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Enhancing therapeutic efficacy by targeting non-oncogene addicted cells with combinations of signal transduction inhibitors and chemotherapy

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The effects of inhibition of the Raf/MEK/ERK and PI3K/Akt/mTOR signaling pathways and chemotherapeutic drugs on cell cycle progression and drug sensitivity were examined in cytokine-dependent FL5.12 hematopoietic cells. We examined their effects, as these cells resemble normal hematopoietic precursor cells as they do not exhibit "oncogene-addicted" growth, while they do display "cytokine-addicted" proliferation as cytokine removal resulted in apoptosis in greater than 80% of the cells within 48 hrs. When cytokine-dependent FL5.12 cells were cultured in the presence of IL-3, which stimulated multiple proliferation and anti-apoptotic cascades, MEK, PI3K and mTOR inhibitors transiently suppressed but did not totally inhibit cell cycle progression or induce apoptosis while chemotherapeutic drugs such as doxorubicin and paclitaxel were more effective in inducing cell cycle arrest and apoptosis. Doxorubicin induced a G_1 block, while paclitaxel triggered a G_2/M block. Doxorubicin was more effective in inducing cell death than paclitaxel. Furthermore the effects of doxorubicin could be enhanced by addition of MEK, PI3K or mTOR inhibitors. Cytokine-dependent cells which proliferate in vitro and are not "oncogene-addicted" may represent a pre-malignant stage, more refractory to treatment with targeted therapy. However, these cells are sensitive to chemotherapeutic drugs. It is important to develop methods to inhibit the growth of such cytokine-dependent cells as they may resemble the leukemia stem cell and other cancer initiating cells. These results demonstrate the enhanced effectiveness of targeting early hematopoietic progenitor cells with combinations of chemotherapeutic drugs and signal transduction inhibitors.

Introduction

Proliferation and suppression of apoptosis in many hematopoietic precursor cells is promoted by interleukin-3 (IL-3) and other cytokines/growth factors.[1-13] Hematopoietic cell lines have been isolated which require IL-3 for cell proliferation and survival.[1,3] The FL5.12 cell line is an IL-3-dependent cell line isolated from the fetal liver of BALB/c mice and is viewed as a model of early hematopoietic progenitor cells.[1] Cytokine-deprivation of these cells results in rapid cessation of growth with subsequent death by apoptosis, (programmed cell death) within 48 hrs.[2,9,10] In the presence of IL-3, these cells proliferate continuously, however, they are non-tumorigenic when injected into immunocompromised mice.[6-8] Spontaneous factor-independent cells are rarely recovered from the FL5.12 cell line ($<10^{-7}$), making it an attractive model to analyze the effects various genes have on signal transduction and leukemogenesis, since abrogation of cytokine-dependence is an important factor in the development of leukemia.[6-8,11] Furthermore, this cell line is a model for examining the effects of signal transduction inhibitors and chemotherapeutic drugs on the induction of death in early hematopoietic precursor cells and potentially leukemia stem cells (LSC) as these cells and their transformed derivative lines, share markers expressed on LSCs and other cancer initiating cells.[5,11,14-21]

IL-3 exerts its biological activity by binding to the IL-3 receptor (IL-3R) which activates the Ras/Raf/MEK/ERK, PI3K/Akt/mTOR and other signaling and anti-apoptotic cascades.[3] Aberrant expression of the Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways have been detected in many AML samples and their joint overexpression is usually associated with a poor prognosis.[22] IL-3R is reported to be expressed on LSCs.[5,23,24] Aberrant expression of PI3K/Akt/mTOR and other signaling pathways have been observed in LSCs and other CICs.[5,16,25-30]

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Relatively little is known regarding the interactions between the Raf/MEK/ERK and PI3K/Akt/mTOR pathways in "non-oncogene" addicted, non-malignant cells and the sensitivity of such cells to signal transduction inhibitors and classical chemotherapy.[31,32] Understanding the roles the Raf/MEK/ERK and PI3K/Akt/mTOR cascades play in the control of normal and malignant cell cycle progression will enhance our knowledge of how these pathways regulate the sensitivity of CICs and the remaining "bulk" cancer cells to various therapeutic approaches.[4,5,31-48] It is important to learn how targeting these pathways may suppress the growth of CICs. These same pathways are also being considered for targeting in aging and decreasing their activities may suppress aging.[49-57] Thus these are critical pathways implicated in various types of human diseases and aging.

