c-myc Down-Regulation Induces Apoptosis in Human Cancer Cell Lines Exposed to RPR-115135 (C$_{31}$H$_{29}$NO$_{4}$), a Non-Peptidomimetic Farnesyltransferase Inhibitor

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ABSTRACT

A therapeutic strategy that relies on the use of c-myc antisense in combination with a farnesyltransferase inhibitor, RPR-115135 (C$_{31}$H$_{29}$NO$_{4}$), was studied in human cancer cell lines carrying different mutations (Ras, p53, myc amplification). Cell proliferation was strongly inhibited by the combination and was observed when c-myc oligo (at a concentration that down-regulates c-myc expression) was followed by RPR-115135. Cell cycle analysis demonstrated an accumulation in G$_0$-G$_1$ phase and a tendency to apoptosis (not detectable in cells treated with a single agent). Morphological examination and DNA fragmentation assays (filter binding and enzyme-linked immunosorbent assay DNA fragmentation) confirmed the induction of apoptosis. Apoptosis was not p53- and/or p21$^{waf-1}$-dependent, and the key effector was caspase activation. The combination induced Bax expression and Bcl-2 inhibition. Down-regulation of c-myc amplification carried out a specific role exclusively when Ras was mutated. Exposure of human proliferating lymphocytes to combination did not result in cytotoxicity, suggesting that mechanisms regulating c-myc gene expression during normal T cell proliferation might not be involved. Because of the high percentage of human tumors overexpressing c-myc mRNA and/or protein and, simultaneously, harboring oncogenic Ras mutants (i.e., colon cancers), interrupting the myc and Ras-signaling pathway would be one of the major focuses on therapy of these types of tumors.

Progress in molecular oncology has led to the identification of different potentially exploitable targets for anti-cancer drug development (Bange et al., 2001). As a result, many compounds in new categories have been developed, such as inhibitors of signal transduction, of cyclin-dependent kinase, and of angiogenesis, as well as gene therapy and immunotherapy. Potential therapeutic strategies and goals range from the total shutdown of the expression of a particular gene, to diminution in overexpression, or the differentiation between a cellular gene required for normal cell growth and its single point mutation Among these there are, as an example, 1) the cellular proto-oncogene Ras, which, when mutated, encodes for oncoproteins insensitive to the inhibitory activity of GTPase-activated proteins (Katz and McCormick, 1997); and 2) c-myc, whose amplification can often be correlated with the disease state (Calabretta et al., 1985). The RAS superfamily proteins are intimately involved in malignant transformation (Bos, 1989). The knowledge that Ras was readily prenylated (a lipid post-translational modification) by protein FTase and that inhibition of this reaction has functional consequences for the transformed phenotype expressing oncogenic Ras provided the rationale for targeted anti-cancer drug development of FTIs (Adjei, 2001).

FTase is responsible for catalyzing farnesylation of several cellular proteins by transfer of a C-15 farnesyl moiety from farnesyl pyrophosphate. Studies have shown that farnesyl-

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tion of Ras is the obligatory first step in a series of posttranslational modifications that lead to the membrane association that, in turn, determines the switch from an inactive to an active Ras-GTP bound form (Katz and McCormick, 1997).

Based on the theoretical assumption that inhibiting Ras farnesylation might result in the inhibition of Ras functions, a range of FTIs have been synthesized or identified (Gibbs, 2000; Hill et al., 2000; Adjei, 2001; Crul et al., 2001; Karp et al., 2001; Purcell and Donehower, 2002).

Their biology is fascinating since, after substantial investigation and their use in at least one Phase III trial, the exact mechanism of action remains unclear. FTIs can block the farnesylation of several additional proteins [such as RhoB, prelamin A and B, and centromere proteins (CENP-E, CENP-F)] (Ashar et al., 2000; Adjei, 2001; Crespo et al., 2001; Crul et al., 2001). Although the FTIs clearly do not or only partly target Ras, these agents appear to have clinical activity in leukemia and in some solid tumors (Adjei, 2001; Karp et al., 2001; Purcell and Donehower, 2002).

Although inhibition of FTase by these compounds has been well documented also in normal tissues, their toxic effects seem to be manageable. However, initial ongoing Phase II-III studies show that the antineoplastic activity of FTIs, administered as a single agent, is not comparable with that obtained by standard cytotoxic drugs (Adjei, 2001; Karp et al., 2001; Purcell and Donehower, 2002). The suggestion of these studies is to use FTIs in combination with cytotoxic agents or with signal transduction inhibitors. Consequently, combination studies are ongoing (Edamatsu et al., 2000; Adjei, 2001; Schwartz et al., 2001; Purcell and Donehower, 2002; Russo et al., 2002a,b). These findings are significant for understanding the mechanism of action of FTIs as well as for clinical use of FTIs.

Cancerogenesis is a multistep process requiring the activation of more than one proto-oncogene and the loss of tumor suppressor genes. Although a drug active against a single oncogene may be sufficient to return a transformed cell to a pretransformed state [see experiments with FTIs in H-Ras-transfected cells or in transgenic H-Ras mice (Gibbs, 2000; Hill et al., 2000; Adjei, 2001; Crul et al., 2001; Karp et al., 2001; Purcell and Donehower, 2002)], the cell will likely still be abnormal because it has other expressed oncogenes. It might be better, or even necessary, to inhibit several oncogenes to return the cell to a stable, nontransformed state. This means that the tumor should be shown to express the oncogene or signaling pathway that is to be inhibited. It may be more appropriate, for the type of drugs considered here, to classify tumors by their complement of oncogenes and tumor suppressor genes, rather than by their histopathological type.

