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14. ABSTRACT In order to define mechanisms by which HER2/neu overexpression drives breast cancer cell growth and chemoresistance, antisense oligodeoxynucleotides (ODNs) have been used to down-regulate HER2/neu expression in human breast cancer cells. Such antisense ODNs suppress HER2/neu mRNA and protein expression in a dose-dependent, sequence-specific manner. Antisense ODN-mediated down-regulation of HER2/neu expression in HER2/neu-overexpressing breast cancer cells inhibits cell cycle progression in G0/G1 and results in apoptotic cell death. In tissue culture studies, combined treatment of HER2/neu overexpressing breast cancer cells with HER2/neu antisense ODNs and conventional chemotherapeutic agents results in synergistic inhibition of cell growth and activation of apoptosis. These studies have been extended to demonstrate synergistic antitumor effects following systemic treatment with HER2/neu antisense ODNs and chemotherapeutic agents in breast cancer xenografts in nude mice.					
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Introduction

Advances in molecular biology have identified mutations in specific genes, termed oncogenes, which play a role in the development of cancer. One of the most common molecular abnormalities seen in breast cancer is overexpression of the oncogene known as HER2/neu (1, 2). This gene encodes a protein, termed p185, which normally plays a vital, but tightly regulated, role in cell proliferation. Overexpression of the HER2/neu oncogene in breast cancer cells leads to overproduction of the p185 protein, excessive stimulation of cell proliferation and uncontrolled cell growth. Overexpression of HER2/neu in breast cancer appears to be associated with poor patient survival and with resistance to chemotherapy and hormonal therapy (1-9).

We initially pioneered studies examining the effects of down-regulating the p185 protein in cancer cells using monoclonal antibodies (10-16). We have shown that monoclonal antibodies specific for the p185 protein can neutralize p185 on the tumor cell surface, resulting in inhibition of cancer cell growth in vitro and in vivo. Based on our initial studies of p185-targeted antibody therapy in rodent model systems, monoclonal antibodies specific for the p185 molecules overexpressed in human breast cancer have been developed (17). One such antibody, Herceptin[®], has been approved for the treatment of patients with breast cancer. We have identified a distinct mechanism for down-regulating p185 expression in human breast cancer cells, using antisense oligodeoxynucleotides (ODNs) (18). The studies performed with support from this award have characterized the effects of such antisense ODNs on human breast cancer cell proliferation and apoptotic cell death in vitro (19-21), and have examined synergistic anti-cancer effects resulting from the exposure of human breast cancer cells to antisense ODNs in combination with conventional chemotherapeutic agents (19).

Body and Key Research Accomplishments

See Manuscripts in Appendices 1-3.

Reportable Outcomes

Publications in Peer-Reviewed Journals

1. Roh, H., Boswell, C., Hirose, C., Pippin, J. and Drebin, J.A. (1999) Synergistic anti-tumor effects of HER2/neu antisense oligodeoxynucleotides and conventional chemotherapeutic agents. *Surgery* 126: 413-421.
2. Roh, H., Pippin, J. and Drebin, J.A. (2000) Down-regulation of HER2/neu expression induces apoptosis in human cancer cells that overexpress HER2/neu. *Cancer Research* 60: 650-655.
3. Roh, H., Pippin, J.A., Green, D.W., Boswell, C.B., Hirose, C., Mokadam, N.A. and Drebin, J.A. (2000) HER2/neu antisense targeting of human breast carcinoma. *Oncogene* 19: 6138-6143.

Published Abstracts:

1. Roh, H., Pippin, J. and Drebin, J.A., (1998) Down-regulation of HER2/neu expression by antisense oligonucleotides induces apoptosis in human breast cancer cells that overexpress HER2/neu. *Surgical Forum* 49: 418-419.

2. Boswell, C.B., Pippin, J. and Drebin, J.A. (1998) Down-regulation of HER2/neu expression using combinations of antisense oligonucleotides and monoclonal antibodies results in enhanced antitumor effects. *Surgical Forum* 49: 456-458.
3. Roh, H., Pippin, J. and Drebin, J.A. (1998) HER2/neu-specific antisense oligodeoxynucleotides induce apoptosis in human breast cancer cells that overexpress HER2/neu. *Breast Cancer Research and Treatment* Vol. 50, p. 294.
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Conclusions

The studies performed with support from this award have examined effects of specifically down-regulating HER2/neu expression in human breast cancer cells. These studies were among the first to demonstrate that specific down-regulation by a non-antibody mechanism could inhibit breast cancer cell growth, and were the first to demonstrate that down-regulation of HER2/neu expression in overexpressing breast cancer cells could induce apoptotic cell death (20,21). They also were the first to demonstrate the potential usefulness of HER2/neu antisense oligonucleotides for in vivo therapeutics in breast tumor xenograft model systems (19, 21), and to define potential cytotoxic synergy between antisense-mediated HER2/neu down-regulation and conventional chemotherapeutic agents. Collectively these studies have added to the growing literature concerning mechanisms of HER2/neu-driven breast carcinogenesis and therapeutic targeting of HER2/neu in human breast cancer cells.

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Appendix 1

Roh, H., Boswell, C., Hirose, C., Pippin, J. and Drebin, J.A. (1999)

Synergistic anti-tumor effects of HER2/neu antisense oligodeoxynucleotides and conventional chemotherapeutic agents. *Surgery* 126: 413-421.

Synergistic antitumor effects of HER2/neu antisense oligodeoxynucleotides and conventional chemotherapeutic agents

Haeri Roh, PhD, Christopher B. Hirose, MD, Craig B. Boswell, MD, James A. Pippin, BS, and Jeffrey A. Drebin, MD, PhD, FACS, St Louis, Mo

Background: The *HER2/neu* oncogene is overexpressed in a substantial fraction of human tumors. *HER2/neu* overexpressing tumors may be intrinsically resistant to chemotherapy. The present study examined the ability of antisense-mediated downregulation of *HER2/neu* expression to enhance the anti-tumor effects of conventional chemotherapeutic agents against human tumor cells that overexpress *HER2/neu*.

Methods: The effects of *HER2/neu* antisense oligodeoxynucleotides (ODNs) on the growth inhibitory and proapoptotic activity of several distinct chemotherapeutic agents were examined *in vitro*. *In vivo* effects of *HER2/neu* antisense ODNs in combination with doxorubicin hydrochloride were assessed by examining the growth of human tumor xenografts implanted into nude mice.

Results: The proliferation of tumor cell lines that overexpress *HER2/neu* was inhibited by antisense ODNs in combination with conventional chemotherapeutic agents in an additive or synergistic fashion. Such combination therapy also demonstrated synergistic activation of apoptosis. *HER2/neu* antisense ODNs in combination with doxorubicin hydrochloride demonstrated synergistic antitumor effects *in vivo* as well.

Conclusions: Downregulation of *HER2/neu* expression can enhance the sensitivity of human cancer cells, which overexpress *HER2/neu* to the cytotoxic effects of chemotherapy. Antisense ODNs targeting the *HER2/neu* gene may play a role in cancer therapy. (*Surgery* 1999;126:413-21.)

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ADVANCES IN MOLECULAR BIOLOGY have identified mutations in specific genes, termed oncogenes, that play a role in the development of cancer. One of the most common molecular abnormalities seen in adenocarcinomas of the breast, lung, ovary, and pancreas is overexpression of the oncogene known as *HER2/neu*.^{1,2} This gene encodes a protein, p185, which normally plays a vital, but tightly regulated, role in cell proliferation. Overexpression of the *HER2/neu* oncogene in cancer cells leads

to overproduction of the p185 protein, excessive stimulation of cell proliferation, and uncontrolled cell growth. Overexpression of *HER2/neu* in malignant tumors appears to be associated with poor patient survival² and with resistance to chemotherapy and hormonal therapy.³⁻⁵

We initially demonstrated the effects of downregulating the p185 protein in cancer cells using monoclonal antibodies. We have shown that monoclonal antibodies specific for the p185 molecule can neutralize p185 on the tumor cell surface, resulting in inhibition of tumor cell proliferation *in vitro* and *in vivo*.^{6,7} On the basis of our initial studies of p185-targeted antibody therapy in rodent models, monoclonal antibodies specific for the p185 molecules overexpressed in human breast cancer cells have been developed. One such antibody has shown significant antitumor effects in initial clinical trials.^{8,9} We have recently identified a more potent means of downregulating *HER2/neu* expression in human cancer cells, using antisense oligodeoxynucleotides (ODNs).^{10,11} Furthermore,

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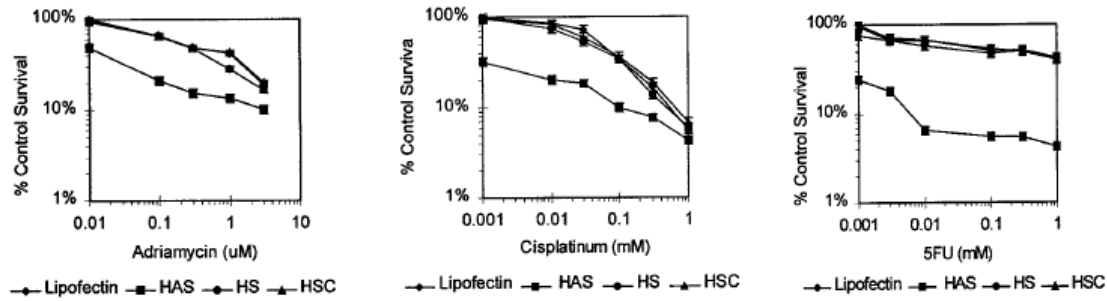


Fig 1. Effects of treatment with ODNs and chemotherapeutic agents on BT474 human breast carcinoma cells. All ODNs were used at a final concentration of 1 $\mu\text{mol/L}$. *HAS*, HER2/neu antisense ODNs; *HS*, HER2/neu sense ODNs; *HSC*, HER2/neu scrambled antisense ODNs.

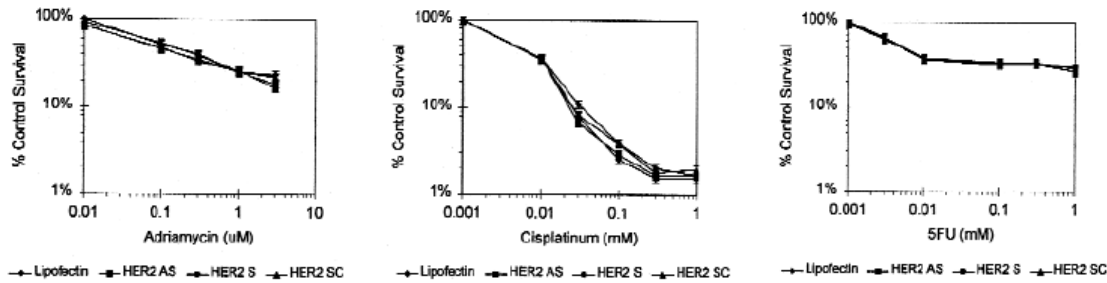


Fig 2. Effects of treatment with ODNs and chemotherapeutic agents on MCF7 human breast carcinoma cells. All ODNs were used at a final concentration of 1 $\mu\text{mol/L}$. *HER2 AS*, antisense ODNs; *HER2 S*, sense ODNs; *HER2 SC*, scrambled antisense ODNs.

we have demonstrated that antisense-mediated downregulation of HER2/neu expression in tissue culture is associated not only with cell cycle arrest but also with activation of programmed cell death (apoptosis) in cancer cells that overexpress HER2/neu.¹¹ On the basis of these observations, we have examined the ability of antisense-mediated HER2/neu downregulation to enhance antitumor activity of conventional chemotherapeutic agents against tumors that overexpress HER2/neu.

METHODS

Cell culture. The human breast carcinoma lines BT474 and MCF7 were purchased from the American Type Culture Collection (Rockville, Md). Cells were cultured in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 2 mmol/L L-glutamine, 10 $\mu\text{g/mL}$ bovine insulin, 100 $\mu\text{g/mL}$ penicillin/streptomycin mixture, and 2.5 $\mu\text{g/mL}$ amphotericin B (all from Mediatech, Herand, Va). Cell cultures were maintained in a 37°C incubator with 5% carbon dioxide humidified air.

