AZD6244 and doxorubicin induce growth suppression and apoptosis in mouse models of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, with no effective treatment for most individuals who succumb to this neoplasm. We report that treatment of primary HCC cells with the mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase 1/2 inhibitor AZD6244 (ARRY-142886) plus doxorubicin led to synergistic growth inhibition and apoptosis. *In vivo* administration of AZD6244, doxorubicin, or the combination of AZD6244 and doxorubicin in mice bearing 5-1318 HCC xenografts resulted in approximately 52% ± 15%, 12% ± 9%, and 76% ± 7% growth inhibition, respectively. AZD6244-inhibited tumor growth was associated with increased apoptosis, inactivation of ERK1/2, inhibition of cell proliferation, and down-regulation of cell cycle regulators, including cyclin D1, cdc-2, cyclin-dependent kinases 2 and 4, cyclin B1, and c-Myc. The AZD6244-doxorubicin combined protocol not only promoted apoptosis but also induced a synergistic effect not seen in single-agent–treated tumors, including increased expression of the *p130 RB* tumor suppressor gene. Our study provides a strong rationale for clinical investigation of combination therapy with the mitogen-activated protein/ERK kinase 1/2 inhibitor AZD6244 and doxorubicin in patients with HCC. [Mol Cancer Ther 2007;6(9):2468–76]

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common primary neoplasm, accounting for ~667,000 deaths annually worldwide (1). Despite the available treatment options, the incidence is still nearly as high as the mortality rate. More than 80% of patients present with advanced or unresectable HCC, and for those undergoing resection, the recurrence rate can be as high as 50% at 2 years (2, 3). Because of frequent recurrence, metastasis, or development of new primary tumors (4, 5), the 5-year survival rate is only 25% to 39% after surgery (6–8). Although several chemotherapeutic agents, such as 5-fluorouracil, epirubicin, cisplatin, nolatrexed, etoposide, mitoxantrone, paclitaxel, gemcitabine, and capecitabine, have been used in treatment of HCC, no single or combination chemotherapy regimen is particularly effective (reviewed in ref. 9). Thus, treatment of HCC remains an urgent health concern. Doxorubicin is perhaps the most widely used agent in HCC. Among 475 patients who received doxorubicin in various studies, a 16% response rate was documented (10). Recent large phase III studies have shown a 4% to 10.5% response rate in HCC treated with doxorubicin in the control group (11, 12). Overall response rate, but not overall survival, was doubled when doxorubicin was given in combination with cisplatin, IFN, and 5-fluorouracil (12).

The Ras/Raf/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway is one of the most critical signaling cascades for liver tumorigenesis (13–18). The product of the hepatitis B virus X gene (HBx protein), the core protein of hepatitis C virus (19–21), down-regulation of Ras inhibitors such as RASSF1A and NORE1A (22), Sprouty 2 (23), and Sprouty-related protein with Ena/vasodilator-stimulated phosphoprotein homology 1 and 2 (24), and up-regulation of interleukin-6 (25) and insulin-like growth factor-II (26, 27) have all been shown to activate the Ras/Raf/MEK/ERK pathway in HCC. Activated Ras turns on Raf kinases that phosphorylate and activate MEK1/2, which in turn activates ERK1/2. ERK1/2 kinases phosphorylate a wide range of downstream effectors involved in cell immortalization, cell motility, angiogenesis, drug resistance, and extracellular matrix remodeling (18, 28). The ligand-independent constitutive activation of the Ras/Raf/MEK/ERK pathway can play an important role in tumor development and is considered another mechanism of resistance of receptor-targeted therapies (29). The use of MEK1/2 inhibitors to treat HCC by arrest of proliferation, angiogenesis, and induction of apoptosis from inappropriate signaling in the Ras/Raf/MEK/ERK cascade is a promising mode of therapeutic intervention. Preclinical studies have proved the potential of MEK inhibition to suppress hepatoma cell proliferation and tumorigenicity (18). We have recently reported that treatment of human HCC xenografts with AZD6244 (ARRY-142886) blocked ERK1/2 activation, reduced *in vivo* tumor growth, and induced apoptosis (30).

The objective of this study was to assess the effects of doxorubicin plus AZD6244 on growth suppression and apoptosis in a mouse xenograft model of human HCC.

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Materials and Methods

Reagents

AZD6244 (AstraZeneca) was dissolved in DMSO to a final concentration of 20 mmol/L and stored at −20°C. Antibodies against cleaved caspase-7 (20,000 daltons), cleaved caspase-3, phosphorylated cyclin D1 at Thr^{286}, phosphorylated ERK1/2 at Thr^{202}/Tyr^{204}, phosphorylated p90RSK at $Ser^{359/363}$, and cleaved poly(ADP-ribose) polymerases (89,000 daltons) were obtained from Cell Signaling Technology. Antibodies against ERK1/2, cdc-2, cyclin-dependent kinase (Cdk)-4, Cdk-2, Cdk-6, cyclin A, cyclin B1, cyclin D1, pRB, E2F1, c-Myc, and α-tubulin were from Santa Cruz Biotechnology, Inc. p130 RB, $p16^{INK4a}$, $p21^{WAF1}$, $p27^{Kip1}$, and Ki-67 antibodies were from Lab Vision Corp. Conjugated secondary antibodies were supplied by Pierce. The chemiluminescent detection system was supplied by Amersham Pharmacia Biotech. Tissue culture dishes and eight-chamber slides were from Lab-Tek Chamber Slide System (Nunc, Inc.). The cell proliferation ELISA (bromodeoxyuridine) and *in situ* cell death detection kit were obtained from Roche Diagnostics Corp.