To test this hypothesis, the combination of a non-peptidomimetic FTI, RPR-115135, and a c-myc antisense oligonucleotide was studied in different human cancer cells well characterized for Ras and c-myc status. The possible role of p53 and p21WAF1/CIP1 was investigated in isogenic cell line systems consisting of HCT-116 cells with disrupted p53 functions or of HCT-116 p21 knock-out cells. The role of c-myc expression was verified in normal T lymphocytes (resting and/or proliferating).

Materials and Methods

Chemical Treatments. RPR-115135 [C32H39NO5, (mol. wt. = 479.58)] is produced by Aventis Pharma (Centre de Recherches de Vitry, Alfortville, France). It was prepared as a 1 mM stock solution in dimethyl sulfoxide, and aliquots were stored at −20°C until needed. A 15-mer antisense [S] ODN (5′-AAAGTGAAGGGCACAT-3′) that was complementary to the translation initiation region of c-myc mRNA and the control scrambled sequence [S] ODN, containing the “G-quartet” motif (5′-AAGCATAAAGGGTGTT-3′), were obtained from TIB Mol Biol (Genoa, Italy).

Cell Culture. Human colon cancer cell line HCT-116 was grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen) and 2 mM glutamine. Cells transfected with a vector containing a dominant-negative mutant p53 transgene (248R/W) (cloned into a pCMV plasmid) to inhibit p53 function (Ottoboni et al., 2001) and HCT-116p21 knockout cells ([Waldman et al., 1995; kindly provided by Dr. B. Volgestein (Johns Hopkins University, Baltimore, MD)] were grown in the same medium. Human leukemia HL-60 and K562 cell lines, human ovarian OVCAR-3, SKOV-3, and Pa-1 (teratocarcinoma) cell lines, and human neuroblastoma LAN-5 cell line were cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (Invitrogen) and 2 mM glutamine.

Human lymphocytes were isolated from heparinized blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation and were resuspended at a density of 1.0 × 106 cells/ml in complete RPMI 1640 medium and cultured in the presence of PHA, 10 mg/ml, as described previously (Russo et al., 1994).

Cell counts were determined using a Coulter counter with Channelizer attachment to monitor cell size (Beckman Coulter Inc., Fullerton, CA). Cell membrane integrity was determined by trypan blue dye exclusion assay.

Cell Treatment. Cells were exposed to oligos for 24 h before, simultaneously with, or 24 h after RPR-115135 administration. Antiproliferative effects in T human lymphocytes were assessed by counting cells treated according to the following schedules: 1) resting T cells (no c-myc detectable) exposed continuously for 24 h to different concentrations of RPR-115135 or for 2 h to 0.1 μM oligo followed by different concentrations of RPR-115135 for additional 24 h; 2) resting T cells in medium for 2 h, then exposed to PHA, and after 7 h [high expression of c-myc mRNA, as determined by RT-PCR (data not shown)], exposed to different concentrations of RPR-115135 for an additional 24 h; 3) resting T cells incubated with 0.1 μM oligo for 2 h, then induced with PHA, and after 7 h, exposed to different concentrations of RPR-115135 for an additional 24 h; and 4) resting T cells induced for 72 h with PHA, then exposed to 0.1 μM oligo for 26 h, RPR-115135 for 24 h, or 0.1 μM oligo for 2 h (at 70 h after PHA) followed by RPR-115135 for additional 24 h.

Cell Cytotoxicity. Cells were plated in log phase into 96-multiwell plates (250 cells/well) with 190 μl of complete medium for 24 h and then treated with various concentrations of drugs for 6 days. At the end of the incubation time (6 days), 40 μl of MTS tetrazolium solution (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI) was added for 2 h, and then absorbance was read at 490 nm with a 96-well plate reader.

The IC50 was calculated as the drug concentration that inhibits 50% of the control cells. IC50 values were estimated by fitting the data with a nonlinear regression to the dose-effect model derived by Chou and Talalay (1977, 1981): \[ f(D) = f_0 + \frac{D \cdot m}{D + m} \] (eq. 1), where \( D \) is the dose of the drug, \( D_m \) is the IC50, and \( f_0 \) is the fraction affected by the dose, \( f_0 \). is the fraction unaffected, and \( m \) is a coefficient that determines the sigmoidicity of the curve.

In a second assay, 6.0 × 105 cells plated on T75 flasks were treated for 24 h with the drug under testing, washed twice, re-fed in drug-free medium, incubated for additional days until they reached confluence, and finally counted.
**Protein Extraction and Western Blot Analysis.** Cells were collected for immunoblot analysis in Hanks’ balanced salt solution. Proteins were extracted according to the method of Vikhanskaya et al. (1997). Equivalent cell extracts were electrophoresed on 10% polyacrylamide gels, transferred to nitrocellulose filters (Hybond-ECL, Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK), and then assessed for c-myc, Bcl-2 or Bax protein levels by immunoblot analysis using anti-c-myc, anti-Bcl-2 or anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA). As a control, immunoblots were reproessed for expression of actin using specific antibodies (Oncogene Science, Paris, France). Immune complexes were detected with the ECL (enhanced chemiluminescence) reagent system (Amersham Biosciences UK Ltd.) after addition with the appropriate immunoglobulin (IgG).

