Modulation of Survival Signaling Pathways and Persistence of the Genotoxic Stress as a Basis for the Synergistic Interaction between the Atypical Retinoid ST1926 and the Epidermal Growth Factor Receptor Inhibitor ZD1839

Chiara Zanchi, Valentina Zuco, Cinzia Lanzi, Rosanna Supino, and Franco Zunino

Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy

Abstract
Strategies targeting apoptotic pathways may have relevance to improve the efficacy of antitumor therapy. Because synthetic atypical retinoids are potent inducers of apoptosis, there is an increasing interest in exploiting their potential in novel therapeutic approaches. In the present study, we have investigated the cellular effects of the combination of a novel atypical retinoid, ST1926, and the epidermal growth factor receptor inhibitor ZD1839. The results indicated a synergistic interaction between the two drugs associated with a dramatic enhancement of apoptotic response, up-regulation of the cell death receptor DR5, and caspase 8 activation. Other molecular events induced by the cotreatment included (a) a stabilization of the ST1926-induced genotoxic stress detected by formation of phosphorylated γ-H2AX foci and (b) a complete inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation associated with activation of the proapoptotic protein BAD (i.e., inhibition of phosphorylation on Ser112). In addition, ZD1839 itself inhibited survival pathways by causing a partial dephosphorylation of Akt and a marked down-regulation of survivin. The role of ERK-mediated survival pathways in the cellular response to the drug combination was further supported by the counteracting effect of stimulation of survival pathways by an alternative receptor tyrosine kinase and by the use of a specific inhibitor of the ERK pathway. In conclusion, the results support that the combination of these well-tolerated agents may have therapeutic potential.

Introduction
Apoptosis is recognized to be an important phenomenon in antitumor drug-induced tumor cell killing and susceptibility to apoptosis of tumor cells is a determinant of efficacy of chemotherapy (1). Apoptosis regulating programs may be impaired in human tumors and the fate of treated cells is likely determined by the balance between proapoptotic and antiapoptotic signals. Thus, modulation of apoptosis-related pathways may be a promising approach to improve the efficacy of antitumor therapy.

Synthetic retinoids, which are reported as potent inducers of apoptosis, represent an emerging class of potentially useful agents (2–5). Whereas the biological effects of natural retinoids are mediated by the retinoid receptors, the proapoptotic activity of atypical synthetic retinoids seems to be independent of retinoid receptors (4, 6). We have recently reported that a novel adamantyl retinoid, ST1926, is a potent inducer of apoptosis in a variety of human tumor cells (7). As for other retinoid-related molecules, the molecular mechanisms involved in apoptosis induction are not clearly defined. In ovarian carcinoma cells several features of the cellular response to ST1926 are reminiscent of the genotoxic stress response because it involves activation of p53 and modulation of p53-target genes and genes involved in DNA damage response (8). Moreover, exposure to ST1926 results in activation of mitogen-activated protein (MAP) kinases, in particular the c-jun-NH2-kinase and p38 stress kinase. The activation of MAP kinases may have a complex and controversial influence in determining the ultimate fate of the cell, depending on the cell type and the context of various signals received by the cell. In ovarian carcinoma cells, MAP kinases, including extracellular signal-regulated kinases (ERK), seem to have a protective role against ST1926-induced cytotoxicity (8). Indeed, these kinases are involved in regulating a number of cellular processes including survival/proliferation and could potentially contribute to influence the ability of cell to survive in stress conditions. Akt is another protein kinase implicated in the cellular response to various stimuli capable of inducing apoptosis (9) and plays a key role in promoting cell survival through integration of pathways triggered by a number of signals including those mediated by growth factor receptors.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase frequently deregulated in cancer cells and has emerged as a molecular target for anticancer therapy (10). EGFR targeting agents include monoclonal antibodies against the extracellular ligand-binding domain of the receptor and small-molecule inhibitors that block the activation of the EGFR tyrosine kinase domain. In the signaling cascade activated by EGFR, the ERKs and Akt play a relevant role regulating cellular processes such as proliferation and survival. Thus, EGFR represents a potential target for modulation of downstream protective events. Indeed, tyrosine kinase inhibitors of the EGFR, such as ZD1839 (Iressa, Gefitinib), have been reported to interact synergistically with a number of cytotoxic agents including DNA damaging agents (11–14).