In the following studies, we sought to determine the effects of Raf/MEK/ERK and PI3K/Akt/mTOR on cell cycle progression and drug resistance by inhibiting the Raf/MEK/ERK and PI3K/Akt/mTOR pathways in cytokine-dependent hematopoietic cells with MEK, PI3K or mTOR inhibitors in the presence and absence of chemotherapeutic drugs. While "non-oncogene" addicted cells were not as sensitive to signal transduction inhibitors as "oncogene-addicted" cells,[58,59] the "non-oncogene addicted" cells were sensitive to chemotherapeutic drugs and the therapeutic efficacy can be enhanced by targeted therapy. Therefore, it may be efficacious to target "non-oncogene addicted" pre-leukemia cells before the development of leukemic cells with combinations of chemotherapy and signal transduction inhibitors.

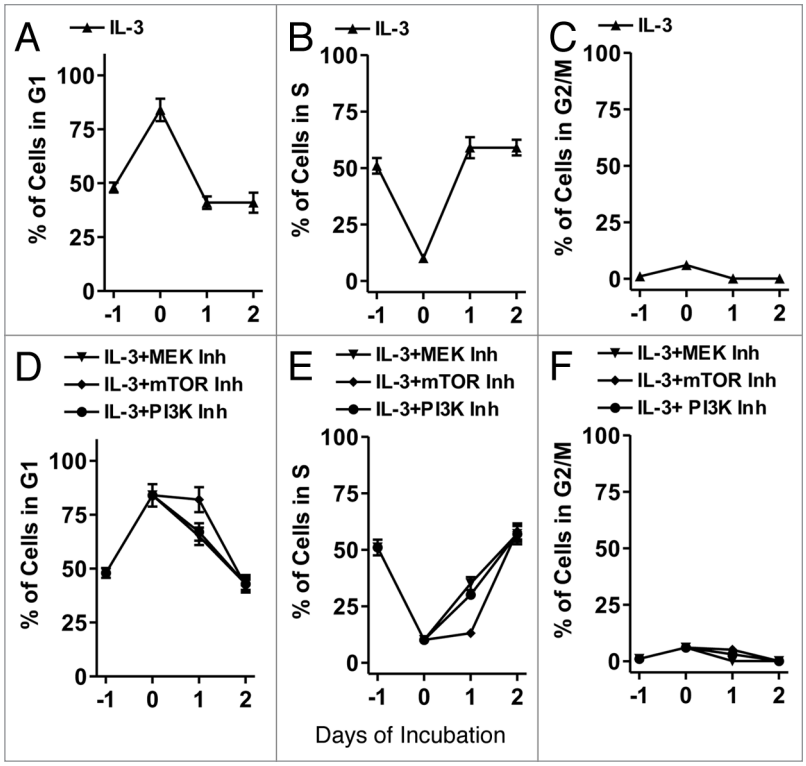

Figure 1. Cell cycle progression in FL5.12 cells in the presence of signal transduction inhibitors. FL5.12 cells were collected, washed with PBS twice and then plated in phenol-red free medium containing 5% CS FBS (Day $_{-1}$). After 24 hrs (Day 0) of incubation, IL-3 and in some cases 10 μM U0126 (MEK inhibitor), 100 nM rapamycin (mTOR inhibitor) or 10 μM PI3K inhibitor (LY294002) were added. The percentage of cells in the different stages of the cell cycle determined after PI staining and FACS analysis with the Modfit computer program. These experiments were repeated three times and averaged together.

Results

Effects of inhibition of the Raf/MEK/ERK and PI3K/Akt/mTOR pathways on cell cycle progression in cytokine-dependent FL5.12 cells. To elucidate the roles of the Raf/MEK/ERK and PI3K/Akt/mTOR pathways on cell cycle progression in cytokine-dependent hematopoietic cells, FL5.12 cells were deprived of IL-3 for 24 hrs and then stimulated with IL-3 in the presence and absence of inhibitors which target Raf/MEK/ERK or PI3K/Akt/mTOR pathways. When FL5.12 cells were deprived of IL-3 for 24 hrs, they accumulated in the G_1 phase (**Fig. 1A**) and exited the S phase (**B**). Upon addition of IL-3 at day 0, the cells exited G_1 (**A**) and entered S phase (**B**).

