Cancer Therapy: Preclinical

Blockade of the Extracellular Signal-Regulated Kinase Pathway Enhances the Therapeutic Efficacy of Microtubule-Destabilizing Agents in Human Tumor Xenograft Models

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Abstract

Purpose: The extracellular signal-regulated kinase (ERK) pathway is upregulated in human cancers and represents a target for mechanism-based approaches to cancer treatment. However, specific blockade of the ERK pathway alone induces mostly cytostatic rather than proapoptotic effects, resulting in a limited therapeutic efficacy of inhibitors that target the mitogen-activated protein kinase/ERK kinase (MEK). Given the cytoprotective role of the ERK pathway, we examined whether its blockade by the MEK inhibitor PD184352 might enhance the therapeutic efficacy of anticancer drugs in human tumor xenograft models.

Experimental Design: We recently showed that blockade of the ERK pathway by MEK inhibitors enhances the induction of apoptosis by microtubule-destabilizing agents, including TZT-1027 and vinorelbine, in various tumor cells with aberrant activation of the ERK pathway in vitro. We here examined the therapeutic efficacy of the combination of PD184352 with TZT-1027 or vinorelbine in nude mice harboring HT-29 or HT1080 tumor xenografts, in which the ERK pathway is activated as a result of mutations of BRAF and NRAS, respectively.

Results: Coadministration of PD184352 markedly sensitized HT-29 or HT1080 tumor xenografts to TZT-1027–induced or vinorelbine-induced cytotoxicity. Low doses of TZT-1027 or vinorelbine that by themselves showed little or moderate cytotoxicity thus suppressed the growth of HT-29 xenografts almost completely and induced essentially complete regression of HT1080 xenografts when administered with PD184352. The enhanced therapeutic efficacy of the drug combinations was achieved by a relatively transient blockade of the ERK pathway.

Conclusions: Administration of both a MEK inhibitor and a microtubule-destabilizing agent represents a promising chemotherapeutic strategy with improved safety for cancer patients.

The extracellular signal-regulated kinase (ERK) pathway is activated in a variety of cell types by diverse extracellular stimuli and mediates signaling between various cell surface receptors and the nucleus. Activation of the ERK pathway is triggered by loading of GTP onto Ras at the plasma membrane, which is followed by sequential activation of a series of protein kinases, including a member of the Raf family, mitogen-activated protein kinase/ERK kinase isoforms 1 and 2 (MEK1/2), and ERK isoforms 1 and 2 (ERK1/2). Activated ERK then phosphorylates various downstream substrates that contribute to regulation of a wide range of cellular processes, such as proliferation, differentiation, survival, and motility (1).

Aberrant activation of the ERK pathway is apparent in and contributes to the pathogenesis of many types of human cancer (2, 3). In particular, overexpression or activating mutation of the epidermal growth factor receptor (EGFR) (4), activating mutation of RAS (5), and activating mutation of RAF (6), all of which culminate in the activation of MEK1/2 and ERK1/2, have been associated with various human cancers. The ERK pathway thus represents a promising target for the development of anticancer drugs, and highly selective small-molecule inhibitors of MEK1/2, including PD98059, PD184352, and AZD6244, have been developed (7, 8).

We have previously shown that specific blockade of the ERK pathway by PD98059 or PD184352 markedly suppressed not only the proliferation (9) but also the invasiveness (10) of tumor cells with aberrant pathway activation. However, blockade of the ERK pathway by itself was mostly...
cytostatic, rather than cytotoxic, resulting in only a moderate induction of apoptosis in these tumor cells (9). Thus, although PD184352 or AZD6244 totally suppressed the proliferation of T24 human bladder carcinoma cells in culture (11) or that of HT-29 human colon adenocarcinoma or BxPC3 human pancreatic cancer xenografts in vivo (12), respectively, these tumor cells remained viable and resumed proliferation after removal of the inhibitor or cessation of drug administration. Recent clinical studies of MEK inhibitors in patients with advanced cancers showed that, although PD184352 or AZD6244 achieved target inhibition at well-tolerated doses, these drugs alone exhibited insufficient antitumor activity (13, 14). Strategies to improve the anticancer activity of MEK inhibitors might prove to be therapeutically beneficial for cancer patients.

