally gave better results than suramin. However, despite the specificity of αIR3 MAb, the potential of anticancer strategies based on IGF-IR blockage by delivery of a murine antibody are of limited practical value in clinical settings because of the emergence of immune responses, the short peptide half-life, and the high cost. The use of suramin may, therefore, be an attractive alternative.

The analysis of the cell cycle indicated that the blockage of IGF-IR induces a blockage of the cells in G_1 phase that joins the G_2-M blockage induced by doxorubicin. The mitogenicity of IGF-IR has been known for a long time (28), and, for an optimal growth, IGF-IR is required in all cell cycle phases. The inhibition of IGF-IR mitogenic activity diminishes cell entry into S phase, but this effect does not antagonize the action of anticancer agents, such as doxorubicin, on the cell cycle. Indeed, agents that block IGF-IR and doxorubicin appeared to act on different phases of the cell cycle, and targeting IGF-IR may potentiate the action of doxorubicin. On the other hand, the analysis of apoptotic nuclei showed that αIR3 MAb or suramin actually have a significant effect in enhancing the apoptotic effects of doxorubicin. These findings confirmed that the disruption of IGF-IR antiapoptotic action may potentiate *in vitro* drug-induced cell death. Because the proapoptotic effects attributable to targeting IGF-IR are more dramatic when cells are in anchorage-independent conditions (28), we ascertained the effects of a combination treatment in semisolid medium. αIR3 MAb treatment induced a highly significant increase in the anchorage-independent growth inhibition of TC-71 cells observed after doxorubicin exposure.

The ability of tumor cells to grow in the absence of contact with extracellular matrix should not be considered as an artifact of cultured cells: anchorage-independence correlates quite well with tumorigenicity (29) and is probably the property that allows tumor cells to infiltrate surrounding tissues and to establish distal metastases. In this context, the favorable effect derived from the inhibition of antiapoptotic signaling of IGF-IR, which

Table 3 Effects on colony formation in soft agar of TC-71 cells by combination treatment[a]

	IMDM 10% FCS		MOPC-21		αIR3	
	No. of colonies ± SE	Inhibition %[b]	No. of colonies ± SE	Inhibition %[b]	No. of colonies ± SE	Inhibition %[b]
Control	1019 ± 46		963 ± 59	13.5	328 ± 42	67.8
DXR, 3 ng/ml	977 ± 137	4.1	816 ± 37	26.7	144 ± 6[c]	85.9
DXR, 10 ng/ml	888 ± 36	12.8	722 ± 25	35.2	79 ± 6[c]	92.2
DXR, 30 ng/ml	711 ± 18	30.2	623 ± 16	44.0	49 ± 6[d]	95.2
DXR, 100 ng/ml	249 ± 37	75.5	231 ± 34	79.3	6 ± 1[c]	99.5
VCR, 300 pg/ml	875 ± 24	14.1	ND[e]	ND	370 ± 33[c]	63.7
VCR, 600 pg/ml	744 ± 82	27.0	ND	ND	293 ± 8[c]	71.2
VCR, 1 ng/ml	290 ± 25	71.5	ND	ND	96 ± 7[c]	90.5

[a] Cells (3300) were plated in 0.33% agarose with medium plus 10% FCS in the absence (control) or in the presence of increasing concentrations of doxorubicin (DXR) or vincristine (VCR) ± αIR3 (1 μg/ml), and colonies were counted after 7 days. Data are expressed as mean of triplicate plates ± SE.

[b] Percentages were calculated with respect to control.

[c] $P < 0.05$; Student's *t* test, compared with the corresponding dose of DXR, or VCR.

[d] $P < 0.001$; Student's *t* test, compared with the corresponding dose of DXR, or VCR.

[e] ND, not determined.

renders the cells more sensitive to the apoptotic action of doxorubicin, might be particularly relevant.

Taken together, our findings indicate that therapeutic strategies aimed at the blockage of IGF-IR, by using either αIR3 MAb or suramin, could yield a potential advantage in combined treatments with conventional therapeutic agents for ES patients.

ACKNOWLEDGMENTS

The authors thank Dr. Alba Balladelli (Laboratorio di Ricerca Oncologica, Istituti Ortopedici Rizzoli, Bologna, Italy) for critical reading of the manuscript.