ODN treatment. Phosphorothioate ODNs targeting the 5' region of the HER2/neu mRNA molecule¹⁰ were obtained from Oligos, Etc (Wilsonville, Ore). The lyophilized ODNs were reconstituted in sterile distilled water to 1 mmol/L, filter sterilized, and stored in aliquots at -20°C as stock solutions. For subsequent antisense experiments, the stock solutions of ODNs were diluted to give final concentration of 1 $\mu\text{mol/L}$. Diluted ODNs were mixed with Lipofectin (Gibco-BRL, Gaithersburg, Md) to yield a final concentration of 10 $\mu\text{g/mL}$, and cells were exposed to the mixture for 4 hours in the presence of serum-free OptiMem (Gibco), after which time the ODN-containing medium was replaced with standard culture media.

Western blotting. Crude cell extracts were obtained by direct lysis of tumor cells in lysis buffer (50 mmol/L Tris-HCl [7.4], 5 mmol/L EGTA, 1% Triton X-100, 150 mmol/L NaCl, 2 mmol/L phenylmethylsulfonyl fluoride, 1% aprotinin, and 5 $\mu\text{g/mL}$ leupeptin). Ten micrograms of the lysate per lane was subjected to 7.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis and elec-

troblotted onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, Mass). Blots were hybridized with specific antibodies, followed by a species-specific horseradish peroxidase-conjugated second antibody, and were developed with the enhanced chemoluminescent system. Cell extracts were prepared 48 hours after the initiation of antisense treatment. Antibodies specific for p185^{her2/neu} and for the 170-kd MDR-1 gene product were obtained from Oncogene Research (Cambridge, Mass) and used according to the supplier's recommendations. An actin-specific monoclonal antibody (Amersham Corp, Arlington Heights, Ill) was used as a control for protein loading.

Chemotherapeutic agents. Chemotherapy drugs used in the studies described here were obtained from Sigma Chemical Company (St Louis, Mo) and from the pharmacy at Barnes-Jewish Hospital.

Chemotherapy-induced cytotoxicity. Cells (1.5×10^6) of exponentially growing tumor cells were plated in 10 mL of standard culture media. After incubation at 37°C for 24 hours, culture medium was changed to medium containing the chemotherapeutic agent to be tested. After 24 hours of incubation with the drug, cells were detached from the culture dish with trypsin/ethylenediamine tetraacetic acid, washed once in tissue culture medium, and resuspended in OptiMem I. Five thousand cells in 40 μ L of OptiMem were plated into each well of 96-welled flat-bottom culture plate in triplicate. Ten microliters each of preformed mixture of lipofectin with or without specific ODNs were added to each well to give a defined final concentration, and cells were incubated at 37°C for 4 hours. Fifty microliters of standard culture medium containing 20% fetal bovine serum was then added to each well to give a final concentration of 10% fetal bovine serum. Cells were further incubated at 37°C for 5 days. Growth of the cells after 5 days was assayed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.¹² Briefly, 5 mg/mL stock solution of MTT was added to each well to give a final concentration of 500 μ g/mL, and plates were incubated in the dark for 4 hours at 37°C. The purple formazan product was solubilized overnight in 10% sodium dodecylsulfate/0.01 N hydrochloric acid at 37°C, and the absorbance at $\lambda = 595$ nm was recorded with $\lambda = 650$ nm as a reference wavelength (Model 3550 Microplate Reader; Bio-Rad, Hercules, Calif). Results are the mean of triplicate samples. Dose response curves were generated across a dose range of the specific chemotherapeutic agent.

CPP32 apoptosis assay. Cells (1×10^6) were

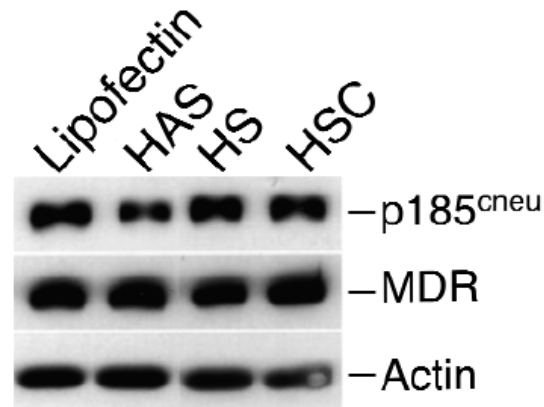


Fig 3. Expression of p 185^{neu} and p 170^{MDR} on western blotting of BT474 lysates after treatment of BT474 cells with HER2/neu antisense or control ODNs. Actin was probed as a control for equal protein loading. HAS, HER2/neu antisense ODNs; HS, HER2/neu sense ODNs; HSC, HER2/neu scrambled antisense ODNs.

treated in a 100-mm culture dish with chemotherapeutic agents, followed 24 hours later by ODNs as described earlier. After incubating for 72 hours from the initiation of chemotherapy treatment, cells were detached from the culture dish and washed once with phosphate-buffered saline solution. Activation of CPP32 protease was determined by using ApoAlert CPP32 Assay kit (Clontech, Palo Alto, Calif) according to the manufacturer's protocol. Absorbance of the DEVD-pNA cleavage product pNA at 405 nm from each sample was converted to the units of CPP32 with the standard curve generated by the absorbance of known amount of pNA.

Tumor cell implantation and measurement of tumor growth. BT474 tumor cells were cultured under standard conditions. Tumor cells were released from tissue culture dishes with trypsin/ethylenediamine tetraacetic acid, washed in serum-free medium, counted, and diluted in serum-free medium to a final concentration of 5×10^7 cells/mL. Tumor cells were then injected into the mid-dorsum of female NCR nude mice (Charles River, Cambridge, Mass) in a total volume of 0.2 mL. Nude mice were implanted with 60-day time-release estrogen pellets (Innovative Research, Sarasota, Fla) with a trocar needle 5 to 7 days before the tumor cell implantation. Animals were inspected daily for tumor development. Growing tumors were measured with vernier calipers, and tumor area was calculated as the product of tumor length and width.⁷

In vivo administration of antisense oligodeoxynucleotides. Stock ODNs were diluted with sterile

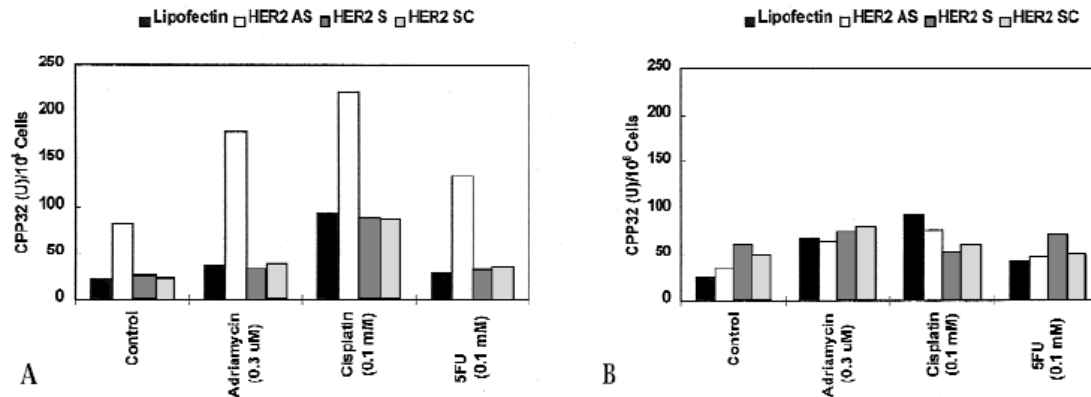


Fig 4. Activation of apoptotic cell death mechanisms in BT474 (A) and MCF7 (B) breast cancer cells treated with ODNs. *HER2 AS*, antisense ODNs; *HER2 S*, sense ODNs; *HER2 SC*, scrambled antisense ODNs.

saline solution to give a final dose of 10 mg/kg in a volume of 0.2 mL. Tumor-bearing animals were injected intraperitoneally with ODN solutions with a 30-gauge needle on days 10 through 24 after tumor cell implantation.

RESULTS

Downregulation of HER2/neu expression chemosensitizes BT474 breast carcinoma cells. Initial studies examining the effects of downregulating HER2/neu expression on sensitivity to chemotherapy were conducted with BT474 human breast carcinoma cells. These cells overexpress HER2/neu to more than 50 times the level seen in normal cells as the result of gene amplification and demonstrate significant in vitro growth inhibition after downregulation of HER2/neu expression with antisense ODNs or monoclonal antibodies.^{10,13} BT474 cells treated with combinations of chemotherapeutic agents and HER2/neu antisense ODNs demonstrate greater suppression of cell growth than do cells treated with either modality alone. Treatment with a variety of agents, including doxorubicin hydrochloride (Adriamycin), cis-platinum, and 5-fluorouracil, results in dose-dependent suppression of BT474 cell growth (Fig 1). HER2/neu antisense treatment by itself also has significant suppressive effects on BT474 growth compared with control ODNs, confirming previous results.^{10,11} However, the combination of chemotherapeutic agents with antisense ODNs results in a displacement of the dose-response curve, suggesting at least an additive antitumor effect and, in some cases, a shift in the slope of the dose-response curve, suggesting a synergistic antitumor effect. Thus downregulation of HER2/neu expression is able to increase the sensitivity of BT474 cells

to the cytotoxic effects of several traditional chemotherapeutic agents. Similar results are obtained with other tumor cell lines that overexpress HER2/neu, including Panc 1 pancreatic carcinoma cells and SKOV3 ovarian cancer cells (data not shown), which suggests that the enhanced chemosensitivity observed after HER2/neu downregulation may apply to tumors of diverse histologic origins that overexpress HER2/neu.

Downregulation of HER2/neu expression does not enhance the chemosensitivity of tumor cells that express low/normal levels of HER2/neu. Tumor cells that overexpress HER2/neu appear to be dependent on continuously elevated expression of this gene and undergo growth arrest and apoptotic cell death after downregulation of HER2/neu expression. In contrast, cells with lower levels of HER2/neu expression do not appear to be as dependent on continuous expression of this gene.^{10,13} The MCF7 breast carcinoma cell line expresses low/normal levels of HER2/neu.^{10,13} We have shown previously that treatment of MCF7 cells with HER2/neu antisense ODNs results in the downregulation of HER2/neu expression but has little effect on MCF7 cell growth.¹⁰ To determine whether the cytotoxic effects of chemotherapy are enhanced by HER2/neu downregulation in tumor cells expressing low levels of this gene, the effects of HER2/neu antisense ODNs on the growth of MCF7 cells after exposure to chemotherapeutic agents have been examined.

Treatment of MCF7 cells with the chemotherapeutic agents Adriamycin, cis-platinum, and 5-fluorouracil results in dose-dependent inhibition of tumor cell growth (Fig 2). However, in contrast to the findings noted earlier for BT474 cells, there is no enhancement of these effects in cells treated