Effects of AZD6244, Doxorubicin, and AZD6244 plus Doxorubicin on the Growth of S.c. HCC Xenografts

The study received ethics board approval at the National Cancer Centre of Singapore and Singapore General Hospital. All mice were maintained according to the ''Guide for the Care and Use of Laboratory Animals'' published by the NIH. They were provided with sterilized food and water *ad libitum* and housed in negative pressure isolators with 12-h light/dark cycles.

HCC xenografts were implanted into male severe combined immunodeficient mice of 9 to 10 weeks of age (Animal Resources Centre). Four lines of HCC xenografts (2-1318, 5-1318, 26-1004, and 30-1004) were minced under sterile conditions as described (31). Except for 26-1004 HCC line, the 2-1318, 5-1318, and 30-1004 lines were derived from hepatitis B virus–positive HCC. The 26-1004 line had wild-type p53. Two lines (2-1318 and 5-1318) exhibited a mutation in codon 249 of the *p53* gene. 30-1004 had a frameshift mutation in codon 270 of the *p53* gene. A mutational analysis of mammalian target of rapamycin pathway genes (*PIK3CA, PTEN, TSC1, TSC2*, and *HIF1A*) reveals that one xenograft line (26-1004) exhibited a 16-bp deletion in exon 8 of the *PTEN* gene. Activation of multiple downstream components of the mammalian target of rapamycin signaling pathway specifically in tumors, including phosphorylated S6K1 and ribosomal protein S6, a target of S6K1, was observed in all four lines. The creation and characterization of these xenograft lines are reported elsewhere (31).

To investigate the effects of AZD6244, doxorubicin, or AZD6244 plus doxorubicin on the growth of HCC xenografts, mice bearing HCC xenografts (14 per group) were orally administered either 200 μL water or 75 mg/kg AZD6244 daily, 1 mg/kg doxorubicin once every 2 days, or doxorubicin plus AZD6244 for 18 days starting from day 7 after tumor implantation. By this time, the HCC xenografts reached the size of ~100 mg. The dose of doxorubicin was ~60% of the dose used in human [60 mg/m^2 or 1.6 mg/kg (12)]. Growth of established xenografts was monitored at least twice weekly, and tumor volume was calculated as described (31). Animals were sacrificed on day 18 during the treatment, body weight and tumor weights were recorded, and tumors were harvested for analysis. To investigate how the tumors responded after the treatments stopped, mice bearing 2-1318 tumors were treated as described above. The treatments were stopped on day 18 and tumors were monitored for another 18 days.

Isolation of Primary HCC Cells, Combination Index Study, and Cell Proliferation Assay

Primary cells from 2-1318, 5-1318, and 26-1004 tumors were isolated as described (30). Primary HCC cells were plated at a density of 2.0×10^4 per well in modified Eagle's medium containing 10% fetal bovine serum (growth medium) for 48 h. For combination index study, 2-1318 cells were treated with escalated doses of AZD6244 (0.1, 0.25, 0.5, 1, 2, 3, 4, 5, and 6 μmol/L) or doxorubicin (0.17, 0.34, 0.51, 0.68, 0.85, 1.02, 1.19, 1.36, 1.53, 1.7, and 3.4 μmol/L) or 0.5 μmol/L AZD6244 plus various doses of doxorubicin (0.17, 0.34, 0.51, 0.68, 0.85, 1.02, 1.19, 1.36, and 1.53 μmol/L) in modified Eagle's medium containing 2.5% fetal bovine serum for 24 h. Apoptosis was determined as described below. Analysis of combined drug effects was determined as described by Chou and Talalay (32). For proliferation assay, cells were treated with 0.5 μmol/L AZD6244 or three different doses of doxorubicin (0.17, 0.34, and 0.51 μmol/L) or the combination of 0.5 μmol/L AZD6244 plus the three doses of doxorubicin for 24 h. Cell proliferation was assayed using a bromodeoxyuridine kit (Roche Diagnostics) as described by the manufacturer. Experiments were repeated at least thrice, and the data were expressed as the mean ± SE.

Detection of Apoptosis

Primary HCC cells were plated in eight-chamber slides at a density of 1.0×10^4 per chamber in growth medium for 48 h. Cells were treated with AZD6244 or doxorubicin or AZD6244 plus doxorubicin as described above. Apoptosis was detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay using the *in situ* cell death detection kit as described by the manufacturer. Apoptotic cells were then visualized under a fluorescent microscope equipped with a FITC filter. The labeling index was obtained by counting the number of positive cells among 500 cells per region. Data were expressed as percentage values.

