Biochemical and Biophysical Research Communications 433 (2013) 456–462

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Blockade of the ERK pathway enhances the therapeutic efficacy of the histone deacetylase inhibitor MS-275 in human tumor xenograft models

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ARTICLE INFO

Article history:
Received 28 February 2013
Available online 15 March 2013

Keywords:
ERK pathway
MEK inhibitor
HDAC inhibitor
Combination therapy
Synergistic drug interaction

ABSTRACT

The ERK pathway is up-regulated in various human cancers and represents a prime target for mechanism-based approaches to cancer treatment. Specific blockade of the ERK pathway alone induces mostly cytostatic rather than pro-apoptotic effects, however, resulting in a limited therapeutic efficacy of the ERK kinase (MEK) inhibitors. We previously showed that MEK inhibitors markedly enhance the ability of histone deacetylase (HDAC) inhibitors to induce apoptosis in tumor cells with constitutive ERK pathway activation in vitro. To evaluate the therapeutic efficacy of such drug combinations, we administered the MEK inhibitor PD184352 or AZD6244 together with the HDAC inhibitor MS-275 in nude mice harboring HT-29 or H1650 xenografts. Co-administration of the MEK inhibitor markedly sensitized the human xenografts to MS-275 cytotoxicity. A dose of MS-275 that alone showed only moderate cytotoxicity thus suppressed the growth of tumor xenografts almost completely as well as induced a marked reduction in tumor cellularity when administered with PD184352 or AZD6244. The combination of the two types of inhibitor also induced marked oxidative stress, which appeared to result in DNA damage and massive cell death, specifically in the tumor xenografts. The enhanced therapeutic efficacy of the drug combination was achieved by a relatively transient blockade of the ERK pathway. Administration of both MEK and HDAC inhibitors represents a promising chemotherapeutic strategy with improved safety for cancer patients.

1. Introduction

Aberrant activation of the extracellular signal-regulated kinase (ERK) signaling pathway contributes to the pathogenesis of many types of human cancer [1,2]. In particular, activating mutations of the epidermal growth factor receptor (EGFR), Ras, and Raf, all of which result in activation of MEK (ERK kinase) isoforms 1 and 2 (MEK1/2) and ERK isoforms 1 and 2 (ERK1/2), have been detected in various human cancers [3–5]. The ERK pathway thus represents a promising target for the development of anticancer drugs, and highly selective small-molecule inhibitors of MEK1/2, including PD184352, PD0325901, and AZD6244, have been developed [6].

We have shown that specific blockade of the ERK pathway by MEK inhibitors markedly suppressed not only the proliferation but also the invasiveness of tumor cells with aberrant activation of this pathway [7,8]. However, blockade of the ERK pathway by itself was largely cytostatic, rather than cytotoxic, resulting in only a moderate induction of apoptosis in these tumor cells [7]. Thus, although PD184352 or AZD6244 totally suppressed the proliferation of T24 bladder carcinoma cells in culture [9] or that of HT-29 colon adenocarcinoma or BxPC3 pancreatic cancer xenografts in vivo [10], 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 have shown that, although PD184352 or AZD6244 achieved target inhibition at well-tolerated doses, these drugs alone exhibited insufficient antitumor activity [11,12]. Efficient induction of apoptotic cell death is essential for the development of effective cancer chemotherapy.

Optimal use of molecularly targeted therapies lies in combination treatment, either with classic cytotoxic drugs or with other targeted therapies [6,13]. In this regard, specific interruption of the cytoprotective ERK pathway by MEK inhibitors has been

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http://dx.doi.org/10.1016/j.bbrc.2013.03.009

proposed as a means to enhance the lethal actions of cytotoxic anticancer agents through a shift in the balance between pro- and anti-apoptotic signaling [14]. Consistent with this notion, MEK inhibitors have been shown to enhance the induction of apoptosis by several anticancer agents, including microtubule inhibitors, in human tumor cells in culture [9] as well as in human tumor xenografts in nude mice [15–17].

We have recently shown that blockade of the ERK pathway by PD184352 markedly enhanced the induction of apoptosis by histone deacetylase (HDAC) inhibitors in a variety of solid tumor cells with aberrant ERK pathway activation in vitro, an effect that appeared to be attributable to the increased accumulation of reactive oxygen species (ROS) [18]. Furthermore, such enhanced cell death induction by the combination of a MEK inhibitor and an HDAC inhibitor was apparent even in non-small cell lung cancer and chronic myelogenous leukemia cells exhibiting resistance to EGFR or Abl tyrosine kinase inhibitors, respectively [19]. We now show that blockade of the ERK pathway with a MEK inhibitor resulted in marked potentiation of the therapeutic efficacy of the HDAC inhibitor MS-275 in human tumor xenograft models.