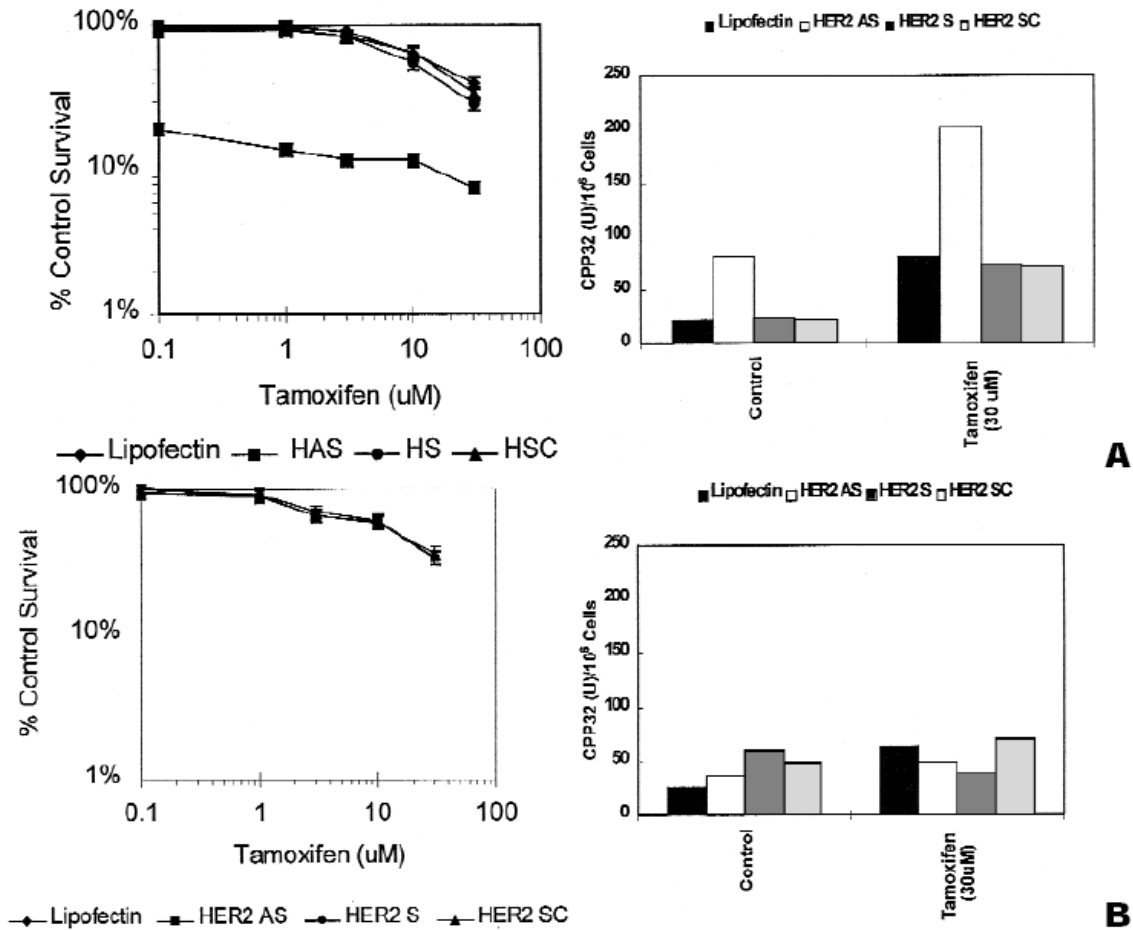


Fig 5. Treatment with tamoxifen plus HER2/neu antisense ODNs results in synergistic inhibition of tumor growth and activation of apoptotic cell death mechanisms in BT474 tumor cells (A) but not MCF7 tumor cells (B). *HAS*, HER2/neu antisense ODNs; *HS*, HER2/neu sense ODNs; *HSC*, HER2/neu scrambled antisense ODNs; *HER2 AS*, antisense ODNs; *HER2 S*, sense ODNs; *HER2 SC*, scrambled antisense ODNs.

with HER2/neu antisense ODNs. The dose-response curves are neither displaced nor are there alterations in the slope as the result of the downregulation of HER2/neu expression. Similar results (that is, failure of HER2/neu downregulation to chemosensitize) are obtained with another low/normal expressing cell line, the WI38 diploid fibroblast line (data not shown). This suggests that the ability of HER2/neu antisense ODNs to potentiate the effects of chemotherapeutic agents is limited to tumor cells that overexpress HER2/neu.

Downregulation of HER2/neu expression does not alter MDR-1 gene expression. Sensitivity or resistance to the effects of anticancer chemotherapeutic agents is often related to changes in the

expression of drug-resistance genes, such as MDR-1.¹⁴ MDR-1 encodes a 170 kd protein, termed *P-glycoprotein*, which functions to pump chemotherapeutic agents out of the cell. To determine whether downregulation of HER2/neu expression results in coordinate downregulation of MDR-1 gene expression, we have examined the expression of the products of these 2 genes in control and HER2/neu antisense-treated BT474 breast carcinoma cells. Treatment of BT474 cells with HER2/neu antisense ODNs can significantly downregulate the expression of the p185 product of the HER2/neu gene (Fig 3). Scanning densitometry analysis on serially diluted samples reproducibly demonstrates 50% to 80% downregulation of p185 after

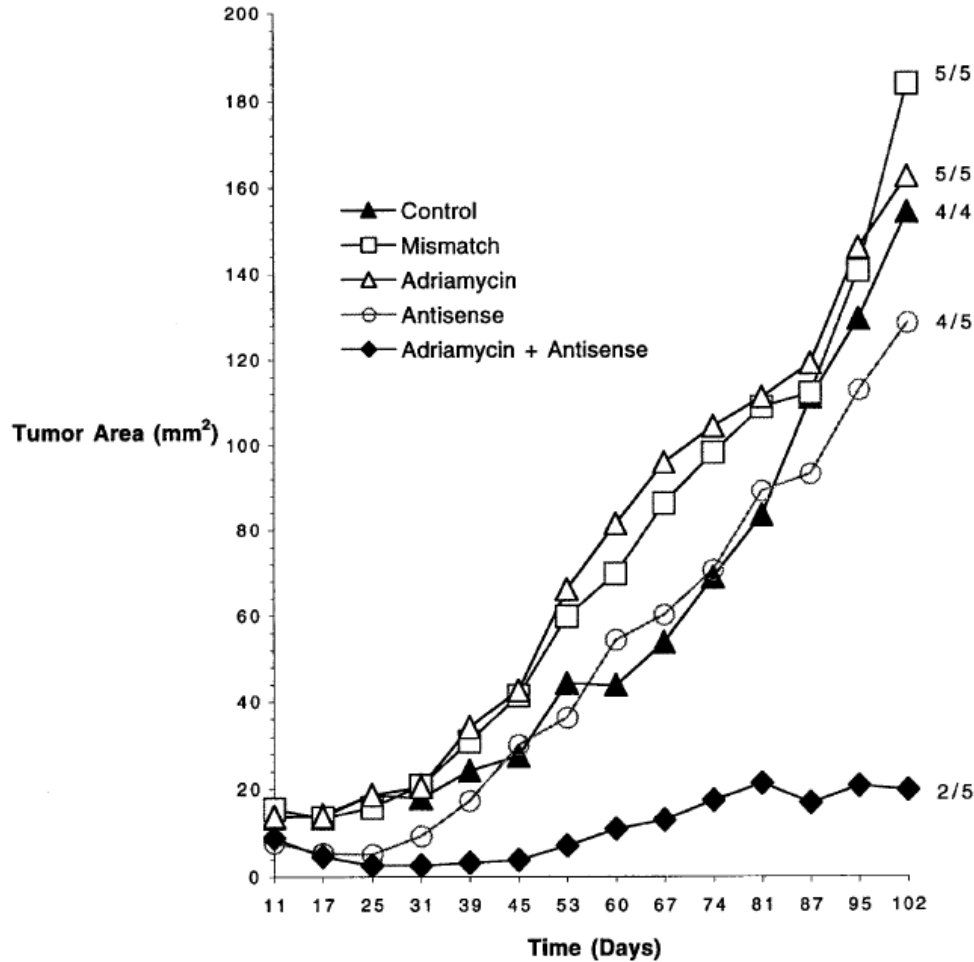


Fig 6. Synergistic antitumor effects of treatment with HER2/neu antisense ODNs in combination with Adriamycin. Fractions indicate tumor-bearing/total-treated animals as determined on day 102.

HER2/neu antisense treatment. In contrast, there is no effect of HER2/neu antisense ODNs on expression of the 170 kd MDR-1 gene product. Thus alterations in chemosensitivity resulting from HER2/neu downregulation are not the result of gross alterations in MDR-1 gene expression.

Effects of HER2/neu downregulation and chemotherapy on apoptotic cell death. The process of apoptotic cell death is a final common metabolic pathway that can result from diverse cellular stimuli.¹⁵ Activation of the caspase CPP32 is 1 of the final common events in apoptotic cell death. Both conventional chemotherapeutic agents¹⁵ and antisense-mediated downregulation of HER2/neu expression¹¹ can trigger apoptotic cell death. To determine whether chemotherapy-induced apop-

toxicity and antisense-mediated apoptosis showed additive or synergistic effects, we examined CPP32 activation in BT474 breast carcinoma cells treated with antisense or control ODNs and the chemotherapeutic agents Adriamycin, 5-fluorouracil and cis-platinum.

HER2/neu antisense treatment itself can cause significant CPP32 activation (Fig 4, A). At the doses used and in the absence of HER2/neu antisense treatment, the conventional chemotherapeutic agents also activate CPP32 to a limited extent. However, the combination of HER2/neu antisense ODNs with chemotherapy results in more than additive increases in CPP32 levels. It appears that the combination of antisense-mediated HER2/neu downregulation and exposure to chemotherapeu-

tic agents is synergistic with respect to activating apoptotic cell death pathways in BT474 cells. Similar results have been obtained in other HER2/neu overexpressing tumor cell lines (data not shown).

In contrast, there are no significant synergistic effects of HER2/neu antisense treatment on chemotherapy-induced apoptosis in MCF7 cells, which express low levels of HER2/neu (Fig 4, B). Similar results have been obtained with the WI38 fibroblast line (data not shown). Thus the ability of HER2/neu downregulation to potentiate the proapoptotic effects of chemotherapy is restricted to tumor cells that overexpress this gene.

HER2/neu downregulation potentiates the activity of tamoxifen. Both BT474 and MCF7 breast cancer cells are estrogen responsive and demonstrate growth inhibition and activation of apoptotic cell death after exposure to the estrogen receptor antagonist tamoxifen. To determine whether HER2/neu downregulation can potentiate the activity of estrogen receptor blockade on breast cancer cells, we have examined the effects of HER2/neu antisense ODNs on the sensitivity of BT474 and MCF7 cells to tamoxifen in cell growth (MTT) and apoptosis (CPP32 activation) assays.

Treatment with HER2/neu antisense ODNs shifts the cell growth dose-response curve after tamoxifen treatment in BT474 breast cancer cells (Fig 5, A). Furthermore, the combination of HER2/neu antisense and tamoxifen results in synergistic activation of apoptosis as determined by CPP32 activation assay. In contrast, there is no significant effect of HER2/neu antisense treatment on the sensitivity of MCF7 cells to tamoxifen (Fig 5, B). Thus downregulation of HER2/neu expression appears to sensitize human breast cancer cells that overexpress HER2/neu to the effects of antiestrogenic compounds and to the activity of cytotoxic chemotherapeutic agents.

HER2/neu antisense ODNs and Adriamycin exert synergistic antitumor effects in vivo. Antisense ODNs targeting oncogene products encoded by *c-myc*,¹⁶ *c-raf*,¹⁷ and *Bcl2*¹⁸ have shown activity in human tumor xenograft models and, in some cases, are undergoing phase I and phase II trials in patients with cancer. To begin to examine the antitumor activity of HER2/neu antisense ODNs, alone and in combination with chemotherapy, on the in vivo growth of human tumor cells that overexpress HER2/neu, we have used a BT474 xenograft model.¹⁹ Nude mice are implanted with controlled-release estrogen pellets and subsequently injected subcutaneously with 1×10^7 BT474 cells. Mice with growing BT474 tumors are randomized to receive HER2/neu antisense ODNs, scrambled sequence control ODNs, Adriamycin alone, HER2/neu anti-

sense plus Adriamycin, or a saline solution control. Treatment with scrambled sequence control ODNs or Adriamycin alone had no effect on the growth of BT474 xenografts, when compared with tumor growth in mice receiving a saline solution control (Fig 6). Mice receiving HER2/neu antisense alone had transient stabilization of tumor growth. One mouse had complete regression of its tumor, but in the other animals the tumors rapidly regrew when treatment stopped.

In contrast, the combination of HER2/neu antisense ODNs and Adriamycin resulted in complete tumor regression in 3 of 5 treated animals and prolonged tumor growth inhibition in the other 2 animals (Fig 6). Thus the synergistic in vitro antitumor effects observed with combinations of HER2/neu antisense ODNs and Adriamycin also can be obtained after the administration of these agents to tumor-bearing animals.

DISCUSSION

The HER2/neu gene is overexpressed in a significant fraction of human tumors. Down-regulation of HER2/neu expression with monoclonal antibodies has already reached the clinic, with promising initial results. However, there are a number of limitations to antibody targeting of HER2/neu expression. The antibody molecules only bind and downregulate p185 expression from the tumor cell surface. Intracellular p185 may stimulate mitogenic signaling before the display on the cell surface. Furthermore, it has been shown that tumors that overexpress HER2/neu shed soluble fragments of the p185 extracellular domain into culture medium in vitro²⁰ and into the systemic circulation in patients with cancer.⁸ These soluble extracellular domains can block antibody binding to p185 on the tumor cell surface and may prevent antibody-mediated downregulation of p185.²⁰ They also dramatically shorten the circulation time of injected antibody and may prevent clinical tumor regression.⁸ Thus antibody therapy is a less than ideal approach to targeting the HER2/neu gene product in human tumors.

