Blockade of constitutively activated ERK signaling enhances cytotoxicity of microtubule-destabilizing agents in tumor cells

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Abstract

The extracellular signal–regulated kinase (ERK) signaling pathway is constitutively activated in many human tumor cell types. Given the cytoprotective role of this pathway, we examined whether its specific blockade might sensitize human tumor cells to the induction of apoptosis by various anticancer drugs. Although blockade of ERK signaling alone did not induce substantial cell death, it resulted in marked and selective enhancement of the induction of apoptosis by microtubule-destabilizing agents in tumor cells in which the ERK pathway is constitutively activated. The synergistic activation of c-Jun NH_2-terminal kinase by the combination of an ERK pathway inhibitor and a microtubule-destabilizing agent appeared to be responsible, at least in part, for this effect. These results suggest that administration of the combination of an ERK pathway inhibitor and a microtubule-destabilizing agent is a potential chemotherapeutic strategy for the treatment of tumor cells with constitutive activation of the ERK pathway.

Key words: MEK inhibitor; microtubule-destabilizing agent; apoptosis; combination therapy; JNK.

Introduction

The extracellular signal–regulated kinase (ERK) pathway is activated in a variety of cell types by diverse extracellular stimuli and is among the most extensively studied of signaling pathways that connect various membrane receptors to the nucleus [1, 2]. Activation of the ERK pathway is triggered by GTP loading of Ras at the plasma membrane, which is followed by sequential activation of a series of protein kinases including a member of the Raf family (such as Raf-1), mitogen-activated protein (MAP) kinase or ERK kinase (MEK) 1 and 2, and ERK1 and ERK2. Activated ERK1/2 then phosphorylates various downstream substrates that contribute to the regulation of a wide range of cellular processes such as proliferation, differentiation, survival, and motility.

Mechanisms for precise spatiotemporal control of intracellular signaling pathways have evolved to ensure homeostasis in multicellular organisms. Inappropriate activation of these pathways underlies several refractory diseases, with aberrant activation of the ERK pathway having been shown to be a key contributing factor to many types of human cancer [3, 4]. In particular, overexpression or activating mutation of the epidermal growth factor receptor (EGFR) gene (in tumors of the lung, breast, colon, ovary, and bladder) [5], activating mutation of *RAS* (in tumors of the pancreas, colon, and lung) [6], and activating mutation of *RAF* (in melanomas and tumors of the colon, ovary, and thyroid) [7] have been associated with cancer and found to result in the activation of MEK1/2 and ERK1/2 in most cases. Inhibition of the ERK pathway thus represents a promising strategy for cancer treatment [8, 9].

We have previously shown that specific blockade of the ERK pathway by MEK

inhibitors results in marked suppression not only of the proliferation [10] but also of the invasiveness [11] of tumor cells in which the pathway is constitutively activated. However, blockade of the ERK pathway by itself was found to be largely cytostatic, rather than cytotoxic, resulting in only a moderate induction of apoptosis in these tumor cells [10]. Efficient induction of apoptotic cell death is essential for the development of effective cancer chemotherapy [9].

Combination therapy based on cytotoxic agents with nonoverlapping toxicities is a key approach to cancer treatment, being generally driven by safety considerations. Interruption of the cytoprotective ERK pathway by MEK inhibitors has thus been proposed as a means to enhance the action of cytotoxic anticancer agents [12]. Consistent with this notion, the chemotherapeutic effects of doxorubicin and paclitaxel were found to be enhanced by the MEK inhibitor U0126 in H460 human non–small cell lung cancer cells [13]. We have also recently shown that the MEK inhibitor PD184352 enhances the induction of apoptosis by histone deacetylase inhibitors in a variety of tumor cells with constitutive activation of the ERK pathway [14].

We now show that blockade of the ERK pathway results in marked enhancement of the cytotoxic effect of microtubule-destabilizing agents in tumor cells in which the pathway is constitutively activated. The combination of an ERK pathway inhibitor with a microtubule-destabilizing drug induced synergistic activation of c-Jun NH_2-terminal kinase (JNK), with this effect appearing to be responsible, at least in part, for the enhanced induction of cell death.

Materials and Methods

Materials. Vincristine, paclitaxel, and cytosine β-D-arabinofuranoside (Ara-C) were obtained from Sigma; etoposide (VP-16), cisplatin, and doxorubicin were from Wako (Osaka, Japan); and SP600125 was from Biomol. PD184352 were synthesized as described previously [11]. TZT-1027 and vinorelbine were kindly provided by Teikoku Hormone (Kawasaki, Japan). Antibodies to ERK1/2 and to EGFR were from Santa Cruz Biotechnology; those to diphosphorylated ERK1/2, to Tyr^{1173}-phosphorylated EGFR, and to β-actin were from Sigma; and those to Asp^{175}-cleaved caspase-3, to JNK, and to diphosphorylated JNK were from Cell Signaling Technology.

