Effects of PRIMA-1 on Wild-type L1210 Cells Expressing Mutant p53 and Drug-resistant L1210 Cells Lacking Expression of p53: Necrosis *vs.* Apoptosis

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Abstract. *The effects of PRIMA-1 on wild-type (WT) mouse leukemia L1210 cells and drug-resistant L1210 cells (Y8) were studied with respect to the induction of apoptosis and necrosis in these cell lines. The WT L1210 cells express mutant p53 while the Y8 L1210 cells do not express p53 mRNA or protein, but do express WAF1/p21 and Gadd 45 mRNA's and proteins. It was found that, in response to treatment with PRIMA-1, the WT L1210 cells became necrotic with little apoptosis while the Y8 L1210 cells showed a much higher level of apoptosis than necrosis. Flavopiridol in combination with PRIMA-1 caused a synergistic increase in necrosis in the WT L1210 cells while LY 294002 in combination with PRIMA-1 caused a synergistic increase in apoptosis in the Y8 L1210 cells. These studies showed that PRIMA-1 had an effect not only on cells expressing mutant p53, but also on cells that do not express p53, suggesting that PRIMA-1 and PRIMA-1-like molecules have multiple sites of action independent of restoring p53 function and that these can interact with other signaling pathways involving CDK's and PI3 kinases.*

In a systematic study of compounds that suppress the growth of human tumor cells expressing mutant p53, a small molecular weight compound, 2, 2-bis (hydroxymethyl)-1-azabicyclo[2,2,2] octan-3-one, was identified as a lead compound. It was designated as PRIMA-1 (p53 reactivation and induction of massive apoptosis) (1, 2). Other studies have been carried out to establish the mechanism(s) by which this was effected (3). Extending these studies, it was shown that a PRIMA-1 analog was more active than PRIMA-1 and was shown to synergize with cisplatin to induce apoptosis (4). Additional studies showed that PRIMA-1 had cytotoxic effects on human chronic lymphocytic leukemia cells with and without hemizygous p53 deletion (5). The results from these studies showed that, in combination with fludarabine, additive and synergistic cytotoxic effects occurred with PRIMA-1 (5). Most recently, a study using a series of analogs, with PRIMA-1 as the reference compound, showed that PRIMA-1 may act through other mechanisms not related to activating mutant p53 (6).

Our studies, in which we compared the effects of PRIMA-1 on the parental WT L1210 cells that express mutant p53 and on a drug-resistant L1210 cell line that does not express p53, showed that there are PRIMA-1-directed effects that are independent of p53 activation. Understanding the mechanism(s) by which PRIMA-1 and similar molecules exert their effects on tumor cells will undoubtedly provide key concepts that could be exploited in small molecule drug design.

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Materials and Methods

Cell culture. The parental mouse leukemia L1210 cell line was originally purchased from the American Type Culture Collection (Rockville, MD, USA). The Y8 and ED2 cell lines were selected for resistance to deoxyadenosine as previously described (7, 8); the hydroxyurea-resistant cells (HU-7) were selected for resistance to hydroxyurea (9); the MQ-580 cells were selected for resistance to 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (10). The cells were grown in RPMI 1640 culture medium supplemented with 10% horse serum, $NaHCO_3$ (2 g/l) and gentamycin (50 mg/l). The cells were incubated at 37˚C in a humidified atmosphere of 95% air/5% CO_2. The drug-resistant cells were maintained in the respective drugs. The cells were subcultured every 2-3 days to maintain the cells in log-phase.

Cell growth inhibitor studies. The WT and Y8 cells, in log-phase, were incubated in the presence and absence of PRIMA-1 for 72 hours. Aliquots of the cultures were taken for cell counts using a Coulter counter, Model Z1.

Cell cycle analysis by flow cytometry. The WT and Y8 cells were treated with different concentrations of PRIMA-1 for various periods of time. Using the method of Krishan (11), the cells (aliquots of $1.0x10^6$ cells in duplicate) were stained with propiduim iodide and kept overnight at 4˚C. Prior to running the samples on a Becton-Dickinson FACSCAN (San Jose, CA, USA), the cells were passed through a 25-gauge needle. Ten thousand events were recorded for each sample and the data analyzed using BD ModFit software. All experiments were carried out at least twice.