**c-myc mRNA Detection by Semiquantitative RT-PCR Analysis.** The mRNA from cell lines was isolated utilizing the Quick Prep Micro mRNA purification kit (Pharmacia AB, Uppsala, Sweden) and quantitated spectrophotometrically. About 200 ng of mRNA from each sample were reverse-transcribed using oligo(dT) as primers, following the manufacturer’s instructions (GeneAmp RNA PCR kit; PerkinElmer Life Sciences, Boston, MA). The primers used were for c-myc (5′-TGG TCT TCC CCT ACC CTC TCA AC-3′/H11032 and for GAPDH (5′-GGT CAT CCC GAC TCT ACC CTC TCA AC-3′). The PCR was carried out according to the method of Vikhanskaya et al. (1997). The protocol for PCR was designed to measure the level of c-myc expression relative to the expression of an internal standard gene (glyceraldehyde-3-phosphate dehydrogenase). Quantitation was performed with PCR CELISA DIG Labeling (Roche Diagnostics, Mannheim, Germany).

**Flow Cytometry.** Cells were plated in log phase in T75 flasks (2700 cells/cm²) in complete medium for 24 h, and then treated. Samples were prepared for flow cytometry essentially as described previously (Russo et al., 2002a). Briefly, cells were washed with 1× phosphate-buffered saline, pH 7.4, and then fixed with ice-cold 70% ethanol. Samples were washed with 1× phosphate-buffered saline and stained with propidium iodide (6 μg/ml) (Sigma-Aldrich, St. Louis, MO) containing RNase (2 μg/ml) (Sigma-Aldrich) for 30 min at 37°C. Cell cycle analysis was performed using a BD Biosciences fluorescence-activated cell analyzer and Cell Quest version 1.2 software (BD Biosciences Immunocytometry Products, San Jose, CA). For each sample at least 15,000 cells were analyzed, and quantitation of the cell cycle distribution was performed using ModFit LT Version 1.01 software (Verity Software House Inc., Topsham, ME).

**DAPI Staining.** Treated cells were harvested, washed in 1× phosphate-buffered saline, and fixed with 4% paraformaldehyde, stained for 5 min in 0.1 mg of DAPI/ml in a methanol solution, and finally analyzed via fluorescence microscopy to assess chromatin condensation and segregation. A total of 1000 cells were scored for each slide.

**DNA Secondary Fragmentation Assay.** Apoptosis-associated DNA fragmentation was analyzed by filter-binding assay as described previously (Russo et al., 2002a). A filter-binding assay was performed under nondeproteinizing conditions using protein-adsorbing filters (vinyl/acrylic copolymers filters, Metrical membrane, 0.8 mm pore size, 25-mm diameter; Pall Gelman Laboratory, Ann Arbor, MI) according to the method of Bertrand et al. (1991). Prelabelled cells (0.5 × 10⁶) with 0.02 μCi/ml [¹⁴C]thymidine were loaded onto polynyl chloride filters and washed with 5 ml of Hanks’ balanced salt solution. Cells were then lysed with 5 ml of solution containing 0.2% sodium sarkosyl, 2 M NaCl, 0.04 M EDTA (pH 10.0). After the lysing solution had dripped through by gravity, it was washed from the filter with 5 ml of 0.02 M EDTA (pH 10.0). Filters were then processed as in the case of alkaline elution (Bertrand et al., 1991). Radioactivity was counted by liquid scintillation spectrophotometry in each fraction (loading fraction, wash, lysing solution + EDTA wash, filter). DNA fragmentation (apoptosis) was determined as the fraction of [¹⁴C]-labeled DNA in the lysis fraction + EDTA washes relatively to total intracellular [¹⁴C]-labeled DNA. Results are expressed as the percentage of DNA fragmented in treated cells compared with DNA fragmented in control untreated cells (background) using the formula: (F₀ - F₁)/(1 - F₀) × 100, where F₀ and F₁ represent DNA fragmentation in treated and control cells, respectively.

**Detection of Apoptosis.** Cellular DNA fragmentation ELISA assay (Roche Diagnostics) was applied to measure apoptotic cell death by detection of BrdU-labeled DNA fragments in culture supernatant and cytoplasm of cell lysates, according to manufacturer’s instructions (catalog number 1585 045).

The assay is based on the quantitative sandwich enzyme immunoassay (ELISA) principle using two mouse monoclonal antibodies directed against DNA and BrdU, respectively. This allows the specific detection and quantification of BrdU-labeled DNA fragments.

**Caspase Activity.** Caspase activity was detected in cells using the EnzCheck Caspase 3 assay kit with Z-DEVD-AMC substrate (Molecular Probes, Eugene, OR).

Treated and untreated cells (10⁶) were harvested, lysed, and assayed as described in the kit protocol. Reactions were carried out at room temperature and fluorescence was measured in a fluorescence microplate reader using excitation at 342 nm and emission detection at 441 nm after different times.

**Results**

**Induction of Cell Cytotoxicity.** Several human cancer cell lines carrying different mutations and normal human cells (T lymphocytes) were used in this study (Table 1).