Antisense compounds represent a distinct approach to downregulating HER2/neu expression in human cancer. Although there have been a number of flawed studies that used antisense approaches to downregulate gene expression,²¹ carefully designed studies, with ODNs at concentrations of 1 $\mu\text{mol/L}$ or less, have demonstrated the validity of this approach.²² A number of antisense compounds are in clinical trials in patients with cancer and other disorders.^{17,18,22} We have previously shown that appropriate HER2/neu-specific antisense

ODNs can selectively downregulate HER2/neu expression.¹⁰ Treatment of human tumor cell lines that overexpress HER2/neu with antisense ODNs results in the inhibition of tumor cell growth and activation of apoptotic cell death.^{10,11}

Here we demonstrate for the first time the synergistic antitumor activity of HER2/neu antisense ODNs in combination with conventional anticancer agents. It is notable that synergistic activation of apoptotic cell death mechanisms results from such combination therapy, and it appears that these results can be obtained not only *in vitro* but also in a tumor xenograft model. Recently 2 laboratories have identified similar synergy between conventional chemotherapeutic agents and HER2/neu-specific monoclonal antibodies in human tumor xenograft models.^{23,24}

The mechanism by which HER2/neu overexpression promotes chemoresistance, and conversely by which HER2/neu downregulation leads to chemosensitization, is not well understood. It appears from data presented here that gross changes in MDR-1 expression do not result from changes in HER2/neu expression. However, it is possible that the function of the MDR-1 gene product is altered as a result of changes in HER2/neu expression, with resulting changes in chemotherapeutic drug accumulation. It is also possible that the expression of other members of the multi-drug resistance gene family is altered as the result of changes in HER2/neu expression.

Several laboratories have suggested that HER2/neu overexpression results in the elevated expression of the p21^{cip} protein, with resulting cell cycle arrest and opportunities for DNA repair.^{24,25} Downregulation of HER2/neu expression in these reports resulted in the downregulation of p21^{cip}. This has led to the suggestion that DNA damaging agents would show the greatest synergy with strategies that downregulate HER2/neu expression.^{9,24} However, we have shown that diverse anticancer agents can synergize with the effects of HER2/neu downregulation. Some of these agents, such as tamoxifen, are not thought to have a direct DNA damaging effect on tumor cells. Furthermore, we have not observed downregulation of p21^{cip} in tumor cells that overexpress HER2/neu after antisense- or monoclonal antibody-mediated downregulation of HER2/neu expression (unpublished observations). It is possible that the synergistic effects of HER2/neu downregulation and chemotherapy occur at the level of activation of apoptotic cell death mechanisms, a final common pathway. We are currently investigating this possibility.

Regardless of the mechanism of action, a growing

body of data suggests that strategies that downregulate HER2/neu expression enhance the antitumor activity of conventional chemotherapeutic agents against tumors that overexpress HER2/neu. Initial clinical trials in this regard have also been promising.⁹ It is hoped that further improvements in our ability to modify HER2/neu expression in human cancer cells that overexpress this oncogene will result in real gains in the treatment of such cancers.

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Appendix 2

Roh, H., Pippin, J. and Drebin, J.A. (2000) Down-regulation of HER2/neu expression induces apoptosis in human cancer cells that overexpress HER2/neu. Cancer Research 60: 650-655.

Down-Regulation of HER2/*neu* Expression Induces Apoptosis in Human Cancer Cells That Overexpress HER2/*neu*¹

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Abstract

The HER2/*neu* oncogene is overexpressed in a significant fraction of human tumors; such overexpression is thought to play a role in the aberrant proliferation of cancer cells. The effects of HER2/*neu*-specific phosphorothioate antisense oligodeoxyribonucleotides on HER2/*neu* expression, tumor cell proliferation, and activation of apoptotic cell death pathways have been examined. Antisense treatment down-regulates HER2/*neu* expression in a dose-dependent and sequence-specific manner. HER2/*neu* antisense treatment specifically inhibits the growth of tumor lines that overexpress HER2/*neu*, but it has little effect on the growth of tumor cells that express low levels of HER2/*neu*. Down-regulation of HER2/*neu* expression is not only cytostatic, but it also results in the activation of apoptotic cell death pathways in cells that overexpress HER2/*neu*. These results suggest that, in addition to stimulating tumor cell proliferation, HER2/*neu* overexpression in cancer cells acts as an antiapoptotic cell survival factor.

Introduction

The HER2/*neu* gene, also called *erbB2*, encodes a M_r 185,000 glycoprotein with intrinsic tyrosine kinase activity, p185 (1–3). The HER2/*neu*-encoded p185 molecule occupies a critical position in the biochemical pathways responsible for the transduction of mitogenic signals from a variety of growth factor receptors (4, 5). In addition to its role in regulating normal cellular proliferation, overexpression of the HER2/*neu* gene appears to play a role in neoplastic cell growth. A significant fraction of human tumors of the breast, lung, ovary, and pancreas overexpress p185 (6). Several laboratories have demonstrated that monoclonal antibodies directed against the p185 protein can inhibit the *in vitro* and *in vivo* growth of certain tumor cell lines that overexpress p185 (7–9). Initial clinical trials of such antibodies in patients with breast cancer have produced promising results (10, 11).

However, monoclonal antibody targeting of p185 appears to result in primarily cytostatic, as opposed to cytotoxic, effects on susceptible tumor cells (9, 12). Furthermore, tumor cells that overexpress p185 can shed soluble antigenic fragments from the cell membrane, which may interfere with monoclonal antibodies reaching the tumor cell surface *in vitro* (13) and *in vivo* (10). These limitations have led to the development of a number of distinct approaches to inhibiting p185 expression or function, including the use of chemical inhibitors of p185 tyrosine kinase activity (14), viral mechanisms of inhibiting HER2/*neu* expression (15), gene transfer of single chain antibody (16) or dominant negative HER2/*neu* mutants (17) and treatment with

antisense oligonucleotides (18–21). Here we describe the effects of a phosphorothioate antisense oligodeoxyribonucleotide directed against the 5' region of the HER2/*neu* mRNA molecule on HER2/*neu* expression, cell proliferation, and apoptotic cell death in HER2/*neu*-overexpressing cancer cells derived from tumors of several tissue types.

Materials and Methods

Cell Culture. The human carcinoma lines BT474 (breast), Calu-3 (lung), CFPAC-1 (pancreas), MCF-7 (breast), NCI-H23 (lung), SK-OV-3 (ovary), T24 (bladder), and the human diploid fibroblast line, WI-38, were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 μ g/ml penicillin/streptomycin mixture, and 2.5 μ g/ml amphotericin B (all purchased from Mediatech, Herand, VA), and were maintained in a 37°C incubator with 5% CO₂ humidified air.

Oligodeoxynucleotide Treatment. Phosphorothioate ODNs³ targeting the 5' region of the HER2/*neu* mRNA molecule were obtained from Oligos, Etc (Wilsonville, OR), bases in bold font indicate substitutions:

Antisense: CTCCATGGTGCAC

Sense: GTGAGCACCATGGAG

Scrambled antisense: CGCCTTATCCGTAGC

One-mismatch: CTCCATGCTGCTCAC

Four-mismatch: CTACCTGCTGCTGAC

The lyophilized ODNs were reconstituted in sterile distilled water to 1 mM, filter-sterilized, and stored in aliquots at –20°C as stock solutions. For subsequent experiments, the stock solutions of ODNs were diluted to give final concentration of 1 μ M. Diluted ODNs were mixed with 10 μ g/ml Lipofectin (Life Technologies, Inc., Gaithersburg, MD), and cells were exposed to the mixture for 4 h, after which the mixture-containing medium was replaced with the culture medium.

Western Blotting. Crude cell extracts were obtained by direct lysis of the cells in lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton X-100, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 1% Aprotinin, and 5 μ g/ml Leupeptin). Twenty μ g of the lysate from each of the cell line were subjected to 7.5% SDS-PAGE and electroblotted onto a PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Blots were hybridized with specific antibodies, followed by a species-specific alkaline phosphatase-conjugated second antibody, and were developed using the CDP-star chemiluminescent system. In antisense experiments, cell extracts were prepared 48 h (unless otherwise noted) after the initiation of treatment as described for Northern blotting, and 2 μ g of lysates were separated by SDS-PAGE. Antibodies specific for p185^{HER2/*neu*} were obtained from Oncogene Research (Cambridge, MA) and used according to the supplier's recommendations. Anti-PARP and anti-cyclin B antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). An actin-specific monoclonal antibody (Amersham Corp., Arlington Heights, IL) was used as a control for protein loading.

Cell Growth Assay. One-ml aliquots containing 2×10^4 cells were plated into the wells of 24-well culture plates and were allowed to adhere for 24 h. After cells were firmly attached, they were washed once with prewarmed OPTIMEM-I (Life Technologies, Inc.), and 10 μ g/ml Lipofectin mixture with or without 1 μ M antisense, sense, or scrambled antisense ODNs were applied to each well for 4 h at 37°C. After the 4-h incubation, the ODN mixture was

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³ The abbreviations used are: ODN, oligodeoxynucleotide; PARP, poly (ADP ribose) polymerase.

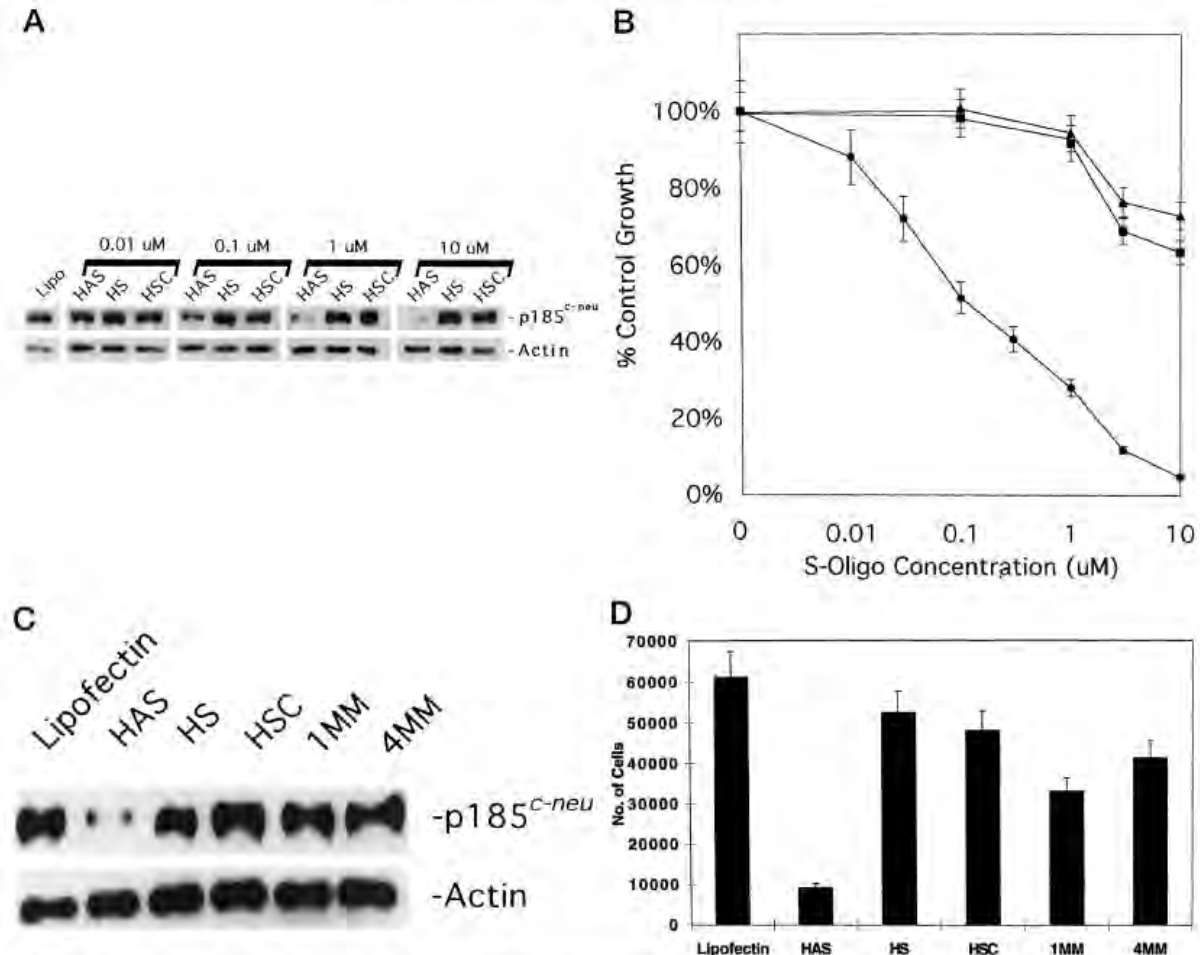


Fig. 1. *A*, HER2/neu antisense ODNs down-regulate p185 expression in a dose-dependent manner. BT474 cells were treated with the indicated concentrations of different ODNs for 4 h in the presence of Lipofectin. Western blots were performed 48 h after treatment. *Lipo*, Lipofectin alone; *HAS*, HER2/neu antisense; *HS*, HER2/neu sense; *HSC*, HER2/neu scrambled antisense. Blots were reprobed with an actin-specific antibody to control for protein loading. *B*, HER2/neu antisense ODNs induce dose-dependent growth inhibition of BT474 cells. BT474 cells were treated with Lipofectin alone (100% control), or with HER2/neu antisense (●), sense (■), or scrambled antisense (▲) ODNs in various doses in triplicate. Cells were counted 5 days after treatment. Results show means; bars, SE. *C*, sequence-specific effects of HER2/neu antisense ODNs on p185 expression—Western blot analysis. Two μg each of protein lysates from cells treated with Lipofectin with or without antisense (*HAS*), sense (*HS*), scrambled antisense (*HSC*), one-base mismatched antisense (*1MM*), or four-base mismatched antisense (*4MM*) ODNs were separated on a 7.5% SDS-PAGE and transferred onto PVDF membrane. The HER2/neu encoded p185 molecule was identified by probing with an anti-HER2/neu monoclonal antibody. The same blot was reprobed with an antiactin antibody to control for protein loading. *D*, sequence-specific effects of HER2/neu antisense ODNs on cell growth. Cells were treated in triplicate as described above and counted 5 days after treatment. Results show means; bars, SE.

replaced with regular serum containing culture medium, and the cells were incubated for an additional 5 days. At the indicated times, cells were detached from the wells with trypsin-EDTA and counted with a hemocytometer. Only viable cells based on trypan blue dye exclusion were counted. Results are the means of triplicate samples.