Determination of apoptotic and necrotic fractions in WT and Y8 L1210 cells. WT and Y8 L1210 cells, in log-phase, were treated with PRIMA-1, PRIMA-1 plus flavopiridol or PRIMA-1 plus LY 294002 for various periods of time. Aliquots of cells ($1.0x10^6$ cells) in duplicate were collected by centrifugation and washed with cold phosphate-buffered saline (PBS). The apoptotic fraction was determined using the Annexin-V-FLUOS kit (Boehringer-Mannhein, Indianapolis, IN, USA), which measures the level of phosphatidylserine that moves to the cell membrane surface. The necrotic fraction was determined by the level of propidium iodide staining of the nucleus. An apoptotic cell stains with Annexin-V-FLUOS, but not with propidium iodide. Conversely, a necrotic cell stains with propidium iodide but not with Annexin-V-FLUOS. The cells were analyzed on a BD-FACSCAN using 488 nm excitation, a 515 nm bandpass filter to measure Annexin binding and a 515 nm filter to measure propidium iodide uptake. Ten thousand events were collected for each sample. BD Lysis III software was used to analyze the data. Each experiment was carried out at least twice with different batches of cells in duplicate in each experiment.

Western blot analysis of protein levels. The WT and Y8 L1210 cells, in log-phase, were treated with PRIMA-1 for 24 hours. The cells were collected by centrifugation and the cell pellet was washed with cold PBS. The cells were resuspended in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 1% SDS in PBS) which contained protease inhibitor cocktail (Roche). The cell suspension was sonicated, centrifuged and the cell-free supernatant fluids quick-frozen and stored at –84˚C. The protein samples were separated on a 12% SDS gel and transferred to Immobilon membranes. The membranes were blocked with 5% milk for 1 hour before the addition of the primary antibody for incubation overnight at 4˚C. The blots were washed and then treated with the second IgG-HRP antibody.

Measurement of caspase-3 activity. WT and Y8 L1210 cells, in log-phase, were treated with PRIMA-1 for 3 and 6 hours. Cell-free extracts were prepared in duplicate from aliquots of $1x10^6$ cells. Caspase-3 activity was determined with Ac-DEVD-AMC as the substrate and the measurement of 1-amino-4-methylcoumarin as the product. In each experiment, controls were run in which the caspase-3 inhibitor, DEVD-CHO, was added to the appropriate wells to demonstrate the specificity of caspase-3 activity being measured.

Materials. The cell culture medium components were purchased from Gibco BRL (Bethesda, MD, USA). PRIMA-1 was purchased from Cayman Chemicals (Ann Arbor, MI, USA). LY 294002 was purchased from Sigma (St. Louis, MO, USA). Flavopiridol was a gift from the National Cancer Institute (USA) through Dr. Edward A. Sausville. The antibody for p21 (purified mouse anti-p21) was purchased from BD Pharmingen. The antibodies for p27 (mouse monoclonal) and MDM2 (rabbit polyclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies, goat anti-rabbit IgG-HRP and anti-mouse IgG-HRP were purchased from Santa Cruz and Amersham Biosciences, respectively. The ECL Western blotting detection reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA).

Results

Effects of PRIMA-1 on cell cycle transit of WT and Y8 L1210 cells. The effects of PRIMA-1 on the cell cycle distribution of the log-phase WT and Y8 L1210 cells were determined after incubation with PRIMA-1 for 24 hours. As seen in Figure 1 and Table I, PRIMA-1, at a concentration of 50 μM-, caused the WT L1210 cells to block in the G2/M-phase of the cell cycle at the expense of the G0/G1 population. On the other hand, there was only a slight increase in the G2/M population of the Y8 cells in response to PRIMA-1 treatment. Higher concentrations of PRIMA-1 caused necrosis and apoptosis, respectively, in the WT and Y8 cells. The difference in the cell cycle effects could not be attributed to differences in the PRIMA-1 IC_{50} values for the two cell lines. The IC_{50} values for PRIMA-1 in the WT and Y8 L1210 cells were 160 μM and 150 μM, respectively.