Cell culture and flow cytometry. Human tumor cell lines T24 [bladder carcinoma; H-Ras(G12V)], HT1080 [fibrosarcoma; N-Ras(Q61K)], PC-9 [lung adenocarcinoma; EGFR(ΔE746–A750)], HeLa S3 (cervical adenocarcinoma), PC3 (prostate adenocarcinoma), Colo320 (colon adenocarcinoma), WiDr [colon adenocarcinoma; B-Raf(V600E)], TCO [colon adenocarcinoma; B-Raf(V600E)], A549 [lung adenocarcinoma; K-Ras(G12S)], H1650 [lung adenocarcinoma; EGFR(ΔE746–A750)], and II-18 [lung adenocarcinoma; EGFR(L858R)] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum [4]. Cells exposed to various reagents were harvested by treatment with trypsin, fixed with 70% ethanol, treated with DNase-free RNase A (100 μg/ml, Sigma), stained with propidium iodide (20 μg/ml), and analyzed for DNA content with the use of a FACSCalibur flow cytometer and Cell Quest Pro software (Becton Dickinson) [10].

Immunoblot analysis. Cell lysates were prepared as described previously [10, 11] and fractionated by SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane and probed with primary antibodies and horseradish peroxidase–conjugated secondary antibodies (Promega). Immune complexes were visualized with enhanced chemiluminescence reagents (GE Healthcare Bio-Sciences).

Immunofluorescence microscopy. Cells were fixed with methanol at –20ºC and stained with antibodies to Asp^{175}-cleaved caspase-3 (1/100 dilution) and Alexa Fluor 488–conjugated secondary antibodies (1/200 dilution, Molecular Probes) [15]. The appearance of phosphatidylserine on the extracellular side of the plasma membrane and membrane permeability were evaluated by staining with annexin V and propidium iodide, respectively, with the use of an Annexin-V-Fluos staining kit (Roche Diagnostics). Condensation of nuclear DNA was also examined by staining with 4',6-diamidino-2-phenylindole (DAPI). Cells were observed with the use of an Axioskop2 Plus fluorescence microscope (Carl Zeiss).

RNA interference. The sequences 5′-GGGCCTACAGAGAGCTAGTTCTTAT-3′ and 5′-CCAACTGTGAGGAATTATGTCGAAA-3′ were designed to generate small interfering RNA (siRNA) duplexes specific for human JNK1 and JNK2 mRNAs, respectively; the scrambled oligoribonucleotide 5′-GGGATACAGAGCGATTGCTTCCTAT-3′ was used as a control (Invitrogen). Subconfluent cultures of T24 cells were transfected with siRNA (50 nM) for 48 h with the use of the LipofectAMINE 2000 reagent (Invitrogen).

Results and Discussion

Blockade of the ERK pathway sensitizes tumor cells to the induction of cell death by microtubule-destabilizing agents. We have previously shown that specific blockade of the ERK pathway by MEK inhibitors such as PD98059 and PD184352 completely suppresses the growth of tumor cells in which the pathway is constitutively activated. However, blockade of the ERK pathway by itself resulted in only a moderate induction of cell death in these tumor cells [10]. Thus, although treatment of T24 cells with PD98059 (50 μM) or PD184352 (10 μM) totally suppressed the activation of ERK1/2 as well as cell proliferation (Fig. 1A), it did not increase the proportion of dead cells with a fractional DNA content (cells in sub-G_1 phase) (Fig. 1B), which is a characteristic feature of apoptotic cell death [16]. Furthermore, the MEK inhibitor–induced suppression of cell growth was reversible, with cells resuming proliferation after removal of the inhibitor (Fig. 1A). Induction of cell death is essential for the development of effective cancer chemotherapy.

To provide a basis for the development of new chemotherapeutic strategies against cancer, we examined whether specific blockade of the ERK pathway might sensitize tumor cells to the cytotoxic action of chemotherapeutic agents by promoting cell death. Each of the anticancer drugs examined (Ara-C, VP-16, cisplatin, doxorubicin, vincristine, and paclitaxel) induced the death of T24 and HT1080 cells, with the proportion of cells in sub-G_1 phase increasing to between 40 and 80% after treatment with optimal drug

concentrations for more than 48 h (Fig. 1D, data not shown). To examine the effect of ERK pathway inhibition on death induction by these anticancer drugs, we therefore treated the tumor cells with suboptimal drug concentrations that increased the proportion of cells in sub-G_1 phase to ~20% after 48 h (Fig. 1B, C).

PD98059 markedly enhanced the induction of cell death by vincristine in both T24 and HT1080 cells (Fig. 1B, C). This effect of PD98059 on death induction by vincristine was most pronounced at low concentrations of the latter drug in T24 (≤3 nM) and PC-9 (≤10 nM) cells; under such conditions, vincristine by itself exhibited only a small cytotoxic effect in these cells (Fig. 1D). In contrast, the effect of PD98059 on the cytotoxicity of Ara-C, VP-16, cisplatin, or doxorubicin was suppressive or differed between tumor cell lines (Fig. 1C). Although PD98059 potentiated the induction of cell death by paclitaxel in these tumor cells [17], the effect was less pronounced than that observed with vincristine (Fig. 1C). In subsequent experiments, we therefore focused on the effect of ERK pathway inhibition on the induction of cell death by microtubule-destabilizing agents.

