Cancer Chemother Pharmacol (1997) 40: 60–64

ORIGINAL ARTICLE

Won G. An · Rodney C. Schnur · Len Neckers
Mikhail V. Blagosklonny

Depletion of p185^{erbB2}, Raf-1 and mutant p53 proteins by geldanamycin derivatives correlates with antiproliferative activity

Received: 6 June 1996 / Accepted: 28 September 1996

Abstract *Purpose*: Recently, it has been shown that geldanamycin (GA), a benzoquinone ansamycin, is able to deplete mutant p53, p185^{erbB2} and Raf-1 proteins in cancer cells. However, the relationship between these activities of GA and its antiproliferative activity is not clear. Here we investigated the effects of 28 GA derivatives in SKBr3, a human breast cancer cell line. *Methods*: We performed Western blot analysis of Raf-1, p185^{erbB2} and mutant p53 proteins following drug treatment and correlated these findings with the cytotoxicity of the various GA derivatives. *Results*: We found that downregulation of Raf-1, p185^{erbB2} and mutant p53 proteins was correlated. Thus, a drug that was active against one oncoprotein was equally active against the two others. Inactive derivatives were identified by their inability to downregulate these oncoproteins, even at a high dose (2 μM). These inactive drugs also had no or minimal antiproliferative activity ($IC_{50} > 3$ μM). All other analogs (at a concentration of 2 μM) downregulated p53, p185^{erbB2}, and Raf-1, and also displayed cytotoxicity (IC_{50} in the range 6–600 nM). This category of drugs was further divided into more- and less-active agents by testing at lower doses (40 nM). The drugs that remained active against their molecular targets had an IC_{50} for antiproliferative activity of less than 40 nM. Maximal effects on mutant p53, p185^{erbB2} and Raf-1 were observed at doses that were 4–5 times greater than the cytotoxic IC_{50}. *Conclusions*: These findings suggest that GA and its derivatives are cytostatic/cytotoxic at concentrations that also downregulate Raf-1, p185^{erbB2} and mutant p53, and raise the possibility that depletion of these proteins and the antiproliferative activities of GA have a common mechanism.

Key words Geldanamycin · p185^{erbB2} · Mutant p53 · Raf-1 · Cancer cells

W.G. An · L. Neckers · M.V. Blagosklonny (✉)
Clinical Pharmacology Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA
Fax (301) 435-8036

R.C. Schnur
Pfizer Central Research, Groton, Connecticut 06340, USA

Introduction

Pharmacologic targeting of specific signal transduction pathways related to oncogenic transformation is a promising approach in cancer treatment. Although current approaches favor specific targeting of individual gene products, cancer cells have multiple genetic alterations involving oncogenes and tumor suppressor genes. Therefore, in order to reverse the transformed phenotype, it would be desirable to identify an agent capable of affecting multiple targets in the signal transduction pathway.

The wild-type p53 gene product is a tumor suppressor protein, and is one of the most commonly mutated proteins found in cancer cells [1, 12, 19]. Besides losing their tumor suppressor function, mutated p53 proteins may also have a dominant positive [7, 8, 11], as well as a dominant negative, role in fostering tumorigenesis [18, 25]. P185^{erbB2} (erbB2) overexpression is common in breast and ovarian cancer, and is associated with poor prognosis and drug resistance (for review see reference 13). Several approaches including monoclonal anti-erbB2 antibodies [10] and erbB2 antisense oligonucleotides [28] have been proposed as anticancer agents. Raf-1 is a central component of the signal transduction pathway utilized by many growth factors via p21^{ras} [14]. Since p21^{ras}-activating mutations are involved in various malignancies, members of this signal transduction pathway also can be considered as targets for anticancer chemotherapy [20].

Geldanamycin (GA), an antibiotic of the benzoquinone ansamycin class, has been shown to bind specifically to heat shock protein 90 (HSP90) and its homolog

localized to the endoplasmic reticulum, GRP94 [6, 17, 26]. This drug is able to destabilize several HSP90-associated proteins, including multiple oncogene and protooncogene products. Rapid degradation of Raf-1 and erbB2 proteins following GA treatment follows the dissociation of these proteins from HSP90 and GRP94, respectively [6, 14, 16, 17, 23]. Mutant but not wt p53 binds members of the HSP90 family in vivo and in vitro [5, 24]. Targeting of HSP90 by GA also results in alteration of the conformation of mutant p53 with subsequent reduction in its stability and steady-state level [4, 5].