Effects of PRIMA-1 on induction of necrosis and apoptosis in WT and Y8 L1210 cells. A comparison of the effects of PRIMA-1 on necrosis and apoptosis in the WT and Y8 L1210 cells was carried out. As seen in Figure 2 and Table II, PRIMA-1 caused a greater necrotic than apoptotic effect in the WT L1210 cells. In contrast, PRIMA-1 had a much greater apoptotic effect on the Y8 L1210 cells than on the WT 1210 cells. There was a steep increase in the apoptotic and necrotic effects between the treatments with 50 μM and 75 μM PRIMA-1, respectively.

Time-course for induction of apoptosis and necrosis in PRIMA-1-treated WT L1210 cells. WT L1210 cells were incubated with PRIMA-1 (150 μM) for various periods of time to determine the time-course in which the necrotic and apoptotic fractions of the WT L1210 cells were generated. As seen in Table III, between the ninth and twelfth hours, there was a sharp increase in the necrotic fraction with little apoptosis seen during that period. Between the twelfth and twenty-fourth hours, there was a continuing large increase in the fraction of cells undergoing necrosis *versus* the fraction undergoing apoptosis. Even at this high concentration of PRIMA-1, the fraction of necrotic cells was greater than the fraction of apoptotic fraction in the WT L1210 cells.

Effects of flavopiridol in potentiating the necrotic effect of PRIMA-1 on WT L1210 cells. As seen in Table IV, PRIMA-1 at a concentration of 50 μM had little effect on the WT L1210 cells with respect to inducing either necrosis or