Therefore, on the basis of evidence that ST1926 induces a DNA damage response and ST1926-induced cytotoxicity can be modulated by inhibitors of survival pathways (7, 8), the present study was done to investigate the cellular effects of a combination of ST1926...
and ZD1839. The results provide evidence of a synergistic interaction between the two drugs. Inhibition of survival pathways was accompanied by a persistence of genotoxic damage and an enhancement of the apoptotic response.

Materials and Methods

Cell Culture and Drugs. The human ovarian carcinoma cell lines (IGROV-1 and SKOV-3), the vulvar squamous carcinoma cell line A431, the breast carcinoma cell line MCF-7, and the lung adenocarcinoma cell line A549 were grown in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) in 5% CO2 at 37°C. The chemical structure and synthesis of the atypical retinoid ST1926 were previously described (7). The compound (Sigma-Tau, Pomezia, Italy) was dissolved in DMSO before further dilution in culture medium. Stock solutions were stored at −20°C. ZD1839 (Iressa) was kindly provided by Astra Zeneca (Macclesfield, Cheshire, United Kingdom). A 10 mmol/L solution in DMSO was prepared before use. Final concentration of DMSO in culture medium was 0.5%. In all experiments involving hepatocyte growth factor (HGF; Sigma, St. Louis, MO), cells were exposed to the growth factor (20 ng/mL) 2 hours before treatment with ZD1839 and ST1926. The MEK1/2 inhibitor UO126 (Sigma) was dissolved in DMSO and added to the culture medium 30 minutes before treatment with ZD1839 and ST1926.

Antiproliferative Activity. Cells were seeded in duplicate into six-well plates. After 24 hours, cells were exposed to ZD1839, ST1926, or to both drugs simultaneously for 72 hours. After treatment, adherent cells were trypsinized and counted by a cell counter (Coulter Electronics, Luton, United Kingdom). Drug concentrations producing 10%, 30%, 50%, and 80% viability of trypsinized and counted by a cell counter (Coulter Electronics, Luton, Belgium) containing 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) in 5% CO2 at 37°C. The chemical structure and synthesis of the atypical retinoid ST1926 were previously described (7). The compound (Sigma-Tau, Pomezia, Italy) was dissolved in DMSO before further dilution in culture medium. Stock solutions were stored at −20°C. ZD1839 (Iressa) was kindly provided by Astra Zeneca (Macclesfield, Cheshire, United Kingdom). A 10 mmol/L solution in DMSO was prepared before use. Final concentration of DMSO in culture medium was 0.5%. In all experiments involving hepatocyte growth factor (HGF; Sigma, St. Louis, MO), cells were exposed to the growth factor (20 ng/mL) 2 hours before treatment with ZD1839 and ST1926. The MEK1/2 inhibitor UO126 (Sigma) was dissolved in DMSO and added to the culture medium 30 minutes before treatment with ZD1839 and ST1926.

Antiapoptotic Activity. Cells were seeded in duplicate into six-well plates. After 24 hours, cells were exposed to ZD1839, ST1926, or to both drugs simultaneously for 72 hours. After treatment, adherent cells were trypsinized and counted by a cell counter (Coulter Electronics, Luton, United Kingdom). Drug concentrations producing 10%, 30%, 50%, and 80% viability of trypsinized and counted by a cell counter (Coulter Electronics, Luton, United Kingdom). Drug concentrations producing 10%, 30%, 50%, and 80% viability of trypsinized and counted by a cell counter (Coulter Electronics, Luton, United Kingdom).

Cell Cycle and Apoptosis. For cell cycle analysis, cells were trypsinized, fixed in 70% ethanol, stained in phosphate-buffered solution (PBS) containing 10 μg/mL propidium iodide (Sigma) and RNase A (66 units/mL; Sigma) for 18 hours, and analyzed by FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

For apoptosis detection, adherent and floating cells were harvested and analyzed for DNA fragmentation by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (Roche, Mannheim, Germany) as previously described (8). Apoptosis was assessed by flow cytometry and the results were analyzed using the CellQuest software (Becton Dickinson, Mountain View, CA).