PD98059 markedly enhanced the induction of cell death by low concentrations of vincristine in a wide variety of tumor cells in which the ERK pathway is constitutively activated, including WiDr and TCO cells with Raf activation; HT1080, A549, and T24 cells with Ras activation; and H1650, PC-9, and II-18 cells with EGFR activation (Fig. 2A, C). It had no such effect in tumor cells in which the ERK pathway is not constitutively activated, including HeLa S3, PC3, and Colo320 cells. Blockade of the ERK pathway not only by PD98059 but also by PD184352 or U0126 (10 μM) enhanced the cytotoxicity

of structurally different microtubule-destabilizing agents (Fig. 2B, data not shown), including vincristine, vinorelbine, and TZT-1027 [18]. Furthermore, treatment of II-18 cells with gefitinib, a potent EGFR tyrosine kinase inhibitor [19], abolished the autophosphorylation of EGFR as well as markedly inhibited ERK1/2 activation and enhanced the induction of cell death by vincristine (Fig. 2C). Together, these results indicated that blockade of the ERK pathway not only by a MEK inhibitor (directly) but also by an EGFR inhibitor (indirectly) sensitizes tumor cells to the induction of cell death by microtubule-destabilizing agents.

The combination of an ERK pathway inhibitor and a low concentration of microtubule-destabilizing agent induces apoptosis mediated in part by JNK. To characterize the cell death induced by the combination of an ERK pathway inhibitor and a low concentration of microtubule-destabilizing agent, we examined several markers of apoptosis in T24 cells. The combination of PD98059 and vincristine (3 nM) induced a marked increase in the proportion of cells manifesting DNA condensation (as revealed by DAPI staining), those displaying phosphatidylserine at the cell surface in the absence of membrane permeability (as revealed by staining with annexin V and propidium iodide), and those expressing the activated form of caspase-3 (Fig. 3A), all of which are characteristics of the early phase of apoptotic cell death [20]. Immunoblot analysis also revealed that the drug combination induced the cleavage (activation) of caspase-3 to yield 19- and 17-kDa fragments (Fig. 3B). These results thus indicate that apoptosis is the major mechanism of cell death induced by the combination of PD98059 and a low

concentration of vincristine in T24 cells.

The net balance of signaling by the cytoprotective ERK1/2 pathway and the stress-related JNK1/2 and p38 MAP kinase pathways has been suggested to determine whether cells survive or undergo apoptotic cell death on exposure to various insults [21]. To investigate the molecular mechanism by which MEK inhibitors enhance the induction of apoptosis by vincristine, we examined the activation status of these kinases in T24 cells exposed to PD98059, 3 nM vincristine, or the combination of these agents. Although vincristine alone, but not PD98059, induced the activation of JNK, the extent of vincristine-induced JNK activation was greatly increased by the presence of the MEK inhibitor (Fig. 3B). Furthermore, the addition of 10 μM SP600125, a specific inhibitor of JNK [22], suppressed the induction of cell death by the combination of vincristine and PD98059 (Fig. 3C). In contrast, vincristine (3 nM) induced a slight activation of p38 MAP kinase in T24 cells and this effect was not enhanced substantially by PD98059 (data not shown). These results thus suggested that JNK mediates the apoptotic cell death induced by the combination of a MEK inhibitor and a low concentration of microtubule-destabilizing agent.

Finally, we examined the effect of RNA interference–mediated depletion of JNK1/2 on the induction of apoptosis by PD98059 and vincristine (3 nM) in T24 cells. Immunoblot analysis revealed that transfection of the cells with siRNAs specific for JNK1 or JNK2 mRNAs resulted in pronounced and preferential depletion of the 46- and 54-kDa JNK isoforms, respectively (Fig. 3C). Depletion of either JNK1 or JNK2 substantially inhibited the induction of apoptotic cell death by the combination of PD98059 and

vincristine; depletion of JNK2 appeared to be more effective in this regard than that of JNK1. Furthermore, depletion of both JNK1 and JNK2 suppressed the induction of cell death by the combination of PD98059 and vincristine more markedly. These results thus suggest that the cell death induced by the combination of PD98059 and vincristine is mediated, at least in part, by JNK1 and JNK2. The precise mechanism by which such a drug combination induces synergistic activation of JNK1/2 remains to be elucidated.