Combination therapy is becoming the norm in cancer treatment, with combination of cytotoxic agents with non-overlapping toxicities being driven by safety considerations. Optimal use of molecularly targeted therapies has also been proposed to lie in combination therapy, either with classic cytotoxic drugs or with other targeted therapies (8, 15). In this regard, specific interruption of the cytotoxic antitumor agents through a shift in the balance between proapoptotic and antiapoptotic signaling (16). Consistent with this notion, the MEK inhibitor U0126 was found to enhance the induction of apoptosis by paclitaxel, a microtubule-stabilizing agent, in human tumor cells in culture (17). Furthermore, enhancement of the therapeutic efficacy of paclitaxel or docetaxel by PD184352 or AZD6244 has been shown for several human tumor xenografts in nude mice (18–20).

We have recently shown that blockade of the ERK pathway by PD98059 or PD184352 markedly enhanced the induction of apoptosis by microtubule destabilizing agents, including vinorelbine and TZT-1027, in a variety of tumor cells with aberrant ERK pathway activation in vitro (11). We have now extended our in vitro findings and shown that PD184352-mediated blockade of the ERK pathway enhanced the induction of apoptosis by TZT-1027 in HT-29 xenografts in vivo as well as markedly potentiated the therapeutic efficacy of TZT-1027 or vinorelbine in HT-29 or HT1080 xenografts.

Materials and Methods

Reagents and antibodies. PD184352 was synthesized as described previously (10). TZT-1027 (Soblicitin) was kindly provided by Aska Pharmaceutical. Vinorelbine ditartrate (Navelbine) was obtained from Kyowa Hakko Kirin. Cremophor EL was from Sigma-Aldrich. Antibodies to ERK1/2 were from Santa Cruz Biotechnology; those to diphosphorylated ERK1/2 were from Sigma-Aldrich; and those to Ki-67 were from Abcam.

Cell culture. The human tumor cell lines HT-29 (colon adenocarcinoma; obtained from the American Type Culture Collection on January 4, 2001) and HT1080 (fibrosarcoma; obtained from Health Science Research Resources Center on April 6, 2004) were cultured in DMEM supplemented with 10% fetal bovine serum for no longer than 10 wk after recovery from frozen stocks.

Animals and tumor cell implantation. Animal care and experimental procedures were done in accordance with the Guidelines for Animal Experimentation of Nagasaki University and approved by the Institutional Animal Care and Use Committee. HT-29 or HT1080 cells (2 × 10⁶) were injected s.c. into the right flank of 5- to 6-wk-old female BALB/c nu/nu mice (Charles River). After the resulting tumors had achieved a size of 200 ± 20 mm³ or 600 ± 50 mm³, mice were randomly assigned to balanced groups of five to seven animals. PD184352 was suspended in an 8:1:1 (v/v/v) mixture of PBS/ethanol/cremophor EL, whereas TZT-1027 and vinorelbine were dissolved in 0.05 mol/L sodium lactate buffer (pH 4.5) and in PBS, respectively. Mice were treated every 7 d with PD184352 (200 mg/kg) or vehicle by oral administration (four times per day, every 6 h) and with TZT-1027 (0.25–2.5 mg/kg), vinorelbine (5-20 mg/kg), or vehicle by i.v. injection (once per day, 1 h after the first PD184352 administration). Tumor volume was measured with digital calipers and calculated according to the following formula: (longest diameter) × (shortest diameter)²/2. Body weight, tumor volume, and toxicities were noted every 2 to 4 d for the duration of the experiment.

Immunoblot analysis. Tumor extracts prepared by mechanical homogenization of excised tumors on ice in hypotonic cell lysis buffer (3) were fractionated by SDS-PAGE and subjected to immunoblot analysis as described.
Immune complexes were detected with an enhanced chemiluminescence system (GE Healthcare Bio-Sciences).