Flow-Cytometric Detection of Apoptosis. BT474 cells (5×10^5) were treated with 1.5 ml of ODN mixture in 60-mm culture dishes as described. After an additional incubation of 72 h, cells were detached from the culture dish and collected via centrifugation. Floating cells from the supernatant were combined with the trypsinized cells. Pooled cells were washed once with the growth medium and stained with Annexin V-FITC and propidium iodide (ApoAlert Annexin V Apoptosis Kit; Clontech), according to the manufacturer's protocol. After cells were stained in the dark for 10 min at 25°C, their fluorescence was analyzed via flow cytometry using a FACScan (Becton Dickinson).

CPP32 Assay. Cells (1×10^6) were treated in 100-mm culture dishes with 5 ml of ODN mixture as described previously. After incubating for 72 h from the initiation of the treatment, cells were detached from the culture dish and

washed once with PBS. Activation of CPP32 protease was determined by the ApoAlert CPP32 Assay kit (Clontech), according to the manufacturer's protocol. Absorbance of the DEVD-pNA cleavage product pNA at 405 nm from each sample was converted to CPP32 units using the standard curve generated by the absorbance of known amount of pNA.

Results

HER2/neu Antisense ODNs Inhibit p185 Expression and BT474 Tumor Growth in a Dose-dependent Manner. Antisense ODNs represent a potent approach to inhibiting the expression of specific genes. However, biological effects that result from antisense ODN exposure may also occur via a variety of non-antisense mechanisms (22, 23). We and others have demonstrated the biological activity of HER2/neu-specific antisense ODNs targeted to the 5' region of the HER2/neu mRNA molecule (18–21). To further characterize the specificity of HER2/neu antisense effects on BT474 breast carcinoma

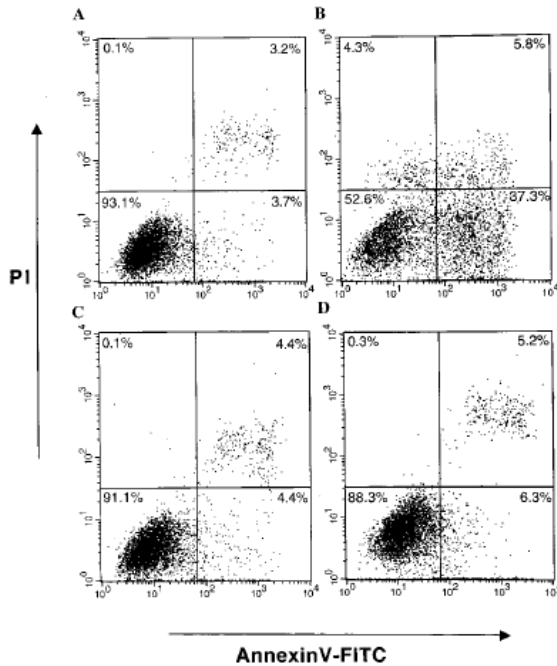


Fig. 2. HER2/neu antisense treatment induces alterations in Annexin V staining. BT474 cells treated with Lipofectin alone (A) or with HER2/neu antisense (B), HER2/neu sense (C), or HER2/neu scrambled antisense (D) ODNs were stained with Annexin V-FITC and propidium iodide after 72 h of treatment. Annexin V-FITC signal is shown on the X axis; propidium iodide (PI) signal is shown on the Y axis. Ten thousand events were collected for each sample.

growth, we have examined the dose-dependence of ODN effects on p185 expression and tumor cell growth.

As shown in Fig. 1A, treatment of BT474 breast carcinoma cells with HER2/neu-specific antisense ODNs results in dose-dependent inhibition of p185 expression. There is no effect of control sense or scrambled antisense ODNs on p185 expression. The biological effects of HER2/neu antisense ODN treatment on BT474 growth parallel the biochemical effects on p185 expression. As shown in Fig. 1B, HER2/neu-specific ODNs have modest but reproducible tumor inhibitory effects at concentrations as low as 30 nM, with more potent effects (>80% inhibition of cell growth) at 1 μ M. In contrast, control sense and scrambled antisense sequences have only minimal nonspecific effects on cell growth at concentrations \leq 1 μ M. To avoid such nonspecific toxic effects, all subsequent experiments have been performed using a 1- μ M treatment dose.

Antisense Effects on HER2/neu Expression and Tumor Cell Proliferation Are Sequence Specific. To demonstrate conclusively that the antiproliferative properties of the HER2/neu-specific antisense sequences studied here are due to Watson-Crick base pairing between the antisense molecule and its target mRNA, we examined the sequence-specificity of antisense effects. The ability of antisense ODNs and of modified antisense molecules containing one or four mismatched bases to down-regulate HER2/neu mRNA, inhibit expression of the p185 protein, and inhibit BT474 tumor cell proliferation were studied and compared to the effects of control ODNs representing sense or scrambled antisense (nine base mismatches). HER2/neu antisense treatment significantly inhibits the expression of the p185 protein after 48 h of treatment (Fig. 1C). There is little effect of control sense and scrambled antisense sequences. Examination of the effects of mismatch sequences demonstrates the stringent sequence-

specificity of the antisense effect. There is marked diminution of the activity of the one-mismatch sequence in terms of its effect on p185 protein levels, whereas the four-mismatch antisense sequence has essentially no effect on p185 expression.

The effects of these sequences on p185 protein levels correlate with their effects on BT474 tumor cell proliferation. As shown in Fig. 1D, antisense ODNs inhibit BT474 cell growth by >80%, whereas control ODNs have only a modest effect on cell growth, confirming earlier results. The one-base mismatch antisense ODN has a markedly less potent effect on BT474 cell growth, and the four-base mismatch sequence has an effect little different from those of sense and scramble control ODNs. Thus, the effects of antisense molecules on p185 expression and on tumor cell proliferation appear to be critically dependent on sequence complementarity with the HER2/neu mRNA molecule.

HER2/neu Antisense Treatment Results in Cell Death and Cell Surface Changes Associated with Apoptosis. It was observed that BT474 cell culture dishes treated with HER2/neu antisense ODNs not only contained fewer viable tumor cells, but also contained an increased fraction of dead floating cells compared with cultures treated with either Lipofectin alone or with control ODNs. It was hypothesized that some of the HER2/neu antisense effect on tumor cell growth was not simply the result of inhibition of tumor cell proliferation, but might actually be due to an antisense-mediated increase in apoptotic cell death. To address this question, the fractions of cells in various stages of apoptotic death were assessed by simultaneous Annexin V and propidium iodide staining, using flow cytometry, 72 h after treatment with antisense or control ODNs. A significant fraction of BT474 tumor cells treated with HER2/neu antisense ODNs were in the early (Fig. 2B, lower right quadrant) or late (Fig. 2B, upper right quadrant) stages of apoptotic cell death. In contrast, there was little effect of sense (Fig. 2C) or scrambled antisense (Fig. 2D) ODNs on apoptotic cell death, which was comparable to that seen in cells treated with Lipofectin alone (Fig. 2A). Cytometric analysis also revealed that HER2/neu antisense-treated cells showed evidence of nuclear condensation and an increased fraction of nuclei containing a sub-2N DNA content, an indicator of apoptotic cell death (Table 1).

HER2/neu Antisense Treatment Results in Activation of CPP32 and Degradation of Caspase Substrates. In addition to changes in cell surface phosphotyrosine expression, which is the basis for Annexin V staining, cells undergoing apoptotic death typically activate proteases (caspases) that degrade specific intracellular proteins. As shown in Fig. 3A, BT474 cells treated with HER2/neu-specific antisense ODNs demonstrated activation of the caspase CPP32, also known as caspase 3, whereas cells treated with control ODNs did not. Moreover, antisense-treated BT474 cells showed evidence of degradation of the proteins PARP and cyclin B, which are substrates of CPP32 and other proteases activated in apoptotic cells (Fig. 3B). Thus, by multiple criteria, it was demonstrated that HER2/neu antisense ODN treatment resulted in activation of apoptotic cell death pathways in BT474 breast carcinoma cell lines.

HER2/neu Antisense Treatment Inhibits Cancer Cell Growth and Activates CPP32 in Cancer Cells of Varied Histological Origin That Overexpress p185. Prior studies of HER2/neu down-regulation, using HER2/neu-specific monoclonal antibodies, have demonstrated that the growth of some tumor cell lines that over-

Table 1 Effects of HER2/neu antisense ODNs on nuclear DNA content

	BT474 cell treatment ^a			
	Lipofectin	Antisense	Sense	Scrambled antisense
Dead cells with DNA content <2N	2%	18%	2%	2%

^a DNA content determined 72 h after ODN treatment by flow cytometry.

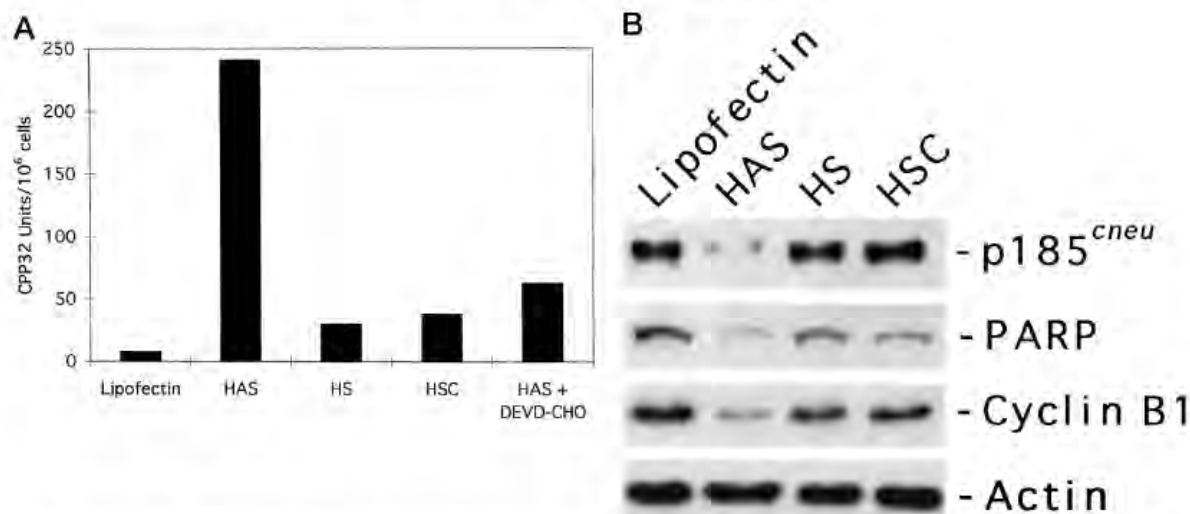


Fig. 3. Activation of the apoptosis specific protease CPP32 and disappearance of its substrates, PARP and cyclin B, after treatment with HER2/neu antisense phosphorothioate ODNs. **A**, the enzymatic activity of CPP32 in lysates of BT474 treated with Lipofectin alone or with HER2/neu antisense (HAS), sense (HS), or scrambled antisense (HSC) phosphorothioate ODNs was determined by *in vitro* cleavage of DEVD-pNA; the amount of the cleavage product pNA from each lysate was converted to units of CPP32 by a standard curve. The activation of CPP32 was specifically inhibited by addition of the CPP32 inhibitor DEVD-CHO to HER2/neu antisense-treated cell lysates (HAS + DEVD-CHO). **B**, lysates from BT474 cells 48 h after treatment with Lipofectin alone or HER2/neu antisense, HER2/neu sense, or HER2/neu scrambled antisense phosphorothioate ODNs were separated on 10% SDS-PAGE and probed with anti-p185, anti-PARP, and anti-cyclin B murine monoclonal antibodies. The blot was reprobed with an antiactin antibody as a control for protein loading.

express p185 can be inhibited by antibody exposure, whereas there is little effect of such treatment on tumors that express more modest levels of p185 (9, 24). To further define the effects of HER2/neu-specific antisense ODNs, we examined the effects of antisense treatment on tumor cell growth and activation of CPP32 in a panel of tumor cell lines of distinct histological origins that express varying levels of p185 (Fig. 4A).