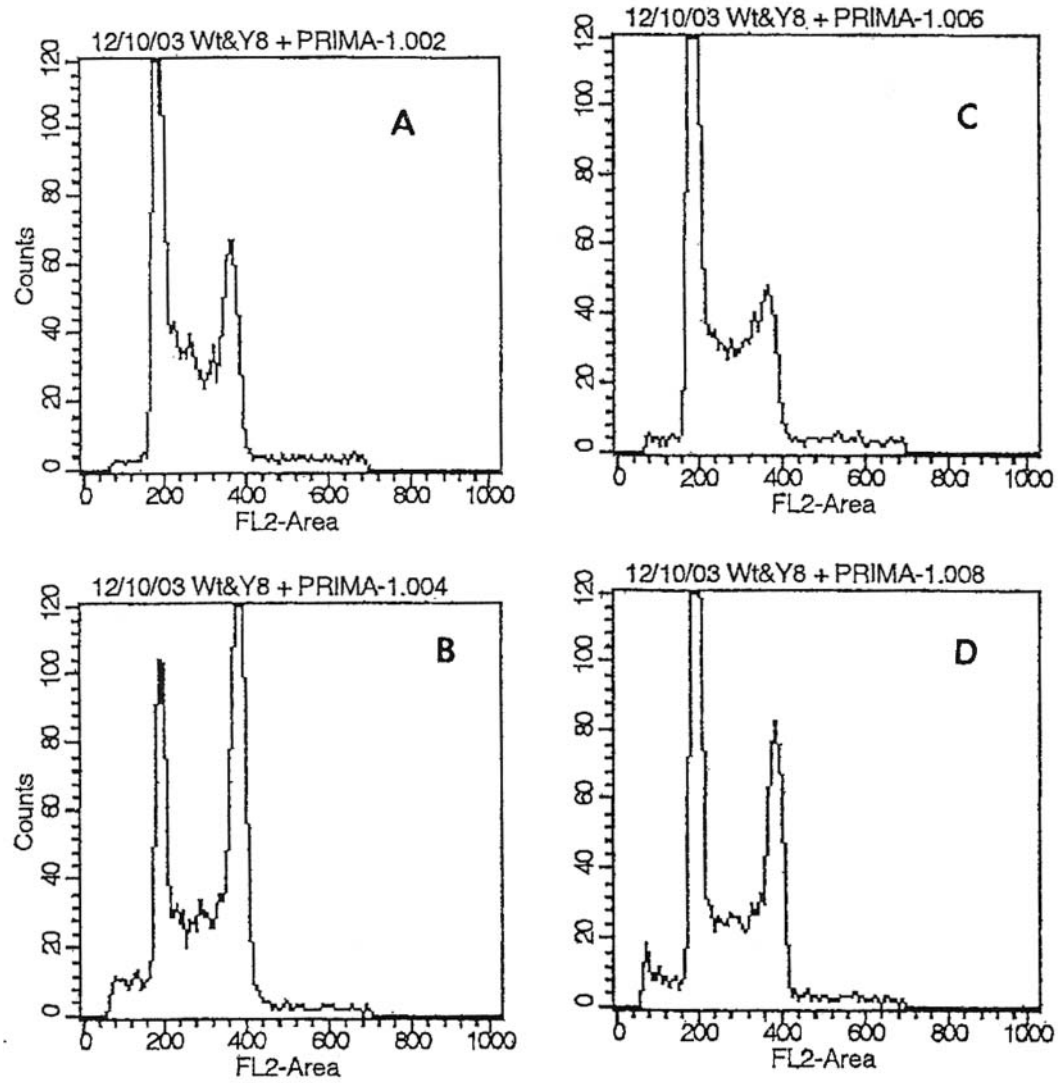

Figure 1. *Effect of PRIMA-1 on the cell cycle of WT and Y8 L1210 cells. WT and Y8 L1210 cells (in log-phase) were incubated in the presence of PRIMA-1 (50 μM) for 24 hours. The cells were processed to determine the cell cycle fractions by the method of KRISHAN (11). Panels: A and C, WT and Y8 cells, respectively, as controls, no drug; B and D, WT and Y8 cells, respectively, treated with PRIMA-1 (50 μM).*

apoptosis. However, when flavopiridol was combined with PRIMA-1, there was a synergistic increase in the necrotic fraction of the WT L1210 cells with no effect on apoptosis.

Effects of LY 294002 on PRIMA-1 in WT and Y8 L1210 cells. The effects of PRIMA-1 in combination with LY 294002 were studied in the WT and Y8 L1210 cell lines. As seen in Table V, LY 294002 alone or in combination with PRIMA-1 had very little effect on the WT L1210 cells. On the other hand, while LY 294002 alone had small effect on the Y8 cells, the combination of PRIMA-1 plus LY 294002 caused a large increase in apoptosis with little effect on necrosis.

Table I. *Comparison of the effects of PRIMA-1 on the cell cycle of WT and Y8 L1210 cells.*

Cell type[a]	G0/G1	S	G2/M
	(% fraction)		
WT	38	49	13
+ PRIMA-1, 50 μM	17	46	38
Y8	45	46	10
+ PRIMA-1, 50 μM	42	37	21

[a]Cells were incubated in the presence of PRIMA-1 for 24 hours before being processed for cell cycle distributions. These data are the average of two entirely separate experiments in which each condition was set up in duplicate.