Immunohistochemical analysis. HT-29 or HT1080 xenografts were harvested, fixed in buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. After removal of paraffin and rehydration, tissue sections were incubated with 3% hydrogen peroxide to quench endogenous peroxidase activity and then exposed to 5% normal goat serum to block nonspecific sites. The sections were then incubated consecutively with primary antibodies and biotinylated secondary antibodies, and immune complexes were visualized with the use of an LSAB2 kit/horse-radish peroxidase (DAKO). The sections were finally counterstained with hematoxylin and examined with an Axiovert 200 M microscope equipped with Axiovision 3.0 software (Carl Zeiss). Detection of apoptotic cells by

Fig. 1. Potentiation of the apoptosis-inducing effect of TZT-1027 by PD184352 in HT-29 xenografts. A, mice harboring subcutaneous HT-29 tumors (∼300 mm³) were dosed once orally with PD184352 (200 mg/kg) and the tumors were excised at the indicated times thereafter. Tumor extracts (20 μg protein) were subjected to immunoblot analysis with antibodies to total or diphosphorylated ERK1/2. B, mice bearing HT-29 xenografts were treated with the combination of PD184352 (200 mg/kg, four times a day) and TZT-1027 (1 mg/kg) as described in Materials and Methods. Tumors excised 24 or 48 h after the first PD184352 dosing were subjected to immunohistochemical analysis with antibodies to diphosphorylated ERK1/2 (top) or lysed and subjected to immunoblot analysis (20 μg protein) with antibodies to total or diphosphorylated ERK1/2 (bottom). C, mice harboring HT-29 xenografts were treated with PD184352 (200 mg/kg) or vehicle every 6 h for a total of four administrations. Tumors were excised 24 h after the first PD184352 dosing and subjected to the TUNEL assay. Bottom, the percentage of TUNEL-positive cells was determined for 10 randomly selected microscopic fields. Columns, mean of two separate experiments, each done in triplicate; bars, SD. *, P < 0.05. Scale bars, 50 μm.
Blockade of the ERK pathway by PD184352 enhances induction of apoptosis by TZT-1027 in an HT-29 tumor xenograft model. Human colon adenocarcinoma HT-29 xenografts, in which the ERK pathway is activated as a result of mutation of BRAF, were chosen for optimization of dosing schedules in vivo. Nude mice harboring subcutaneous HT-29 tumors (∼300 mm³) were treated orally with PD184352 at a dose of 200 mg/kg. Immunoblot analysis of extracts of the excised tumors revealed that a single dose of PD184352 almost completely suppressed the phosphorylation (activation) of ERK1/2 for 6 hours, after which ERK1/2 phosphorylation gradually increased, returning to control levels by 9 to 12 hours (Fig. 1A). These results indicated that administration of PD184352 every 6 hours would be required for continuous suppression of ERK1/2 activation in HT-29 tumors in nude mice.

To examine the potential of PD184352 to enhance the induction of apoptosis by TZT-1027, we treated mice bearing subcutaneous HT-29 tumors (∼300 mm³) with PD184352 (200 mg/kg, orally) every 6 hours for a total of four administrations and, at 1 hour after the first PD184352 dosing, injected them i.v. with TZT-1027 (0.5 or 1 mg/kg). Immunoblot analysis of tumor extracts as well as immunostaining of tumor sections with antibodies to phosphorylated ERK1/2 revealed that phosphorylation of ERK1/2 was suppressed completely for up to 24 hours.
after the initial PD184352 administration, with the phosphorylation returning to control levels by 48 hours in most of the treated mice (Fig. 1B). Coadministration of TZT-1027 did not interfere with the PD184352-induced suppression of ERK1/2 phosphorylation. Immunostaining for Ki-67 as a marker for proliferating cells confirmed that the number of such cells in tumor sections was decreased greatly at 24 hours after the initial dosing with PD184352 compared with that apparent for vehicle-treated tumors (Fig. 1C).

TUNEL staining of tumor sections for cells undergoing apoptotic death revealed that PD184352 dosing alone did not increase the number of apoptotic cells (Fig. 1D), consistent with our results obtained in vitro (11). TZZT-1027 treatment alone increased the number of TUNEL-positive cells in HT-29 xenografts by 24 hours in a dose-dependent manner, and this effect was enhanced by coadministration of PD184352 compared with that apparent for vehicle-treated tumors (Fig. 1C).