However, GA may exert other effects on cells [2], and its cytotoxicity could be unrelated to the targeting of these oncoproteins. In order to determine the relationship between the cytotoxicity of GA and its ability to downregulate selected oncoproteins, we compared 28 derivatives of GA in this regard. The human breast cancer cell line, SKBr3, was used for investigation since these cells express high levels of erbB2, detectable Raf-1, and mutant p53 (see references 4 and 6). We found a correlation between the ability of the various GA derivatives to destabilize oncoproteins and their cytotoxicity. Thus, we propose that there is a common mechanism responsible both for depletion of mutant p53, erbB2 and Raf-1 proteins, and for the antiproliferative activity of GA and its derivatives.

Materials and methods

Materials

GA was obtained from the Developmental Therapeutics Program, NCI. Geldanamycin and dihydrogeldanamycin derivatives were obtained from Pfizer Central Research and have been described previously [21, 22]. These analogs have been tested for their ability to inhibit erbB2 autophosphorylation in cell culture [21, 22]. Modifications to the 17–19 positions of the quinone ring have revealed a broad structure – activity relationship in vitro. Optimum substitutions at the 17 position of ansamycins have identified small alkylamino groups unfunctionalized or bearing hydroxyl or amino groups as the best inhibitors. 17, 18-Bicyclic and tricyclic ring quinones are similarly active. Modifications at the 19 position are not tolerated [21, 22]. All drugs were dissolved in DMSO as 5 m*M* stock solutions.

Cell culture

SKBr3, a human breast cancer cell line, was obtained from the American Type Culture Collection (Rockville, Md.). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 m*M* L-glutamine.

Western blotting

Cells were lysed in TNES buffer as previously described [4]. The protein concentration was determined with the BCA reagent (Pierce, Rockford, Ill.). The protein (25 μg) was subjected to electrophoresis on 8% polyacrylamide gels (for p53 and Raf-1 determination), transferred to nitrocellulose by electroblotting, and probed with anti-p53 (pAb-2, clone 1801, Oncogene Science, Cambridge, Mass.), or anti-Raf-1 (Santa Cruz, Santa Cruz, Cal.) antibodies, as previously described [4, 23]. For erbB2 determination, lysates were subjected to electrophoresis on 7% polyacrylamide gels, transferred to nitrocellulose by wet electroblotting overnight, and probed with anti-erbB2 antibody (Oncogene Science). Protein band intensities were quantified by computerized densitometry using image analysis software. Equal loading was confirmed by HSP90 Western blot analysis and by Coomasie Blue staining of the membrane.

Antiproliferative (cytotoxic/cytostatic) assay.

Cells were seeded in 96-well flat-bottomed plates (Costar) at a density of 3000/well in 0.1 ml DMEM with 10% FBS. Next day 0.1 ml of medium containing drugs was added to triplicate wells. MTT assays assessing viable cell numbers were performed after 3 days, as described previously [3].

Results

Raf-1, p53 and erbB2 downregulation

As had been previously shown, GA was able to decrease steady-state levels of Raf-1 and mutant p53 proteins in SKBr3 cells. In comparing the abilities of the different GA derivatives to downregulate these proteins, a correlation was found between the ability of the drugs to decrease the levels of mutant p53 and Raf-1 after 16 h treatment (Fig. 1A). Although downregulation of Raf-1 protein was more pronounced than that of p53 protein, the five compounds that failed to downregulate Raf-1 protein also showed no activity against p53 (Fig. 1A). The same five compounds were also inactive against erbB2 protein (Fig. 1B), whereas a perfect match was observed with other drugs in their ability to downregulate all three proteins (Fig. 1B). Thus active drugs were similarly active against all three molecular targets.