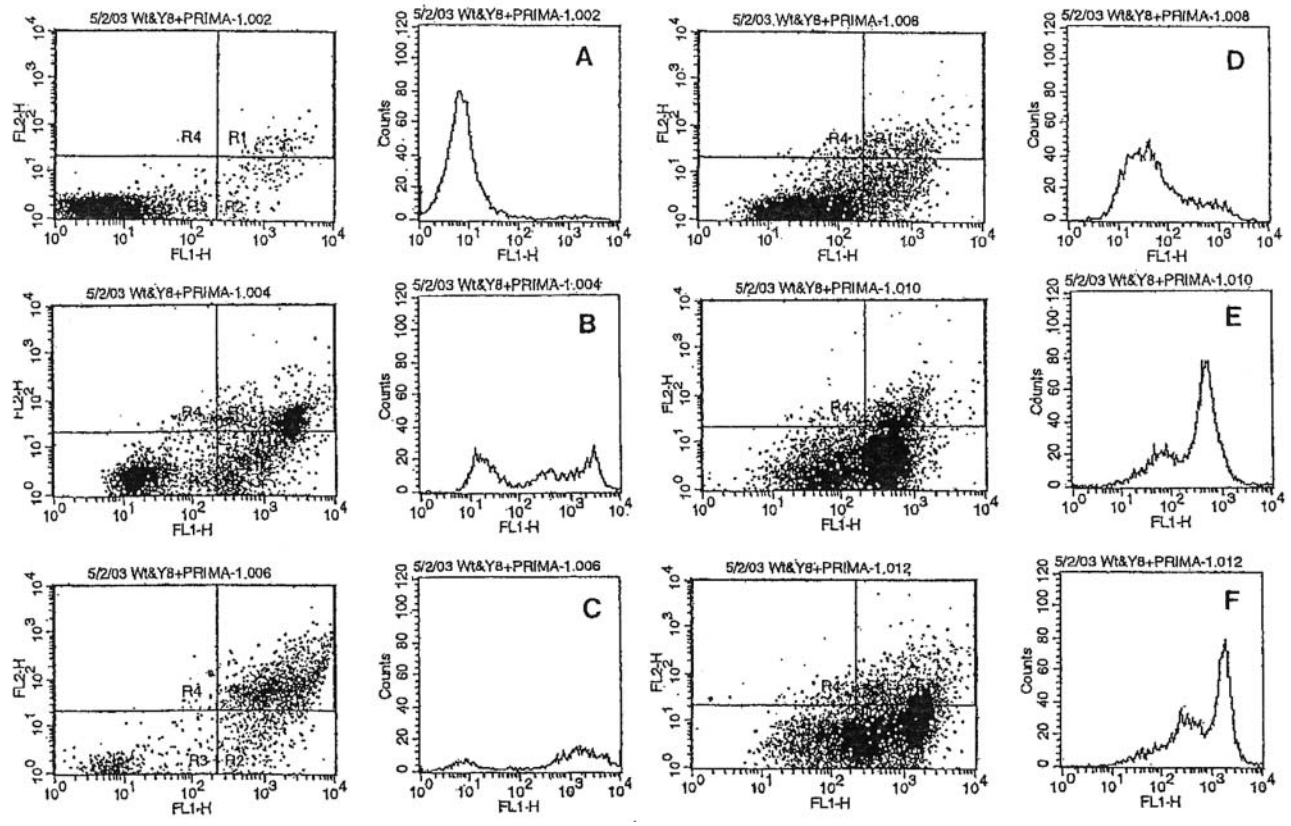

Figure 2. *Effect of PRIMA-1 on apoptosis and necrosis in WT and Y8 L1210 cells. WT and Y8 L1210 cells were treated with PRIMA-1 (75 and 100 μM) for 24 hours and the cells analyzed for the apoptotic and necrotic fractions using Annexin-V and propidium iodide straining. The quadrants represent: R1, necrotic cells, R2, apoptotic cells and R3, viable cells.*

Table II. *Comparison of the effects of PRIMA-1 on apoptosis and necrosis in WT and Y8 L1210 cells.*

Cell type[a]	PRIMA-1 (μM)	Apoptotic (% fraction)	Necrotic (% fraction)
WT	–	1	1
	75	22	32
	100	12	49
Y8	–	11	5
	75	59	7
	100	66	12

[a]Cells in log-phase were incubated in the presence of PRIMA-1 for 24 hours.

Table III. *Time-course for PRIMA-1-induced necrosis in WT L1210 cells.*

Time[a] (h)	Apoptotic (% fraction)	Necrotic (% fraction)
0	2	1
3	2	1
6	1	1
9	1	1
12	1	25
24	23	58

[a]WT L1210 cells, in log-phase, were incubated in the presence of PRIMA-1 (150 μM) for the times indicated. The cells were processed for determination of the apoptotic and necrotic fractions.

Caspase-3 activity in PRIMA-1-treated WT and Y8 L1210 cells. WT and Y8 cells were treated with PRIMA-1 (100 μM) for 0, 3 and 6 hours. Cell-free extracts were prepared from these cells and the caspase-3 activity determined. As seen in Table VI, as a result of PRIMA-1 treatment there was little increase in caspase-3 activity in the cell-free extracts from either cell line and that there was essentially no difference in the caspase-3 activity between the WT L1210 cells that undergo necrosis *versus* the Y8 L1210 cells that preferentially undergo apoptosis.

Table IV. *Necrotic effects of PRIMA-1 in combination with flavopiridol on WT L1210 cells.*

Drugs	Apoptotic (% fraction)[a]	Necrotic (% fraction)[a]
None	2	2
PRIMA-1, 50 μM	3	4
Flavopiridol, 60 μM	2	2
PRIMA-1 + FP	3	65

[a]WT L1210 cells in log-phase were incubated with the drugs, alone and in combination, for 24 hours.

Table V. *Apoptotic and necrotic effects of PRIMA-1 in combination with LY 294002 in WT and Y8 L1210 cells.*

Cell type[a]	Apoptotic (% fraction)	Necrotic (% fraction)
WT	3	1
PRIMA-1, 50 μM	8	8
LY 294002, 25 μM	5	0
PRIMA-1 + LY	18	12
Y8	12	2
PRIMA-1, 50 μM	39	18
LY 294002, 25 μM	14	1
PRIMA-1 + LY	78	6

[a]cells in log-phase were incubated in the presence of the drugs, alone and in combination for 24 hours.