The expression of c-myc protein levels, as assayed by Western blot analysis, in human cancer cell lines is shown in Fig. 1. HL-60 and LAN-5 cells represented, respectively, positive and negative controls for c-myc expression (Galderisi et al., 1999; Pan and Simpson, 1999). Among the cell lines tested, HL-60, HCT-116, Pa-1, and K652 cells expressed high levels

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Status of Ras and myc in several human cancer cell lines and normal human cells</th>
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<tbody>
<tr>
<td>Subpanel</td>
<td>Cell Line†</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>HCT-116</td>
</tr>
<tr>
<td>Leukemia</td>
<td>HL-60</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>K562</td>
</tr>
<tr>
<td>Normal human</td>
<td>OVCAR-3</td>
</tr>
<tr>
<td></td>
<td>Pa-1</td>
</tr>
<tr>
<td></td>
<td>SKOV-3</td>
</tr>
</tbody>
</table>

<sup>a</sup> p53 status was: wt in HCT-116 and Pa-1 cells; mutated (mu) or deleted in HL-60, K562, OVCAR-3, and SKOV-3 cells (Debernardis et al., 1997; O’Connor et al., 1997).
<sup>b</sup> According to Koo et al. (1996).
<sup>c</sup> As evaluated by RT-PCR.
<sup>d</sup> According to Pan and Simpson (1999).
<sup>e</sup> According to Ceballos et al. (2000).
<sup>f</sup> Expression of K-Ras protein (normal gene) according to Hung et al. (1996).
<sup>g</sup> After a 7-h incubation with 10 mg/ml PHA (RT-PCR data not shown; Harel-Bellan et al., 1988).
Table 2

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>mRNA c-myc/GAPDH</th>
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<tbody>
<tr>
<td>HL-60</td>
<td>0.165 ± 0.012</td>
</tr>
<tr>
<td>Pa-1</td>
<td>0.148 ± 0.024</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>0.081 ± 0.010</td>
</tr>
<tr>
<td>LAN-5</td>
<td>0.071 ± 0.018</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>0.0710 ± 0.018</td>
</tr>
</tbody>
</table>

Expression of c-myc proteins in human cancer cell lines. A, left: expression of c-myc proteins in HL-60 (lane 1), LAN-5 (lane 2), and OVCAR-3 (lane 3) cell lines. Expression of actin proteins was as described in the text. A, right: expression of c-myc proteins in SKOV-3 (lane 1), Pa-1 (lane 2), LAN-5 (lane 3), HCT-116 (lane 4), and K-562 (lane 5) cell lines. Expression of actin proteins was as described in the text. C-myc protein expression in HL-60 (lane 1), LAN-5 (lane 2), and HCT-116 cells (Fig. 1B) and HL-60 cells (Fig. 1C), whereas scramble oligo did not affect the expression of c-myc proteins (Fig. 1B).

Looking at the sequence of exposure, it appeared that a synergistic effect was obtained when RPR-115135 was added to the medium, after a preincubation for 24 h with c-myc antisense oligo, for an additional 5 days (Table 3). The simultaneous administration resulted in an additive effect, whereas the sequence RPR-115135 followed by c-myc oligo determined an antagonistic effect. Scramble oligo was devoid of any effect in combination with RPR-115135 (Table 3).

The sequence c-myc oligo for 24 h followed by RPR-115135 was used for all of the following experiments. The nontoxic concentration of 0.1 μM both for c-myc oligo and for RPR-115135 was used for combination experiments.

RPR-115135, like other FTIs (Gibbs, 2000), is a cytostatic drug; thus, drug removal allows HCT-116 cells exposed to 10 μM RPR-115135 for 24 h to grow until confluence. In contrast, the combination c-myc oligo/RPR-115135 at 0.1 μM was strongly cytotoxic; after drug removal (total exposure 24 + 24 h), HCT-116 cells were completely unable to proliferate (survival fraction after 5 days = 1.7%).

Cell Cycle and Apoptosis Induction. We have reported previously that the growth inhibition induced by RPR-115135 in exponentially growing HCT-116 cells could not easily be accounted for on the basis of a specific cell cycle arrest phenotype or induction of apoptosis (Russo et al., 2002a,b). When HCT-116 cells were incubated for 24 h with 0.1 μM c-myc antisense oligo and then for an additional 24 h with 0.1 μM RPR-115135, a G0-G1 arrest and a tendency to apoptosis (sub-G1 population) was observed (Fig. 2).

To confirm the tendency to apoptosis, 1000 cells were scored after DAPI staining. A significant number of cells showing chromatin condensation (p < 0.002 according to Student’s t test) were observed only in samples exposed to the combination c-myc oligo/RPR-115135 (Table 4). The combination was specific; no induction of apoptotic cells was observed when treatment with RPR-115135 was preceded by exposure to scramble oligo (Table 4).

Filter binding assay (Fig. 3) revealed that DNA secondary fragmentation (apoptosis-related) started 3 h after addition of 0.1 μM RPR-115135 to cells previously exposed for 24 h to 0.1 μM c-myc oligo and increased with increasing incubation time, until it reached the maximum (81.2 ± 2.8% over control) after 48 h. (Fig. 3). No DNA fragmentation was evaluated in cells exposed to either RPR-115135 or c-myc oligo (Fig. 3). Cellular DNA fragmentation ELISA assay detecting apoptosis confirmed these observations (Fig. 4).

Role of Caspases in Mediating Apoptosis. To gain insights into the mechanism by which c-myc oligo in combination with RPR-115135 induced apoptosis, effects on caspases (-3, -6, and -7) were investigated by measuring protease activity using the fluorogenic substrate Z-DEVD-AMC. Active caspases cleave Z-DEVD-AMC between the second aspartic acid (D) and AMC, resulting in the release of the fluorescent AMC. This causes an increase in fluorescent activity.

c-myc oligo (for 25, 28, or 30 h) or RPR-115135 (for 1, 3, or
6 h) treatment alone did not determine any increase in fluorescence (Fig. 5), whereas the combination caused a great increase in fluorescence (Fig. 5), suggesting that caspase activation is induced only by c-myc oligo/RPR-115135 treatment.

