Resistance to Mitogen-Activated Protein Kinase Kinase (MEK) Inhibitors Correlates with Up-Regulation of the MEK/Extracellular Signal-Regulated Kinase Pathway in Hepatocellular Carcinoma Cells

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ABSTRACT

The extracellular signal-regulated (ERK), mitogen-activated protein kinase (p42/p44 MAPK) pathway is up-regulated in hepatocellular carcinoma (HCC). Molecular targeting of this critical mitogenic pathway may have therapeutic potential for the treatment of HCC; however, chemoresistance to long-term therapy may develop. In the present study, we employed small-molecule MAPK kinase (MEK) inhibitors, including U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] and PD184161 (Neoplasia 8:1–8, 2006), in HepG2 and Hep3B human HCC cell lines to identify potential mechanism(s) of resistance. U0126 dose-dependently suppressed ERK phosphorylation at both 1- and 24-h time points in HepG2 cells, previously shown to be sensitive to growth inhibition by U0126. In contrast, ERK phosphorylation was only decreased at the 1-h time point but not at 24 h in the more resistant Hep3B cells. It is interesting that the lack of prolonged phospho-ERK suppression was associated with MEK hyperphosphorylation in Hep3B cells. Several MEK/ERK pathway intermediates were up-regulated in Hep3B cells; furthermore, transfection of Raf-1 small interfering RNA to suppress MEK/ERK pathway activation sensitized Hep3B cells to U0126. MEK inhibitor resistance was independent of p53 or hepatitis Bx protein status. Finally, we showed that combining two chemically distinct MEK inhibitors enhanced growth inhibition and apoptosis compared with the single agents. Taken together, these results suggest that up-regulated expression or activity of the MEK/ERK pathway contributes to MEK inhibitor resistance in HCC cells. Our findings also provide preclinical evidence suggesting that the status of the MEK/ERK pathway in patients may predict response to MEK/ERK-targeted therapeutics.

Although substantial strides have been made in the pharmacologic treatment of many malignancies, some tumor types exhibit significant resistance to chemotherapy. Hepatocellular carcinoma (HCC) is one of the most common causes of solid organ cancer mortality worldwide and also one of the most chemoresistant (Llovet et al., 2003; Burroughs et al., 2004). The incidence of HCC is on the rise and has doubled in the last 20 years in the United States (El-Serag et al., 2003; Gomaa et al., 2008). Furthermore, mean survival time (8 months) has not increased substantially over the past 20 years (El-Serag, 2004). The best treatment for HCC currently involves surgical resection and/or transplantation, which can only be performed in select patients who present early in the course of their disease (Olthoff, 1998). Traditional chemotherapeutics have been largely unsuccessful due to chemoresistance (Carr, 2004).

Molecular targeted therapies are of recent interest for the treatment of cancer (Sebolt-Leopold and Herrera, 2004). These agents can disrupt tumorigenesis by specifically targeting molecular signals regulating cellular proliferation and/or enhancing apoptosis. A major advantage of molecularly targeted therapy is that such treatments are well tol-
erated with little measurable systemic toxicity compared with traditional cytotoxic chemotherapy. Furthermore, molecularly targeted therapies may overcome mechanisms by which cancer cells resist traditional cytotoxic chemotherapeutic agents. Despite these advantages, chemoresistance may still arise with molecularly targeted agents, but such mechanisms of chemoresistance have not been extensively studied to date.

A class of molecular targeted compounds that has shown promise includes the small-molecule protein kinase inhibitors that disrupt mitogenic signaling pathways. The extracellular signal-regulated (ERK), mitogen-activated protein kinase (p42/p44 MAPK) pathway is a target that has received significant attention (Sebolt-Leopold, 2004; Sebolt-Leopold and Herrera, 2004). The ERK/MAPK phosphorylation cascade is central to the regulation of differentiation, development, growth, and survival (Seger and Krebs, 1995). ERK is activated by MAPK kinase (MEK), which itself is activated via phosphorylation by MEK kinase (or Raf) (van Biesen et al., 1995).