The growth of several different tumor lines that overexpress p185 was inhibited by antisense treatment, whereas there was little effect on the growth of tumor cell lines, as well as a nontumorigenic diploid fibroblast line, that express more modest levels of p185 (Fig. 4B). The effects on cell growth mirrored the effects of antisense treatment in triggering apoptosis as demonstrated by CPP32 activation (Fig. 4C). These findings demonstrate that a variety of tumor cell types are sensitive to the pro-apoptotic effects resulting from antisense-mediated down-regulation of HER2/neu expression. These results also suggest that the growth inhibitory effects of HER2/neu-specific antisense ODNs result in part from triggering apoptotic cell death in cells that overexpress HER2/neu.

It is of interest that the CFPAC cell line, which only moderately overexpresses p185, was quite sensitive to the effects of HER2/neu down-regulation, whereas the MCF-7 cell line, which expresses somewhat lower levels of p185, was not affected by antisense ODN exposure. Examination of the sensitivity of these cell lines to growth inhibition after p185 down-regulation by a distinct mechanism, using p185-specific monoclonal antibodies, confirmed their relative sensitivity and resistance to HER2/neu-directed therapy.[†] This may reflect a threshold level of p185 overexpression required for sensitivity to HER2/neu down-regulation.

Discussion

The effects of a 15-base antisense ODN molecule, targeted against the 5' region of the HER2/neu molecule, on HER2/neu expression,

tumor cell proliferation, and apoptotic tumor cell death were examined. It was demonstrated that such HER2/neu-directed antisense ODNs exert sequence-specific effects on HER2/neu expression and tumor cell growth. Mismatched antisense ODNs altered at a single base are markedly less effective at down-regulating HER2/neu expression and inhibiting BT474 tumor cell growth; sequences containing four or more mismatches have minimal effects on either HER2/neu expression or tumor cell growth. The biological effects of HER2/neu-specific antisense ODNs are dose-dependent, occurring 2–3 logs below the concentrations at which nonspecific effects occur. Collectively, these results strongly support the view that antisense-mediated down-regulation of HER2/neu expression results in inhibition of BT474 tumor cell proliferation.

A number of reports of antisense effects on tumor cell proliferation have been published; in several cases it was subsequently demonstrated that non-antisense mechanisms accounted for the tumor inhibitory effects (22, 23, 25, 26). ODNs can bind growth factors (23) and their receptors in sequence non-specific fashion (25). They may also bind other cellular proteins, resulting in aptameric effects (26). The results presented here are unlikely to be due to such non-antisense mechanisms for a number of reasons, including avoidance of sequences known to cause non-antisense effects (23, 25, 26); the use of relatively short (15 base) ODNs at concentrations of 1 μ M and below; demonstration of parallel effects of antisense compounds on protein expression as well as tumor cell growth; and the use of multiple control ODNs and tumor targets that define the effect in terms of antisense sequence and target cell susceptibility.

It is interesting to compare the results presented here with prior studies of HER2/neu down-regulation using monoclonal antibodies or antisense ODNs. Monoclonal antibodies specific for p185 have been demonstrated to have inhibitory effects on the growth of some tumor lines in cell culture (9), including several of the lines tested here. In general, these antibodies appear to be exclusively active in inhibiting the growth of cancer cells that strongly overexpress HER2/neu, sug-

[†]Roh *et al.*, unpublished results.

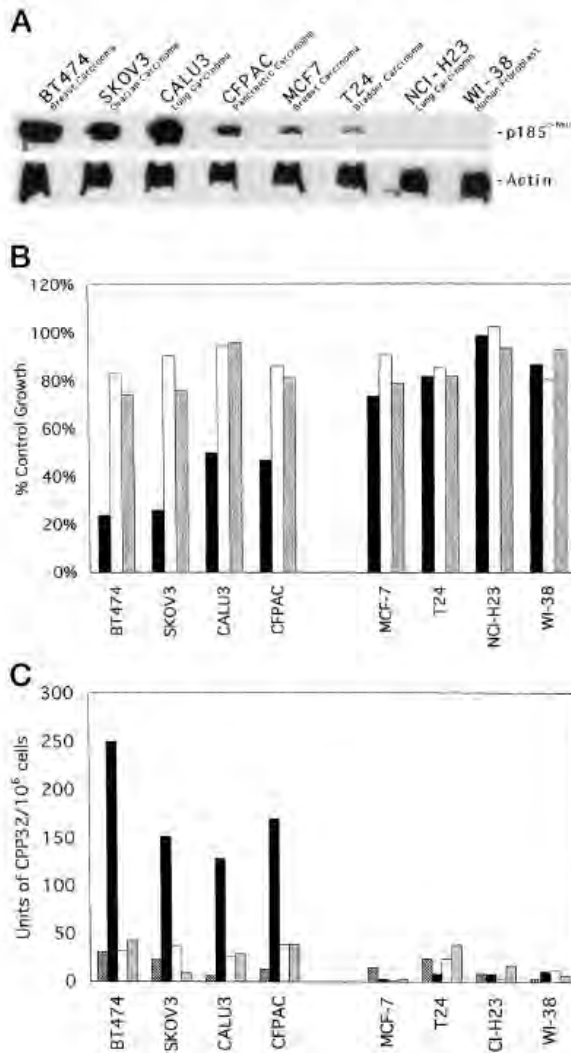


Fig. 4. HER2/neu antisense ODNs inhibit tumor growth and activate apoptosis in tumor cells of various histological origins which overexpress p185. *A*, Western blot demonstrating relative p185 expression in diverse cell lines (20 μ g protein/lane). *B*, HER2/neu antisense ODNs (filled columns) inhibit the growth of tumor cells that overexpress p185. Sense (open columns) and scrambled antisense (stippled columns) ODNs have little or no effect. *C*, HER2/neu antisense ODNs induce apoptosis in tumor cells that overexpress p185. Cells were treated with Lipofectin alone (striped columns), HER2/neu antisense ODNs (filled columns), sense ODNs (open columns), and scrambled antisense ODNs (stippled columns), and assayed for CPP32 activation after 72 h.

gesting that such tumor cells are critically dependent on p185-mediated mitogenic signaling.

Antisense compounds directed against the HER2/neu oncogene have also been shown to inhibit the growth of some breast carcinoma and ovarian carcinoma cell lines (18–21). These agents also appear to be most effective against tumor cells that overexpress HER2/neu. We have demonstrated that other tumor cell types, including lung and pancreatic adenocarcinoma cell lines, can be inhibited by HER2/neu-specific antisense ODNs. Preliminary data from our laboratory suggest that antisense ODNs directed against HER2/neu sequences are superior to monoclonal antibodies specific for p185 at both down-regulating HER2/neu expression and inhibiting the *in vitro* growth of

tumors that overexpress HER2/neu.⁴ Whether HER2/neu antisense ODNs are more potent than p185-specific monoclonal antibodies *in vivo*, and whether the combination of such agents can result in enhanced antitumor effects will require further study.

Perhaps the most intriguing finding presented here is the observation that antisense-mediated down-regulation of HER2/neu expression in tumor cell lines that overexpress HER2/neu is not simply cytostatic, but actually results in activation of apoptotic cell death mechanisms. Standard monoclonal antibodies reactive with p185 have not been shown to activate cell death pathways in tumor cells that overexpress HER2/neu (9, 12).⁴ However, multimeric antibodies directed against p185, as well as intracellular expression of a single-chain p185-specific antibody, have been shown to activate apoptotic cell death pathways in tumor cells that overexpress HER2/neu (16, 27). These prior reports suggested that factors specific to the altered anti-p185 antibodies, rather than the result of interfering with p185 expression, were critical in inducing apoptosis (16, 27). The studies presented here demonstrate that down-regulation of p185 by a non-antibody mechanism, using antisense ODNs, can also trigger cell death in cancer cells that overexpress the p185 protein. Thus, cancer cells that overexpress HER2/neu are dependent on p185 for cell survival as well as proliferation, and strategies that interfere with p185 expression or function can induce apoptotic cell death.

The molecular pathways by which HER2/neu overexpression interacts with cell death/survival signaling have not been defined. Several of the cell lines studied here, including BT474, have known p53 mutations (28). Thus, the effects of HER2/neu-specific antisense ODNs in activating apoptosis must be p53-independent. It has been demonstrated previously that increased HER2/neu expression resulting from gene transfection can increase Bcl-2 levels (29). Furthermore, p185 physically associates with FAS ligand on tumor cell surfaces and may interfere with FAS signaling by this mechanism (30). Thus, it is possible that modulation of Bcl-2 and/or FAS ligand function links HER2/neu overexpression to cell survival signaling. Regardless of the underlying molecular pathways involved, the triggering of apoptotic cell death following p185 down-regulation may prove a useful approach to the therapy of tumors that overexpress HER2/neu.

HER2/neu overexpression has also been shown to play a role in resistance to the lethal effects of tumor necrosis factor (24) and chemotherapy (31, 32). Because these both are activators of apoptotic cell death, this may provide yet another example in which the level of HER2/neu expression contributes to altering the balance between tumor cell survival and cell death signals. We have demonstrated that antisense-mediated down-regulation of HER2/neu expression can, by itself, shift this balance in favor of tumor destruction. Antisense compounds are beginning to enter clinical trials in patients with cancer and other diseases (33–35). The ability of HER2/neu antisense ODNs to inhibit tumor growth, using *in vivo* xenograft models, and to potentiate the cytotoxic effects of other modalities, such as chemotherapy, currently is under investigation (36).

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Appendix 3

Roh, H., Pippin, J.A., Green, D.W., Boswell, C.B., Hirose, C., Mokadam, N.A. and Drebin, J.A. (2000) HER2/neu antisense targeting of human breast carcinoma. Oncogene 19: 6138-6143.



HER2/*neu* antisense targeting of human breast carcinoma

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Overexpression of the HER2/*neu* oncogene is observed in approximately 30% of human breast carcinoma specimens. HER2/*neu* overexpression is a negative prognostic factor in breast cancer patients. Cancer cells that overexpress HER2/*neu* may also be less sensitive to chemotherapy. In order to further define mechanisms by which HER2/*neu* overexpression drives neoplastic cell growth and chemoresistance, antisense oligonucleotides (ODNs) have been utilized to selectively down-regulate HER2/*neu* expression in human breast cancer cells. Such antisense ODNs suppress HER2/*neu* mRNA and protein levels in a dose-dependent, sequence-specific manner. Down-regulation of HER2/*neu* expression in HER2/*neu* overexpressing breast cancer cells inhibits cell cycle progression in G₀/G₁ and results in apoptotic cell death. In tissue culture studies, combined treatment of HER2/*neu* overexpressing breast cancer cells with HER2/*neu* antisense ODNs and conventional chemotherapeutic agents results in synergistic inhibition of cancer cell growth and activation of apoptotic cell death mechanisms. These studies have been extended to demonstrate synergistic antitumor effects following systemic treatment with antisense ODNs plus doxorubicin in nude mice bearing human breast carcinoma xenografts. Collectively these findings demonstrate that HER2/*neu* overexpression stimulates anti-apoptotic cell survival mechanisms and suggest that HER2/*neu* antisense ODNs may be of use in cancer therapeutics. *Oncogene* (2000) 19, 6138–6143.