The role of caspases was further investigated by the use of caspase-3 peptide inhibitor Z-DEVD-fmk (Suzuki et al., 1998). Z-DEVD-fmk blocks c-myc oligo/RPR-115135-induced caspase activation (Fig. 5) as well as c-myc oligo/RPR-115135-induced apoptosis. The percentage of apoptotic cells (scoring DAPI-stained slides) in the presence of 50 mM Z-DEVD-fmk was 21.3 ± 2.6. Caspase inhibition resulted in a 66% inhibition of apoptosis. These results might suggest that caspase activation is required for c-myc oligo/RPR-115135-induced apoptosis.

Role of p53 and p21\textsuperscript{waf1} in Mediating Cell Cycle Arrest and Apoptosis. The growth-inhibitory effect induced by RPR-115135, as reported for other FTIs (Gibbs, 2000; Hill et al., 2000), is not p53-dependent (Ottoboni et al., 2001; Russo et al., 2002a,b). To investigate the possible role of p53 in c-myc oligo increasing susceptibility to RPR-115135, some experiments were performed in two different clones with p53-disrupted activity. As shown in Table 5, pre-exposure of Mu-p53-2 or Mu-p53-4 clone to 0.1 μM c-myc oligo (subtoxic

Table 3

<table>
<thead>
<tr>
<th>Drug Treatment Schedule</th>
<th>IC\textsubscript{50} \textsuperscript{a}</th>
<th>Statistically Significant CI (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous treatment (6 days)</td>
<td>100.0</td>
<td>0.05 (0.034–0.389)</td>
</tr>
<tr>
<td>Scramble oligo</td>
<td>0.41 ± 0.15</td>
<td>N.S.</td>
</tr>
<tr>
<td>RPR-115135</td>
<td>0.009 ± 0.003</td>
<td>1.776 (1.064–4.225)</td>
</tr>
<tr>
<td>c-myc oligo</td>
<td>0.43 ± 0.21</td>
<td>N.S.</td>
</tr>
<tr>
<td>Oligo + RPR-115135</td>
<td>0.78 ± 0.11</td>
<td>N.S.</td>
</tr>
<tr>
<td>Scramble oligo + RPR-115135</td>
<td>0.58 ± 0.25</td>
<td>N.S.</td>
</tr>
<tr>
<td>c-myc oligo + RPR-115135</td>
<td>0.07 ± 0.04</td>
<td>0.98 (0.720–1.006)</td>
</tr>
<tr>
<td>Scramble oligo + RPR-115135</td>
<td>0.41 ± 0.27</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The drug concentration (millimolar concentration) was expressed as mean ± S.E. of two independent experiments performed at least in triplicate.

\textsuperscript{b} To analyze the interactions between the two agents, for each tested combination of the two drugs, CI (confidence interval) values were calculated as proposed by Chou and Talalay (1977, 1981), for mutually exclusive drugs: CI = (D\textsubscript{1}/(D\textsubscript{1} + D\textsubscript{2}))/1/2, where D\textsubscript{1} represent the estimated dose of the drug alone capable of producing the same effect of the combined drugs, as estimated from the median-effect eq. 1. In outleline form, CI near to 1 indicates additivity, CI > 1 indicates antagonism, CI < 1 indicates synergism, CI < 0.03 indicates strong synergism. To extrapolate confidence limits (CL), i.e. statistical significance for CI values, we conducted a parametric bootstraping where we assumed the distribution of parameters (m and D\textsubscript{m}) as formulated in eq. 1 to be approximately gaussian, with estimated mean and asymptotic standard error as calculated using nonlinear regression commands of SPSS software.

\textsuperscript{c} Cells were treated for 24 h with 0.1 mM oligos and then for an additional 5 days with different concentrations of RPR-115135 (from 0.001 to 1.0 mM).

\textsuperscript{d} Cells were treated for 24 h with different concentrations of RPR-115135 (from 0.001 to 1.0 mM) and for an additional 5 days with 0.1 mM oligos.

\textsuperscript{e} Cells were treated simultaneously with 0.1 mM oligos and with different concentrations of RPR-115135 (from 0.001 to 1.0 mM) for 6 days.

Fig. 2. Effects of drugs on cell cycle progression of HCT-116 cells. Cells were untreated (A), or treated with 0.1 μM RPR-115135 for 24 h (B), 0.1 μM c-myc antisense oligo for 48 h (C), or 0.1 μM scramble oligo for 24 h followed by 24 h with 0.1 μM RPR-115135 (D) and analyzed by flow cytometry. The figure is one representative of three independent experiments, performed in duplicate.
TABLE 4
Percentage of apoptotic cells induced by different drug treatments in HCT-116 cells

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>% of Apoptotic Cells</th>
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<tbody>
<tr>
<td>Controls</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>Scramble oligo</td>
<td>18.5 ± 0.2</td>
</tr>
<tr>
<td>RPR-115135</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>c-myc oligo</td>
<td>16.2 ± 1.2</td>
</tr>
<tr>
<td>c-myc oligo + RPR-115135</td>
<td>62.8 ± 7.4</td>
</tr>
<tr>
<td>Scramble oligo + RPR-115135</td>
<td>8.2 ± 0.2</td>
</tr>
</tbody>
</table>

* One thousand cells were scored for each DAPI-stained slide. Values are mean ± S.E. of two independent experiments performed at least in duplicate.