Effects of PRIMA-1 on cellular levels of p21, p27 and MDM 2 in WT and Y8 L1210 cells. As previously reported, the WT L1210 cells express a mutant p53, while Y8 cells no longer express p53, but express basal levels of WAF1/p21 mRNA and protein (12) in the absence of p53. PRIMA-1 treatment of the WT and Y8 L1210 cells resulted in different effects on the levels of p21, p27 and MDM2 proteins, as shown in Figure 3 (a typical experiment). PRIMA-1 treatment of the WT cells had no effect on the expression of p21 in the WT cells; that is, there was no induction of p21 expression. In the Y8 cells, PRIMA-1 treatment resulted in a 40% decrease in p21 and a 50% decrease in p27 (these are the average values for four separate Western blot analyses). In addition, PRIMA-1 treatment of the WT cells had essentially no effect on the expressed levels of MDM2 protein and did not induce expression of MDM2 protein in the Y8 cells.

Effects of PRIMA-1 on L1210 cell lines having resistance to other ribonucleotide reductase inhibitors. As previously reported, a series of L1210 cell lines was selected for resistance to inhibitors directed at the specific subunits of ribonucleotide reductase (7-10). The effects of PRIMA-1 on these L1210 cell lines were compared. As seen in Table VII, the ED2 cells responded to PRIMA-1 treatment in a manner very similar to the Y8 cells. On the other hand, the HU cells, in response to this concentration of PRIMA-1, showed very little apoptosis or necrosis, resembling the responses seen in the WT L1210 cells. Interestingly, the

Table VI. *Effects of PRIMA-1 on caspase-3 activation in WT and Y8 L1210 cells.*

Cell type	Time[a] (h)	Caspase-3 activity (net)[b]
WT	0	330
	3	615
	6	820
WT + PRIMA-1	3	565
+ PRIMA-1	6	990
Y8	0	300
Y8	3	670
Y8	6	805
Y8 + PRIMA-1	3	476
Y8 + PRIMA-1	6	813

[a]WT or Y8 cells were incubated in the presence of PRIMA-1 (100 μM) for the time indicated; cell-free extracts were prepared and caspase-3 activity determined.
[b]Controls were run in which the caspase-3 inhibitor Ac-DEVD-CHO was added to the extract to specifically assay for caspase-3 activity.

Table VII. *Effects of PRIMA-1 on L1210 cell lines with resistance to other ribonucleotide reductase inhibitors.*

L1210 cell type[a]	PRIMA-1 (75 μM)	Apoptosis % fraction[b]	Necrosis % fraction[b]
WT	–	2.3	1.3
	+	6.3	7.4
Y8	–	16.0	2.7
	+	21.4	37.7
ED2	–	11.9	2.5
	+	17.3	41.3
HU	–	2.8	2.2
	+	3.4	3.7
MQ-580	–	1.9	2.2
	+	4.3	27.1

[a]Y8 cells were selected for resistance to deoxyadenosine (8); ED2 cells were selected for resistance to deoxyadenosine and IMPY (9); HU cells were selected for resistance to hydroxyurea (9); MQ-580 cells were selected for resistance to thiosemicarbazone (10).
[b]Values are the average of two separate experiments with duplicates in each experiment.

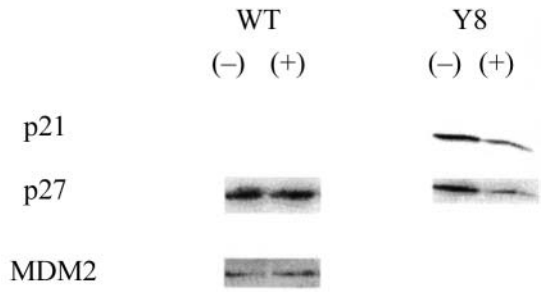

Figure 3. *Western blot analyses showing levels of p21, p27 and MDM2 in WT and Y8 L1210 cells. WT and Y8 L1210 cells were incubated in the presence and absence of PRIMA-1 (100 μM) for 24 hours. Cell-free extracts were prepared for SDS-PAGE and the Western blots treated as described in the "Materials and Methods" section.*

MQ-580 cell response to PRIMA-1 treatment involved a much greater effect on necrosis than on apoptosis.