Keywords: HER2/*neu*; breast cancer; antisense oligonucleotides; oncogenes; apoptosis; cell cycle

Introduction

Overexpression of the HER2/*neu* oncogene (also called erbB2), and its protein product, p185^{HER2/*neu*}, are seen in approximately 30% of human adenocarcinomas of the breast, as well as in a significant fraction of adenocarcinomas of the lung, ovary and pancreas (Menard *et al.*, 2000). Overexpression of HER2/*neu* is a negative prognostic factor following tumor resection (Slamon *et al.*, 1987; Berchuck *et al.*, 1990; Kern *et al.*, 1990) and may be associated with increased resistance to cancer chemotherapy (Baselga *et al.*, 1997; Pegram *et al.*, 1997). The mechanism(s) by which HER2/*neu* overexpression stimulates neoplastic cell growth and renders cells chemoresistant have not been completely defined. The recent evolution of therapeutic approaches to targeting HER2/*neu* overexpressing tumors in

patients with metastatic breast cancer highlights the importance of further characterizing the role of HER2/*neu* overexpression in cancer biology.

We initially demonstrated the direct growth-inhibitory effects of monoclonal antibodies targeting p185^{HER2/*neu*} on the surface of HER2/*neu* transformed cells (Drebin *et al.*, 1984, 1985, 1986, 1988; Katsumata *et al.*, 1995). Subsequent studies from multiple laboratories have verified these observations and have led to the development of monoclonal antibodies targeting the human HER2/*neu* product (Hudziak *et al.*, 1989; Harwirth *et al.*, 1993; Stancovski *et al.*, 1991). One such antibody (HERCEPTIN[®]) has demonstrated significant clinical activity in patients with breast cancer—particularly when administered in conjunction with conventional chemotherapeutic agents (Baselga *et al.*, 1996; Pegram *et al.*, 1998).

Numerous other approaches to inhibiting cancer cell growth by down-regulating HER2/*neu* expression or inhibiting p185^{HER2/*neu*} tyrosine kinase activity have shown promise in the laboratory. Several laboratories, including our own, have demonstrated that antisense oligonucleotides (ODNs) can specifically down-regulate HER2/*neu* mRNA expression in cancer cells with resulting inhibitory effects on the proliferation of cancer cells than overexpress HER2/*neu* (Roh *et al.*, 1999, 2000). Antisense ODNs bind to complementary mRNA sequences through Watson-Crick base pairing, and can activate the degradation of the complementary mRNA by the endogenous nuclease RNAaseH (Green *et al.*, 2000). Antisense ODNs targeting HER2/*neu* appear to be more potent than monoclonal antibodies at inhibiting HER2/*neu*-stimulated neoplastic proliferation *in vitro* (unpublished results). We have utilized antisense approaches to down-regulate HER2/*neu* expression in studying the role of HER2/*neu* overexpression in breast cancer on apoptotic cell death mechanisms and sensitivity to chemotherapeutic agents.

*Antisense ODNs specifically inhibit the expression of HER2/*neu* and inhibit BT474 breast carcinoma cell growth*

Antisense ODNs can inhibit HER2/*neu* gene expression in human breast cancer cells, resulting in significant dose-dependent suppression of p185^{HER2/*neu*} levels and a resultant inhibition of cancer cell growth (Roh *et al.*, 1998, 2000). The result of a representative experiment, demonstrating antisense suppression of p185^{HER2/*neu*} expression, is shown in Figure 1a. Such antisense-mediated inhibition of the HER2/*neu* gene product has no effect on expression of other proteins, including actin (Figure 1a) or the p185^{HER2/*neu*}-related protein products of the genes ErbB-1, ErbB-3 and ErbB-4

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a.



b.

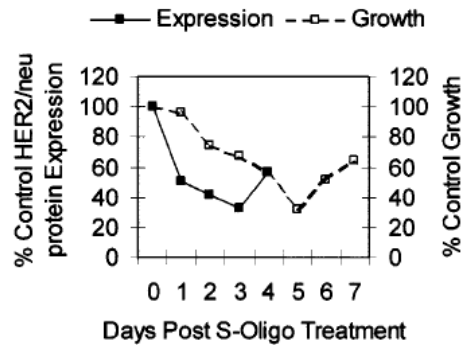


Figure 1 Antisense-mediated down-regulation of HER2/neu expression (a) Down-regulation of p185^{HER2/neu}. Replicate BT474 breast carcinoma cultures were treated with phosphorothioate modified ODNs (1 μ M) and Western blots performed on protein lysates after 24 h, as described (Roh *et al.*, 1998). Lipofectin = 10 μ g/ml Lipofectin alone; HER2 Antisense = 15 base HER2/neu antisense sequence; HER2/neu Scramble = 15 base ODN consisting of scrambled antisense sequence nucleotides. (b) Kinetics of p185^{HER2/neu} and cancer cell growth inhibition following HER2/neu antisense ODN treatment. Replicate samples of BT474 cells were treated with HER2/neu antisense ODNs or Lipofectin on day 0. On the days indicated cell lysates were prepared for Western blot analysis and triplicate samples were counted using a hemocytometer. Western blots were quantitated by densitometry. Levels of p185^{HER2/neu} and cell numbers following antisense treatment are expressed as a percentage of the levels/numbers observed in BT474 cultures treated with Lipofectin alone

(unpublished results). Furthermore, there is an excellent correlation between the effects of antisense ODNs on p185^{HER2/neu} expression and biological effects on BT474 breast cancer cell growth. As shown in time course studies (Figure 1b), down-regulation of p185^{HER2/neu} slightly precedes and generally parallels the biological effects of antisense ODNs in inhibiting the proliferation of BT474 breast carcinoma cells. As p185^{HER2/neu} levels recover, there is a corresponding recovery of cell proliferation.

The antiproliferative effects of HER2/neu antisense ODNs are sequence-specific as well (Roh *et al.*, 2000). When BT474 breast carcinoma cells are treated with HER2/neu antisense ODNs, there is a significant inhibition of HER2/neu expression at the mRNA and protein levels, and a corresponding inhibition of cell growth (Figure 2). When ODNs mismatched at a single base (out of 15 bases) are substituted for the intact antisense sequence, there is a moderate reduction in activity both in inhibiting HER2/neu expression and in inhibiting BT474 cell growth. A four base mismatched sequence has almost no activity at either inhibiting HER2/neu expression or cancer cell growth. Thus the ability of HER2/neu-specific antisense ODNs to inhibit HER2/neu expression, and to interfere with cancer cell growth, are both target-specific and sequence-specific.

The antiproliferative effects of HER2/neu antisense ODNs appear to be limited to cancer cells that overexpress HER2/neu. As shown in Table 1, the proliferation of several breast cancer cell lines that overexpress HER2/neu is inhibited by HER2/neu antisense ODN treatment. In contrast, the proliferation of cells with more moderate levels of HER2/neu expression is not significantly altered by HER2/neu antisense ODN treatment. Similar results have been obtained in studies on high and low HER2/neu expressing cancer cells of other histologic types (Roh *et al.*, 2000). It is important to note that all of the cell

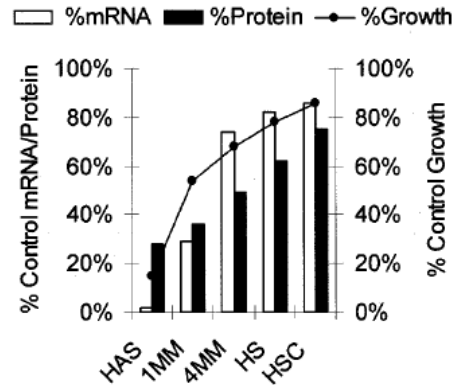


Figure 2 Sequence specificity of HER2/neu antisense effects. Replicate BT474 cultures were treated with phosphorothioate ODNs as indicated at 1 μ M in the presence of 10 μ g/ml Lipofectin for 24 h. Cell lysates were analysed for HER2/neu RNA and protein levels by Northern and Western blotting. Blots were quantitated by densitometry. Cell growth was determined by counting triplicate samples. Results are normalized to levels seen in cells treated with Lipofectin alone. HAS = HER2/neu antisense ODN; 1MM = one base mismatched HER2/neu antisense ODN; 4MM = four base mismatched HER2/neu antisense ODN; HS = HER2/neu sense sequence ODN; HSC = HER2/neu scrambled sequence ODN. Similar results were presented in (Roh *et al.*, 2000)

lines, regardless of baseline HER2/neu expression levels, undergo down-regulation of HER2/neu expression in response to HER2/neu antisense ODN treatment (Roh *et al.*, 1998, data not shown). These results suggest that cancer cells that overexpress HER2/neu require continuous high level expression of p185^{HER2/neu}. In contrast, cancer cells that express lower levels of HER2/neu have presumably undergone neoplastic transformation by distinct molecular mechanisms, and

thus are not sensitive to antisense-mediated down-regulation of p185^{HER2/neu}.

Mechanisms of growth inhibition following antisense-mediated down-regulation of HER2/neu expression

The ability of antisense ODNs to specifically down-regulate HER2/neu expression has been utilized to investigate the mechanisms by which HER2/neu overexpression facilitates neoplastic cell growth. As shown in Figure 3, down-regulation of p185^{HER2/neu} using antisense ODNs results in the accumulation of BT474 cells in the G0/G1 phase of the cell cycle. There is a corresponding decrease in cells in the S and G2/M phases of the cell cycle. Similar results are obtained following antisense ODN treatment of the distinct HER2/neu overexpressing breast cancer cell line SKBR3 (Figure 3). These results suggests that HER2/neu overexpression in breast cancer cells alters cell cycle regulatory mechanisms controlling G1 progression or the G1/S transition.

In addition to inhibiting cell cycle progression, antisense-mediated down-regulation of HER2/neu expression in HER2/neu overexpressing breast cancer cells results in activation of apoptotic cell death mechanisms (Roh et al., 2000). As shown in Figure 4, antisense treatment of HER2/neu overexpressing BT474 and SKBR3 cells results in activation of CPP32

(caspase 3), a final common effector of apoptotic cell death (Webb et al., 1997). Treatment with a control scrambled sequence ODN has no such effect on CPP32 activity. There is no effect of HER2/neu antisense ODN treatment on CPP32 activity in the MCF7 breast cancer cell line, which expresses lower levels of HER2/neu (Figure 4). Thus HER2/neu overexpression in breast cancer cells appears to act as an anti-apoptotic cell survival factor in cancer cells that overexpress this oncogene.

Synergistic effects of HER2/neu antisense ODNs and conventional chemotherapeutic agents

The ability of HER2/neu overexpression to inhibit apoptotic cell death mechanisms suggests that it might also interfere with the activation of apoptotic cell death in response to treatment with cancer chemotherapeutic agents. Such a mechanism might be responsible for the marked resistance to chemotherapy seen in cancer cells that overexpress HER2/neu (Baselga et al., 1997; Pegram et al., 1997). In order to test this

Table 1 Expression of p185^{HER2/neu} and antisense-mediated inhibition of cell proliferation in breast cancer cell lines

Cell line	p185 ^{HER2/neu} expression	Antisense ODN inhibition of proliferation
BT474	++++	Yes
SKBR3	++++	Yes
T47D	++	Minimal
MCF7	++	No
BT549	+	No
BT20	+	No
MDA468	±	No

Summary of multiple experiments examining effects of HER2/neu antisense ODNs on the growth of human breast carcinoma cell lines

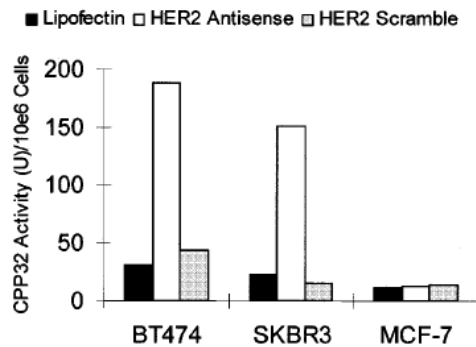


Figure 4 Down-regulation of HER2/neu expression activates CPP32 in HER2/neu overexpressing breast cancer cells. Cells were treated with ODNs (1 μM) or Lipofectin alone and CPP32 activity was determined as described (Roh et al., 2000). Similar results were presented in Roh et al. (2000)