Effects on Raf-1, p53 and erbB2 correlate with antiproliferative activity

Antiproliferative activity of all compounds was determined using an MTT test. In this assay, GA had an IC_{50} of 7 n*M*. The IC_{50} of the five inactive drugs exceeded 3 μ*M*, whereas the IC_{50} of the remaining active drugs ranged from 6 to 600 n*M* (Fig. 2). The maximal antiproliferative effect of the active drugs was achieved at a concentration five fold greater than their IC_{50} values, and a further increase in drug concentration did not exert any additional antiproliferative effect (data not shown).

Despite 100-fold differences in their cytotoxicity IC_{50} values, all active drugs appeared to be similarly effective as anti-Raf-1 agents when tested at a concentration of 2 μ*M* (Fig. 2A). Since this concentration far exceeded the IC_{50} of these compounds, we also determined drug effects on Raf-1 downregulation at lower drug concentrations. At a dose of 40 n*M*, compounds with a cytotoxicity IC_{50} below 40 n*M* still downregulated Raf-1. In contrast, compounds with a cytotoxicity IC_{50}

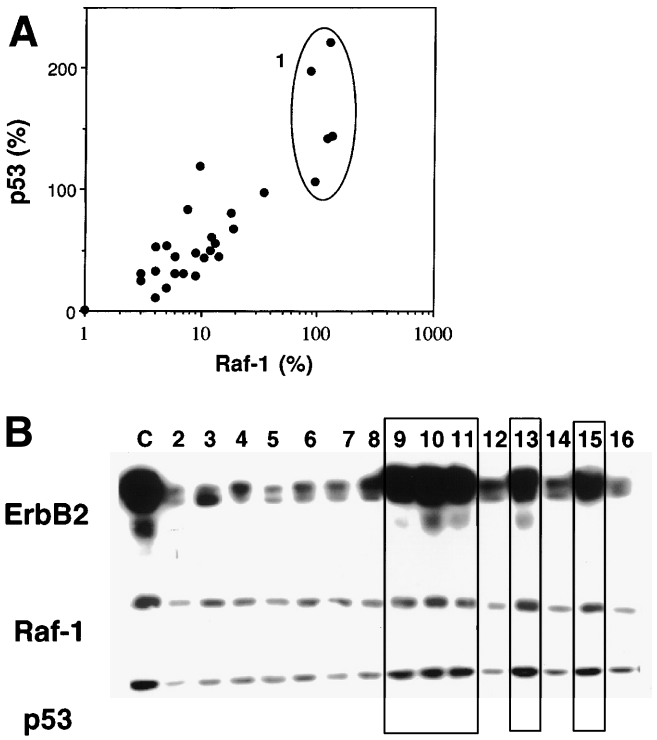

Fig. 1A,B Downregulation of Raf-1, mutant p53 and erbB2. **A** Correlation between effects of GA derivatives on p53 and Raf-1 proteins. SKBr3 cells were incubated with 2 μ*M* of each drug overnight (16 h). Cells were lysed and p53 and Raf-1 protein levels were measured as described in Methods. Levels of mutant p53 and Raf-1 are expressed as percent of control (untreated cells) and are plotted against each other. Each point on the graph represents an individual drug. The group of inactive drugs is outlined (group 1). **B** Effects of some active and inactive GA derivatives on erbB2, Raf-1 and mutant p53 levels. SKBr3 cells were incubated with 2 μ*M* of each drug for 8 h and Western blots were performed as described in Methods (*C* control, *boxed lanes* (9, 10, 11, 13, 15) inactive drugs)