The functional importance of ERK/MAPK pathway signaling in experimental HCC has been well documented (McKillop et al., 1997, 1999a,b; Schmidt et al., 1997a,b, 1999). We and others have demonstrated increased expression and activity of ERK/MAPK signaling intermediates in human HCC tissues ex vivo (Schmidt et al., 1997b; Ito et al., 1998; Huynh et al., 2003). Hepatitis B and C have been shown more recently to activate the ERK/MAPK pathway (Fukuda et al., 2001; Tsutsumi et al., 2003). Activation and overexpression of ERK/MAPK in HCC does not appear to be associated with Ras mutations, which are rare in HCC (Schwarz et al., 1995; Stahl et al., 2005). Similarly, B-Raf mutations have not been detected in human HCC (Tannapfel et al., 2003); however, Raf-1 has been shown to be overexpressed in human HCC (Hwang et al., 2004). EGFR and its ligand, TGF-α, are also up-regulated in HCC, which may be responsible, in part, for downstream activation of ERK/MAPK (Harada et al., 1999; Höpfner et al., 2004; Moon et al., 2004). Multiple MAPK-mediated processes promote human HCC including increased proliferation, cell cycle progression, enhanced tumorigenicity, and inhibition of apoptosis (Wiesenauer et al., 2004). In contrast, surrounding normal hepatocytes are usually quiescent; therefore, they are not dependent upon ERK/MAPK mitogenic signaling or susceptible to its inhibition. Thus, the ERK/MAPK pathway is an attractive pharmacotherapeutic target for the treatment of HCC (Duesbery et al., 1999; Köhno and Pouyssegur, 2003).

We and others have shown the potential for successful treatment of liver and colon cancer with small-molecule MEK inhibitors that disrupt the ERK/MAPK signaling pathway (Allen et al., 2003; Schmidt et al., 2003; Wiesenauer et al., 2004). However, we also discovered a lack of ERK suppression after long-term MEK inhibitor treatment in some resistant human HCC tumors upon ex vivo analysis (Klein et al., 2006). In the present study, we examine possible mechanisms that may contribute to this signaling resistance in vitro. We demonstrate that HCC cells show differential sensitivity to long-term MEK inhibitor treatment. In resistant HCC cells (i.e., Hep3B), resistance is associated with a lack of prolonged phospho-ERK (P-ERK) suppression and increased MEK phosphorylation. Resistance correlates with up-regulation of ERK/MAPK pathway intermediates but is not dependent upon p53 or hepatatitis Bx protein. Silencing the MEK/ERK pathway with Raf-1 siRNA sensitizes Hep3B cells to U0126. Furthermore, combining chemically distinct MEK inhibitors to dually target MEK provides sustained inhibition of MEK/ERK signaling in the chemoresistant Hep3B cells, resulting in enhanced growth inhibition and apoptosis.

Materials and Methods

Cell Culture. HepG2 and Hep3B cells were obtained from the American Type Culture Collection (Manassas, VA). Adherent cells were maintained in modified Eagle's media-α containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Hepatitis Bx protein-expressing HepG2 cells were a gift from Dr. Mark Feitelson (Thomas Jefferson University, Philadelphia, PA). Stably transfected p53-positive Hep3B cells were a gift from Dr. Paul Lai (The Chinese University of Hong Kong, Hong Kong, China). MEK inhibitors, PD098059 and U0126 (Calbiochem, San Diego, CA) or PD184161 (Pfizer, Ann Arbor, MI) (Klein et al., 2006), were administered 24 h after plating HCC cells for the indicated time periods.

Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay buffer (phosphate-buffered saline, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mM sodium vanadate). Cell lysates were centrifuged at 10,000 rpm for 10 min. Supernatants (10 μg of total protein) were resolved by SDS-polyacrylamide gel electrophoresis on 4 to 20% gradient gels (Invitrogen, Carlsbad, CA). Separated proteins were transferred to Immobilon P membranes (Millipore Corporation, Billerica, MA), then incubated for 1 h in blocking solution (Tris-buffered saline (TBS), 0.05% Tween 20, and 5% nonfat dry milk). Membranes were washed in TBS-Tween 20 and incubated with primary antibody according to the manufacturer’s recommendations. After washing with TBS-Tween 20, they were incubated with appropriate secondary antibody (horseradish peroxidase-conjugated IgG) for 60 min at room temperature. Membranes were washed before detection by enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, UK). The primary antibodies employed included specific phospho-p42/44 mitogen-activated protein kinase (Thr202/Tyr204) and phospho-MEK1/2 antibodies (Cell Signaling Technology Inc., Danvers, MA), total ERK1/2 (K-23), total MEK1/2, K-ras-2 (C-19), B-raf, and Raf-1 (C-12) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the pan-Ras (Ab-3) antibody (Calbiochem). Membranes were stripped and reprobed with actin (C-11) (Santa Cruz Biotechnology, Inc.), to confirm equal protein loading. Densitometric analysis was performed using a Hewlett-Packard scanner and Scion Image software (Scion Corporation, Frederick, MD).