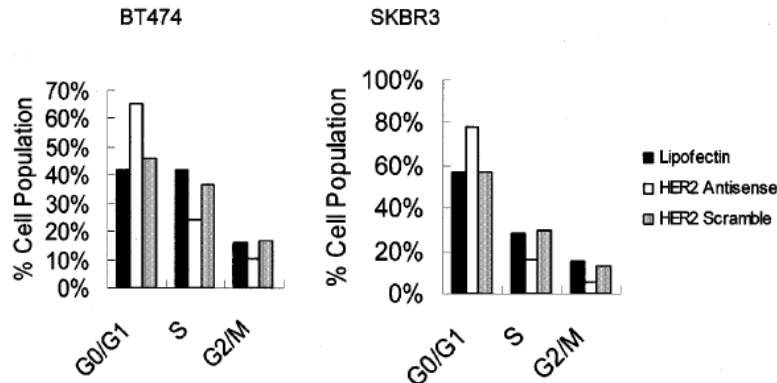


Figure 3 Effect of HER2/neu down-regulation on cell cycle progression in HER2/neu overexpressing breast cancer cell lines. Cells were treated with ODNs or Lipofectin alone for 24 h and then were pulsed with Bromodeoxyuridine (BrdU) for 30 min. Cells were harvested, fixed and stained with fluorescein-labeled anti-BrdU antibody in the presence of propidium iodide. Dual channel cytometry was used to determine cell cycle distribution

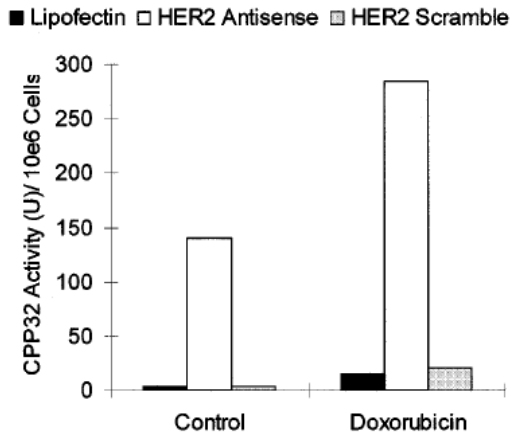


Figure 5 Synergistic activation of CPP32 activity in BT474 cells treated with HER2/neu antisense ODNs and doxorubicin. BT474 cells were treated with ODNs (0.3 μ M) \pm doxorubicin (0.2 μ M). CPP32 activity was determined as described (Roh et al., 2000). Similar results were presented in Roh et al. (1999)

hypothesis, we investigated the ability of moderate doses of HER2/neu antisense ODNs in combination with moderate doses of the chemotherapeutic agent doxorubicin to activate apoptotic cell death, as measured by CPP32 activation (Roh et al., 1999). As shown in Figure 5, treatment with either antisense ODNs (0.3 μ M) or doxorubicin (0.2 μ M) moderately activates CPP32. However, when the two agents are combined there is a greater than additive activation of CPP32, suggesting a synergistic effect on cell death. There is a similar synergistic effect when inhibition of cell proliferation is examined (Roh et al., 1999). These findings support the possibility that HER2/neu overexpression renders cancer cells resistant to chemotherapeutic agents by altering the balance of cell survival/cell death signaling.

In vivo synergy of antisense ODNs and doxorubicin in BT474 human breast carcinoma xenografts

Antisense ODNs have entered clinical trials in a variety of human disorders, most prominently cancer (O'Dwyer et al., 1999; Nemunaitis et al., 1999; Waters et al., 2000). Based on the synergistic activity of HER2/neu antisense ODNs and doxorubicin in inhibiting BT474 cell proliferation and activating apoptotic cell death mechanisms *in vitro*, we were interested in examining the ability of systemic treatment with HER2/neu antisense ODNs to inhibit the growth of BT474 tumor xenografts in nude mice (Roh et al., 1999). As shown in Figure 6, treatment with HER2/neu antisense ODNs results in significant inhibition of BT474 tumor growth; a scrambled sequence control ODN has no such effects. At the doses utilized, doxorubicin has no effect on BT474 tumor growth. Most strikingly, the combination treatment using HER2/neu antisense ODNs and doxorubicin results in enhanced antitumor effects, with complete resolution of tumors in some treated animals (Roh et al., 1999). Thus HER2/neu antisense ODNs can synergize with conventional chemotherapeutic agents in mediating antitumor effects *in vivo*.

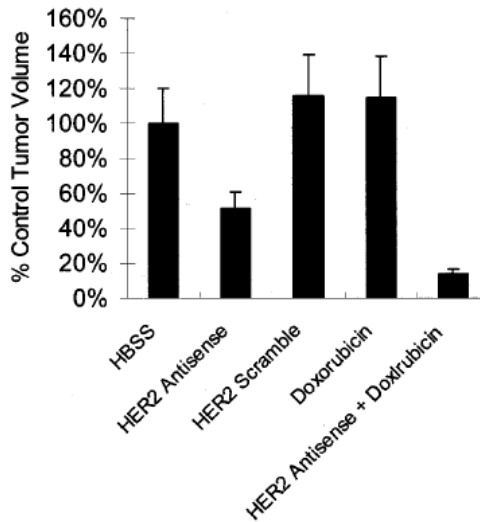


Figure 6 Synergistic inhibition of BT474 xenografts by the combination of HER2/neu antisense ODNs and doxorubicin. Nude mice received BT474 cell implants in the mid-dorsum on day 0. When small tumor nodules were palpable (day 10), mice were randomized into groups of five animals and treated as indicated. Tumor size was measured on day 30 and is expressed as the product of tumor length and width. Results represent means \pm s.e. of the mean. HBSS=daily injections with Hanks balanced salt solution days 10–23; HER2 Antisense=10 mg/kg daily, days 10–23; HER2 Scramble=10 mg/kg daily, days 10–23; Doxorubicin=10 mg/kg, single dose, day 10; HER2 Antisense+Doxorubicin=antisense ODNs 10 mg/kg, days 10–23 plus doxorubicin 10 mg/kg single dose, day 10. Similar results were presented in Roh et al. (1999)

Discussion

In recent years the HER2/neu oncogene has received a great deal of attention as a potential therapeutic target in breast cancer. HER2/neu is an attractive target for molecular therapeutics both because it is overexpressed in a significant fraction of human tumors, and because its overexpression appears to play a critical role in the biological behavior of the cancer cell. Thus interference with HER2/neu expression or function can inhibit the neoplastic growth of cancer cells. The results of clinical trials using monoclonal antibody approaches to targeting HER2/neu in human breast cancer represent one of the first examples of the successful translation of modern molecular biological research into clinical cancer treatment (Drebin et al., 1984, 1985, 1986, 1988; Katsumata et al., 1995; Hudziak et al., 1989; Stancovski et al., 1991; Baselga et al., 1996; Pegram et al., 1998; Roh et al., 1998).

However, monoclonal antibodies may not be the ideal mechanism for therapeutic manipulation of HER2/neu signaling pathways. Antibodies only react with cell surface p185HER2/neu; elevated levels of intracellular p185^{HER2/neu} may deliver mitogenic signals prior to display on the cell membrane (Graus-Porta et al., 1995). Furthermore, soluble antigenic p185^{HER2/neu} fragments shed from the tumor cell membrane may interfere with antibody binding to p185^{HER2/neu} on the tumor cell surface. Such shed tumor antigens have been demonstrated to interfere with the antiproliferative

effects of p185^{HER2/neu}-specific monoclonal antibodies *in vitro* (Brodowicz *et al.*, 1997), and the presence of p185^{HER2/neu} antigenic fragments in patient serum appears to be a predictor of a poor clinical response to p185^{HER2/neu} monoclonal antibody therapy (Baselga *et al.*, 1996). Thus the exploration of distinct approaches to targeting HER2/neu in cancer therapy appears warranted.

Antisense oligonucleotides represent a potentially powerful method of selectively inhibiting gene expression (Green *et al.*, 2000; O'Dwyer, 1999; Nemunaitis *et al.*, 1999; Waters *et al.*, 2000). However, the study of antisense compounds has been plagued by a number of factors resulting in flawed or incorrect experimental results (Stein, 1995). ODNs, and particularly the more stable phosphorothioate derivatives used in most experiments, are highly charged molecules that, if utilized at high concentrations (>1 μ M), may bind to cellular proteins and interfere with biological functions via non-antisense mechanisms (Webb *et al.*, 1997; Stein, 1995). Furthermore, high concentrations of ODNs may be directly toxic to cells. Again, biological activity attributed to such antisense ODNs may not be the result of the specific inhibition of a target gene.

The experimental findings in the HER2/neu antisense studies reviewed here are unlikely to be the result of such non-antisense artifacts. It has been demonstrated that the HER2/neu antisense ODN utilized exerts dose-dependent, sequence-specific effects on HER2/neu expression. The anticancer effects of the antisense ODN molecule closely parallel effects on gene expression, and occur with a similar time course. Furthermore, experiments have been conducted at ODN concentrations of 1 μ M and below to minimize non-specific toxicity and multiple control ODNs have been utilized to confirm the specificity of antisense effects. Finally, the selective effects of HER2/neu-specific antisense ODNs in inhibiting only the growth of cancer cells that overexpress HER2/neu are quite similar to the results obtained when monoclonal antibodies are utilized to target HER2/neu in such cells (Shepard *et al.*, 1991). Thus it appears that antisense ODNs represent a distinct and potent mechanism for targeting HER2/neu in breast cancer cells that overexpress this oncogene. A comparison of the activities of HER2/neu-specific antisense ODNs and p185^{HER2/neu}-specific monoclonal antibodies is presented in Table 2.

In addition to cytostatic effects, antisense-mediated down-regulation of HER2/neu expression results in the activation of apoptotic cell death mechanisms in cancer cells that overexpress HER2/neu. Exposure to some, but not all, p185^{HER2/neu}-specific monoclonal antibodies also results in apoptotic cell death (Ghetie *et al.*, 1997; Kita *et al.*, 1996). Thus, cancer cells that overexpress HER2/neu are dependent on p185^{HER2/neu} for cell survival as well as cell proliferation. The molecular mechanisms by which HER2/neu overexpression alters cell survival/cell death signaling have not been completely defined, but the elevated expression of p185^{HER2/neu} has been linked to two key regulators of apoptotic cell death: Bcl2 and Fas ligand (Kumar *et al.*, 1996; Shen and Novak, 1997).

The cytotoxic effects of most cancer chemotherapeutic agents result from the activation of apoptotic cell death mechanisms following chemotherapy-induced

Table 2 Comparison of HER2/neu antisense ODNs and p185^{HER2/neu}-specific monoclonal antibodies

Pharmacologic property	Antisense ODN	Antibody
Inhibition of cell growth specific for		
HER2/neu overexpressing cancer cells	Yes	Yes
G1 arrest	Yes	Yes
Apoptosis	Yes	Some
Synergy with chemotherapy	Yes	Yes
Immune activation	No	Yes
Inhibited by soluble p185 ^{HER2/neu}	No	Yes
Activity <i>in vivo</i> – animal models	Yes	Yes
Activity <i>in vivo</i> – clinical trials	?	Yes

DNA damage. Previous studies have suggested that HER2/neu overexpression facilitates DNA repair mechanisms, and that down-regulation of HER2/neu may reverse these effects (Pietras *et al.*, 1998; You *et al.*, 1998). It has been demonstrated here that antisense-mediated down-regulation of HER2/neu expression can result in synergistic activation of apoptotic cell death pathways in combination with conventional chemotherapeutic agents. Such synergy might result from effects of HER2/neu down-regulation on DNA repair mechanisms, with resulting increased chemotherapy-mediated toxicity (You *et al.*, 1998) or may reflect direct effects of enhancing apoptotic cell death mechanisms resulting from the down-regulation of HER2/neu (Roh *et al.*, 2000). It is possible that both of these mechanisms, and possibly additional mechanisms as yet undefined, play a role in the synergistic effects of HER2/neu down-regulation and conventional chemotherapeutic agents. The characterization of such mechanisms is an important area for additional study and may lead to further improvements in cancer therapy.

It has been demonstrated here that HER2/neu antisense ODNs, administered systemically, can synergize with doxorubicin in the experimental therapy of human breast carcinoma xenografts in nude mice (Roh *et al.*, 1999). Current studies in our laboratory are extending these results, investigating distinct antisense compounds and the use of antisense ODNs in conjunction with other chemotherapeutic agents as well as in combination with p185^{HER2/neu}-specific monoclonal antibodies. Antisense compounds targeting several distinct oncogenes and growth stimulatory genes have reached phase I and II clinical trials, with promising evidence of target gene down-regulation and therapeutic efficacy in some treated patients (O'Dwyer *et al.*, 1999; Nemunaitis *et al.*, 1999; Waters *et al.*, 2000). It is possible that HER2/neu-specific antisense compounds may eventually play a role in the management of breast cancer patients whose tumors overexpress this oncogene.

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