Similar experiments were performed with the FL5.12 cells that were treated with IL-3 and MEK1 (10 μM U0126), PI3K (10 μM LY294002) and mTOR (100 nM) inhibitors. These concentrations of MEK1, PI3K and mTOR inhibitors inhibit activation/phosphorylation of ERK1,2, Akt, p70S6K and S6 in FL5.12 cells and other cell lines.[11,54,55] Addition of the MEK1 or PI3K inhibitors increased the percentage of cells in G_1 (**D**) compared to untreated cells for the first 24 hrs of treatment (**A**). Furthermore these inhibitors suppressed the entry of FL5.12 cells into the S phase after the first 24 hrs of treatment (**E**). After treatment with the mTOR inhibitor for 24 hrs, there was a more profound block in G_1 (**D**) and inhibition of entry into S phase (**E**). After two days of treatment, similar levels of cells treated with the MEK, PI3K and mTOR inhibitors were in S phase as compared to untreated cells. Thus after a single inhibitor treatment, a transient suppression in cell cycle progression was observed.

Effects of doxorubicin and paclitaxel on cell cycle progression in FL5.12 cells. The effects of two chemotherapeutic drugs on cell cycle progression in FL5.12 cells were examined to determine the ability of these agents to induce cell cycle arrest and apoptosis in cytokine-dependent cells (**Fig. 2**). Addition of doxorubicin, which inhibited DNA synthesis, at concentrations between 10 and 100 nM, resulted in a blockage of the cells in the G_1 phase of the cell cycle (**A**). This arrest in G_1 was more readily observed at day 1 and day 2 when 100 nM doxorubicin was used. Doxorubicin prevented entry into S phase in a dose-dependent fashion (**B**).

In contrast, treatment of FL5.12 cells with IL-3 and either 1 or 10 nM paclitaxel, which prevented microtubule disassembly, resulted in an exit of the cells from the G_1 (**D**) and S (**E**) phases and a block in G_2/M. The blockage in G_2/M occurred after 2 days of paclitaxel addition (**F**). Thus doxorubicin and paclitaxel

differed in how they affected cell cycle progression in cytokine-dependent FL5.12 cells.

Effects of MEK and mTOR inhibitors on prevention of cell cycle progression induced by doxorubicin and paclitaxel. The effects of combining signal transduction inhibitors with chemotherapeutic drugs on cell cycle progression were determined (**Fig. 3**). When cells were treated with the MEK inhibitor and either doxorubicin or paclitaxel for 2 days, the cells had exited S phase (**B**) a dramatic arrest in G_2/M occurred (**C**). In contrast, when they were treated with the mTOR inhibitor and either doxorubicin or paclitaxel, they were blocked from entering G_2/M (**F**) and many of the cells remained in S phase (**E**).

Effects of MEK and mTOR inhibitors on the induction of apoptosis induced by doxorubicin. The ability of targeted therapy and classical chemotherapy to induce apoptosis in FL5.12 cells was determined by annexin V/PI binding (**Table 1**). As a control, some cells were deprived of IL-3 for 48 hrs which resulted in a 2.5-fold increase in apoptotic cells compared to cells continuously cultured in IL-3. When the cells were grown in medium containing IL-3, the MEK1 inhibitor by itself did not have a significant effect on the induction of apoptosis. In contrast, treatment with either the PI3K or mTOR inhibitors did have greater effects on the induction of apoptosis, consistent with the involvement of this pathway on the prevention of apoptosis.