PD184352 potentiates the therapeutic efficacy of microtubule-destabilizing agents in an HT-29 tumor xenograft model. Mice bearing subcutaneous HT-29 tumors (~200 mm³) were dosed every 7 days with TZT-1027 (0.5 or 1.0 mg/kg) for a total of four cycles. This dosing regimen was based on ongoing clinical trials in which many anticancer drugs are administered weekly. Under such conditions, TZT-1027 inhibited the growth of HT-29 xenografts in a dose-dependent manner (Fig. 2A and B). To evaluate the effect of blockade of the ERK pathway on the therapeutic efficacy of TZT-1027, we coadministered PD184352 (200 mg/kg, four times a day) on each day that TZT-1027 was injected. Such weekly dosing of the MEK inhibitor by itself inhibited the growth of HT-29 xenografts only slightly. However, coadministration of PD184352 markedly enhanced the therapeutic efficacy of TZT-1027. Indeed, the combination of PD184352 and TZT-1027 at 1 mg/kg induced essentially complete suppression of tumor growth. Furthermore, H&E staining of tumors revealed that this drug combination induced the disappearance of tumor cells as well as a spongy appearance of the interstitium associated with residual mucin, characteristics that were not apparent or much less prominent in tumors treated with either drug alone (Fig. 2C). These results suggested that the therapeutic efficacy of this drug combination is even greater than that suggested by its effect on tumor volume and...
weight. None of such treated mice showed weight loss or other overt clinical signs of toxicity, including gastrointestinal toxicity.

Even if the dosing regimen was initiated when tumors had reached a size of $\sim 600 \text{ mm}^3$, the combination of PD184352 and TZT-1027 exhibited therapeutic efficacy. Essentially complete growth suppression of HT-29 xenografts was thus achieved by weekly dosing with PD184352 and TZT-1027 (1 mg/kg), again with no evidence of drug toxicity including weight loss (Fig. 3A and B). The histopathology of such treated tumors revealed a reduction in tumor cellularity that was apparent after administration of only two treatment cycles (Fig. 3C). Treatment with TZT-1027 alone at a higher dose (2.5 mg/kg) totally inhibited the growth of HT-29 xenografts, but all such treated mice showed weight loss, which was most evident after the second drug dosing and was followed by death in four of six mice by days 10 to 14.

We next examined whether coadministration of PD184352 would potentiate the therapeutic efficacy of vinorelbine (Navelbine), a microtubule-destabilizing agent that has been evaluated in clinical trials. TZT-1027 and vinorelbine exhibit low neurotoxicity in animal models and humans, respectively (22, 23). Weekly i.v. administration of vinorelbine exhibited no or only a slight inhibitory effect on the growth of HT-29 xenografts at doses of 5 mg/kg or 10 or 20 mg/kg, respectively (Fig. 4A and B). However, coadministration of PD184352 (200 mg/kg, four times a day every 7 days) sensitized HT-29 xenografts to vinorelbine-induced cytotoxicity. Under such conditions, the growth of HT-29 xenografts was inhibited by vinorelbine in a dose-dependent manner, with almost complete growth suppression being achieved at the dose of 20 mg/kg. Furthermore, the drug combination induced prominent histopathologic changes in the tumors (Fig. 4C) that were similar to those induced by the combination of PD184352 and TZT-1027. Overall, these results indicated that blockade of the ERK pathway by PD184352 markedly potentiated the therapeutic efficacy of microtubule-destabilizing agents in the HT-29 xenograft model.

PD184352 potentiates the therapeutic efficacy of TZT-1027 in an HT1080 tumor xenograft model. We examined...
whether the combination of PD184352 and TZT-1027 might also exhibit enhanced therapeutic efficacy in the HT1080 fibrosarcoma xenograft model, in which the ERK pathway is activated as a result of mutation of NRAS.

In contrast to HT-29 xenografts, weekly treatment with PD184352 (200 mg/kg, four times a day) alone substantially inhibited the growth of HT1080 xenografts (Fig. 5A). However, the growth of HT1080 tumors resumed 2 to 3 days after drug administration, most likely as a result of the recovery of ERK1/2 phosphorylation to the control level by 48 hours (Fig. 5A, inset). Whereas weekly administration of TZT-1027 (0.25-1 mg/kg) alone inhibited the growth of HT1080 xenografts in a dosedependent manner, coadministration of PD184352 markedly enhanced the therapeutic efficacy of TZT-1027 at each dose examined (Fig. 5A and B). Tumors had thus shrunk to the limits of visible detection by 2 to 4 days after the second administration of the combination of PD184352 and TZT-1027 at doses of 0.5 or 1 mg/kg. The drug combination induced a prominent infiltration of immune cells (such as macrophages and neutrophils) and interstitial fibrosis in the tumors (Fig. 5C), indicating that aggressive elimination of tumor cells was under way. None of the mice treated with the combination of PD184352 and TZT-1027 showed signs of drug toxicity, including weight loss or gastrointestinal toxicity; they instead appeared healthier than the vehicle-treated animals. The latter mice did exhibit weight loss in association with tumor growth, with HT1080 xenografts having previously been shown to induce cachexia in animals (24).