Western Blot Analysis

Tissue lysates were prepared and subjected to Western blot analysis as described previously (31). All primary antibodies were used at a final concentration of 1 μg/mL.

Immunohistochemistry

Sections (5 μm) were dewaxed, rehydrated, and subjected to antigen retrieval. After blocking endogenous peroxidase activity, and blocking nonspecific staining, the sections

were incubated with the primary antibodies against Ki-67 and cleaved caspase-3 (overnight at 4°C). Immunohistochemistry was done as described previously (31). For Ki-67, only nuclear immunoreactivity was considered positive. The number of labeled cells among at least 500 cells per region was counted and then expressed as percentage values.

Statistical Analysis

Body weight at sacrifice, s.c. tumor weight, Ki-67 index, and percentage of cleaved caspase-3–positive cells were compared using ANOVA. The differences in tumor growth among the treatment groups were determined by ANOVA.

Results

To study the effects of AZD6244 plus doxorubicin on proliferation, primary HCC cells were treated with 0.1% DMSO, 0.5 μmol/L AZD6244, three doses of doxorubicin (0.17, 0.34, and 0.51 μmol/L), or doxorubicin plus 0.5 μmol/L AZD6244 for 24 h. Figure 1A shows that AZD6244 and three doses of doxorubicin caused a 57% ± 8%, 18 ± 5%, 25 ± 6%, and 38% ± 5% reduction in DNA synthesis, respectively ($P < 0.01$; all treated groups versus control). Approximately 82% reduction in DNA synthesis was observed when 0.5 μmol/L AZD6244 was combined with 0.1 μg doxorubicin ($P < 0.01$).

As shown in Supplementary Table S2,[3] the median effect dose for AZD6244, doxorubicin, and AZD6244 plus doxorubicin to cause 50% apoptosis was 4.48, 3.37, and 0.5 μmol/L of AZD6244 + 1.02 μmol/L doxorubicin, respectively. Based on the analysis described by Chou and Talalay (Supplementary Data;[3] ref. 32), the 0.5 μmol/L AZD6244 plus 1.02 μmol/L doxorubicin was synergistic (0.420) by 58% under the specified conditions where the combination caused 50% apoptosis.

To investigate whether AZD6244-doxorubicin–induced growth arrest and apoptosis in primary HCC cells were associated with the inactivation of ERK and activation of caspases, lysates from AZD6244-treated, doxorubicin-treated, and AZD6244-doxorubicin–treated cells were subjected to Western blot analysis. Figure 1B shows that AZD6244 treatment caused a loss of ERK1/2 phosphorylation. Apoptotic markers such as cleaved caspase-7, caspase-3, and cleaved poly(ADP-ribose) polymerase fragments were absent in vehicle- or doxorubicin-treated samples but readily detected in the AZD6244-treated cells (Fig. 1B). These markers were ~3.5-fold higher in AZD6244-doxorubicin–treated cells compared with AZD6244-treated cells. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay revealed that AZD6244-doxorubicin caused 2.5 times more cell death than AZD6244 alone ($P < 0.01$; Fig. 1C).

We first determined the optimal dose of AZD6244 for *in vivo* combined therapy. Treatment of mice bearing 5-1318 xenografts with 50 mg and 75 mg/kg AZD6244 once daily for 18 days resulted in 32% ± 9% and 43% ± 7% growth inhibition, respectively. We selected the dose of 75 mg for our combined studies. Mice bearing 2-1318, 5-1318, 26-1004, and 30-1004 xenografts were treated with 75 mg/kg AZD6244, 1 mg/kg doxorubicin, and AZD6244 plus doxorubicin. Both animal toxicity and the ability of these treatments to inhibit tumor formation and progression were determined. Doxorubicin alone suppressed the growth of 2-1318 and 5-1318 xenografts, but not 26-1004 or 30-1004 xenografts, by approximately 27% and 21%, respectively (Table 1; Fig. 2). AZD6244, when given at a dose of 75 mg/kg, suppressed the growth of 2-1318, 5-1318, 26-1004, and 30-1004 xenografts by approximately 53%, 41%, 48%, and 28%, respectively (Table 1; Fig. 2). Reduction of tumor growth rates without causing regression was observed in four of four xenograft lines treated with AZD6244 (Fig. 2). As shown in Table 1 and Fig. 2, AZD6244 plus doxorubicin resulted in enhanced antitumor activity as observed by a long period of tumor growth inhibition after the treatment stops (Fig. 2A). The differences in tumor activity seen were statistically significant ($P < 0.01$). AZD6244, doxorubicin, and the combined therapy prevented mice from gaining weight during the course of treatment (Table 1). The body weight of treated mice at sacrifice was ~11% less than that of control mice.

To examine the antiproliferative and apoptotic effects of AZD6244, doxorubicin, or AZD6244 plus doxorubicin *in vivo*, sections from treated tumors were stained with Ki-67 and cleaved caspase-3 antibodies. Table 1 shows that the Ki-67 labeling index in all four AZD6244-treated xenografts was significantly decreased compared with vehicle- and doxorubicin-treated tumors ($P < 0.01$). A further decrease in the number of cells stained with Ki-67 antibody was observed in all xenografts treated with AZD6244 plus doxorubicin (Table 1). The percentage of cells stained for cleaved caspase-3 was significantly increased in AZD6244-treated and AZD6244-doxorubicin–treated tumors ($P < 0.01$; Table 1). A slight increase in apoptosis was also observed in doxorubicin-treated xenografts (Table 1). These results support the view that the antitumor effects of AZD6244 and AZD6244 plus doxorubicin are associated with inhibition of cell proliferation and increased apoptosis.

