ANTICANCER RESEARCH *25*: 101-106 (2005)

Blockage of Cyclin cdk's, PKC and Phosphoinositol 3-kinase Pathways Leads to Augmentation of Apoptosis in Drug-resistant Leukemia Cells: Evidence for Interactive Effects of Flavopiridol, LY 294002, Roscovitine,Wortmannin and UCN-01

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Abstract. *A mouse leukemia L1210 cell line (Y8), selected for resistance to deoxyadenosine, has a markedly altered phenotypic expression that includes loss of sensitivity to dATP as an allosteric inhibitor of ribonucleotide reductase, increased expression of c-myc, c-fos and WAF1/p21, but decreased expression of p53. In addition, the Y8 cells have a very strong apoptotic response to a variety of agents under conditions in which the parental wild-type cells do not apoptose. In these studies, we show that flavopiridol (a cdk inhibitor) causes the Y8 cells to undergo apoptosis via a caspase-3 activation process. The apoptotic response to flavopiridol is markedly enhanced by LY294002. Data also show that the apoptotic response of the Y8 cells to roscovitine (a cdk inhibitor) is enhanced by UCN-01 (a PKC inhibitor). These data show that simultaneous blockage of specific pathways leads to increased apoptosis in the Y8 cells with essentially no effects on the parental wild-type L1210 cells.*

When it was found that the two non-identical protein subunits of ribonucleotide reductase (RR) could be easily and reproducibly separated using blue-dextran Sepharose columns (1), it became possible to determine whether the then known RR inhibitors independently and selectively inhibited/inactivated one or both RR subunits (2, 3). These studies showed that compounds such as hydroxyurea and IMPY (3) and the thiosemicarbazones (2) specifically inactivated the subunit that contains the non-heme iron-tyrosyl free radical subunit (NHI, R2), while the large subunit that contains the active site and the allosteric binding sites (EB, R1) was inhibited by dATP, Inox and pyridoxal phosphate (2). Since Thelander (4) had shown that the genes encoding the two subunits of RR were on separate chromosomes, it became apparent that cell lines selected for resistance to drugs directed at each subunit would be important in understanding the nature of the biochemical interactions between the non-identical subunits and in elucidating the role each played in the overall ribonucleotide reductase activity in the intact tumor cell. Mouse leukemia L1210 cell lines were selected for resistance to hydroxyurea (5, 6), deoxyadenosine (7) and 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIQ) (8) and the properties of these cells were studied with respect to ribonucleotide reductase.

In examining further the phenotypic expressions of the L1210 cell lines selected for resistance to the specific ribonucleotide reductase inhibitors, it was found that these cells had alterations that could not be overtly related to changes at the ribonucleotide reductase site. For example, the thiosemicarbazone-resistant L1210 cell line (MQ-580) over-expressed protein-disulfide isomerase, p-glycoprotein and MRP (9, 11), while the deoxyadenosine-resistant L1210 cell line (Y8) overexpressed c-myc, c-fos and p21/WAF1 but no p53 (12, 13).

Further studies of Y8 cells showed that, in response to a variety of agents each having different modes of action, the Y8 cells became highly apoptotic under conditions in which the parental wild-type cells showed essentially no apoptosis (14-24).

In this report, we present data that show that blockage of pathways involving cyclin cdk's, PKC or PI-3-kinase leads to apoptosis in Y8 cells and, importantly show that these pathways are interactive and suggest that they converge at a step leading to caspase-3 activation and apoptosis.

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Key Words: Cyclin cdk's, PKC, phosphoinositol 3-kinase, apoptosis, deoxyadenosine resistance, p53, flavopiridol, LY294002, roscovitine, wortmannin, UCN-01, leukemia cells.

Table I. *Effect of flavopiridol on cell cycle distribution of WT and Y8 L1210 cells.*

Cell type[a]	Addition	Cell cycle, %		
		G0/G1	S	G2/M
WT control	none	46	45	9
WT	FP, 300 μM	29	33	32
Y8 control	none	44	48	9
Y8	FP, 300 μM	44	28	27

[a]Cells in log-phase were treated with and without flavopiridol for 24 hours. Flasks were set up in duplicate for each sample and the experiment was repeated with a different batch of cells. Cell cycle analysis was carried out as described in the Materials and Methods section.