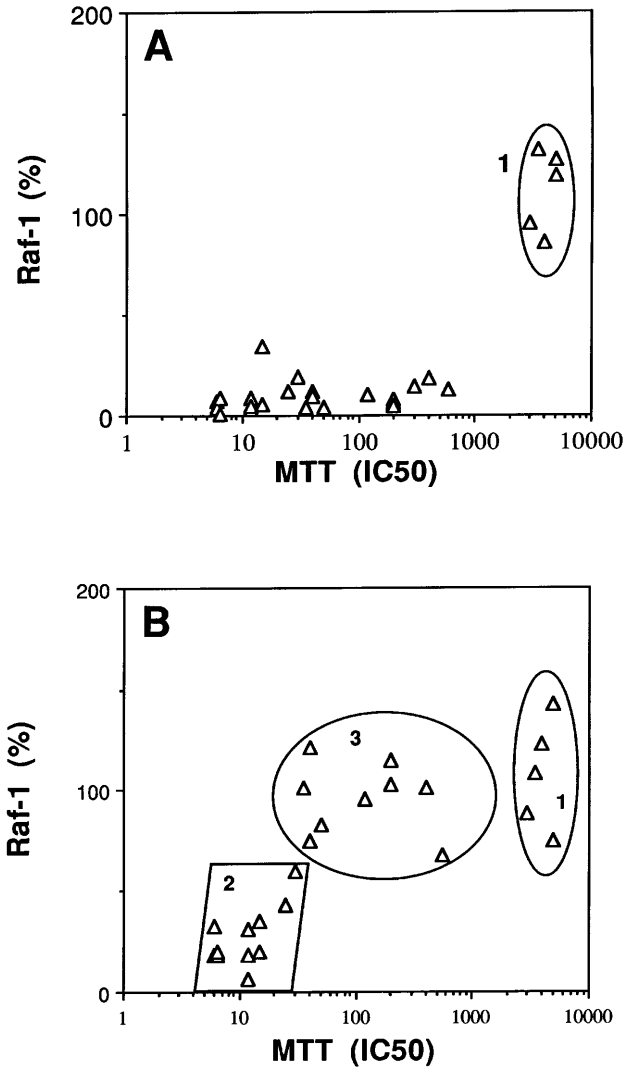

Fig. 2A,B Relationship between Raf-1 depletion by high (2 μ*M*) or low (40 n*M*) drug concentration and antiproliferative IC_{50} values. Raf-1 protein levels were measured by Western blot as described in Methods. Levels of Raf-1 are expressed as percent of control (untreated cells). IC_{50} was determined using the MTT assay as described in Methods. **A** Levels of Raf-1 protein after exposure of SKBr3 cells to the drugs at a concentrate of 2μ for 8 h were plotted against the antiproliferative IC_{50} values (determined by the MTT assay) of the same drugs. Each point on the graph represents an individual drug *Group 1* inactive drugs. **B** Levels of Raf-1 protein after exposure of SKBr3 cells to the drugs at a concentration of 40 n*M* for 8 h were plotted against the antiproliferative IC_{50} values (determined by the MTT assay) of the same drugs. Each point on the graph represents an individual drug. Three groups of drugs are outlined: *group 1* inactive drugs, *group 2* drugs active at low concentration, *group 3* drugs *active the high* concentration

between 40 and 600 n*M* failed to downregulate Raf-1 when tested at 40 n*M*, demonstrating a correlation between Raf-1 downregulation and cytotoxicity (Fig. 3B).

The cytotoxicity IC_{50} of these drugs also correlated with their ability to downregulate mutant p53 and erbB2 proteins (Fig. 3). When tested at 40 n*M*, all drugs which had a cytotoxicity IC_{50} less than 10 n*M* were maximally active against all oncoproteins (Fig. 2B, Fig. 3).

Discussion

GA, a benzoquinone ansamycin antibiotic, possesses potent tumoricidal activity [26, 27]. Although originally described as tyrosine kinase inhibitors, benzoquinone ansamycins have been shown to be inactive when added directly to purified tyrosine kinases [9, 26]. Recently, we have shown that GA downregulates Raf-1, mutant p53 and erbB2 proteins [4, 16, 22], but not wild-type p53 [4]. The conformation of wild-type p53, in contrast to mutant p53, does not require functional HSP90 [5], one of the targets of GA. However, it is not clear how the ability to deplete these proteins relates to the antiproliferative effects of GA. For example, it has been demonstrated that GA can induce wild-type p53 in A2780 cells, but this induction appears unrelated to GA cytotoxicity [15]. Cancer cells have multiple genetic alterations, involving, overexpression of oncoproteins and