REFERENCES

1. Bacci, G., Toni, A., Avella, M., Manfrini, M., Sudanese, A., Ciaroni, D., Boriani, S., Emiliani, E., and Campanacci, M. Long-term results in 144 localized Ewing's sarcoma patients treated with combined therapy. Cancer (Phila.), *63:* 1477–1486, 1988.

2. Bacci, G., Picci, P., Ferrari, S., Mercuri, M., Brach del Prever, A., Rosito, P., Barbieri, E., Tienghi, A., and Forni, C. Neoadjuvant chemotherapy for Ewing's sarcoma of bone. No benefit observed after adding iphosphamide and etoposide to vincristine, actinomycin, cyclophosphamide, and doxorubicin in the maintenance phase. Results of two sequential studies. Cancer (Phila.), *82:* 1174–1183, 1998.

3. Craft, A., Cotterill, S., Malcolm, A., Spooner, D., Grimer, R., Souhami, R., Imeson, J., and Lewis, I. Ifosfamide-containing chemotherapy in Ewing's sarcoma: the Second United Kingdom Children's Cancer Study Group and the Medical Research Council Ewing's Tumor Study. J. Clin. Oncol., *16:* 3628–3633, 1998.

4. Horowitz, M. E., Kinsella, T. J., Wexler, L. H., Belasco, J., Triche, T., Tsokos, M., Steinberg, S. M., McClure, L., Longo, D.L. Steis, R. G., Glatstein, E., Pizzo, P. A., and Miser, J. S. Total-body irradiation and autologous bone marrow transplant in the treatment of high-risk Ewing's sarcoma and rhabdomyosarcoma. J. Clin. Oncol., *11:* 1911–1918, 1993.

5. Paulussen, M., Ahrens, S., Craft, A. W., Dunst, J., Frohlich, B., Jabar, S., Rube, C., Winkelmann, W., Wissing, S., Zoubek, A., and Jurgens, H. Ewing's tumors with primary lung metastases: survival analysis of 114 (European Intergroup) Cooperative Ewing's Sarcoma Studies patients. J. Clin. Oncol., *16:* 3044–3052, 1998.

6. Yee, D., Favoni, R. E., Lebovic, G. S., Lombana, F., Powell, D. R., Reynolds, C. P., and Rosen, N. Insulin-like growth factor I expression by tumors of neuroectodermal origin with the t(11;22) chromosomal translocation. A potential autocrine growth factor. J. Clin. Investig., *86:* 1806–1814, 1990.

7. Scotlandi, K., Benini, S., Sarti, M., Serra, M., Lollini, P-L., Maurici, D., Picci, P., Manara, M. C., and Baldini, N. Insulin-like growth factor I receptor-mediated circuit in Ewing's sarcoma/peripheral neuroectodermal tumor: a possible therapeutic target. Cancer Res., *56:* 4570–4574, 1996.

8. Resnicoff, M., Coppola, D., Sell, C., Rubin, R., Ferrone, S., and Baserga, R. Growth inhibition of human melanoma cells in nude mice by antisense strategies to the type 1 insulin-like growth factor receptor. Cancer Res., *54:* 4848–4850, 1994.

9. Resnicoff, M., Sell, C., Ribini, M., Coppola, D., Ambrose, D., Baserga, R., and Rubin, R. Rat glioblastoma cells expressing an antisense RNA to the insulin-like growth factor I (IGF-I) receptor are nontumorigenic and induce regression of wild-type tumors. Cancer Res., *54:* 2218–2222, 1994.

10. Long, L., Rubin, R., Baerga, R., and Brodt, P. Loss of the metastatic phenotype in murine carcinoma cells expressing an antisense RNA to the insulin-like growth factor I receptor. Cancer Res., *55:* 1006–1009, 1995.

11. Arteaga, C. L., Kitten, L. J., Coronado, E. B., Jacobs, S., Kull, F. C., Allred, D. C., Osborne, C. K. Blockade of the type 1 somatomedin receptor inhibits growth of human breast cancer cells in athymic mice. J. Clin. Investig., *84:* 1418–1423, 1989.

12. Kalebic, T., Tsokos, M., and Helman, L. J. *In vivo* treatment with antibody against IGF-I receptor suppresses growth of human rhabdomyosarcoma and down-regulates $p34^{cdc2}$. Cancer Res., *54:* 5531–5534, 1994.

13. Prager, D., Li, H. L., Asa, S., Melmed, S. Dominant negative inhibition of tumorigenesis *in vivo* by human insulin-like growth factor receptor mutant. Proc. Natl. Acad. Sci. USA, *91:* 2181–2185, 1994.