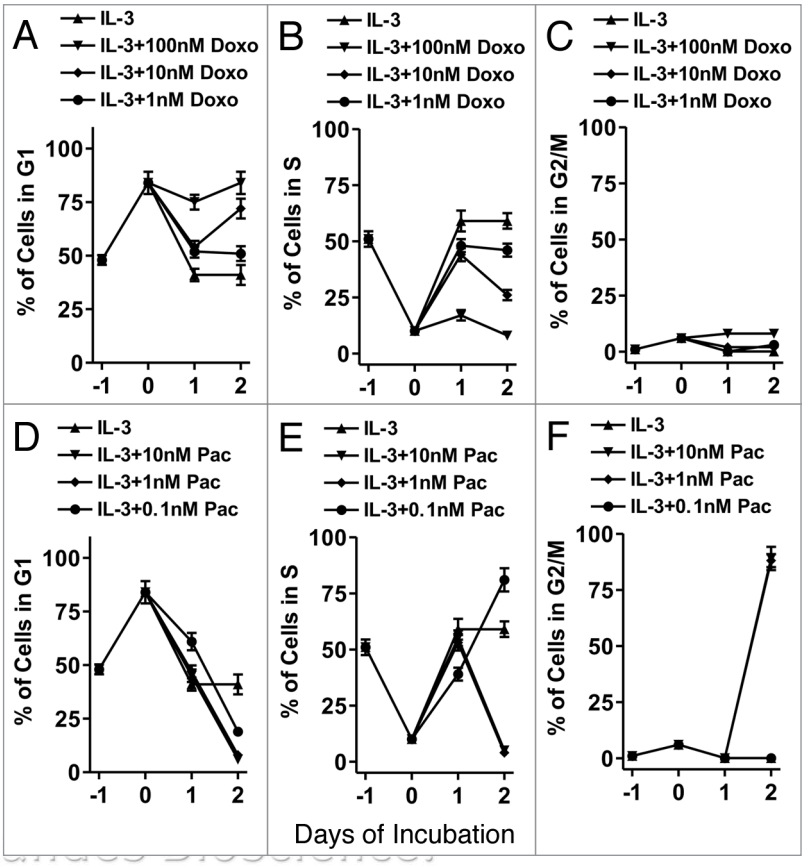

Figure 2. Cell cycle progression in FL5.12 cells in the presence of chemotherapeutic drugs. In FL5.12 cells were collected, washed with PBS twice and then plated in phenol-red free medium containing 5% CS FBS (Day $_{-1}$). After 24 hrs (Day 0) of incubation, IL-3 and the indicated concentrations of doxorubicin (A–C) or paclitaxel (D–F) were added. 1 ml aliquots (1 x 10^5 cells) were removed at the indicated time points and the percentage of cells in the different stages of the cell cycle determined after PI staining and FACS analysis with the Modfit computer program. These experiments were repeated three times and averaged together.

Both doxorubicin and paclitaxel induced apoptosis in FL5.12 cells in a dose-dependent fashion. The effects of co-addition of signal transduction pathway inhibitors on the induction of apoptosis by doxorubicin and paclitaxel were determined. Co-addition of MEK and mTOR inhibitors, but not the PI3K inhibitor LY294002, increased the extent of apoptosis induced by 10 nM doxorubicin. In contrast, co-addition of MEK, mTOR or PI3K inhibitors with paclitaxel did not increase the extent of apoptosis as compared to the level of apoptosis induced by paclitaxel by itself. Thus the apoptosis inducing ability of certain chemotherapeutic drugs can be enhanced by MEK and mTOR inhibitors.

Effects of IL-3 on induction of MAPK pathway and cell cycle regulatory gene expression in FL5.12 cells. The effects of IL-3 activation on the MAPK pathways and cell cycle regulatory gene expression were examined in FL5.12 cells. In these experiments, FL5.12 cells were collected, washed with PBS twice to remove IL-3 and cultured in the absence of IL-3 in medium containing FBS before treatment with a signal transduction inhibitor or doxorubicin for one hour and then subsequently stimulated with IL-3 for the varying time points. In the absence of treatment with targeted therapy or chemotherapy, IL-3 induced the activation of MEK1 and the MAPKs, ERK1/2 and JNK (**Fig. 4**) after 5 minutes of IL-3 stimulation. Interestingly it appeared that after IL-3 stimulation of IL3-deprived cells, the predominant ERK that was being detected was ERK1 and not ERK2. The JNK MAPK has been associated with both proliferation and cell stress.[60-63] Treatment with IL-3 resulted in activation of JNK. When the cells were stimulated with IL-3, essentially no p53 or $p21^{Cip-1}$ were detected.

The effects of the MEK inhibitor U0126, the PI3K inhibitor LY294002 and the chemotherapeutic drug doxorubicin on MAPK and cell cycle regulatory gene expression were examined. In these experiments, the IL3-deprived cells were treated with UO126, LY294002 or doxorubicin, 1 hour prior to the addition of IL-3 and aliquots of the cells were removed at the indicated time intervals. As expected 10 μM U0126 suppressed ERK1/2 activation from the 5 min to 8 hr after IL-3 treatment but did not inhibit the detection of activated MEK1/2. U0126 has been previously shown to inhibit MEK1/2 activity, but not MEK1/2 activation. There is a complicated negative feedback loop which is suppressed by the MEK1/2 inhibitors which actually results in higher levels of activated MEK1/2 as the negative feedback ERK1,2 loop on MEK1/2 is suppressed. Interesting and consistent with the cell cycle data presented earlier, activated ERK1/2 levels did rebound 24 hrs after IL-3 and U0126 treatment.