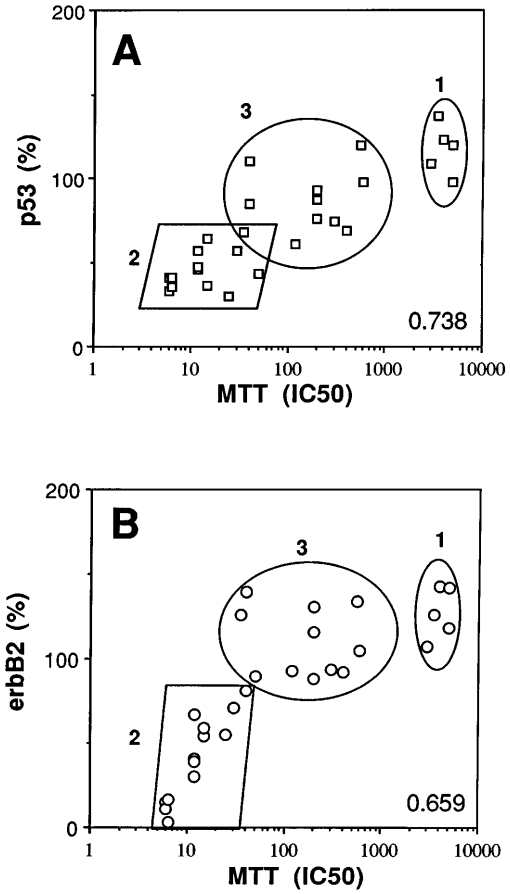

Fig. 3 Downregulation of mutant p53 and erbB2 is correlated with antiproliferative effects. Levels of mutant p53 (**A**) or erbB2 (**B**) protein after exposure to the drugs at a concentration of 40 n*M* for 8 h were plotted against the antiproliferative IC_{50} values (determined by the MTT assay) as described for Fig. 2 (*group 1* inactive drugs, *group 2* drugs active at low concentration, *group 3* drugs active at high concentration

mutations or deletions of tumor suppressor genes. Thus, SKBr3 breast cancer cells express high levels of erbB2 oncoprotein and mutant p53 tumor suppressor protein. Here, we found by testing 28 GA derivatives against SKBr3 cells that a drug that was active against one oncoprotein (Raf-1, mutated p53, or erbB2) was also active against the two others. These results suggest a common mechanism for depletion of Raf-1, erbB2 and mutated p53 by GA and its active derivatives, perhaps involving drug binding to HSP90/GRP94 [4, 6, 16, 23, 25]. In this study, we have presented evidence that a similar mechanism may play a role in the antiproliferative activity of GA.