siRNA. Hep3B cells were plated into 12-well plates (10⁵ cells/well). The next day, cells were transfected with either 100 nM control nontargeting (NT) or Raf-1 siRNA (Dharmacon RNA Technologies, Lafayette, CO) using Dharmafect 4 (Dharmacon RNA Technologies) according to the manufacturer’s protocol. Media were replaced with complete media the next day. For protein expression analyses, cells were harvested 96 h post-transfection. For cell growth studies, U0126 was added 24 h post-transfection, and trypsin blue-excluded counts were performed 96 h post-transfection.

p53 Transient Transfection. Hep3B cells were seeded into six-well plates, and after 24 h, cells were transfected with plasmids expressing wild-type p53, mutant-p53, or vector alone using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. Twenty-four hours post-transfection, the media were changed, and cells were treated with U0126 for 24 h and then harvested.

Apoptosis Assay. Apoptosis was measured using the Cell Death Detection DNA fragmentation enzyme-linked immunosorbent assay (Roche Diagnostics) to quantitate the amount of cytoplasmic histone-
associated DNA fragments induced during apoptosis. Cells were plated in 96-well plates (1 × 10^4 cells) and then treated for 48 h with either U0126 and/or PD184161 (1–20 μM). The assay was conducted according to the manufacturer’s protocol. In brief, cell lysates were prepared and placed into streptavidin-coated microtiter plates. After incubation for 2 h at room temperature with anti-histone biotin and anti-DNA proxy antibodies, the wells were washed and incubated with substrate. Absorbance was read at 405 nm.

**Proliferation Assay.** Cellular proliferation was determined using a colorimetric assay, CellTiter 96 AQ™ One Solution Cell Proliferation Assay (Promega, Madison, WI), in which a tetrazolium compound is bioreduced by cells into a soluble, colored formazan product. Cells were plated in triplicate in 96-well plates at a density of 2 × 10^3/cm². Twenty-four hours later, MEK inhibitors or vehicle control (DMSO) were administered. After treatment for the indicated time period, cells were incubated with 20 μl of CellTiter 96 AQ™ One Solution Reagent. Absorbance was recorded at 490 nm on a 96-well plate reader. Percentage cell growth was determined from a ratio of average absorbances of the treatment wells to the control wells.

**Statistics.** Statistical methods including Student’s t test or one-way analysis of variance with Tukey post-hoc were employed for data analysis.

**Results**

**Differential MEK/ERK Signaling Response to the MEK Inhibitor U0126.** The relative responsiveness of two human HCC cell lines, Hep3B and HepG2, to treatment with small-molecule MEK inhibitors was investigated. Hep3B and HepG2 cells were treated with the MEK inhibitor U0126 for 1 or 24 h to determine the effects of short- or long-term treatment. Cell lysates were analyzed by Western blot to detect the levels of P-ERK and P-MEK and total ERK and MEK as shown in a representative blot. Lysates were prepared and analyzed by Western blot to detect P-ERK and P-MEK levels (Fig. 1). U0126 effectively inhibited ERK activation in both HepG2 and Hep3B cell lines as shown by the dose-dependent decrease in P-ERK levels after 1 h of treatment. However, after 24 h, P-ERK levels in the Hep3B cells returned to or exceeded basal levels; this was also observed as early as 6 h after treatment (data not shown). In contrast, P-ERK levels in HepG2 cells did not rebound but remained suppressed after treatment for 24 h. P-MEK levels dose-dependently increased in Hep3B cells at both the 1- and 24-h time points. In HepG2 cells, P-MEK levels did not increase after 1 h and only increased modestly after 24 h of treatment. Total ERK and total MEK expression were unchanged with MEK inhibitor exposure in either cell line.