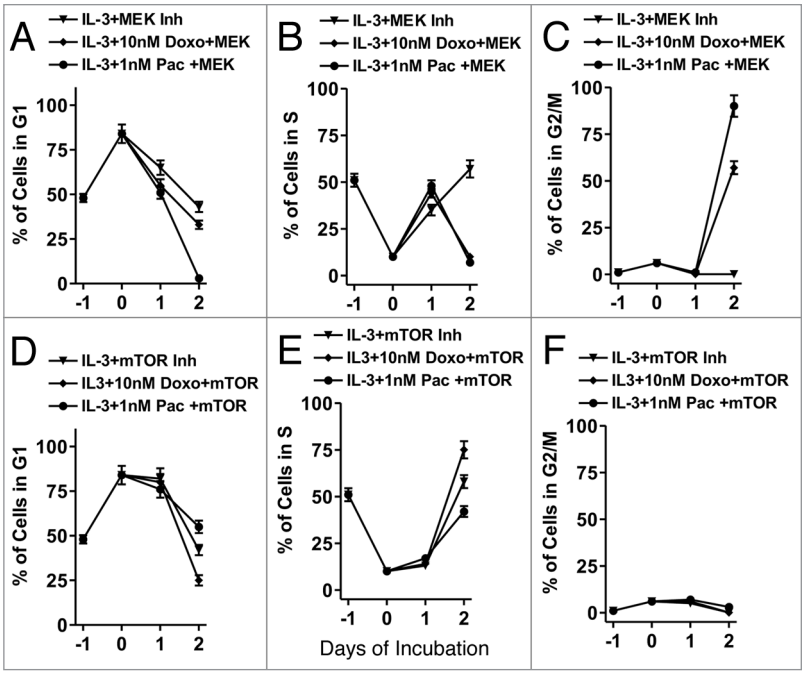

Figure 3. Cell cycle progression in FL5.12 cells in the presence of signal transduction inhibitors and chemotherapeutic drugs. In FL5.12 cells were collected, washed with PBS twice and then plated in phenol-red free medium containing 5% CS FBS (Day $_{-1}$). After 24 hrs (Day 0) of incubation, IL-3 and 10 μM MEK inhibitor and either doxorubicin or paclitaxel (A–C) or -3 and 100 nM mTOR inhibitor and either doxorubicin or paclitaxel (D–F) were added. 1 ml aliquots (1 x 10^5 cells) were removed at the indicated time points and the percentage of cells in the different stages of the cell cycle determined after PI staining and FACS analysis with the Modfit computer program. These experiments were repeated three times and averaged together.

Treatment with IL-3 plus the MEK inhibitor UO126 or IL-3 plus the PI3K inhibitor LY294002 resulted in activation of JNK. Higher levels of activated JNK were detected after IL-3 plus PI3K inhibitor treatment. Slightly higher levels of activated JNK were detected after U0126 and IL-3 treatment than after stimulation with IL-3 by itself.

In contrast and as expected, the PI3K inhibitor did not suppress ERK1/2 activation from 5 minutes after IL-3 treatment until 8 hrs, however, no activated ERK1/2 was detected 24 hrs after treatment with the PI3K inhibitor and IL-3. Treatment with the PI3K inhibitor and IL-3 actually resulted in higher levels of activated JNK than detected in the other treatment conditions suggesting that either inhibition of the PI3K pathway could result in JNK activation. It has been reported that the PI3K pathway can negatively regulate JNK.[66] Thus inhibition of PI3K may result in activation of JNK.

The effects of the chemotherapeutic drug doxorubicin on activation of ERK and JNK were also examined. After IL-3 & doxorubicin treatment and until 8 hrs, activated ERK1/2 was detected, however, no activated ERK1/2 was detected 24 hrs after treatment with doxorubicin and IL-3. After 24 hrs of doxorubicin treatment, p53 and $p21^{Cip-1}$ were induced. Doxorubicin treatment did not result in increased induction of JNK compared to cells just treated with IL-3. Likewise it did not appear to suppress ERK or MEK induction.