Table II. *Apoptotic effect of flavopiridol on WT and Y8 L1210 cells.*

Cell type[a]	Addition	Apoptotic fraction, %
WT	none	2.9
WT	FP, 300 μM	5.1
Y8	none	12.6
Y8	FP, 300 μM	44.7

[a]Cells in log-phase were incubated in the presence and absence of flavopiridol for 24 hours. Flasks were set up in duplicate for each sample and the experiment was repeated with a different batch of cells. Apoptosis was determined using the Annexin V assay as described in the Materials and Methods section.

Table III. *Time course for induction of apoptosis in Y8 L1210 cells.*

Time of incubation, h	Apoptotic fraction (%)
0	5
3	6
6	20
9	30
12	35

Y8 L1210 cells were incubated in the presence of flavopiridol (300 μM) for the times indicated. Apoptosis was assayed using the Annexin V assay.

Materials and Methods

Cell culture. The mouse leukemia L1210 cell line was originally purchased from the American Type Culture Collection (Rockville, MD, USA). The deoxyadenosine-resistant (Y8), hydroxyurea-resistant (HuR) and thiosemicarbazone-resistant (MQ-580) L1210 cells were selected and maintained as previously described (5-8). The cells were grown in RPMI 1640 culture medium supplemented with 10% horse serum, $NaHCO_3$ (2 g/l) and gentamycin (50 mg/l) and maintained at 37˚C in a humidified atmosphere of 95% air/5% CO_2. The cells were subcultured every two to three days to maintain the cells in log-phase.

Cell growth inhibition studies. WT and Y8 cells in log-phase were incubated in the presence and absence of drug for 72 hours. Aliquots of cells 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 drugs for various periods of time. Using the method of Krishan (25), the cells (aliquots of 1.0×10^6 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.

Measurement of apoptosis and necrosis by flow cytometry. WT and Y8 cells, in log-phase, were treated with drug or drugs for various periods of time. Duplicate aliquots of cells (1.0×10^6 cells) were collected by centrifugation and washed with cold phosphate-buffered saline. Apoptosis was determined using the Annexin-V-FLUOS kit (Boehringer-Mannheim, Indianapolis, IN, USA). The necrotic fraction was determined simultaneously by the uptake of propidium iodide. The cells were analyzed on a BD FACSCAN, using 488 nm excitation, a 515 nm bandpass filter for fluorescein detection and a 560 nm filter for propidium iodide detection. Ten thousand events were collected for each sample. BD Lysis II software was used to analyze the data. Each experiment was carried out at least twice with different batches of cells.

Measurement of caspase-3 activity. WT and Y8 cells were treated with a drug or drugs, for three and six hours. Cell-free extracts were prepared in duplicate from aliquots of cells (1×10^6 cells). Caspase-3 activity was determined using a fluorometric assay that measured the release of 7-amino-4-methylcoumarin from the substrate, Ac-DEVD-AMC (caspase-3 kit, PharMingen). In each experiment, controls were run in which the caspase-3 inhibitor Ac-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. Flavopiridol and UCN-01 were obtained from the National Cancer Institute, Bethesda, MD, USA, through the assistance of Dr. Edward A. Sausville, Assistant Director, Developmental Therapeutics Program, NCI. Roscovitine and wortmannin were purchased from Calbiochem, La Jolla, CA, USA. LY-294,002 was purchased from Sigma, St. Louis, MO, USA.

Results

Effect of flavopiridol on WT and Y8 L1210 cells. As shown in Table I, flavopiridol caused both the WT and Y8 L1210 cells to block in G2/M-phase of the cell cycle to the same extent. However, in the WT cells this was accomplished by the decrease in the G0/G1- and S-phase populations, while in the Y8 cells it appeared that it was due to the decrease

Table IV. *Activation of caspase-3 activity by flavopiridol.*

Sample[a]	Time (h)	Net fluorescence[b]
Control	0	120
Control	3	190
Flavopiridol (500 μM)	3	2100
Flavopiridol (500 μM)	6	750

[a]average of two independent experiments with duplicate samples per experiment.
[b]cell-free extracts equivalent to 250,000 cells used per well; substrate used was Ac-DEVD-AFC and the specific inhibitor Ac-DEVD-CHO used to specifically measure caspase-3 activity.