We have now shown that, whereas specific blockade of the ERK pathway by itself does not induce substantial apoptotic cell death, it markedly sensitizes tumor cells to the induction of cell death by microtubule-destabilizing agents. The synergistic induction of cell death by the combination of an ERK pathway inhibitor and a low concentration of microtubule-destabilizing agent was observed in many tumor cell types in which the ERK pathway is constitutively activated. Furthermore, the combination of an ERK pathway inhibitor and a microtubule-destabilizing agent induced synergistic activation of JNK1/2, which appeared to mediate, at least in part, the synergistic induction of apoptotic cell death by the combination of these agents. Together, our results indicate that administration of such a drug combination is a promising chemotherapeutic strategy for the treatment of tumors in which the ERK pathway is constitutively activated.

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References

[1] R. Seger, E.G. Krebs, The MAPK signaling cascade, FASEB J. 9 (1995) 726-735.

[2] T.S. Lewis, P.S. Shapiro, N.G. Ahn, Signal transduction through MAP kinase cascades, Adv. Cancer Res. 74 (1998) 49-139.

[3] H. Oka, Y. Chatani, R. Hoshino, O. Ogawa, Y. Kakehi, T. Terachi, Y. Okada, M. Kawaichi, M. Kohno, O. Yoshida, Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma, Cancer Res. 55 (1995) 4182-4187.

[4] R. Hoshino, Y. Chatani, T. Yamori, T. Tsuruo, H. Oka, O. Yoshida, Y. Shimada, S. Ari-I, H. Wada, J. Fijimoto, M. Kohno, Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors, Oncogene 18 (1999) 813-822.

[5] N.E. Hynes, H.A. Lane, ERBB receptors and cancer: the complexity of targeted inhibitors, Nat. Rev. Cancer 5 (2005) 341-354.

[6] J. Downward, Targeting RAS signaling pathways in cancer therapy, Nat. Rev. Cancer 3 (2003) 11-22.

[7] C. Wellbrock, M. Karasarides, R. Marais, The RAF proteins take centre stage, Nat. Rev. Mol. Cell Biol. 5 (2004) 875-885.

[8] J.S. Sebolt-Leopold, R. Herrera, Targeting the mitogen-activated protein kinase cascade to treat cancer, Nat. Rev. Cancer 4 (2004) 937-947.

[9] M. Kohno, J. Pouyssegur, Targeting the ERK pathway in cancer therapy, Ann. Med. 38 (2006) 200-211.

[10] R. Hoshino, S. Tanimura, K. Watanabe, T. Kataoka, M. Kohno, Blockade of the extracellular signal-regulated kinase pathway induces marked G_1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of $p27^{Kip1}$, J. Biol. Chem. 276 (2001) 2686-2692.

[11] S. Tanimura, K. Asato, S. Fujishiro, M. Kohno, Specific blockade of the ERK pathway inhibits the invasiveness of tumor cells: down-regulation of matrix metalloproteinase-3/-9/-14 and CD44, Biochem. Biophys. Res. Commun. 304 (2003) 801-806.

[12] P. Dent, S. Grant, Pharmacologic interruption of the mitogen-activated extracellular-regulated kinase/mitogen-activated protein kinase signal transduction pathway: potential role in promoting cytotoxic drug action, Clin. Cancer Res. 7 (2001) 775-783.

[13] Y. Hu, M. Bally, W.H. Dragowska, L. Mayer, Inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase enhances chemotherapeutic effects on H460 human non-small cell lung cancer cells through activation of apoptosis, Mol. Cancer Ther. 2 (2003) 641-649.

[14] K. Ozaki, A. Minoda, F. Kishikawa, M. Kohno, Blockade of the ERK pathway markedly sensitizes tumor cells to HDAC inhibitor-induced cell death, Biochem. Biophys. Res. Commun. 339 (2006) 1171-1177.

[15] S. Tanimura, A. Hirano, J. Hashizume, M. Yasunaga, T. Kawabata, K. Ozaki, M. Kohno, Anticancer drugs up-regulate HspBP1 and thereby antagonize the prosurvival function of Hsp70 in tumor cells, J. Biol. Chem. 282 (2007)

35430-35439.

[16] Z. Darzynkiewicz, G. Juan, X. Li, W. Gorczyca, T. Murakami, F. Traganos, Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis), Cytometry 27 (1999) 1-20.

[17] H.M. McDaid, S.B. Horwitz, Selective potentiation of paclitaxel (Taxol)-induced cell death by mitogen-activated protein kinase kinase inhibition in human cancer cell lines, Mol. Pharmacol. 60 (2001) 290-301.

[18] M.A. Jordan, L. Wilson, Microtubules as a target for anticancer drugs, Nat. Rev. Cancer 4 (2004) 253-265.

[19] A.E. Wakeling, S.P. Guy, J.R. Woodburn, S.E. Ashton, B.J. Curry, A.J. Barker, K.H. Gibson, ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy, Cancer Res. 62 (2002) 5749-5754.

[20] C. Diaz, A.T. Lee, D.J. McConkey, A.J. Schroit, Phosphatidylserine externalization during differentiation-triggered apoptosis of erythroleukemic cells, Cell Death Differ. 6 (1999) 218-226.