Discussion

Cytokine-dependent FL5.12 cells are highly sensitive to cytokine deprivation. Cytokine deprivation resulted in the rapid exit from the cell cycle and accumulation of cells in the G_1 phase. After two days of cytokine-deprivation, the cells underwent apoptosis. Furthermore the cytokine-dependent cells are not particularly sensitive to treatment with MEK1, PI3K or mTOR inhibitors, by themselves, indicating that suppression of a single pathway will not prevent cell cycle progression or induce apoptosis in cells which remain cytokine-dependent and their growth does not appear to be "addicted" to a particular oncogene.

In our study, the effects of classical chemotherapy were compared with signal transduction inhibitors which target the Raf/MEK/ERK or PI3K/Akt/mTOR pathways. Classical chemotherapeutic drugs such as doxorubicin or paclitaxel are very effective in inducing cell cycle arrest and apoptosis in cytokine-dependent cells. Doxorubicin normally induces a block in G_1 while paclitaxel induces a block in G_2/M. Thus agents which inhibit DNA synthesis such as doxorubicin inhibit entry into S phase while agents such as paclitaxel, which affect the disassembly of the mitotic spindle apparatus, induce a block in G_2/M. The effects of these chemotherapeutic drugs on inhibition of cell cycle progression can be enhanced with inhibitors which target MEK and mTOR.

Both doxorubicin (**Fig. 4**) and paclitaxel (unpublished observations) induce the activation of ERK1/2. Activation of ERK1/2 can have pro-proliferative, anti-apoptotic effects on cells and contribute to drug resistance.[11] Hence, it is rational to combine certain chemotherapeutic treatments with agents which will suppress ERK1/2 activity.

Inhibition of MEK, PI3K or mTOR by themselves only partially suppressed cell cycle progression and weakly induced apoptosis in cytokine-dependent cells. Single inhibitor treatment may be ineffective in cytokine-dependent cells as the cytokines induce multiple signaling pathways which have overlapping anti-apoptotic effects.

Clearly signal transduction pathways induced by cytokines and chemotherapeutic drugs interact to result in the regulation of cell cycle progression and apoptosis. Devising mechanisms to inhibit these interactions may result in more effective anticancer therapies. Some of these signaling pathways are associated with chemotherapeutic drug resistance.[11,20,21,31,32,37-43,58,59,67,68] Furthermore, the p53 tumor suppressor is a common target of many chemotherapeutic drugs and can interact with many signaling pathways.[69-72] Approaches that combine prolongation of wild-type p53 activity may enhance the effects of chemotherapeutic drugs, either in the presence of absence of signal transduction inhibitors.[73-78] Elucidating the mechanisms of interaction of chemotherapy, p53 activators and signal transduction inhibitors and how they enhance cell death by various mechanisms may yield more effective therapy.

Table 1. Fold increase in apoptosis due to chemotherapeutic drugs and signal transduction inhibitors in FL5.12 cells

	No inhibitor	+10 μM U0126 (MEK Inh)	+10 μM LY294002 (PI3K Inh)	+100 nM rapamycin (mTOR Inh)
		Fold Induction of Apoptosis		
No IL-3	2.5 ± 0.23			
IL-3	1 ± 0.10	1.1 ± 0.14	1.4 ± 0.14	1.6 ± 0.18
IL-3 + 1 nM Doxorubicin	1.1 ± 0.14	1.2 ± 0.13	1.4 ± 0.18	1.6 ± 0.19
IL-3 + 10 nM Doxorubicin	1.3 ± 0.17	1.7 ± 0.17	1.4 ± 0.14	1.9 ± 0.08
IL-3 + 100 nM Doxorubicin	1.8 ± 0.22	2.0 ± 0.22	2.0 ± 0.03	2 ± 0.22
IL-3 + 0.1 nM Paclitaxel	1.4 ± 0.15	1.4 ± 0.14	1.5 ± 0.14	1.6 ± 0.16
IL-3 + 1 nM Paclitaxel	1.8 ± 0.23	1.8 ± 0.22	1.7 ± 0.19	1.9 ± 0.21
IL-3 + 10 nM Paclitaxel	1.8 ± 0.22	1.8 ± 0.20	1.9 ± 0.21	2.1 ± 0.25

The effects of doxorubicin on the induction of apoptosis were measured by the Annexin V/PI assay in the presence of and absence of different concentration of the MEK1 inhibitor U0126 or the mTOR inhibitor Rapamycin. These experiments were repeated 5 times and similar results were observed. Results were normalized to IL-3-treated cells.