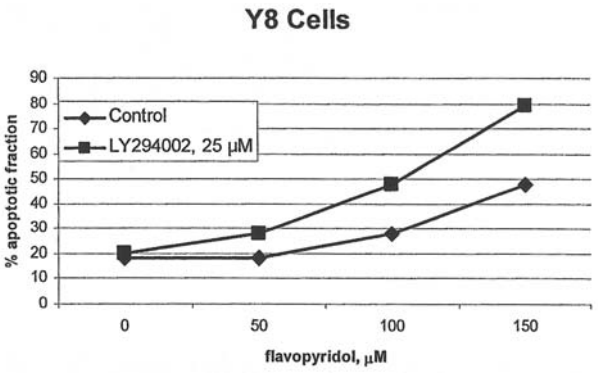

Figure 1. *Effect of LY 294002 on induction of apoptosis in Y8 cells by flavopiridol. Y8 L1210 cells in log phase were treated with flavopiridol and LY294002 for 24 hours. Cells were taken in duplicate and analyzed for the apoptotic fraction using the Annexin V assay.*

in the S-phase population. The IC_{50} values for flavopiridol in the WT and Y8 L1210 cells were 57 and 72 μM, respectively.

The Y8 cells were much more sensitive to the apoptotic effects of flavopiridol than were the parental WT cells as seen in the data in Table II. Flavopiridol (300 μM) had very little effect on the WT cells, but caused 45% of the Y8 cells to undergo apoptosis after 24 hours.

Time course for induction of apoptosis in WT and Y8 cells and caspase-3 activity in cell-free extracts. As seen in Table II, there was extensive apoptosis in the Y8 cells under conditions in which the WT cells underwent very little apoptosis. Significant apoptosis was seen as early as 6 hours after drug treatment was initiated in the Y8 cells (Table III).

The induction of apoptosis reported in Table II was accompanied by an increase in caspase-3 activity as measured in cell-free extracts prepared from flavopiridol-treated Y8 cells. As seen in Table IV, caspase-3 activity increased within three hours of treatment with flavopiridol, but had already decreased by six hours (Table IV).

Interactive pathways leading to apoptotic responses in Y8 cells. In the current study, it has been shown that flavopiridol causes a marked induction of apoptosis in Y8 cells. Previous studies had shown that roscovitine (19), UCN-01 (18) and wortmannin (16), each blocking specific steps, all caused apoptosis in Y8 cells with little or no effect on WT cells. Studies were carried out to determine if there were interactions between or among these pathways. As seen in Figure 1, the addition of LY294002 markedly enhanced the apoptotic effect of flavopiridol. Since LY294002 alone had no apoptotic effect on the Y8 cells, this augmentation of

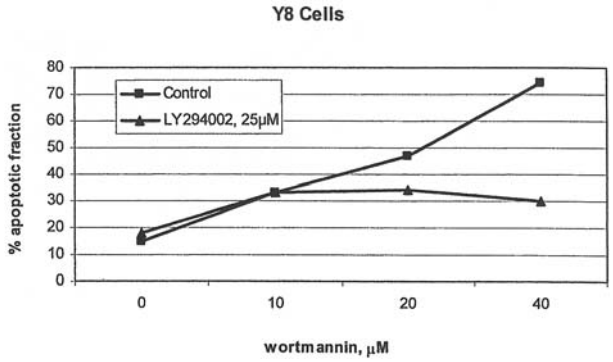

Figure 2. *Effect of LY 294002 on induction of apoptosis in Y8 cell by wortmannin. Y8 L1210 cells were treated with wortmanin and LY 294002 (25 μM) for 24 hours. Cells were taken in duplicate and analyzed for the apoptotic fraction using the Annexin V assay.*

apoptosis in the combination of flavopiridol and LY294002 has to be considered as a synergistic effect.