To eliminate serum-related effects, duplicate experiments were done in serum-free media, and similar results were obtained (data not shown). Two additional MEK inhibitors (PD098059 and PD184161) chemically distinct from U0126 were also used. As with U0126, P-ERK levels consistently returned to basal levels by 24 h in Hep3B cells (data not shown). Taken together, these findings demonstrate that Hep3B cells are relatively resistant to suppression of the MEK/ERK pathway by MEK inhibitors compared with HepG2 cells. We have shown previously that Hep3B cells are more resistant to growth inhibition by U0126 than HepG2 cells (IC50 ~5 versus 0.5 μM, respectively) (Wiesenauer et al., 2004). Thus, the lack of prolonged ERK suppression and MEK hyperphosphorylation by U0126 in Hep3B cells correlates with less effective growth inhibition.

**Chemoresistance of Hep3B to U0126.** To determine whether the relative resistance of Hep3B cells to MEK inhibition may be due to the instability of U0126 in culture or to changes induced within the treated cells, the activity of “conditioned” U0126 (24 h in culture) and the response of conditioned Hep3B cells to the addition of fresh U0126 were evaluated, respectively, as follows: 1) naive Hep3B cells were treated with conditioned media from Hep3B cells treated with 10 μM U0126 for 24 h (conditioned U0126) and then harvested 1 h later; and 2) Hep3B cells treated with U0126 for 24 h (conditioned cells) had the media replaced with conditioned drug-free media, to which fresh 10 μM U0126 was added and then harvested 1 h later. The levels of P-ERK and P-MEK were determined by Western immunoblot (Fig. 2A). The level of P-ERK after 1 h of U0126 treatment was undetectable in Hep3B cells but returned to control levels after 24 h in parallel with increased MEK phosphorylation, as previously shown in Fig. 1. Short-term treatment (1 h) of naive Hep3B cells with conditioned U0126 suppressed P-ERK levels, demonstrating that U0126 is still effective after 24 h in media and that inhibitory factors have not been secreted into the conditioned media. In contrast, Hep3B cells previously exposed to 10 μM U0126 for 24 h (conditioned cells) did not respond to the addition of fresh 10 μM U0126 at a dose and time that completely suppressed P-ERK signaling in naive cells; P-MEK levels also remained elevated. Total ERK and MEK levels were not changed (data not shown). These results suggest that pretreated Hep3B cells remain resistant to further MEK inhibition because of intracellular changes induced by the initial drug exposure.

To further explore this phenomenon, Hep3B cells were pretreated with U0126 for 24 h (conditioned cells) and then allowed to recover in drug-free media for either 1 or 24 h.
before the addition of fresh U0126. After the addition of fresh drug, cell lysates were prepared 1 h later and analyzed for P-ERK levels by Western immunoblot (Fig. 2B). The results show that Hep3B retains their resistant phenotype if allowed to recover for only 1 h in drug-free media; however, by 24 h, the cells are no longer resistant to U0126 and exhibit P-ERK suppression. Total ERK levels were the same (data not shown). This suggests that the resistance mechanism induced in Hep3B cells pre-exposed to U0126 is dynamic, reversible, and not a paracrine effect. It is interesting that P-MEK levels induced by U0126 exposure returned to basal levels in Hep3B cells after 24 h of recovery, corresponding with restoration of the U0126 response; P-MEK levels in Hep2G cells did not change, nor did total MEK levels (Fig. 2C). Thus, the extent of MEK phosphorylation appears to be at least one determinant of resistance to U0126 in HCC cells.