This therapeutic strategy is relevant in those solid and hematological neoplasias which show upfront resistance to classical cytostatic drugs e.g., acute myeloid leukemia (AML) of the elderly, or which become resistant during ongoing treatment. In addition targeted therapy alone has only been a breakthrough in a few entities such as Gleevec (Imatinib) in chronic myeloid leukemia (CML) and gastro-intestinal stroma tumor (GIST) in which neoplastic transformation relies on a singular activating hit (BCR-ABL in CML, c-Kit in GIST).[3-5,79,80] As in many other cancers pathway activation is more complex and interdependent, it can be anticipated, that combined therapeutic strategies will be of increasing importance to enhance the efficiency of classical cytostatic drugs, to limit their side effects and to overcome resistance.

Cells which proliferate continuously like cytokine-dependent FL5.12 cells represent a very appropriate model cell system to evaluate novel signal transduction inhibitors and chemotherapeutic drugs as they are not addicted on a particular oncogene for growth and they do not normally form tumors upon injection into immunocompromised mice. Targeting these cytokine-dependent, non-malignant cells, which can readily be transformed to cytokine-independent, tumorigenic cells, by various activated oncogenes (BCR-ABL, JAK, Flt-3) could result in the discovery of therapeutic approaches which are effective against LSCs or CICs.

Materials and Methods

Cell lines and growth factors. Cells were maintained in a humidified 5% CO_2 incubator with RPMI-1640 [(RPMI) Invitrogen, Carlsbad, CA, USA] supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA). The IL-3 dependent FL5.12 murine cell line was cultured in this medium supplemented with 10% WEHI-3B(D^-) conditioned medium (WCM) as a source of IL-3.[1,2,11]

In some cases, cells were treated with the MEK1 inhibitor U0126 (Promega, Madison, WI), the PI3K inhibitor LY294002 (Calbiochem, Los Angeles, CA) or the mTOR inhibitor rapamycin (Sigma-Aldrich, Saint Louis, MO) as described.[11] These inhibitors were dissolved in dimethyl-sulfoxide (DMSO, Sigma-Aldrich). Some cells were treated with different concentrations of doxorubicin, or paclitaxel (Sigma-Aldrich).

Analysis of cell cycle distribution. Cells were incubated at approximately 2 x 10^5 cells/ml in 3 mls of phenol-red free RPMI containing FCS but lacking IL-3. This initial time point is designated (Day_{-1}). Twenty-four hours later at the T_0 point, IL-3 (10% WEHI clear conditioned medium), and various concentrations of doxorubicin, paclitaxel, U0126, LY294002 or rapamycin were added to the cultures. Aliquots of approximately 1 x 10^5 cells (0.5 mls) were subsequently removed at the indicated time points. Analysis of cell cycle distribution was performed as described previously.[81]

Annexin V apoptotic assays. Annexin V/PI binding assays were performed as previously described with kits purchased from Roche (Indianapolis, IN).[9,11]

Western blot analysis. Cells were washed twice with PBS and then cultured in the presence of phenol red free RPMI 1640 containing 5% charcoal stripped (CS) FBS for 24 hrs. An aliquot of cells was removed (T_{-1} Hr). Cells were treated with the vehicle (DMSO) or the indicated signal transduction inhibitors or doxorubicin for 1 hr and an aliquot was removed (T_0). Cells were then incubated with IL-3 in the presence and absence of the chemotherapeutic drugs and signal transduction inhibitors for the indicated time intervals and aliquots removed. Western blots were performed with antibodies specific for phospho and total MEK, ERK, JNK, p53 and $p21^{Cip-1}$ as we have previously described.[9-11] All antibodies used in this study were purchased from Cell Signaling (Beverly, MA).