* Cells were treated for 48 h with 0.1 mM oligo (c-myc or scramble oligo) or with 0.1 mM RPR-115135.

* Cells were treated for 24 h with 0.1 mM oligo (c-myc or scramble, respectively) and then for an additional 24 h with 0.1 mM RPR-115135.

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**Fig. 3.** Kinetics of induction of DNA secondary fragmentation (apoptosis-related) in HCT-116 cells evaluated by filter-binding assay. Cells were incubated for different times with 10 μM RPR-115135 or 0.1 μM c-myc oligo alone, or with the combination 0.1 μM c-myc oligo for 24 h followed by 0.1 μM RPR-115135 for different times. Data are expressed as mean ± S.E. of three independent experiments performed at least in duplicate.

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The proportion of cells in S phase, whereas the proportion of cells in G<sub>2</sub>-M increased. These experiments supported the hypothesis that the G<sub>0</sub>-G<sub>1</sub> arrest induced by the combination c-myc oligo + RPR-115135 was p21<sup>waft1</sup>-dependent. However the accumulation of the sub-G<sub>0</sub>-G<sub>1</sub> population suggested that the induction of apoptosis might not be p21<sup>waft1</sup>-dependent.

The requirement for p53 and/or p21<sup>waft1</sup> in the induction of apoptosis was further investigated on Mu-p53-2 clone (p53 disrupted) and on HCT-116 p21 knockouts (p21<sup>−/−</sup>). Induction of apoptosis was evaluated by the mean of the DNA fragmentation ELISA assays (Fig. 7). Single-agent exposure did not induce DNA fragmentation (Fig. 7), whereas the combination induced a large extent of DNA fragmentation in both two-cell systems. Consistently, caspase activity was detected only in cells exposed to the combination (Fig. 8, A and B). Taken together, these experiments support the hypothesis that the induction of apoptosis by the combination c-myc oligo/RPR-115135 is not p53- and/or p21<sup>waft1</sup>-dependent, and the key effector might be caspases.

**Role of c-myc Amplification and Ras Mutation.** To examine whether the potentiation effect is more general, a series of human tumor cell lines with differing c-myc, p53, and Ras status were examined (Table 1).

In Fig. 1C we have shown that exposure to 0.1 μM c-myc oligo for 24 h reduced significantly the amount of c-myc proteins in HL-60 cells. When HL-60 cells were preincubated with 0.1 μM c-myc oligo and then with RPR-115135 for 5 additional days, a strong cytotoxic effect was observed [MTS assay (Table 6)], with a potentiation ratio of RPR-115135 ~42 times. As in HCT-116 cells, the combination induced a severe induction of DNA fragmentation (apoptosis), as evaluated by ELISA (Fig. 9).

The concentration of 0.1 μM c-myc oligo was chosen to expose for 24 h several cells (Table 6) before treatment for 5 continuous days with different concentrations of RPR-115135; cytotoxicity was evaluated by MTS assay. The concentration of 0.1 μM c-myc oligo was nontoxic in all cell lines examined [the survival fraction was ~75% (continuous exposure for 6 days)]. Table 6 shows the IC<sub>50</sub> values. The inhibitory effect induced by c-myc oligo was higher in cells overexpressing c-myc, such as K-562 and Pa-1, than in cells in which c-myc was not overexpressed (OVCAR-3 and SKOV-3). All cells were sensitive to RPR-115135 independently of the status of Ras gene; however, there was a tendency for cell lines that harbor K-Ras or N-Ras mutation (HCT-116 and HL-60) or express high levels of K-Ras protein to be more sensitive to RPR-115135. Looking at the potentiation effect, it was high in Pa-1 cells (~67 times [c-myc amplification, high level of Ras protein]), medium in K-562 cells (~18 times [c-myc amplification, Ras wt]), and null in OVCAR-3 and SKOV-3 cell lines (~0.8–1 times [no c-myc amplification, high levels of Ras protein in OVCAR-3 cells; no c-myc amplification, low levels of Ras protein for SKOV-3 cells]).

**Role of Anti- and Pro-apoptotic proteins.** The BCL-2 family of proteins is comprised of pro-apoptotic as well as anti-apoptotic members. The prominent death agonist is Bax, whereas Bcl-2 is the antagonist (Korsmeyer, 1999). HCT-116 and HL-60 cells show a basal level of Bcl-2 and Bax proteins (Fig. 10). When both cells were preincubated for 24 h with 0.1 μM c-myc oligo and then for an additional 24 h with 0.1 μM RPR-115135, a quantitative analysis of the intensity of the bands (Western blotting) revealed that the apparent Bax/
Bcl-2 ratio was 5 times higher (Fig. 10), thus demonstrating a change in relative amount of Bax and Bcl-2 in favor of Bax. Single treatment did not change the Bax/Bcl-2 ratio (Fig. 10).