**Role of p53 in Hep3B Chemoresistance to MEK Inhibition.** The tumor suppressor p53 is responsible for a multitude of cellular changes, including regulating the expression of the multidrug-resistant gene, P-glycoprotein, which is often inversely correlated with p53 expression. The lack of p53 may cause an increase in the expression of P-glycoprotein or other p53-regulated proteins and, therefore, contribute to resistance. Because Hep3B cells are p53-negative, whereas HepG2 cells are p53-positive, we tested the possibility that p53 may play a role in resistance to MEK inhibition. Hep3B cells were transiently transfected with plasmids expressing wild-type p53, mutant p53, or vector alone. Expression of p53 after transfection of the parental, p53-null Hep3B cells was confirmed (Fig. 3A). The expression of wild-type or mutant p53 did not affect P-ERK or P-MEK levels after U0126 treatment for 24 h; total MEK and ERK levels were also unchanged (data not shown). This was confirmed by additional experiments using a stable p53-expressing Hep3B cell line, which was still resistant to U0126 treatment (Fig. 3B). Furthermore, the addition of the P-glycoprotein inhibitor verapamil had no effect on the response of resistant Hep3B cells to U0126 (Fig. 3C). Western blot analysis revealed that P-glycoprotein expression was unchanged in treated Hep3B cells (data not shown). These results suggest that p53 expression does not play a major role in determining the sensitivity of HCC cells to MEK inhibitor treatment.

**Role of Hepatitis B.** There is significant evidence that the hepatitis B virus X protein (HBx) can activate MAPK; however, its role in HCC drug resistance is unknown. Hep3B cells are hepatitis B positive, whereas HepG2 cells are not. To elucidate the potential role of hepatitis B, a HepG2 cell line that stably expresses the HBx protein was employed. Control and HepG2-HBx cells were treated with U0126; P-ERK levels were examined at 1 and 24 h (Fig. 4). Although the HBx-producing HepG2 cells had a slightly higher level of P-ERK, they were no less sensitive to the drug. Total ERK levels were the same (data not shown). HBx, or the lack thereof, does not appear to be integral to the sensitivity of HCC cells to MEK inhibitor treatment.
Role of the Ras/Raf/MEK/ERK Pathway in Resistance. To explore a possible mechanism for the hyperphosphorylation of MEK and the rebound of P-ERK in the resistant Hep3B cell line, the upstream signal that positively regulates MEK was targeted. MEK kinase, also known as Raf, lies upstream of MEK in the ERK/MAPK cascade and phosphorylates MEK. MEK inhibitor-resistant (Hep3B) cells were transfected with either control NT or human Raf-1 siRNA. Raf-1 siRNA-transfected cells showed almost complete inhibition of Raf-1 expression and suppression of downstream P-MEK and P-ERK levels (Fig. 5A). Raf-1 siRNA-transfected cells were also significantly more responsive to U0126 treatment with U0126 for 1 or 24 h as indicated. Cell lysates were prepared and analyzed by Western blot to detect P-ERK as shown in a representative experiment.

Fig. 4. Hepatitis B virus X protein and sensitivity to U0126. HepG2 control and HepG2 cells stably expressing the HBx protein were treated with U0126 for 1 or 24 h as indicated. Cell lysates were prepared and analyzed by Western blot to detect P-ERK as shown in a representative experiment.

Effect of MEK Inhibitor Combination on MEK/ERK Signaling, Proliferation, and Apoptosis. To determine the efficacy of combining two chemically distinct MEK inhibitors to dually target the MEK/ERK pathway, Hep3B cells were treated with U0126 and PD184161. In contrast to the individual inhibitors, the combination of U0126 and PD184161 effectively suppressed P-ERK levels and prevented the P-ERK rebound at the 24-h time point in Hep3B cells (Fig. 6A). Although these treatments did not abrogate MEK phosphorylation, P-MEK levels did not dose-dependently increase with the cotreatment, as was observed with the single agents. Total ERK and MEK levels were similar (data not shown).

Treatment with U0126 in combination with PD184161 significantly inhibited Hep3B cell growth more than either agent alone (Fig. 6B). Furthermore, combination MEK inhibitor treatment significantly enhanced apoptosis in Hep3B
cells relative to the single agents (Fig. 6C). Thus, more effective suppression of ERK phosphorylation by the combination in the resistant Hep3B cells correlates with greater growth inhibition and enhanced apoptosis.