Cell cycle regulators play an important role in the development and progression of HCC. Of the positive cell cycle regulators, alterations in cyclin D1, cyclin A, and cyclin B1 expression relative to normal tissue have been associated with increased cellular proliferation and clinical outcome (reviewed in ref. 33). To understand the potential mechanisms of AZD6244-doxorubicin action, we then did a molecular analysis of AZD6244-treated, doxorubicin-treated, or AZD6244-doxorubicin–treated tumors. Because the combination effect was most profound in the 5-1318 tumors, these were selected for further analysis. First, we investigated the status of the ERK1/2 and cell cycle regulators in tumors after 18 days of treatment. No significant alterations in the levels of total ERK1/2, Cdk-6,

[3] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

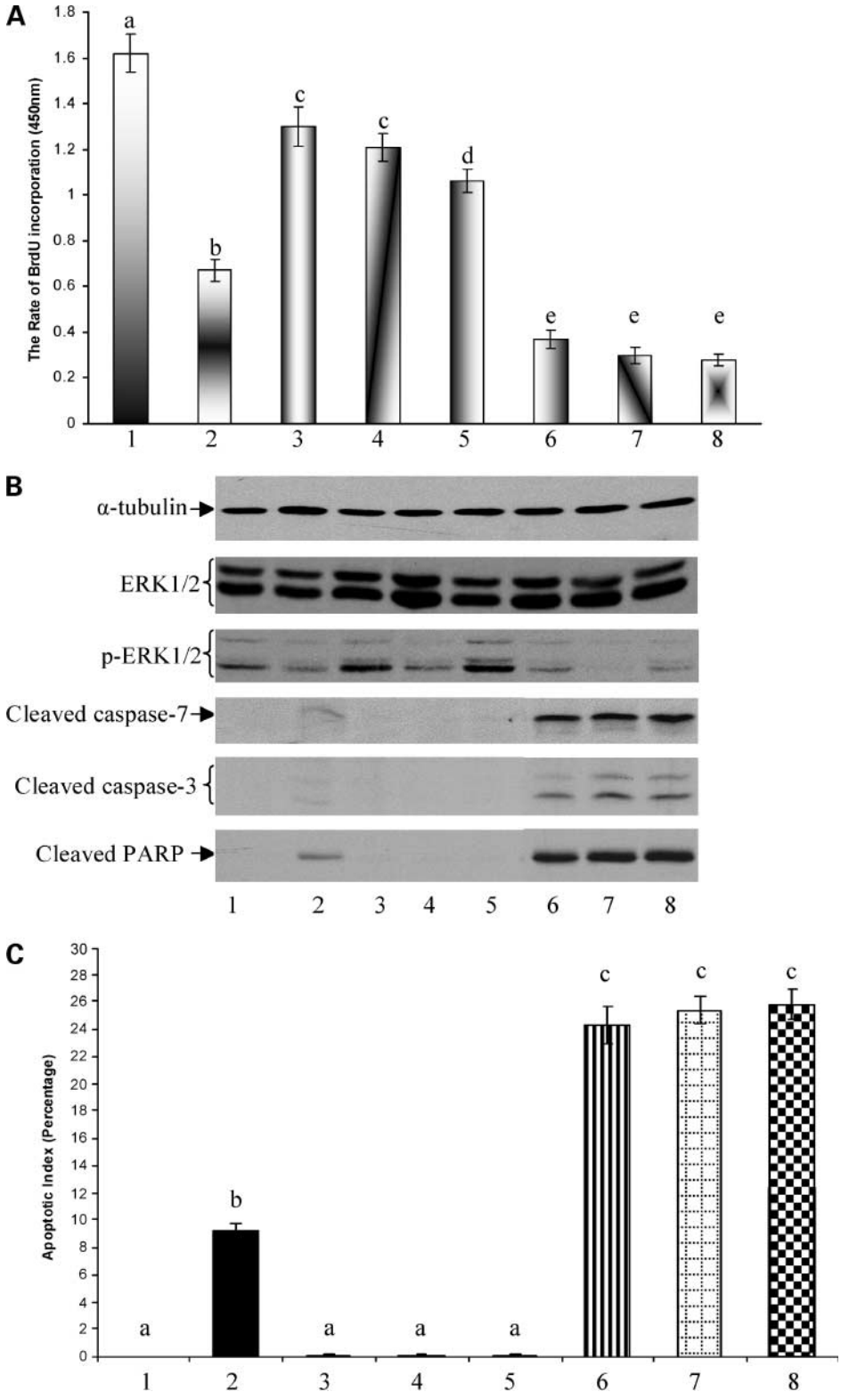

Figure 1. Effects of AZD6244, doxorubicin, or AZD6244 plus doxorubicin on cell proliferation, phosphorylation of ERK1/2, and apoptosis in primary HCC cells. Primary cells from 2-1318 tumors were isolated as described in Materials and Methods. They were grown and treated with vehicle (*lane 1*), 0.5 μmol/L AZD6244 (*lane 2*), 0.17 μmol/L doxorubicin (*lane 3*), 0.34 μmol/L doxorubicin (*lane 4*), 0.51 μmol/L doxorubicin (*lane 5*), 0.5 μmol/L AZD6244 plus 0.17 μmol/L doxorubicin (*lane 6*), 0.5 μmol/L AZD6244 plus 0.34 μmol/L doxorubicin (*lane 7*), and 0.5 μmol/L AZD6244 plus 0.51 μmol/L doxorubicin (*lane 8*) for 24 h in modified Eagle's medium containing 2.5% fetal bovine serum. Cell proliferation was determined by bromodeoxyuridine (*BrdU*) incorporation as described in Materials and Methods. **A,** cell proliferation at 24 h. Experiments were done in quadruplicate. *Columns,* mean; *bars,* SE. Cells were treated as described above, and Western blot analysis was done as described in Materials and Methods. **B,** blots were incubated with the antibodies indicated and representative samples are shown. Apoptotic cells were determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. **C,** apoptotic index. Similar results were obtained when 5-1318 and 26-1004 cells were used. *Bars with different letters* are significantly different from one another ($P < 0.01$) as determined by ANOVA. *Columns,* mean of three experiments; *bars,* SE.

and cyclin A were observed in any of the treatment arms. However, both AZD6244 and AZD6244-doxorubicin induced significant reductions in the levels of cyclin D1, phosphorylated cyclin D1, phosphorylated p90RSK, and c-Myc ($P < 0.01$), consistent with inhibition of the ERK pathway in the treated xenografts (Fig. 3). No significant reduction in E2F1 was observed. The levels of cdc-2, Cdk-2, Cdk-4, and cyclin B1 were significantly reduced ($P < 0.01$),