References

1. Bartek J, Iggo R, Gannon J, Lane DP (1990) Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene 5: 893
2. Benchekroun MN, Schneider E, Safa AR, Townsend AJ, Sinha BK (1994) Mechanisms of resistance to ansamycin antibiotics in human breast cancer cell lines. Mol Pharmacol 46: 677
3. Blagosklonny MV, Neckers LM (1994) The role of Bcl-2 protein and autocrine growth factors in a human follicular lymphoma-derived B cell line. Eur Cytokine Netw 6: 21
4. Blagosklonny MV, Toretsky J, Neckers L (1995) Geldanamycin selectively destabilizes and conformationally alters mutated p53. Oncogene 11: 933
5. Blagosklonny MV, Toretskey J, Bohen S, Neckers LM (1996) Mutant conformation of p53 translated in vitro or in vivo requires functional HSP90. Proc Natl Acad Sci USA 93: 8379
6. Chavany C, Mimnaugh E, Miller P, Bitton R, Nguyen P, Trepel J, Whitesell L, Schnur R, Moyer JD, Neckers L (1996) $p185^{erbB2}$ binds to GRP94 in vivo. J Biol Chem 271: 4974
7. Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C, Levine AJ (1993) p53 gain of function mutations. Nat Genet 4: 42
8. Hsiao M, Low J, Dorn E, Ku D, Pattengale P, Yeargin J, Haas M (1994) Gain-of-function mutations of the p53 gene induce lymphohematopoietic metastatic potential and tissue invasiveness. Am J Pathol 145: 702
9. June CH, Fletcher MC, Ledbetter JA, Schieven GL, Siegel JN, Phillips AF, Samelson LE (1990) Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. Proc Natl Acad Sci USA 87: 7722
10. Katsumata M, Okudaira T, Samanta A, Clark DP, Drebin JA, Jolicoeur P, Greene Ml (1995) Prevention of breast tumour development in vivo by downregulation of the p185neu receptor. Nature Med 1: 644
11. Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW, Vogelstein B (1992) Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 256: 827
12. Levine AJ, Momand J, Finlay CA (1991) The p53 tumor suppressor gene. Nature 351: 453
13. Lupu R, Cardillo M, Harris L, Hijazi M, Rosenberg K (1995) Interaction between erbB-receptors and heregulin in breast cancer tumor progression and drug resistance. Semin Cancer Biol 6: 135
14. Magnuson NS, Beck T, Vahidi H, Hahn H, Smola U, Rapp UR (1994) The Raf-1 serine/threonine protein kinase. Sermin Cancer Biol 5: 247
15. Mcllwrath AJ, Brunton VG, Brown R (1996) Cell-cycle arrest and p53 accumulation induced by geldanamycin in human ovarian tumour cells. Cancer Chemother Pharmacol 37: 423
16. Miller P, DiOrio C, Moyer M, Schnur RC, Bruskin A, Cullen W, Moyer JD. (1994) Cancer Res 54: 2724
17. Miller P, Schnur RC, Barbacci E, Moyer MP, Moyer JD (1994) Binding of benzoquinoid ansamycins to p100 correlates with their ability to deplete the erbB2 gene product p185. Biochem Biophys Res Commun 201: 1313
18. Milner J, Medcalf EA (1991) Cotranslation of activated mutant p53 with wild type drives the wild-type p53 protein into the mutant conformation. Cell 65: 765
19. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC, Vogelstein B (1989) Mutations in the p53 gene occur in diverse human tumour types. Nature 342: 705
20. Prendergast GC, Gibbs JB (1994) Ras regulatory interactions: novel targets for anti-cancer intervention? Bioessays 16: 187
21. Schnur RC, Corman ML, Gallaschun RJ, Cooper BA, Dee MF, Doty JL, Muzzi ML, DiOrio CI, Barbacci EG, Miller PE, Pollack VA, Savage DM, Sloan DE, Pustilnik JD, Moyer JD, Moyer MP (1995) erbB-2 oncogene inhibition by geldanamycin derivatives: synthesis mechanism of action, and structure-activity relationships. J Med Chem 38: 3813
22. Schnur RC, Corman RJ, Gallaschun RJ, Cooper BA, Dee MF, Doty JL, Muzzi ML, Moyer JD, DiOrio CI, Barbacci EG, Miller PE, O'Brien AT, Morin MJ, Foster BA, Pollack VA, Savage DM, Sloan DE, Pustilnik LR, Moyer MP (1995) Inhibition of the oncogene product p185/erbB-2 in vitro and

in vivo by geldanamycin and dihydrogeldanamycin derivatives. J Med Chem 38: 3806
23. Schulte TW, Blagosklonny MV, Ingui C, Neckers L (1995) Disruption of the Raf-1-hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. J Biol Chem 270: 24585
24. Sepehrnia B, Paz IB, Dasgupta G, Momand J (1996) Heat shock protein 84 forms a complex with mutant p53 protein predominantly within a cytoplasmic compartment of the cell. J Biol Chem 271: 15084
25. Srivastava S, Wang S, Tong YA, Hao ZM, Chang EH (1993) Dominant negative effect of a germ-line mutant p53: a step fostering tumorigenesis. Cancer Res 53: 4452
26. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM (1994) Inhibition of HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci USA 91: 8324
27. Whitesell L, Shifrin SD, Schwab G, Neckers LM (1992) Benzoquinonoid ansamycins possess selective tumoricidal activity unrelated to src kinase inhibition. Cancer Res 52: 1721
28. Wiechen K, Dietel M (1995) c-erbB-2 anti-sense phosphorothioate oligonucleotides inhibit growth and serum-induced cell spreading of P185c-erbB2-overexpressing ovarian carcinoma cells. Int J Cancer 63: 604