Extending these studies, the effect of LY294002 on wortmannin-induced apoptosis was studied. As seen in Figure 2, the addition of LY294002 to wortmannin-induced apoptosis in the Y8 cells caused a decrease in apoptosis.

The increase in apoptosis caused by flavopiridol plus LY 294002 and the decrease in apoptosis by the combination of wortmannin plus LY294002 were essentially confirmed by the determination of caspase-3 activity in the Y8 cells. As shown in Table V, there was increased caspase-3 activity in cell-free extracts prepared from Y8 cells treated with flavopiridol and LY294002 and, conversely, decreased caspase-3 activity in cell-free extracts prepared from wortmannin/LY294002-treated cells.

Table V. *Effect of LY294002 on caspase-3 activity in flavopiridol and wortmannin-treated Y8 cells.*

Sample	Time (h)	Caspase-3 activity (net fluorescence)
Control	0	370
Control	3	280
LY, 25 μM	3	450
Flavopiridol, 150 μM	3	370
Flavopiridol + LY	3	5300
Wortmannin, 40 μM	3	1170
Wortmannin + LY	3	600

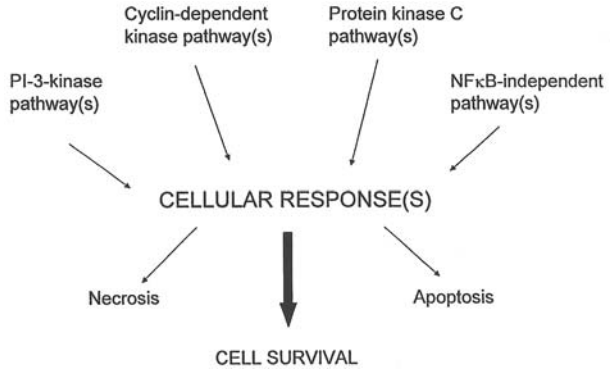

Figure 3. *Pathways leading to cell survival. Blockage of one or more of these pathways leads to augmentation of apoptosis in the Y8 cells but has little or no effect on the parental wild-type L1210 cells.*

Previous studies had shown that roscovitine (19) and wortmannin (16) caused apoptosis in Y8 cells with little effect on WT cells. As shown in Table VI, when UCN-01 was added in combination with roscovitine, there was a significant increase in the apoptotic fraction in the Y8 cells with essentially no effect in the WT L1210 cells.

Response of L1210 cell lines to induction of apoptosis by flavopiridol. To determine the extent to which the various L1210 cell lines were responsive to flavopiridol-induced apoptosis, the apoptotic effects of flavopiridol on the hydroxyurea-resistant and thisemicarbazone-resistant L1210 cells were compared with the flavopiridol effects on the deoxyadenosine-resistant L1210 cells. As seen in Table VII, flavopiridol-induced apoptosis was seen only in the Y8 cells indicating that there are specific unidentified changes in the Y8 cells that promote the apoptotic response. A similar pattern was seen in the response of these same cell lines to Gemcitabine. That is, in response to Gemcitabine treatment, only the Y8 cells became apoptotic (24).

Discussion

Previous studies comparing the effects of various agents on the parental wild-type (WT) L1210 mouse leukemia cells and the deoxyadenosine-resistant (Y8) L1210 cells derived from the WT cells showed that the Y8 cells were particularly sensitive and underwent an apoptotic response under conditions in which the WT cells did not apoptose. These agents included radiation (14), anisomycin (15) roscovitine (19), kinase inhibitors (16), drugs directed at NFkappa B (22), inhibitors of nucleotide metabolism (20) and sodium salicylate (21). In each of these cases, it could be shown that the apoptotic response was mediated through the activation of caspase-3 activity. Curiously, Gemcitabine induced a strong apoptotic response in the Y8 cells but caspase-3 activation could not be demonstrated (24).

In the studies presented here, we show that Y8 cells are much more sensitive to the apoptotic-inducing effects of flavopiridol in a concentration-dependent and time-dependent process. Flavopiridol had been shown to be an effective drug not only in inhibiting tumor cell growth, but also in inducing apoptosis (26, 27).