suggesting that AZD6244 and AZD6244-doxorubicin treatment also affect the cell cycle. No significant differences in the levels of $p16^{INK4a}$, $p21^{WAF1}$, and $p27^{Kip1}$ were observed among treatment groups (data not shown). We next investigated the AZD6244-doxorubicin–treated tumors for potential synergistic effects elicited by this therapeutic combination that might not be observed in tumors treated with single-agent AZD6244 or doxorubicin. At the protein level, we found that AZD6244-doxorubicin treatment induced the expression of p130 RB, a second RB gene product ($P < 0.01$; Fig. 3), whereas such effects were not observed when AZD6244 or doxorubicin was used as monotherapy.

Discussion

HCC is highly resistant to available chemotherapy agents administered either alone or in combination (34). In many cases, no effective therapy can be offered to patients with HCC (reviewed in ref. 35), making new therapeutic strategies for HCC treatment an urgent requirement. Given that ERK1/2 can be activated by several growth factors and receptors, including hepatocyte growth factor and its receptor, the c-met proto-oncogene (13, 36), hepatitis B virus, and hepatitis C virus, inhibition of this pathway in HCC could have profound effects on the development and progression of HCC. The use of a multitargeted kinase inhibitor, sorafenib (Nexavar, Bayer and Onyx Pharmaceuticals), to treat HCC by arresting proliferation and angiogenesis resulting from inappropriate signaling in Ras/Raf/MEK/ERK, vascular endothelial growth factor receptor 2, and platelet-derived growth factor receptor β cascades, respectively, has been reported to have some efficacy (37). In our previous study, we report the establishment and characterization of several patient-derived HCC xenografts. By means of these xenografts, we have shown that the majority of chemotherapeutic drugs currently used in the treatment of HCC have little or no antineoplastic activity *in vivo* (31). We further showed that treatment of mice bearing HCC xenografts with MEK inhibitor, AZD6244, resulted in dose-dependent growth suppression and apoptosis (30). In the present study, we showed that AZD6244 alone is able to inhibit the growth of four HCC xenografts, which agrees with our previous study (30). We also confirm the results of our previous study (31) showing that doxorubicin has mild or no antitumor activity against HCC *in vivo*. However, the growth of HCC xenografts is significantly inhibited when AZD6244 and doxorubicin are given simultaneously. Figure 3 shows that the AZD6244 plus doxorubicin combination induced growth inhibition associated with inactivation of MEK/ERK pathway (MEK1/2, ERK1/2, and p90RSK) as well as a reduction in the expression of positive cell cycle regulators, including Cdk-2, Cdk-4, cyclin D1, c-Myc, cyclin B1, and cdc-2. In addition, AZD6244-doxorubicin therapy also up-regulates p130 RB and promotes apoptosis as shown by an increase in both cleaved poly(ADP-ribose) polymerase and caspase-3 and up-regulates p130 RB, a synergistic effect not seen in single-agent–treated tumors.