Role of c-myc in Normal Cells. Previous studies (Harel-Bellan et al., 1988) clearly supported the role for c-myc protein in the proliferation process of human T lymphocytes. The gene is almost silent in the G0 and early G1 phases and is activated after induction of PHA, during entry into the proliferative phases, and its expression remains high throughout the cell cycle. c-myc protein expression in these cells is

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**Fig. 4.** Kinetics of drug-induced apoptotic cell death in HCT-116 cells were evaluated by ELISA. A total of 10⁴ cells/well were incubated for 24 h with 0.1 μM c-myc oligo followed by 0.1 μM RPR-115135 for different times. After the times indicated, 100 μL/well of supernatant (SN; filled symbols) and 100 μL/well of lysates (open symbols) were removed and tested by ELISA. Data are expressed as mean ± S.E. of two independent experiments performed at least in duplicate.

**Fig. 5.** Induction of caspase activity. Caspase activity was evaluated for c-myc oligo after 24 + 1, 24 + 3, or 24 + 6 h, for RPR-115135 after 1, 3, or 6 h. For the combination, the caspase activity was evaluated after 1-, 3-, or 6-h addition of RPR-115135 to cells exposed previously for 24 h to c-myc oligo. Caspase activity was evaluated in cells exposed to the combination and to 50 mM Z-DEVD-fmk, administered simultaneously with RPR-115135. Data are expressed as mean ± S.E. of two independent experiments performed at least in duplicate.
specifically inhibited by a deoxy-oligonucleotide. The oligo penetrated the cells, reaching the plateau in 2 h, and, specifically, blocked de novo synthesis of c-myc protein induced by PHA (given after 2 h of oligo exposure) in human resting peripheral T cells (Harel-Bellan et al., 1988).

To determine whether the induction of c-myc protein was relevant to the potentiation effect, experiments were performed in human T-lymphocytes. In T resting cells, exposure to 30 μM RPR-115135 (maximum concentration achievable) or to combination did not induce any antiproliferative effect (SF > 98%). No effect was seen in T cells incubated for 7 h with PHA (maximum expression of c-myc) and then for an additional 24 h with 30 μM RPR-115135 (SF 1084%). When cells were preincubated for 2 h with 0.1 μM oligo, before exposure to PHA, then for 7 h in medium containing PHA and, finally, to RPR-115135 (10 or 30 μM), a small potentiation effect was seen. The SF was ~70.5% and 63.2%, respectively. In proliferating lymphocytes (72 h after PHA), all treatments failed to induce cytotoxicity (SF ~71.5%).

**Discussion**

In this work we have shown that down-regulation of c-myc protein levels by nontoxic concentrations of c-myc antisense oligonucleotides resulted in a massive cell death when cells were subsequently exposed to nontoxic concentrations of RPR-115135. Under these experimental conditions, drug removal did not allow any cell recovery. The mechanism by which c-myc oligo increased RPR-115135 cell death was a severe induction of apoptosis mediated by caspase activation. Upstream of caspase activation, the combination treatment induced pro-apoptotic Bax proteins and reduced the amount of anti-apoptotic Bcl-2 proteins. Data obtained in isogenic cell line systems (HCT-116 cells, p53 wt or p53 disrupted; HCT-116 cells, p21\(^{kip1}\)-1 or p21\(^{-}\)-deficient) supported the hypothesis that the induction of apoptosis was not p53- and/or p21\(^{kip1}\)-dependent, whereas the G_0-G_1 arrest was p21\(^{kip1}\)-dependent. In all cellular systems, caspase activation was induced only by the combination treatment. When caspase activation was blocked, no induction of apoptosis was seen. Because the only difference among these cell lines was the absence or presence of a single gene, the interpretation of results was particularly straightforward and uncomplicated by the overexpression of exogenous genetic elements.

**TABLE 5**

Cytotoxic effect induced by different drugs in HCT-116 with disrupted p53 activity

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>IC_{50}a</th>
<th>Mu-p53-2</th>
<th>Mu-p53-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc oligo(^b)</td>
<td>0.47 ± 0.1</td>
<td>0.71 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>RPR-115135(^b)</td>
<td>0.78 ± 0.4</td>
<td>0.58 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>c-myc oligo(^c) + RPR-115135</td>
<td>0.014 ± 0.01</td>
<td>0.011 ± 0.002</td>
<td></td>
</tr>
</tbody>
</table>

* The drug concentration (millimolar concentration) was expressed as mean ± S.E. of two independent experiments performed at least in duplicate.
* Cells were treated continuously for 6 days. The percentage of cell survival fraction after treatment with 0.1 mM c-myc oligo was 77.5 ± 2.4% in Mu-p53-2 and 78.5 ± 1.7% in Mu-p53-4 clone, respectively.
* Cells were treated for 24 h with 0.1 mM c-myc oligo and then for an additional 5 days with different concentrations of RPR-115135 (from 0.001 to 1.0 mM).

**Fig. 6.** Effects of drugs on cell cycle progression of μ-p53-2 clone (panel A) or HCT-116 p21 knockout cells (panel B). Cells were untreated, or treated with 0.1 μM RPR-115135 for 24 h, 0.1 μM c-myc antisense oligo for 48 h, or 0.1 μM c-myc antisense oligo for 24 h followed by 24 h with 0.1 μM RPR-115135 and analyzed by flow cytometry. Data are expressed as mean ± S.E. of two independent experiments performed at least in duplicate.
HCT-116 cells are considered not to be prone to apoptosis induced by gamma radiation (Ottoboni et al., 2001), drug (Russo et al., 2002a,b) or serum deprivation (Russo et al., 2001). It is known that the ability to undergo apoptosis, after extracellular stimuli, depends on the genetic alterations of cells. HCT-116 cells display different mutations affecting important downstream target pathways. Two of these targets (c-myc and Ras) provide a link to enhanced cellular proliferation and resistance to induction of apoptosis.