Western Blot Analysis. Adherent and floating cells were harvested and analyzed for DNA fragmentation by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (Roche, Mannheim, Germany) as previously described (8). Apoptosis was assessed by flow cytometry and the results were analyzed using the CellQuest software (Becton Dickinson, Mountain View, CA).

The following primary antibodies were used: anti-phospho-p44/42 MAP kinase (Thr202/Tyr204; New England Biolabs, Beverly, MA); anti-Bcl-2, anti-caspase 3, anti-phospho-Bad (Ser112; Cell Signaling Technology, Beverly, MA); anti-PBKs/Akt, anti-Bad (Transduction Laboratories, Lexington, KY); anti-caspase 8, anti-Bcl-X (PharMingen, Becton Dickinson); anti-phospho-EGFR pY1086 (Biosource, Camarillo, CA); anti-survivin (ab469; Abcam, Cambridge, United Kingdom); anti-actin and anti-tubulin (Sigma).

Analysis of DR4 and DR5 Expression. For the analysis of death receptors, cells were harvested and incubated at 4°C with biotinylated-conjugated anti-human tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL R1; DR4) or anti-human TRAIL R2 (DR5) monoclonal antibody (R&D Systems, Minneapolis, MN) for 30 minutes, and then for 1 hour with streptavidin-phycocerythrin conjugate (BD PharMingen, Heidelberg, Germany). After washing, cells were resuspended in PBS and antibody binding was detected by flow cytometry (FACScan). Cells incubated only with the phycocerythrin conjugate served as negative controls.

Assessment of Genotoxic Damage. Phosphorylated H2AX histone (γ-H2AX) at the DNA damage sites was detected by immunofluorescence staining (17). Cells were grown on coverslips, treated with drugs as indicated, and fixed in 2% paraformaldehyde in PBS for 5 minutes. After washing in PBS, cells were permeabilized in methanol at −20°C for 20 minutes and blocked with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (PBA) for 15 minutes. Samples were then incubated with anti-γ-H2AX mouse monoclonal antibody (Upstate Biotechnology) for 1 hour, followed by AlexaFluor 594-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR) for 1 hour. Slides were then washed in PBA, incubated with 2 mg/mL Hoechst 33342 (Sigma) for 2 minutes, mounted with Mowiol, and examined by a fluorescence microscope. Cells displaying at least 10 foci were considered as positive.

Results

Cell Growth Inhibition. The combination of the EGFR inhibitor ZD1839 and the atypical retinoid ST1926 was investigated in a panel of human cancer cell lines of different tissue origin and characterized by a differential sensitivity to ZD1839. Using a simultaneous combination treatment with a moderately antiproliferative concentration of ZD1839 (IC50) and two concentrations of ST1926, a supra-additive effect was consistently observed in all tested cell lines (Table 1). Owing to the synergistic interaction being more marked in the IGROV-1 ovarian carcinoma cell line, this cell line was chosen for further studies.

<table>
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<tr>
<th>Cell lines</th>
<th>ZD1839 (IC50)</th>
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<tr>
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<td>1.50</td>
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<tr>
<td>0.01</td>
<td>0.08</td>
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* D.I., Drewinko index calculated for the drug combination. D.I. > 1 indicates synergistic interaction.

Table 1. Analysis of combined treatment of ST1926 and ZD1839 (72 hours) in a panel of human cancer cell lines

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The dose-response curves for the antiproliferative effects of ST1926 and ZD1839 on IGROV-1 cells were constructed in Fig. 1A. Cells were treated simultaneously with ST1926 (in the concentration range of 0.008-0.3 µmol/L) and ZD1839 at toxic and sub-toxic concentrations (i.e., 0.01, 0.04, and 0.1 µmol/L, corresponding to the IC_{10}, IC_{30}, and IC_{50}, respectively). A supra-additive growth inhibitory effect was clearly documented by the dose-response curve of ST1926 in the presence of a sub-toxic dose of ZD1839 (0.01 µmol/L). The analysis of drug interaction (15) confirmed that the ST1926/ZD1839 combination was synergic (Fig. 1B). Drug interaction investigated and analyzed according to Chou and Talalay (16) supported the synergism (not shown).