14. D'Ambrosio, C., Ferber, A., Resnicoff, M., and Baserga, R. A soluble insulin-like growth factor I receptor that induces apoptosis of tumor cells *in vivo* and inhibits tumorigenesis. Cancer Res., *56:* 4013–4020, 1996.

15. Dunn, S. E., Ehrlich, M., Sharp, N. J. H., Reiss, K., Solomon, G., Hawkins, R., Baserga, R., and Barrett, J. C. A dominant negative mutant of the insulin-like growth factor-1 receptor inhibits the adhesion, invasion and metastases of breast cancer. Cancer Res., *58:* 3353–3361, 1998.

16. Scotlandi, K., Benini, S., Nanni, P., Lollini, P-L., Nicoletti, G., Landuzzi, L., Serra, M., Manara, M. C., Picci, P., and Baldini, N. Blockage of insulin-like growth factor-I receptor inhibits the growth of Ewing's sarcoma in athymic mice. Cancer Res., *58:* 4127–4131, 1998.

17. Stein, C. A., Suramin. a novel antineoplastic agent with multiple potential mechanisms of action. Cancer Res., *53:* 2239–2248, 1993.

18. Toretsky, J. A., Thakar, M., Eskenazi, A. E., and Frantz, C. N. Phosphoinositide 3-hydroxide kinase blockade enhances apoptosis in the Ewing's sarcoma family of tumors. Cancer Res., *59:* 5745–5750, 1999.

19. Rowinsky, E. K., and Donehower, R. C. Antimicrotubule agents. *In:* B. A. Chabner and D. L. Longo (eds), Cancer Chemotherapy and Biotherapy. Principles and Practice, pp. 263–296. Philadelphia: Lippincott-Raven, 1996.

20. Doroshow, J. H. Antracyclines and anthracenediones. *In:* B. A. Chabner and D. L. Longo (eds), Cancer Chemotherapy and Biotherapy. Principles and Practice, pp. 409–434. Philadelphia: Lippincott-Raven, 1996.

21. Eastman, A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells, *2:* 275–280, 1990.

22. Friesen, C., Herr, I., Krammer, P. H., and Debatin, K-M. Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat. Med., *2:* 574–577, 1996.

23. Waldman, T. Zhang, Y., Dillehay, L., Yu, J., Kinzler, K., Vogelstein, B., and Williams, J. Cell-cycle arrest versus cell death in cancer therapy. Nat. Med., *3:* 1034–1036, 1997.

24. Sell, C., Baserga, R., and Rubin, R. Insulin-like growth factor I (IGF-I) and the IGF-I receptor prevent etoposide-induced apoptosis. Cancer Res., *55:* 303–306, 1995.

25. Turner, B. C., Haffty, B. G., Narayanan, L., Yuan, J., Havre, P. A., Grumbs, A. A., Kaplan, L., Burgaud, J. L., Carter, D., Baserga, R., and Glazer, P. M. Insulin-like growth factor 1 receptor overexpression mediates cellular resistance and local breast cancer recurrence after lumpectomy and radiation. Cancer Res., *57:* 3079–3083, 1997.

26. Nakamura, S., Watanabe, H., Miura, M., and Sasaki, T. Effect of the insulin-like growth factor 1 receptor on ionizing radiation-induced cell death in mouse embryo fibroblast. Exp. Cell Res., *235:* 287–294, 1997.

27. Dunn, S. E., Hardman, R. A., Kari, F. W., and Barrett, C. J. Insulin-like growth factor 1 (IGF-I) alters drug sensitivity of HBL 100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. Cancer Res., *57:* 2687–2693, 1997.

28. Baserga, R., Hongo, A., Rubini, M., Prisco, M. and Valentinis, B. The IGF-I receptor in cell growth, transformation and apoptosis. Biochim. Biophys. Acta, *1332:* F105–126, 1997.

29. Aaronson, T. A., and Todaro, G. J. Basis for the acquisition of malignant potential by mouse cells cultivated *in vitro*. Science (Wash. DC), *162:* 1024–1026, 1968.

Clinical Cancer Research

Inhibition of Insulin-like Growth Factor I Receptor Increases the Antitumor Activity of Doxorubicin and Vincristine against Ewing's Sarcoma Cells

Stefania Benini, Maria Cristina Manara, Nicola Baldini, et al.

Clin Cancer Res 2001;7:1790-1797.