[21] K. Valerie, A. Yacoub, M.P. Hagan, D.T. Curiel, P.B. Fisher, S. Grant, P. Dent, Radiation-induced cell signaling: inside-out and outside-in, Mol. Cancer Ther. 6 (2007) 789-801.

[22] B.L. Bennett, D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J.C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, D.W. Anderson, SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase, Proc. Natl. Acad. Sci. USA 98 (2001) 13681-13686.

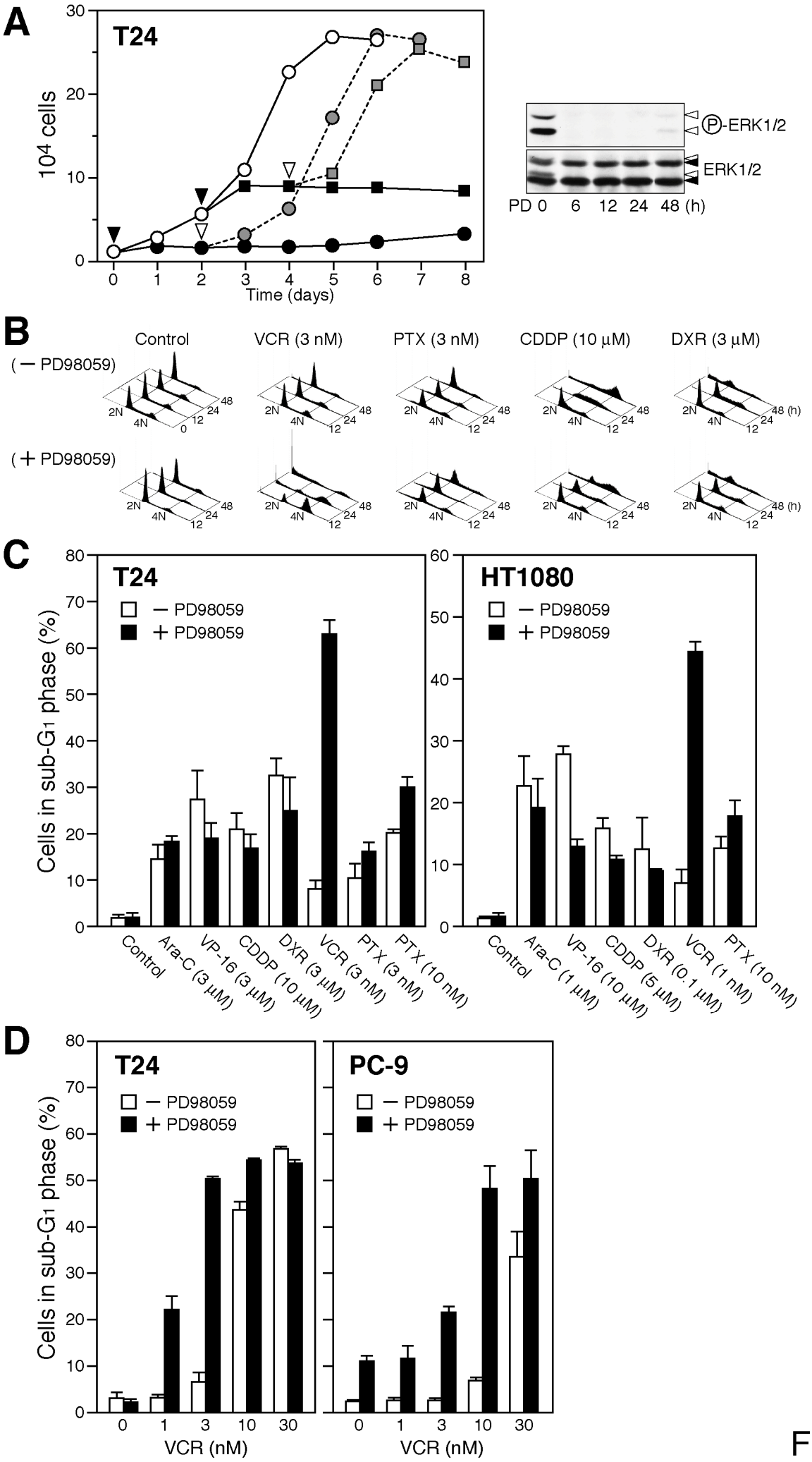
Figure Legends

Figure 1. Selective potentiation by MEK inhibitors of the death-inducing effect of vincristine in tumor cells. (**A**) T24 cells were incubated for the indicated times in the absence (open circles) or presence (closed circles) of 10 μM PD184352. The cells in some dishes were first exposed to PD184352 after culture for 2 days (closed squares), whereas the PD184352-containing medium of some dishes was replaced with drug-free medium after culture for 2 days (gray circles) or 4 days (gray squares), as indicated by the arrowheads. Cells were harvested by exposure to trypsin, and viable cells were counted with the use a hemocytometer after staining with trypan blue (*Left*). T24 cells were treated with 10 μM PD184352 (PD) for the indicated times, after which cell lysates (20 μg of protein) were subjected to immunoblot analysis with antibodies to total or phosphorylated (P) forms of ERK1/2 (*Right*). Open and closed arrowheads indicate phosphorylated and nonphosphorylated forms, respectively, of ERK1/2. (**B**) T24 cells were incubated for 12, 24, or 48 h with the indicated agents in the absence (–) or presence (+) of 50 μM PD98059 and were then analyzed for DNA content by flow cytometry. VCR, vincristine; PTX, paclitaxel; CDDP, cisplatin; DXR, doxorubicin. (**C**) T24 or HT1080 cells were incubated for 48 h with the indicated agents in the absence or presence of 50 μM PD98059 and were then analyzed as in (**B**) for the proportion of cells in sub-G_1 phase. (**D**) T24 or PC-9 cells were incubated for 48 h with the indicated concentrations of vincristine in the absence or presence of 50 μM PD98059 and were then analyzed for the proportion of cells in sub-G_1 phase. Data in (**A**) and (**B**) are representative of three

separate experiments; those in (**C**) and (**D**) are means ± SD from three separate experiments, each performed in duplicate.

Figure 2. Potentiation of the death-inducing effect of microtubule-destabilizing agents by blockade of the ERK pathway in tumor cells in which the pathway is constitutively activated. (**A**) The indicated tumor cell lines were incubated in the absence (Control) or presence of 50 μM PD98059 (PD), vincristine (VCR: 3 nM for HeLa S3, TCO, and A549 cells; 10 nM for PC3, Colo320, and H1650 cells; 30 nM for WiDr cells), or both agents for 48 h, after which the proportion