**Cell Cycle Analysis and Apoptosis.** The analysis of cell cycle perturbations was done on cells exposed to each agent, ST1926 (IC_{50}) or ZD1839 (IC_{50} and IC_{80}), or to their combination. Simultaneous exposure to the two drugs for 72 hours did not determine modifications of the cell cycle distribution compared with that of control cells or of cells exposed to each drug (Fig. 2A). However, a marked increase in the sub-G1 DNA content was found in cells exposed to the drug combination (4-5% in ZD1839- or caspase 8, caspase 3, and, to a lesser extent, caspase 9 was observed in the presence of each single agent, whereas the combined treatment produced a remarkable increase of apoptosis which was dependent on the ZD1839 concentration.

To determine whether the drug interaction resulted in an enhancement of apoptosis, cells were further analyzed by the TUNEL assay (Fig. 2B). A marginal induction of apoptosis was observed in the presence of each single agent, whereas the combined treatment produced a remarkable increase of apoptosis which was dependent on the ZD1839 concentration.

**Modulation of Apoptotic Pathways.** Apoptotic pathways activated by the combined treatment were investigated by Western blot analysis. Cells were treated with ST1926 or ZD1839, alone or in combination, at concentrations corresponding to the IC_{50} values (0.15 and 0.1 µmol/L, respectively) for 48 or 72 hours. In these conditions, barely detectable caspase activation was induced by the two drugs given alone (Fig. 3) according to a marginal induction of apoptosis detected by the TUNEL assay (Fig. 2B). By contrast, in cells treated with the drug combination, a marked cleavage of caspase 8, caspase 3, and, to a lesser extent, caspase 9 was associated with cleavage of the caspase substrate PARP, thus confirming an enhanced activation of caspase-dependent apoptotic pathways. To get insights into the mechanisms responsible for enhancement of apoptosis by the cotreatment, we examined the expression of negative regulators of apoptosis belonging to inhibitors of apoptosis protein family and to the Bcl-2 family. ZD1839 produced a marked reduction in survivin levels after 48 and 72 hours of treatment (Fig. 3). Such an effect, already detectable after 24 hours of treatment (not shown), was maintained in the combined treatment. Similar effects, although to a lesser degree, were observed on the expression levels of Bcl-2 and Bcl-X_{L} (not shown).

**Effect on the Expression of Death Receptors.** Owing to caspase 8, which is implicated in cell death receptor-mediated apoptosis, markedly activated by the combination of ST1926 and ZD1839, the drug effects on the expression of death receptors DR4 and DR5 were examined. Whereas ST1926 or ZD1839 alone did not enhance the expression of DR5 (Fig. 4A), an up-regulation of this receptor was found after simultaneous treatment with ST1926 plus ZD1839 for 48 hours (Fig. 4B). The synergistic effect on this receptor likely reflected the high level of caspase 8 activation and therefore the involvement of the extrinsic pathway in the apoptotic response. No modulation of DR4 expression was found (not shown).

**Effects on Proliferation/Survival Pathways.** To investigate the nature of the cooperation between ZD1839 and ST1926, we examined the effects of the two drugs on proliferation/survival pathways sustained by the EGFR signaling. At first, to check the effect of ZD1839 on its target in our experimental conditions, we examined the tyrosine phosphorylation of EGFR in IGROV-1 cells exposed to ST1926 (IC_{50}) and this effect was maintained when cells were exposed to ZD1839 and ST1926 simultaneously for 24 hours. ST1926 alone did not affect EGFR autophosphorylation. The activation state of ERKs and Akt was examined in cells treated with the two compounds alone or in combination. As shown in Fig. 5A, EGFR autophosphorylation was inhibited after treatment with ZD1839 (IC_{50}) and this effect was maintained when cells were exposed to ZD1839 and ST1926 simultaneously for 24 hours. ST1926 alone did not affect EGFR autophosphorylation. The activation state of ERKs and Akt was examined in cells exposed to ST1926 (IC_{50}) and/or to ZD1839 (IC_{50} and IC_{80}), for 6 or 24 hours. Akt activation, detected as Ser473 phosphorylation following 24 hours of treatment (53% and 23% versus control with 0.1 and 1 µmol/L ZD1839, respectively) and the
combination with the atypical retinoid did not essentially modify this effect (Fig. 5B). A marked dose-dependent inhibition of ERK1/2 phosphorylation was induced by ZD1839, already evident after 6 hours of treatment and more pronounced after 24 hours. The activation of the two ERKs was not substantially modulated by ST1926 alone. Nonetheless, the combination with ZD1839 induced an apparent supra-additive inhibition of ERKs phosphorylation at either 6 or 24 hours (Fig. 5B).