Table 1. Effects of AZD6244, doxorubicin, and AZD6244 plus doxorubicin on body weight and tumor weight at sacrifice, cell proliferation, and apoptosis in four lines of HCC xenografts

Lines of xenografts	Treatments	Body weight (g) at sacrifice	Tumor weight (mg)	Ki-67 index (%)	Cleaved caspase-3 (%)
2-1318	Vehicle	23 ± 1.4	850 ± 96	18.6 ± 6*	10.2 ± 4*
	AZD6244	21 ± 1.4	318 ± 40	6.4 ± 2.1*	18.0 ± 3*
	Doxorubicin	21 ± 0.9	629 ± 74	12.5 ± 3*	13.4 ± 1.7*
	AZD6244 + doxorubicin	20 ± 1.3	208 ± 31	3.8 ± 1.9*	24.1 ± 5*
5-1318	Vehicle	22 ± 1.1	753 ± 64	8 ± 3*	7.1 ± 2.4*
	AZD6244	20 ± 1.2	425 ± 37	4 ± 1.6*	14.5 ± 3*
	Doxorubicin	19.6 ± 1.2	550 ± 64	7 ± 2.4*	9.8 ± 2.4*
	AZD6244 + doxorubicin	19.5 ± 1.3	112 ± 27	3 ± 1.7*	17.1 ± 2.4*
26-1004	Vehicle	24 ± 1.5	889 ± 79	14.5 ± 3.9*	9.8 ± 3*
	AZD6244	22 ± 1.1	460 ± 60	7.6 ± 2.4*	16.8 ± 2.4*
	Doxorubicin	21.7 ± 1.0	890 ± 84	10.6 ± 1.9*	11.2 ± 1.8*
	AZD6244 + doxorubicin	21 ± 1.4	130 ± 27	4.2 ± 2.1*	24.4 ± 3.6*
30-1004	Vehicle	25 ± 1.3	890 ± 102	13.1 ± 3*	7.4 ± 2.5*
	AZD6244	22 ± 1.4	640 ± 70	6.1 ± 2.7*	13.4 ± 2.1*
	Doxorubicin	22 ± 1.7	805 ± 86	11.4 ± 2*	9.7 ± 2.1*
	AZD6244 + doxorubicin	21.6 ± 1.8	430 ± 52	3.2 ± 1.9*	18.6 ± 2.5*

NOTE: Indicated xenografts were s.c. implanted on the right side of male severe combined immunodeficient mice as described in Materials and Methods. Mice bearing HCC xenografts were randomized to one of the four treatment groups ($n = 14$) and treated with either 200 μL water or 75 mg/kg AZD6244 daily, 1 mg/kg doxorubicin once every 2 d, or doxorubicin plus AZD6244 daily for 18 d starting from day 7 after tumor implantation as described in Materials and Methods. By this time, HCC xenografts had reached the size of ~100 mg. Ki-67 index and apoptosis in the tumors were determined by immunohistochemical staining with antibodies against Ki-67 and cleaved caspase-3, respectively. Differences in body weight and tumor weight at sacrifice, Ki-67 index, and cleaved caspase-3 among treatment groups were analyzed by ANOVA.

*$P < 0.01$.