**Discussion**

Multiple lines of evidence implicate the ERK/MAPK pathway in the development and progression of HCC. The dependence of rapidly dividing cells, such as those found in HCC, on mitogenic signaling pathways has fueled treatment strategies aimed at ERK/MAPK targets (Duesbery et al., 1999; Kohno and Pouyssegur, 2003). The small-molecule inhibitors PD098059, U0126, CI-1040, and PD184161 were developed to interact with MEK and specifically target this pathway (English and Cobb, 2002). We have characterized previously the effect of MEK inhibitors in human HCC cells, both in vitro and in vivo (Wiesenauer et al., 2004; Klein et al., 2006). It is interesting that we observed that P-ERK suppression was not sustained in human HCC xenografts after multiple dosing with PD184161, suggesting that these tumors may develop resistance to long-term MEK inhibitor administration (Klein et al., 2006).

In the present study, we examined factors that could potentially contribute to the resistance of HCC to MEK inhibitor treatment. The levels of P-ERK after MEK inhibitor treatment (U0126, PD098059, and PD184161) in human HCC cells showed a short-term decrease; however, after 24 h, P-ERK levels in the resistant Hep3B cells rebounded to baseline. In contrast, sensitive HepG2 cells displayed sustained P-ERK suppression at both time points. Furthermore, P-MEK levels increased in a dose-dependent fashion with MEK inhibitor treatment at both early and late time points in the Hep3B cells. These results suggest that ERK reactivation and MEK hyperphosphorylation are associated with resistance to MEK inhibition. It is interesting that increased P-MEK levels have also been reported after treatment with other MEK inhibitors, such as CI-1040 and AZD6244; therefore, MEK phosphorylation appears to be a common response to MEK inhibitors and may be due to interference with the feedback regulation of the Ras-Raf-MEK-ERK pathway (Wang et al., 2005; Huynh et al., 2007). The increase in MEK phosphorylation may overwhelm the ability of the MEK inhibitor to suppress P-ERK, thus allowing reactivation of the targeted pathway and abrogating the effect of the drug in resistant cells such as Hep3B.

**Fig. 5.** Role of Ras/Raf/MEK/ERK pathway in resistance. A, Hep3B cells were transfected with either control NT or human Raf-1 siRNA. Lysates were prepared 96 h post-transfection and analyzed by Western blot to detect expression of Raf-1, P-ERK, P-MEK, and actin as shown in the representative blot. B, Hep3B cells were transfected with either control NT or Raf-1 siRNA. After 24 h, transfected cells were treated with either vehicle or U0126 for 72 h, and percentage cell growth determined. Data represent the mean ± S.E.M. from three independent experiments. *, P < 0.05 versus 5 µM U0126, NT siRNA-transfected cells. C, Hep3B cells were transfected with either NT or Raf-1 siRNA. After 72 h, U0126 was added to duplicate wells; lysates were prepared after 24 h and analyzed by Western blot to detect P-MEK, P-ERK, total MEK, and total ERK as shown. D, lysates were prepared from exponentially growing HepG2 and Hep3B cells to detect the basal level of expression of proteins in the Ras/Raf/MEK/ERK pathway as shown. Relative expression of the proteins is also presented.
MEK hyperphosphorylation may be a key mediator of MEK inhibitor resistance in HCC cells. Supporting the role of MEK up-regulation in drug resistance is our finding that suppression of the MEK/ERK pathway with Raf-1 siRNA increased the sensitivity of Hep3B cells to U0126. Conversely, it was reported previously that MEK1-transfected HepG2 cells expressing activated MEK were more resistant than wild-type HepG2 cells to U0126-induced apoptosis (Huynh et al., 2003). In addition, P-MEK and activated K-Ras levels were found to be elevated in a mouse colon carcinoma cell line resistant to the MEK inhibitor CI-1040 (Huynh et al., 2007). Similarly, we showed higher levels of pan-Ras, Raf-1, B-Raf, and P-ERK and lower levels of Raf kinase inhibitor protein in Hep3B compared with HepG2 cells, demonstrating overall up-regulation of the MEK/ERK pathway in Hep3B cells. Up-regulation or “priming” of this pathway may facilitate MEK hyperphosphorylation after MEK inhibitor treatment, thus abrogating the inhibitory effect and ultimately leading to resistance. The association between MEK/ERK signaling and HCC sensitivity to MEK inhibitors was supported further by our finding that the combination of two chemically distinct MEK inhibitors, U0126 and PD184161, was more effective than the single agents. U0126 and PD184161 in combination caused a sustained suppression of P-ERK in Hep3B cells that was not observed with single inhibitor treatment. This also correlated with enhanced apoptosis and decreased cell proliferation in combination-treated Hep3B cells.