**Role of Survival/Proliferation Pathways.** To provide additional support to the role of survival/proliferation pathways in the synergistic interaction between ZD1839 and ST1926, we did combination experiments in the presence of HGF as a survival/proliferation stimulus. Cellular response to HGF stimulation is known to protect cells from apoptosis, induced by a variety of stimuli, through activation of the PI3K/Akt and ERK pathways (18). Therefore cellular response to HGF was determined by the induction of phosphorylation of Met receptor, ERKs, and Akt (Fig. 6A). The stimulation with the growth factor resulted in loss of the synergistic interaction of the drugs at their respective IC50 and strongly reduced apoptosis after 72 hours of combination treatment (Fig. 6D). In addition, HGF attenuated the inhibitory effects of the drugs on activation of ERKs and, to a lesser extent, of Akt observed after 24 hours of treatment (Fig. 6B). Owing to the proapoptotic factor BAD, a Bcl-2 family member, being a target of both the MEK/ERK and the PI3K/Akt pathways, we investigated its phosphorylation status. Indeed, phosphorylation of BAD on the specific serine residues, Ser112 (through the ERK signaling) or Ser 136 (through Akt), is known to suppress its proapoptotic function by inhibiting the interaction with the antiapoptotic proteins, Bcl-2 and Bcl-XL (26). According to the effect of ZD1839 and ST1926 on ERK and Akt activation, BAD phosphorylation on Ser112 was reduced by ZD1839 either alone or in combination (Fig. 6B) and phosphorylation on Ser136 seemed barely affected (not shown). Again, the effects of the two drugs were attenuated in the presence of HGF. The counteracting action of HGF on the biochemical effects of the drug combination was still evident after 72 hours of treatment (Fig. 6C). In addition,
in cells exposed to the growth factor, drug-induced cleavage of caspases (8 and 3) and PARP was decreased together with a reduced down-regulation of survivin.

Owing to the above results indirectly supporting that the enhancement of apoptosis induced by the ST1926/ZD1839 combination was mainly related to the enhanced inhibition of the survival pathways involving the ERKs, we explored the effects of ERK pathway inhibition by an alternative approach. ST1926 treatment was done in the presence of the MEK1/2 inhibitor UO126. Figure 7 shows that, in a range of UO126 concentrations inhibiting ERK activation, a supra-additive effect on induction of caspases and PARP cleavage was obtained in cells exposed to the ST1926/UO126 combined treatment as compared with each single-agent treatment. Moreover, a more marked survivin down-regulation was observed in cells exposed to the cotreatment as compared with treatment with the ERK inhibitor alone. Altogether, these results support a major role of survival/proliferation pathways in the modulation of cell response to the ST1926/ZD1839 combination, and indicate that inhibition of the ERK pathway by the EGFR inhibitor provides a relevant contribution to the synergistic interaction between the two drugs.

Genotoxic Damage. To analyze whether EGFR blockade finally affected the genotoxic damage induced by ST1926, we examined the effects of the cotreatment with ZD1839 on the phosphorylation of H2AX histone (γ-H2AX), which can be visualized as foci at the DNA damage sites by immunofluorescence with phospho-specific antibodies. Indeed, this approach is the most sensitive method to detect double-strand DNA breaks (19, 20). Cells were treated with ST1926 (IC_{50}) or ZD1839 (IC_{50}) alone or in combination and washed after 3 hours of drug exposure. A short-term exposure was employed to avoid overlapping of apoptotic DNA fragmentation. γ-H2AX foci were examined after the drug washout and after 3 hours of recovery in the presence or absence of ZD1839 (Fig. 8A). No γ-H2AX foci were observed in control cells (Fig. 8A) and in ZD1839 treated cells (not shown). About 40% of cells were highly positive for γ-H2AX after treatment with ST1926 alone or in combination with ZD1839 (Fig. 8A). Such DNA lesions were reversible upon drug removal in cells treated with ST1926 alone. In contrast, in the cells exposed to the combined treatment, γ-H2AX positivity was still evident in 25% to 30% of cells in the presence of ZD1839 during the recovery time. The absence of ZD1839 during the 3 hours recovery resulted in a low level of positivity (3%). These results suggest that ZD1839 is able
to promote a persistence of ST1926-induced genotoxic stress. Analysis of DNA fragmentation detected by alkaline elution technique also supported the accumulation of DNA lesions in the presence of both agents (data not shown).