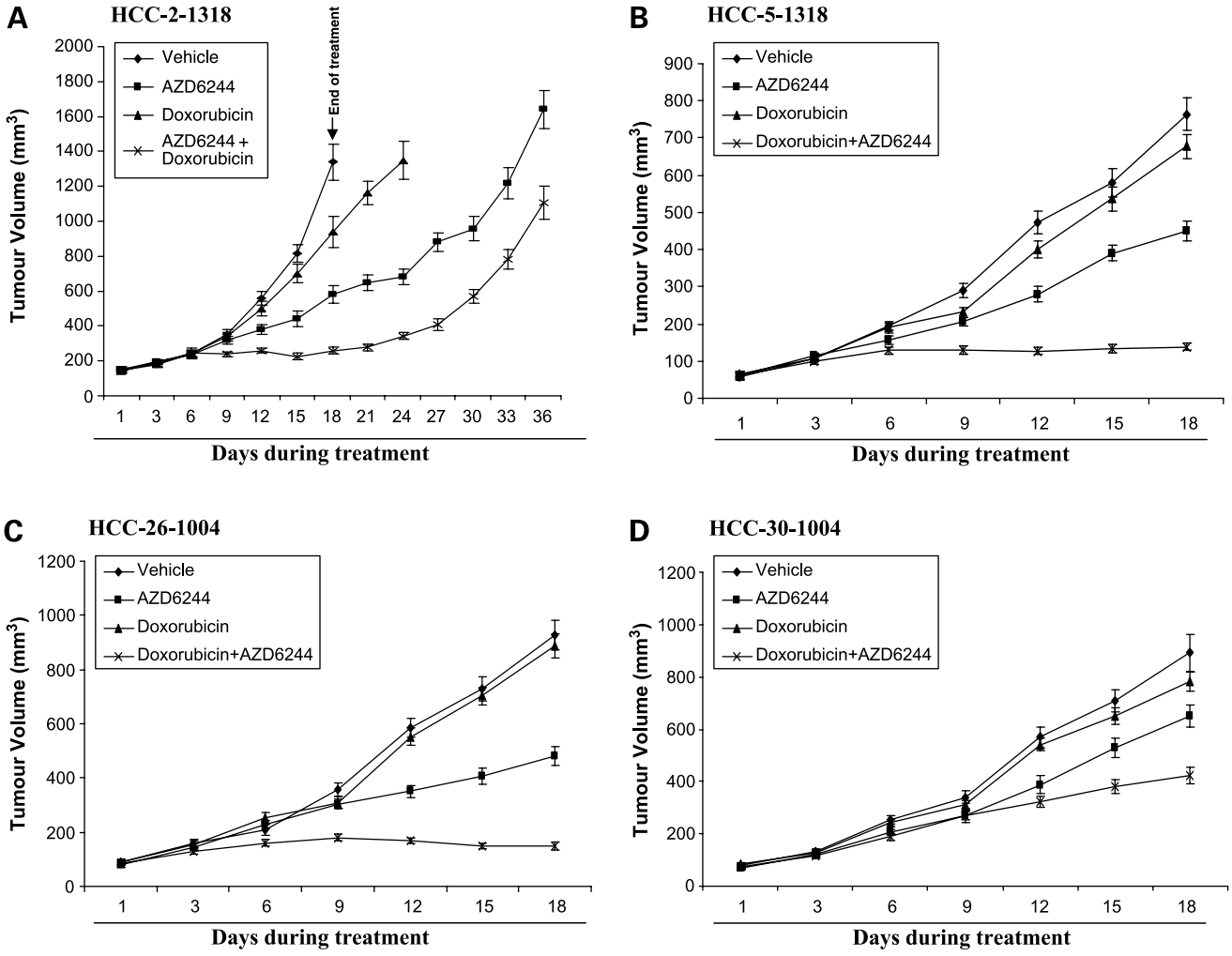

Figure 2. Effects of AZD6244, doxorubicin, or AZD6244 plus doxorubicin on growth rate of HCC xenografts. 2-1318 (**A**), 5-1318 (**B**), 26-1004 (**C**), and 30-1004 (**D**) xenograft lines were s.c. implanted in severe combined immunodeficient mice as described in Materials and Methods. Mice bearing HCC xenografts were treated with vehicle, AZD6244, doxorubicin, or doxorubicin plus AZD6244 daily for 18 d as described in Materials and Methods. Tumor growth was measured and calculated as described in Materials and Methods. *Points,* mean tumor volume at a given time for vehicle, AZD6244, doxorubicin, or AZD6244 plus doxorubicin of the indicated xenografts; *bars,* SE. Note that AZD6244 and doxorubicin plus AZD6244 resulted in enhanced antitumor activity as observed by a long period of tumor growth inhibition after the treatment stops. The differences seen were statistically significant ($P < 0.01$). Experiments were repeated at least thrice with similar results.

In the present study, we have shown that both AZD6244 and doxorubicin decreased the number of proliferating cells compared with vehicle alone. These were further decreased by the combined AZD6244 plus doxorubicin treatment. The exact mechanisms by which the combined AZD6244 plus doxorubicin treatment induces growth inhibition are not well understood. Cell cycle arrest due to up-regulation of p130 RB, hypophosphorylation of pRB, and reduction in positive cell cycle regulators may be, at least in part, responsible for the observed growth inhibition. In addition, reduction in c-Myc expression could also contribute to the antiproliferative activity of AZD6244 and AZD6244-doxorubicin treatments. This assumption is supported by the demonstration that conditional expression of the c-Myc oncogene in mouse liver results in the development of HCC (38).

In this study, doxorubicin acts synergistically with AZD6244 to promote apoptosis in HCC cells. AZD6244-induced and AZD6244-doxorubicin–induced apoptosis is associated with activation of caspases, which agrees with our previous study (30). It remains to be determined whether AZD6244 also causes the release of cytochrome *c*, which is responsible for the activation of caspase-3. Because the Ras/Raf/MEK/ERK pathway is essential for the secretion of factors required for growth, angiogenesis, and survival of the HCC cells (39, 40), *in vivo* inhibition of MEK/ERK by AZD6244 and AZD6244-doxorubicin treatments may also inhibit neovascularization by reducing