of cells in sub-G_1 phase was determined by flow cytometry. Lysates of untreated cells (20 μg of protein) were subjected to immunoblot analysis with antibodies to total or phosphorylated forms of ERK1/2 (*Inset*). (**B**) T24 cells were incubated with the indicated agents for 48 h, after which the proportion of cells in sub-G_1 phase was determined. PD98, PD98059; PD18, PD184352; VNR, vinorelbine; TZT, TZT-1027. (**C**) II-18 cells were incubated for 48 h with the indicated concentrations of vincristine in the absence (–) or presence (+) of 50 μM PD98059 (PD) or 100 nM gefitinib (Gef), after which the proportion of cells in sub-G_1 phase was determined. II-18 cells were also incubated in the absence (Control) or presence of 50 μM PD98059 or 100 nM gefitinib for 24 h, after which cell lysates (20 μg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated or total forms of EGFR or ERK1/2 (*Inset*). All quantitative data are means ± SD from three separate experiments, each performed in duplicate.

Figure 3. Role of JNK in apoptotic cell death induced by the combination of PD98059 and vincristine in T24 cells. (**A**) Cells were incubated for 24 h in the absence (Control) or presence of 50 μM PD98059 (PD), 3 nM vincristine (VCR), or both agents and were then fixed and subjected to staining with DAPI, with annexin V and propidium iodide (PI), or with antibodies to the cleaved form of caspase-3. Bar, 100 μm. (**B**) Cells were incubated for the indicated times in the absence (C) or presence of 50 μM PD98059, 3 nM vincristine, or both agents, after which cell lysates (20 μg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated or total forms of JNK or ERK1/2, to the cleaved form of caspase-3, or to β-actin (loading control). Open arrowheads indicate phosphorylated forms of JNK1/2, phosphorylated forms of ERK1/2, or cleaved fragments (19 and 17 kDa) of caspase-3; closed arrowheads indicate β-actin and nonphosphorylated forms of JNK1/2 and ERK1/2. (**C**) Cells were incubated for 48 h with or without 10 μM SP600125 and in the absence (Control) or presence of 50 μM PD98059, 3 nM vincristine, or both agents, after which they were analyzed for DNA content by flow cytometry. (**D**) Cells transfected with siRNAs specific for JNK1 or JNK2 mRNAs (alone or together) or with a control siRNA were incubated for 24 h in the absence (Control) or presence of 50 μM PD98059, 3 nM vincristine, or both agents. The cells were then analyzed for DNA content by flow cytometry. Lysates (20 μg of protein) of the transfected cells were subjected to immunoblot analysis with antibodies to JNK or to β-actin (*Inset*). Data in (**A**) and (**B**) are representative of three separate experiments; quantitative data in (**C**) and (**D**) are means ± SD from three separate experiments, each performed in duplicate