Acknowledgements

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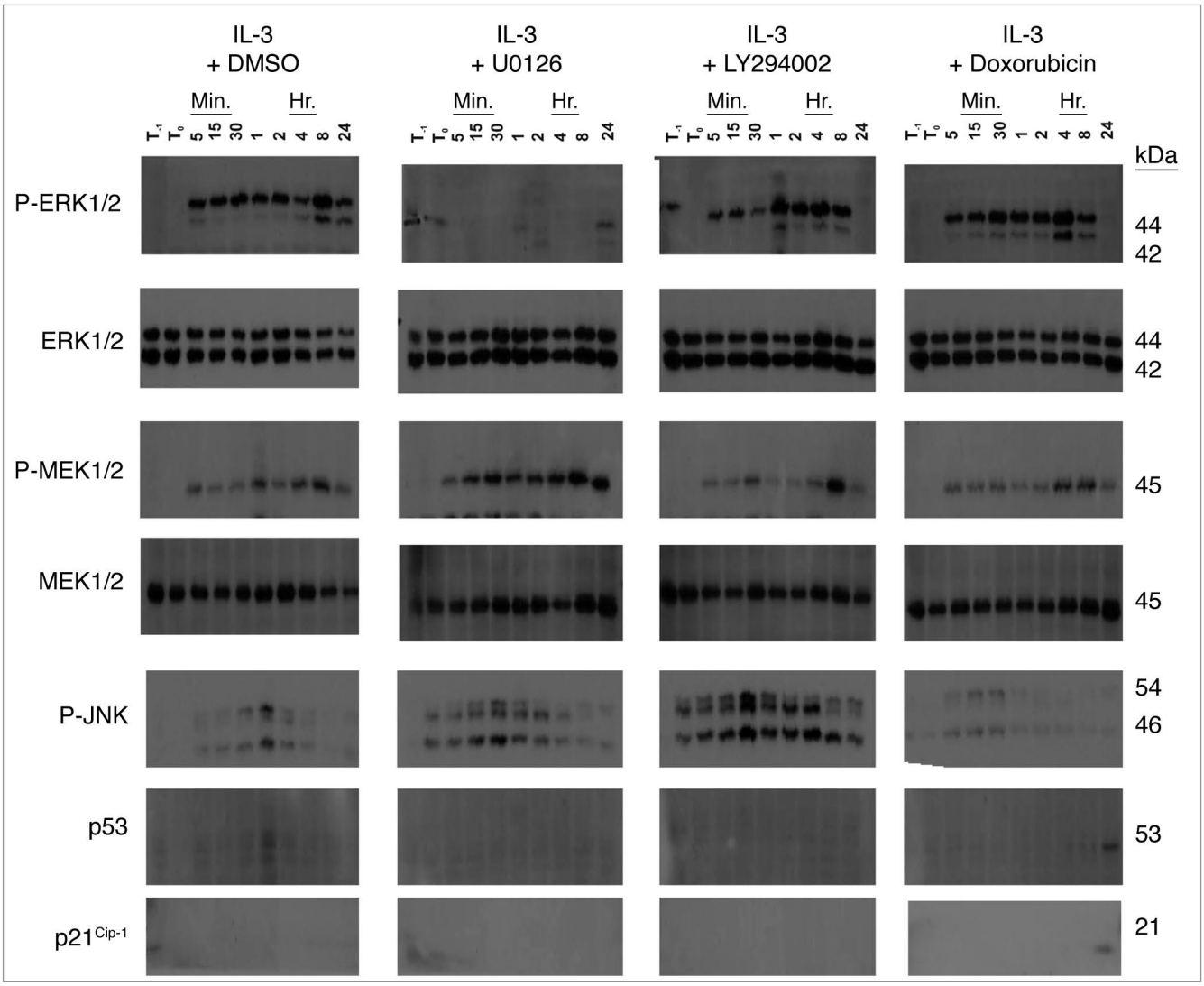

Figure 4. Effects of MEK1, PI3K inhibitors and doxorubicin on MAPK and cell cycle gene expression in FL5.12 cells. The effects of MEK1, PI3K inhibitors and doxorubicin on MAPK and cell cycle gene expression was examined in FL5.12 cells that had been deprived of IL-3 for 24 hrs (T_{-1}) and then treated with the indicated inhibitors (10 μM U0126 or 10 μM LY294002) or 100 nM doxorubicin for 1 hr (T_0) and then stimulated with IL-3 for the indicated time periods. Western blot analysis was performed with antibodies which recognized activated ERK1/2, MEK1/2, JNK, p53 and $p21^{Cip-1}$. These experiments were repeated three times and similar results were obtained.

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