HCT-116 cells have lost ARF normal functions (Yang et al., 2000). ARF is involved in tumor surveillance [ARF antagonizes murine double minute-2 to activate p53 (Lloyd, 2000)], and its expression is activated by abnormal mitogenic signals induced by overexpression of oncoproteins such as myc and Ras (Lloyd, 2000). Loss of the ARF checkpoint subverts this form of cell-autonomous tumor surveillance and allows proteins such as Ras and myc to function as “pure” proliferation enhancers. Consequently, HCT-116 cells can grow moderately well in the condition of serum withdrawal and do not arrest in the G0-G1 phase of the cell cycle (Russo et al., 2001). Under this growth condition, RPR-115135 induced an increased percentage of G0-G1 cells, but not induction of apoptosis, suggesting that RPR-115135 might down-regulate cell cycle factors that would normally impede G0-G1 arrest. However, when c-myc activity was impaired before RPR-115135 exposure, a severe induction of apoptosis occurred, suggest-
ing that blocking myc and Ras signals in HCT-116 cells resulted in induction of apoptosis.

The determination of sequence-dependent effect is of significant interest for this combination. Concomitant administration has additive cytotoxicity, whereas pre-exposure to c-myc oligo has a synergic effect and the opposite combination an antagonistic effect.

One of the classic paradigms of cellular transformation, and the original basis for the multi-hit theory of cancer, is the collaborative effects of Myc and Ras coexpression in primary fibroblasts (Bos, 1989). However, with the complex and diverse signals emanating from Ras, it is not surprising that the molecular mechanisms underlying Myc/Ras collaboration, both for normal cell proliferation and oncogenesis, have remained elusive despite many years of intensive research. Recent experiments (Sears et al., 2000) show that Ras signaling stabilizes and increases the accumulation of functional Myc transcription factor. Two Ras effector pathways contribute to the stabilization of Myc, the Raf/mitogen-activated protein kinase kinase/ERK kinase cascade and the phosphatidylinositol 3-kinase/AKT signaling pathway. These Ras effector pathways control the phosphorylation of two sites in the N terminus of Myc, which have opposing effects on Myc stability (Sears et al., 2000). Specifically, activation of ERK kinases results in the direct phosphorylation of serine 62, which stabilizes Myc protein, and activation of AKT phosphorylates and inactivates GSK-3, which is responsible for phosphorylation of threonine 58, which destabilizes Myc and targets it for ubiquitin-mediated degradation. In addition, there is a hierarchical relationship between these two phosphorylation sites where phosphorylation of threonine 58 requires prior phosphorylation of serine 62. Thus, Ras activation of ERKs leads to the phosphorylation of the newly synthesized Myc on serine 62, and activation of AKT downregulates GSK-3, inhibiting the destabilizing phosphorylation of threonine 58, thus allowing rapid and high-level accumulation of Myc. Then, as the cell cycle progresses and AKT activity falls, GSK-3 becomes active, leading to the phosphorylation of threonine 58 and the increased degrada-

**TABLE 6**

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>IC50a</th>
<th>HL-60</th>
<th>K-562</th>
<th>Pa-1</th>
<th>OVCAR-3</th>
<th>SKOV-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc oligo</td>
<td>2.1 ± 0.7</td>
<td>0.74 ± 0.8</td>
<td>0.28 ± 0.02</td>
<td>3.20 ± 0.70</td>
<td>2.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>RPR-115135b</td>
<td>0.98 ± 0.8</td>
<td>3.2 ± 1.5</td>
<td>0.54 ± 0.1</td>
<td>0.61 ± 0.3</td>
<td>3.7 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>c-myc oligo ± RPR-115135</td>
<td>0.05 ± 0.02</td>
<td>0.18 ± 0.05</td>
<td>0.0076 ± 0.005</td>
<td>0.78 ± 0.1</td>
<td>3.8 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

a The drug concentration (millimolar concentration) was expressed as mean ± S.E. of two independent experiments performed at least in duplicate.
b Cells were treated continuously for 6 days.
c Cells were treated for 24 h with 0.1 mM c-myc oligo and then for an additional 5 days with different concentrations of RPR-115135 (from 0.001 to 1.0 mM).
tion of Myc. As such, Myc protein levels decline later in G1 and then persist at this low level as a cell continues to grow. It is reasonable that blocking this cooperation pathway may result in enhanced apoptosis.

Looking at the data obtained in different human cancer cell lines (HCT-116, HL-60, K562, OVCAR-3, Pa-1, and SKOV-3), the basic role of blocking c-myc signals, when they were amplified, was straightforward. On the other hand, the role of Ras was not unequivocally clarified. However, when Ras is mutated (or Ras proteins are overexpressed), the effect induced by the combination is stronger. This conclusion is not surprising since, as generally reported, FTIs target many different farnesylated proteins other than Ras.

The substantially negative results obtained in human normal T lymphocytes suggested that mechanisms regulating c-myc gene expression during normal T cell proliferation might not be involved in the mechanism of potentiation.

These data support the initial hypothesis that inhibition of more than one oncogene is crucial to have the power to completely inhibit tumor growth; consequently a combination of drugs may be needed. This means, also, that the tumor should be shown to express the oncogene or the signaling pathway that is to be inhibited.

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References


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