A
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104 cells
Time (days)
Ⓟ-ERK1/2
ERK1/2
PD 0 6 12 24 48 (h)
B
Control
VCR (3 nM)
PTX (3 nM)
CDDP (10 μM)
DXR (3 μM)
(− PD98059)
(+ PD98059)
2N
4N
C
T24
HT1080
− PD98059
+ PD98059
Cells in sub-G1 phase (%)
Control
Ara-C (3 μM)
VP-16 (3 μM)
CDDP (10 μM)
DXR (3 μM)
VCR (3 nM)
PTX (3 nM)
PTX (10 nM)
Ara-C (1 μM)
VP-16 (10 μM)
CDDP (5 μM)
DXR (0.1 μM)
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D
T24
PC-9
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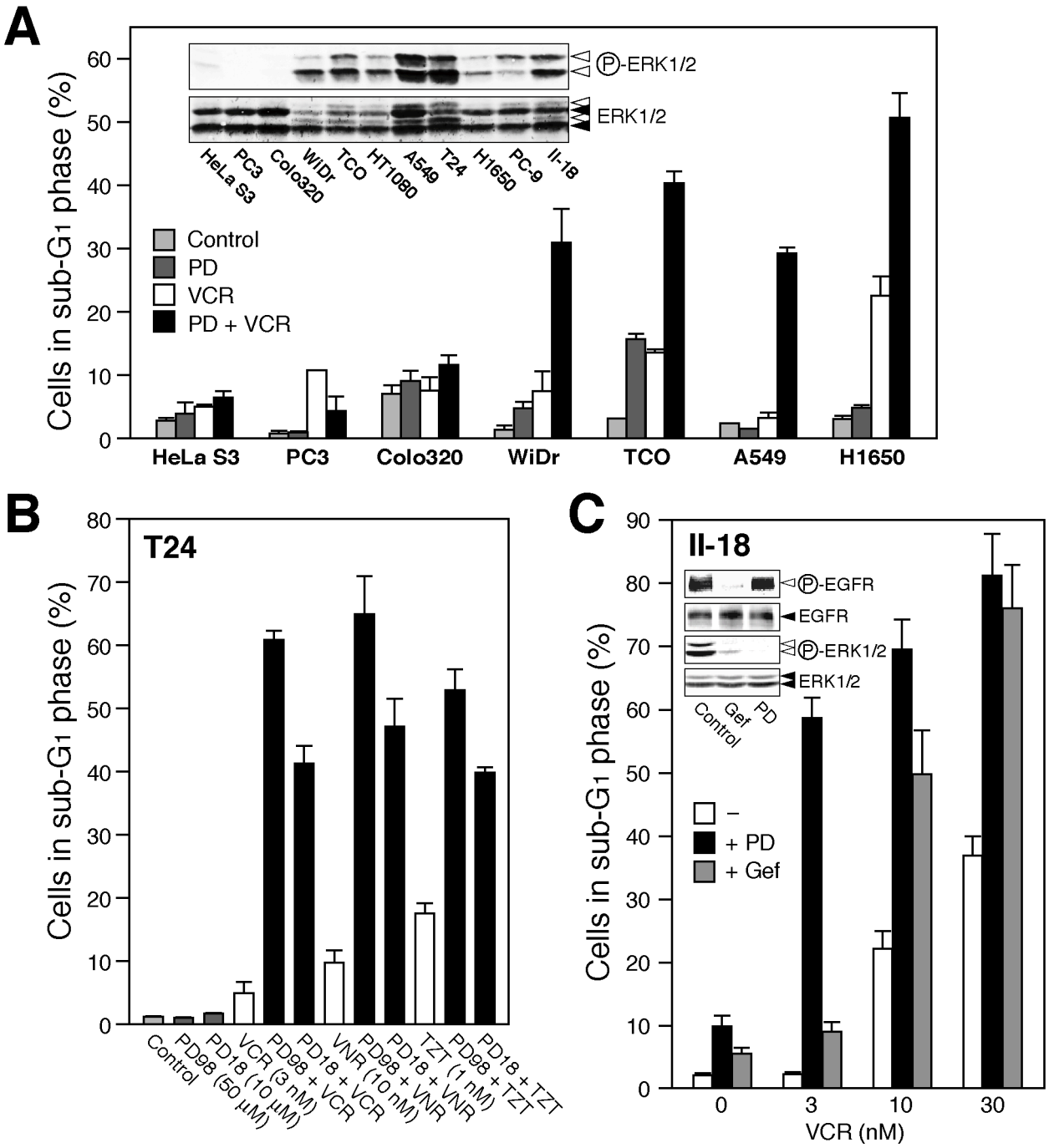
Figure 1