**Discussion**

The ability of tumor cells to survive under stress conditions (e.g., cytotoxic treatment) may critically influence their sensitivity and response to therapy. Resistance to drug-induced apoptosis may reflect defects in the apoptotic programs and/or overactivation of protective signal transduction pathways which promote survival. For example, ERK activation has been implicated as a survival factor and constitutive activation of MEK/ERK cascade found in several tumors is likely involved in supporting malignant progression and proliferation (21). Again, several components of the antiapoptotic PI3K/Akt pathway are deregulated in a wide spectrum of human cancers contributing to promote cell survival (22). Because of these protective pathways also involved in the cellular stress response, in particular in DNA damage response, apoptosis induced by cytotoxic agents could be enhanced by inhibition of growth factor receptor–mediated signal transduction promoting survival. In this context, EGFR might represent a potential target not only for the control of tumor growth but also to improve the efficacy of cytotoxic agents (23, 24).

In the present study, we obtained evidence that in ovarian carcinoma cells treated with ST1926, the concomitant inhibition of survival signaling pathways by ZD1839 resulted in a marked induction of apoptosis and in synergistic cytotoxicity. Although ST1926 is not a conventional cytotoxic agent, several direct and indirect lines of evidence support the drug ability to induce a genotoxic stress (7, 8). Indeed, the pattern of cellular response and modulation of genes implicated in DNA damage response support that ST1926-induced genotoxic stress is implicated in mediating apoptosis induction (8). Moreover, early detection of DNA breaks, p53 activation, and up-regulation of p53-target genes in treated cells are consistent with this interpretation. The present study provides additional support to the proposed mechanism involving...
the formation of DNA lesions as a primary event in apoptosis induction by ST1926. Indeed, we present evidence that ZD1839/ST1926 combination was able to induce an early DNA damage detectable as γ-H2AX foci under conditions producing a synergistic effect on apoptosis induction. Using this approach, we found that ZD1839 produced a persistence of genotoxic lesions induced by ST1926, which were otherwise reversible following drug removal. A proposed rationale for the observed synergistic effects between ZD1839 and DNA-damaging agents is that DNA repair processes might be impaired by the EGFR inhibitor (12, 13). Indeed, by promoting cellular proliferation, EGFR activation might also favor DNA repair. The molecular determinants of such a connection are not clearly defined. However, recent evidence suggests the involvement of an ERK-dependent pathway in this process (25). ERK activation plays a central role in signal transduction, due to its implication in pathways activated by growth factor receptors and, therefore, in promoting survival and proliferation. We previously reported that the inhibition of the MEK/ERK pathway by the MEK inhibitor PD98059 enhanced the antiapoptotic and proapoptotic effects induced by ST1926 (8). Here, we report that ZD1839, a potent inhibitor of EGFR tyrosine kinase activity, reduced in a dose-dependent manner the phosphorylation of the ERKs, and this inhibition was strengthened in combination with ST1926. This effect resulted in activation of BAD, as a result of dephosphorylation on Ser112, which is known to be phosphorylated downstream to the ERKs (26). BAD activation could be further enhanced by partial dephosphorylation on Ser136 (not shown) as a result of a partial inhibition of Akt pathway by ZD1839. The experiments done with the MEK1/2 inhibitor UO126 again support the involvement of inhibition of ERK-mediated pathways in sustaining the enhancement of apoptosis in the drug combination. Consistent with such interpretation is the observation that in conditions in which the activation of survival/proliferation pathways, including ERKs and Akt, was promoted by a receptor alternative to EGFR (i.e., the Met receptor), apoptosis induced by the ZD1839/ST1926 combination was strongly reduced and the synergistic interaction was lost.