Discussion

It is generally accepted that the tumor suppressor, p53, is involved in cell cycle control and apoptosis as the result of various stress factors that include DNA damage and hypoxia (13-15). It has been reported that approximately 50% of human tumors express mutant p53 that prevents the required p53-DNA complex from forming and attenuates the subsequent down-stream effects. On this basis, studies were undertaken to determine if there were compounds available that would restore tumor suppressor function to mutant p53 (1). It was found, based on a clever assay system, that a small molecule, 2,2-bis (hydroxymethyl)-1-azabicyclo [2,2,2] octan-3-one (PRIMA-1) inhibited the growth of several human tumor cell lines carrying mutant p53 (1). In these studies, it was found that PRIMA-1 caused the cells to undergo apoptosis as a result of p53 transactivation. Follow-up studies showed that the sensitivity to PRIMA-1 correlated with the expression levels of mutant p53 (2). Further studies by Wiman's group showed that an analog of PRIMA-1, in which one of the hydroxyl groups was methylated (PRIMA-1 met), synergized the effects of cisplatin in inducing apoptosis in tumor cells carrying mutant p53 (4). These studies also showed that PRIMA-1 met in combination with camptothecin, vinblastine and cisplatin acted synergistically to inhibit tumor cell growth in tumor cells (HCT116 and H1299) that lack p53. Using clinical samples of chronic lymphocytic leukemia cells, it was found that PRIMA-1 acted either in an additive or synergistic manner with fludarabine (5). Most importantly, though, these studies showed that the cytotoxic effects of PRIMA-1 were as great on B-CLL cells from patient samples with hemizygous p53 deletion as on those without this deletion.

Most recently, a series of small molecules (based on the structure of PRIMA-1) were synthesized and studied as probes for the reactivation of mutant p53 (6). While a compound was identified as more active than PRIMA-1 in the test system used by previous authors (1), no evidence was obtained to show that wild-type p53 function was restored (6). It was suggested that PRIMA-1 and similar molecules exert their actions by mechanisms other than activating mutant p53.

The results of our studies, reported here, compared the effects of PRIMA-1 on the WT L1210 cells that express mutant p53 and on the deoxyadenosine-resistant L1210 cells that do not express p53, but express p21 and GADD 45 proteins. These studies showed that there may be much more effects of PRIMA-1 than just the reactivation of mutant p53. Clearly, the WT L1210 cells (that express mutant p53) in response to PRIMA-1 treatment preferentially undergo necrosis rather than apoptosis. On the other hand, the deoxyadenosine-resistant cells (that constitutively express p21 in the absence of p53) preferentially undergo apoptosis rather than necrosis in response to PRIMA-1 treatment. Additionally, the WT and Y8 L1210 cells responded differently to PRIMA-1 in terms of cell cycle responses. The WT L1210 cells were blocked in G2/M in response to PRIMA-1 at the expense of the G0/G1 cells. The same extent of cell cycle block was not seen in the Y8 cells in response to PRIMA-1 treatment. Additional support for the possibility that PRIMA-1 and its analogs have effects on tumor cells independent of p53 function comes from the data presented in Tables IV and V, showing that flavopiridol, an inhibitor of cyclin-dependent kinases, in combination with PRIMA-1, caused a synergistic necrotic response in the parental WT L1210 cells without an increase in apoptosis. Likewise, LY 294002, an inhibitor of PI3-kinase, in combination with PRIMA-1, caused a marked increase in apoptosis independent of an increase in the necrotic fraction. Even though the Y8 cells, in response to PRIMA-1, underwent apoptosis, there was not a corresponding increase in caspase-3 activity, suggesting that the apoptotic pathway induced by PRIMA-1 treatment is not caspase-3 activity-dependent.

The results of these studies using the parental WT L1210 cells and Y8 L1210 cells, provide strong evidence that PRIMA-1 and PRIMA-1-like compounds may have unique and useful modes of action that can be exploited in terms of a better understanding molecular differences between necrotic and apoptotic pathways and also in terms of devising protocols for the treatment of human cancers independent of the p53 status, mutant or null.

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