A
Cells in sub-G1 phase (%)
Ⓟ-ERK1/2
ERK1/2
HeLa S3
PC3
Colo320
WiDr
TCO
HT1080
A549
T24
H1650
PC-9
Il-18
Control
PD
VCR
PD + VCR
B
T24
Cells in sub-G1 phase (%)
Control
PD98 (50 μM)
PD18 (10 μM)
VCR (3 nM)
PD98 + VCR
PD18 + VCR
VNR (10 nM)
PD98 + VNR
PD18 + VNR
TZT (1 nM)
PD98 + TZT
PD18 + TZT
C
Il-18
Ⓟ-EGFR
EGFR
Ⓟ-ERK1/2
ERK1/2
Control
Gef
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Cells in sub-G1 phase (%)
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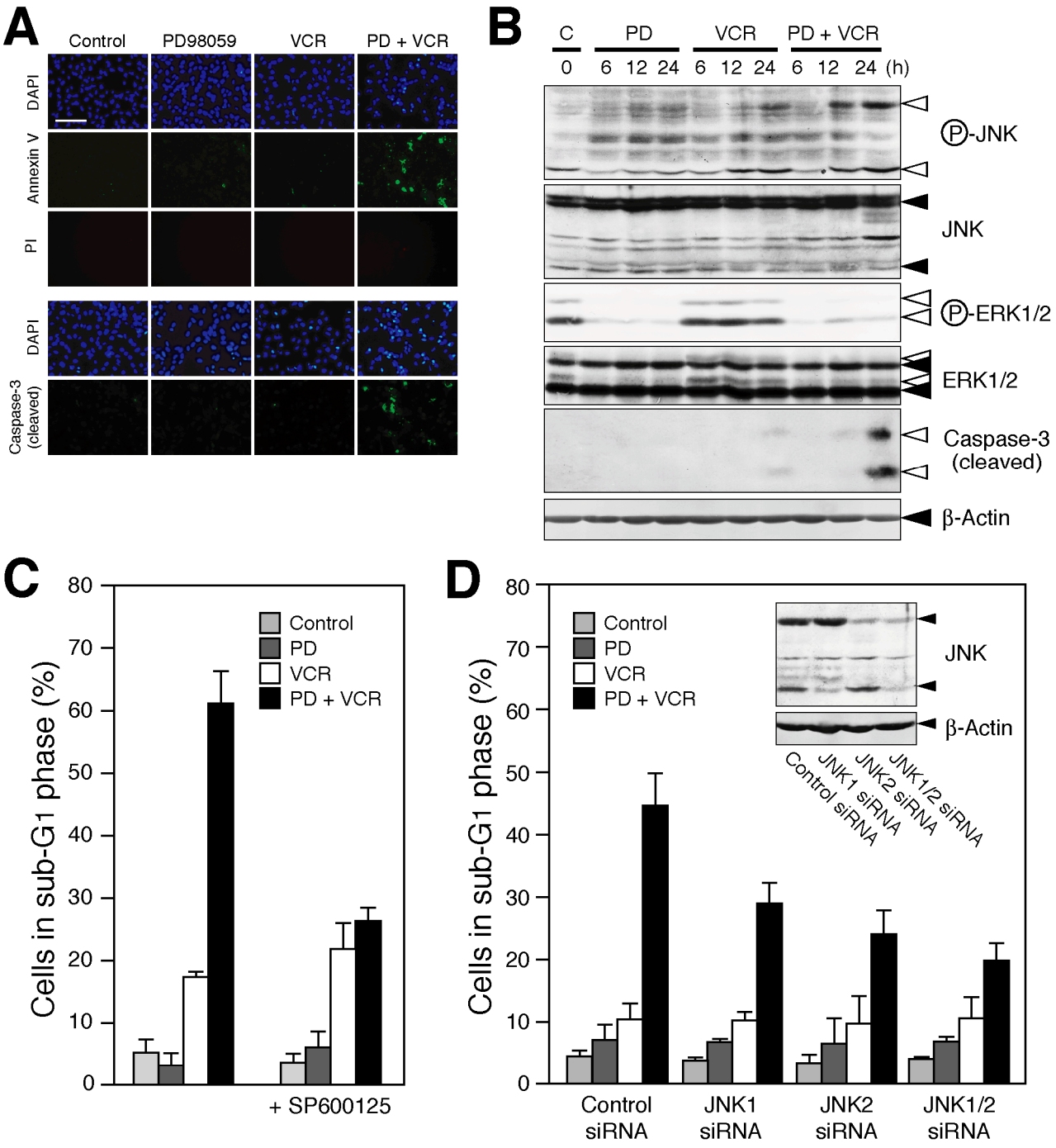
+ PD
+ Gef
VCR (nM)

Figure 2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Control
PD98059
VCR
PD + VCR
DAPI
Annexin V
PI
DAPI
Caspase-3 (cleaved)
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Ⓟ-JNK
JNK
Ⓟ-ERK1/2
ERK1/2
Caspase-3 (cleaved)
β-Actin
C
Cells in sub-G1 phase (%)
Control
PD
VCR
PD + VCR
+ SP600125
D
Cells in sub-G1 phase (%)
Control
PD
VCR
PD + VCR
JNK
β-Actin
Control siRNA
JNK1 siRNA
JNK2 siRNA
JNK1/2 siRNA
Control siRNA
JNK1 siRNA
JNK2 siRNA
JNK1/2 siRNA

Figure 3