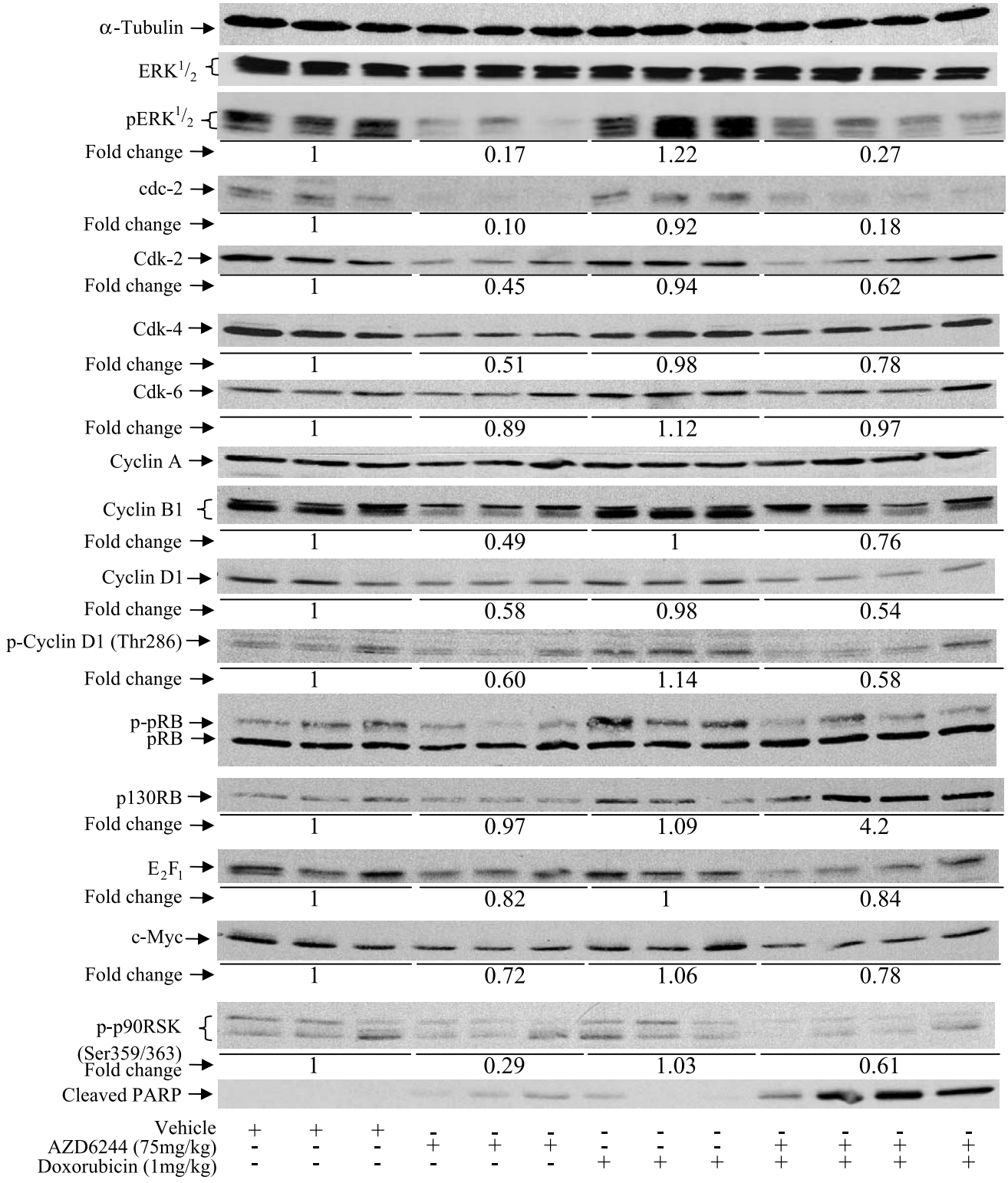

Figure 3. Effects of AZD6244, doxorubicin, or AZD6244 plus doxorubicin on the levels of phosphorylated ERK1/2, cell cycle regulators, p130 RB, pRB, and cleaved poly(ADP-ribose) polymerase in hepatocellular xenografts. 5-1318 xenografts were s.c. implanted in severe combined immunodeficient mice as described in Materials and Methods. Mice bearing HCC xenografts were treated with vehicle, AZD6244, doxorubicin, or doxorubicin plus AZD6244 daily for 18 d as described in Fig. 2. Lysates from vehicle and treated tumors were subjected to Western blot analysis as described in Materials and Methods. Blots were incubated with the indicated antibodies. Representative blots are shown. Densitometric data (fold change) are shown below each group. Similar results were obtained for 2-1318, 26-1004, and 30-1004 xenografts. Experiments were repeated at least thrice with similar results.

their production. Experiments are under way to investigate this possibility.

In HCC, multiple molecular alterations ensure the progressive growth of tumor cells. High tumor proliferation induced by ERK activation is closely linked to chemotherapy resistance (28). Because the blockage of a MEK/ERK pathway can be overcome by other molecular abnormalities, including Janus-activated kinase/signal transducers and activators of transcription (22), focal adhesion kinase (41), and the mammalian target of rapamycin (42), it is likely that HCC could develop resistance to MEK inhibitors. The clinical effectiveness of inhibitors of the MEK/ERK pathway may be limited when administered alone, as HCC tumors possess more than one genetic defect (43). For maximal therapeutic benefit, it may therefore be necessary to combine the MEK1/2 inhibitor AZD6244 with other signal transduction inhibitors, such as inhibitors of the mammalian target rapamycin, or conventional chemotherapeutic drugs, such as doxorubicin. Here, we show that targeted therapy against MEK renders HCC more sensitive to doxorubicin.

In conclusion, we provide evidence that AZD6244 can be combined successfully with doxorubicin in HCC xenograft models based on increased antitumor activity when compared with either agent alone. This observation has a clinical implication because AZD6244 may not be optimally effective as a monotherapy in HCC tumors (43). Therefore, the use of AZD6244-doxorubicin in combination may have great value as an alternative approach in the treatment of this fatal disease.

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