These studies using flavopiridol and our previous studies with roscovitine (19) showed that blockage of the cdk pathways led to increased apoptosis in Y8 cells. Earlier studies had shown that blockage of other pathways also led to increased apoptosis in Y8 cells. These included protein kinase C with UCN-01 (18), phosphoinositol 3-kinase with wortmannin (16), NFkappa B with parthenolide (22) and inhibitors of nucleotide synthesis (20).

The data shown in Figures 1 and 2 and Tables V and VI show that there are interactions between/among these pathways. LY294002 (directed at phosphoinositol 3-kinase) markedly potentiated the apoptotic effect of flavopiridol which inhibits cdk's and UCN-01, which inhibits protein kinase C, potentiated the apoptotic effect of roscovitine (a cdk inhibitor). Interestingly, LY294002 attenuated the apoptotic effects of wortmannin on Y8 cells. This was unexpected since both drugs inhibit phosphoinositol 3-kinase. However, because of the difference in mechanisms of inhibition of PI-3-kinase by these drugs, the results are explainable. Wortmannin does not appear to be as specific an inhibitor of PI-3 kinase as LY294002, also showing inhibition of phospholipase A2 and other kinases (29-32). In addition, the mechanism of inhibition by wortmannin appears to be somewhat complex. Initially, the

Table VI. *Effects of roscovitine and UCN-01 on apoptosis in WT and Y8 cells.*

Cell type[a]	Apoptotic fraction (%)
WT control	1
+ UCN-01, 0.25 μM	2
+ roscovitine, 60 μM	1
+ UCN-01 + ros	1
Y8 control	7
+UNC-01, 0.25 μM	11
+ roscovitine, 60 μM	44
+ UCN-01 + ros	77

[a]Cells in log-phase were treated with UCN-01 (0.25 μM), roscovitine (60 μM) and the combination of UCN-01 (0.25 μM) and roscovitine for (60 μM) 24 h. Apoptosis was determined using Annexin-V assay.

Table VII. *Mouse leukemia L1210 cell line specific induction of apoptosis by flavopiridol.*

Cell line	Treatment (% fraction)	Apoptosis[b]
WT	control	1.4
	FP, 300 μM	1.8
Y8	control	5.6
	FP, 300 μM	43.4
HU-R	control	3.7
	FP, 300 μM	6.7
MQ-580	control	10.5
	FP, 300 μM	12.2

[a]WT, wild-type L1210 cells; Y8, deoxyadenosine-resistant; HU-R, hydroxyurea-resistant; MQ-580, thiosemicarbazone-resistant.
[b]average of two independent experiments with duplicate samples in each experiment.

binding of wortmannin to the ATP-binding site of PI 3-kinase is competitive with respect to ATP but, once bound in the active site, irreversibly modifies and inactivates the enzyme (33). Further, in comparison with LY294002, wortmannin is much less stable in the culture medium and cells.

Other studies had shown that the combinations of roscovitine and parthenolide (22) and parthenolide and lactacystin (23) led to the augmentation of apoptosis in Y8 cells. A summary of these interactions is shown in Figure 3.

Clearly, from the data presented here and in earlier papers (18-23), concurrent blockage of these pathways leading to specific cellular responses (undefined at this time) promote apoptosis in Y8 cells with little or no effect on the parental WT cells. The questions then become: a) Why are the parental WT cells not subject to the same sensitive apoptotic response seen in the Y8 cells?; b) what protective factors are present in the WT cells to decrease sensitivity to these agents?; or c) what factors are decreased or missing in the Y8 cells that allow this increased apoptotic response?; and d) is it a simple case of quantity of the critical factor(s)? Comparing the expression of mRNAs in WT and Y8 cells in preliminary microarray analysis data (Cory JG *et al.*, unpublished) do not give a hint as to what the critical difference(s) may be. It is clear that the apoptotic response(s) can be initiated at the cell surface, the mitochondria and the most recently identified site, the endoplasmic reticulum (34, 35). It is possible that, in these drug combinations that augment apoptosis in Y8 cells, these drugs are simultaneously involving multiple initiation programs leading to apoptosis.

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Received November 2, 2004
Accepted December 20, 2004