MEK inhibitor metabolism, or degradation in the media, did not contribute to resistance after long-term exposure because naive Hep3B cells were sensitive to 24-h conditioned drug-containing media. In addition, repeated dosing was not effective in previously treated Hep3B cells; however, when allowed to recover in drug-free media for 24 h, conditioned Hep3B cells were once again sensitive to redosing with fresh drug. Thus, drug treatment did not generate a “resistant” population of cells; rather, the signaling changes responsible for the refractory response to the drug challenge were transient and reversible. This may have clinical relevance regarding dosing strategies, whereby in-

**Fig. 6.** Effect of MEK inhibitor combination on MEK/ERK signaling, proliferation, and apoptosis in Hep3B cells. A, Hep3B cells were treated with U0126 and/or PD184161 for 24 h. Lysates were prepared for analysis by Western blot to detect P-ERK and P-MEK levels as shown in the representative blot. B, Hep3B cells were treated with U0126 and/or PD184161 for 72 h. Percentage cell growth was determined relative to DMSO-treated cells. Data represent the mean ± S.E.M. from at least three independent experiments. *, P < 0.05 versus the single agents. C, Hep3B cells were treated with U0126 and/or PD184161 for 48 h. Relative apoptosis was determined by enzyme-linked immunosorbent assay compared with DMSO-treated cells. Data represent the mean ± S.E.M. from at least two independent experiments. *, P < 0.05 versus the single agents.
tertiary down-stream may suppress the MEK-ERK pathway more effectively.

Both human HCC cell lines used in this study have constitutively active ERK/MAPK pathways, yet they respond very differently to MEK inhibition. One key difference between these cell lines is p53 expression; the Hep3B cell line is p53-negative, and the HepG2 cell line is p53-positive. The tumor suppressor p53 regulates the expression of the multidrug-resistant gene, P-glycoprotein, which is inversely correlated with p53 expression. The relationship between p53 and P-glycoprotein depends upon the cellular environment, drug used, and type/function of status of p53 mutation (Bush and Li, 2002). P-glycoprotein inhibitors such as verapamil can increase the sensitivity of resistant cell lines to treatment with chemotherapeutics. In the present study, transient and stable p53 expression in Hep3B cells did not alter their signaling resistance to MEK inhibitor treatment. Likewise, cotreatment with the P-glycoprotein inhibitor verapamil had no effect on P-ERK signaling in Hep3Bs. Thus, the lack of p53 expression in Hep3B cells does not seem to be involved in the signaling resistance of these cells to MEK inhibitor treatment.

Hep3B and HepG2 cells not only differ in their p53 status, but Hep3B cells are also hepatitis B-positive and resistant to Fas-mediated apoptosis. Viral proteins from the two most common etiologies of HCC, hepatitis B and C, have been shown to activate the p44/p42 MAPK pathway (Fukuda et al., 2001; Tsutsui et al., 2003). There is significant evidence that the HBx activates MAPK enzymes; however, its role in HCC drug resistance is unknown (Tarn et al., 2001). We report that a HepG2 cell line stably expressing the HBx protein was equally sensitive to MEK inhibitor treatment as the HepB-negative parental cell line. Although it is possible that the viral status of HCC could influence sensitivity to MEK inhibitor treatment, this does not appear to occur in vitro.

In conclusion, we have shown that the chemoresistance of Hep3B cells to MEK inhibitor treatment in vitro is associated with up-regulation of the MEK/ERK pathway. Our findings provide preclinical evidence suggesting that the status of the MEK/ERK pathway in patients may predict response to MEK-based therapeutics. Moreover, further targeting of the MEK pathway combined with optimal dosing strategies may improve the efficacy of such agents. Future studies to identify the mediators of MEK inhibitor chemoresistance will provide insight into factors that affect the efficacy of MEK/ERK targeted therapies.

References