Although Akt pathway is known to play a role in cell survival and its activation is mediated through growth factor receptors, the effect of ZD1839 on this pathway was less marked. A recent study has reported that a closely related compound, 3-Cl-AHPC, was able to decrease Akt kinase activity associated with EGFR protein expression in prostate and breast carcinoma cells (27). However, in our experimental conditions we did not detect any effect of ST1926 itself or ST1926/ZD1839 combination on the level of EGFR protein.

In addition to BAD, other apoptosis-related proteins are known to be regulated by the ERK MAP kinases and inhibition of MEK/ERK pathway has downstream effects on antiapoptotic proteins (28–30). Our results indicate that inhibition of EGFR-ERK pathway by ZD1839 or UO126 produced a down-regulation of Bcl-2 and Bcl-XL (not shown), as previously reported (13, 14), and of survivin, which were maintained in the combined treatment. Although ST1926 is known to activate p53 (8) and p53 may repress the transcription of survivin (31), the reduced expression of the survivin protein was an effect of ZD1839 alone. Survivin down-regulation, as a consequence of inhibition of the MEK/ERK signaling, was previously described (28). Our finding is the first evidence that survivin down-regulation can be induced by an EGFR inhibitor. Survivin is involved in the regulation of both cell death and proliferation because it not only protects cells from apoptosis as an endogenous repressor of the mitochondrial pathway but it is also implicated in cell division and plays a role in the mitotic checkpoint following cellular damage (32).

Therefore, survivin overexpression in tumor cells may represent a mechanism of resistance to genotoxic agents (33). Our data thus suggest that the synergistic interaction between ZD1839 and ST1926 implicates an enhancement of the intrinsic mitochondria-regulated pathway with BAD activation and down-regulation of survivin and Bcl-2/Bcl-XL. In addition, a relevant cooperation by the extrinsic pathway was indicated by the DR5 death receptor up-regulation associated with caspase 8 activation. Owing to DR5 as a DNA-damage inducible gene (34), it is conceivable that a sustained activation of this pathway might be favored by the persistent DNA damage in cells exposed to the cotreatment. The activation of apoptotic signaling pathway mediated by death receptors is recognized to play an important role in atypical retinoid-induced apoptosis (35).

In conclusion, the synergistic antiproliferative and apoptotic effects exhibited by the combination of the atypical retinoid ST1926 and the EGFR receptor inhibitor ZD1839 were related to inhibition of protective survival pathways, in particular ERK-mediated processes, and likely to modulation of cellular pathways controlling DNA repair. The activation of survival signaling pathways mediated by EGFR seems to be a critical event in determining the susceptibility to ST1926-induced apoptosis. Finally, our results support that targeting survival pathways may have pharmacologic implications in an attempt to improve the efficacy of stress-inducing agents. Although clinical studies of ZD1839/platinum compound combinations have been negative and the clinical use of ZD1839 has shown efficacy only as a single-agent therapy in a subset of treated patients (36), recent progress in understanding the molecular basis of clinical
responsiveness to this drug suggests the possibility of selecting patients who likely could benefit from treatment with the drug alone and, possibly, in rationally designed combinations (37, 38). In such a context, the combination of ZD1839 and ST1926 may have a therapeutic potential considering the good tolerability profiles of both agents.

Figure 8. Effects of ZD1839 on DNA damage induced by ST1926 in IGROV-1 cells. Cells were treated for 3 hours with ST1926 alone or with ZD1839/ST1926 combination. After drug washout, ST1926-treated cells were incubated for additional 3 hours in the absence of the drug. Cells treated with the combination were reincubated for 3 hours in the presence (+ZD) or absence (–ZD) of ZD1839. A, immunofluorescence staining of γ-H2AX foci (red). Nuclei were stained with Hoechst 33342 (blue). B, quantification of cells exhibiting γ-H2AX foci. The percentages were obtained by fluorescence microscope counting from smears prepared as in A. Cells displaying 10 or more foci were counted as positive.

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