Discussion

Whereas the MEK inhibitor PD184352 alone exhibited little or limited growth-inhibitory effects in HT-29 and...
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HT1080 tumors, respectively, it markedly sensitized these human xenografts to TZT-1027-induced or vinorelbine-induced cytotoxicity. Low doses of these microtubule-destabilizing agents (TZT-1027 at 1 mg/kg or vinorelbine at 20 mg/kg) that by themselves showed little or moderate cytotoxicity were thus able to suppress the growth of HT-29 xenografts almost completely when combined with PD184352. Moreover, these drug combinations induced the disappearance of tumor cells as well as a spongy appearance of the interstitium associated with residual mucin in the remaining tumor tissue. Alternatively, two cycles of treatment with PD184352 and TZT-1027 at a dose as low as 0.5 mg/kg induced a prominent infiltration of immune cells and interstitial fibrosis in HT1080 tumor tissue, which seemed to result in essentially the complete regression of the xenografts. The difference in the phenotypes of the two xenografts in the treated mice most likely reflects the different types of tumor cells, HT-29 cells being derived from colon adenocarcinoma and HT1080 cells from fibrosarcoma. Regardless of these phenotypic differences, however, administration of the combination of PD184352 and low doses of microtubule-destabilizing agents exhibited prominent therapeutic efficacy in both xenograft models. Coadministration of a MEK inhibitor might thus be expected to contribute to the development of safer anticancer strategies with sufficient therapeutic efficacy by lowering the required dose of cytotoxic microtubule-destabilizing agents.

Our results also show that a relatively transient blockade of the ERK pathway enhanced the therapeutic efficacy of microtubule-destabilizing agents. In our xenograft experiments, we aimed to suppress the phosphorylation of ERK1/2 in tumor cells for the initial 24 hours after the administration of TZT-1027 or vinorelbine. Mice were thus dosed with PD184352 only on the day that they received the cytotoxic drug. Given the relative metabolic instability of PD184352 (25), mice were dosed with the MEK inhibitor every 6 hours for a total of four times to ensure suppression of ERK1/2 phosphorylation for at least 24 hours. Under such conditions, although phosphorylation of ERK1/2 remained totally suppressed for at least 24 hours, it returned to control levels by 48 hours. Continuous blockade of the ERK pathway by administration of PD184352 or AZD6244 twice or thrice a day has previously been shown to be required for enhancement of the therapeutic efficacy of paclitaxel or docetaxel in animal models (18, 20). Recent clinical trials of MEK inhibitors in patients with advanced cancers have shown that daily administration of PD184352 (800 mg twice a day) or AZD6244 (100 mg twice a day) for up to several months is well tolerated, the most common treatment-related toxicities being mild rash, diarrhea, asthenia, nausea, and vomiting (13, 14). However, given the essential role of the ERK pathway in regulation of a wide range of cellular processes including the immune response (26), shortening of the period during which an administered MEK inhibitor suppresses the ERK pathway might be expected to be beneficial in terms of reducing potential side effects in patients.

The molecular mechanism of the interaction between MEK inhibitors and microtubule-destabilizing agents remains unknown. However, MEK inhibitors selectively enhance the cytotoxicity of microtubule-destabilizing agents (11) and microtubule-stabilizing agents (17–20), both of which induce mitotic catastrophe (27). The ERK pathway plays an essential role not only in the G0-G1 transition of the cell cycle but also in the G2-M transition (28). The combination of a MEK inhibitor and a microtubule inhibitor might thus act synergistically to induce mitotic catastrophe in tumor cells. Furthermore, the enhanced cell death induced by such drug combinations is likely mediated, at least in part, by c-Jun NH2-terminal kinase (11, 29).

In summary, we have shown that blockade of the ERK pathway by PD184352 markedly enhanced the therapeutic efficacy of microtubule-destabilizing agents in nude mice bearing HT-29 or HT1080 xenografts by sensitizing the tumor cells to the cytotoxicity of the latter drugs. Combinations of a MEK inhibitor and a microtubule-destabilizing agent may provide a basis for the development of safer anticancer chemotherapies with enhanced efficacy.

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No potential conflicts of interest were disclosed.

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