2. Materials and methods

2.1. Reagents and antibodies

PD184352 [8], AZD6244 [10], and MS-275 [20] were synthesized as described previously. Cremophore EL was obtained from Sigma–Aldrich (St. Louis, MO). Antibodies to ERK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), those to diphosphorylated ERK1/2 were from Sigma–Aldrich, those to acetyl–histone H3 (Lys^9) were from Merck–Millipore (Billerica, MA), those to histone H3 were from Active Motif (Carlsbad, CA), and those to 8-hydroxy-2′-deoxyguanosine (8-OHdG) were from Japan Institute for the Control of Aging (Shizuoka, Japan).

2.2. Animals and tumor cell implantation

The human tumor cell lines HT-29 (colon adenocarcinoma) and H1650 (lung adenocarcinoma), obtained from American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. These tumor cells (2×10^6) were injected subcutaneously into the right flank of 5- to 6-week-old female BALB/c nu/nu mice (CLEA Japan, Tokyo). After the resulting tumors had achieved a size of $\sim$200 mm^3, mice were randomly assigned to balanced groups of five to seven animals. PD184352, AZD6244, and MS-275 were each suspended in an 8/1/1 (v/v/v) mixture of phosphate-buffered sa-line/ethanol/Cremophore EL. Mice were treated every 3 or 4 days (twice per week) with PD184352 (200 mg/kg), AZD6244 (50 mg/kg), or vehicle by oral administration (twice per day with an interval of 6 h) as well as with MS-275 (10–40 mg/kg) or vehicle by oral administration (once per day, 1 h after the first administration of PD184352 or AZD6244). Tumor volume was measured with digital calipers and calculated according to the formula: (longest diameter) $\times$ (shortest diameter)^2/2. Body weight, tumor volume, and toxicities were noted every 2–4 days for the duration of the experiment.

2.3. Immunoblot analysis

Tumor extracts were prepared by mechanical homogenization of excised tumors in a hypotonic cell lysis buffer on ice, fractionated by SDS–polyacrylamide gel electrophoresis, and subjected to immunoblot analysis as described [17]. Immune complexes were detected with an enhanced chemiluminescence system (GE Healthcare Bio-Sciences, Piscataway, NJ).

2.4. Immunohistochemical analysis

HT-29 or H1650 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 consecutively with primary antibodies and horseradish peroxidase-conjugated secondary antibodies and were then stained with 3,3′-diaminobenzidine. The sections were counterstained with hematoxylin and examined with a microscope equipped with Axiovision software (Carl Zeiss, Jena, Germany) [17]. Apoptotic cells were detected with the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay as described [21].

2.5. Statistical analysis

Data are presented as means ± SD. Differences between means were analyzed with the two-tailed Student's *t* test or two-way analysis of variance (ANOVA). A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Blockade of the ERK pathway by a MEK inhibitor enhances induction of apoptosis by MS-275 in tumor xenograft models

Nude mice harboring subcutaneous HT-29 or H1650 tumors ($\sim$200 mm^3), in which the ERK pathway is activated as a result of mutation of B-Raf or EGFR, respectively, were treated orally with PD184352 at a dose of 200 mg/kg [17] or AZD6244 at 50 mg/kg [10], respectively. Immunoblot analysis of tumor extracts revealed that a single dose of these MEK inhibitors almost completely suppressed the phosphorylation of ERK1/2 for 6 h, after which the phosphorylation level gradually returned to control levels by 12 h (Fig. 1A). These results indicated that administration of PD184352 or AZD6244 every 6 h would be required for continuous suppression of ERK1/2 activation in these tumors in nude mice.

For examination of the effects of the HDAC inhibitor MS-275 in tumor xenografts, mice bearing subcutaneous HT-29 tumors were treated orally with MS-275 at a dose of 20 or 40 mg/kg. Whereas the acetylation of histone H3 at Lys^9 was virtually undetectable in untreated tumors, treatment with MS-275 increased the acetylation of histone H3 in a dose-dependent manner and this effect was apparent more than 24 h after drug administration (Fig. 1B).

To examine the potential of AZD6244 to enhance the induction of apoptosis by MS-275 in vivo, we treated mice bearing subcutaneous H1650 tumors ($\sim$200 mm^3) with AZD6244 (50 mg/kg, orally) or vehicle twice, with an interval of 6 h between the two administrations, and with MS-275 (40 mg/kg, orally) at 1 h after the first AZD6244 treatment. Immunoblot analysis of tumor extracts as well as immunostaining of tumor sections with antibodies to phosphorylated ERK1/2 revealed that ERK1/2 phosphorylation was suppressed completely for up to 12 h after the initial AZD6244 administration and that it had returned to control levels by 24–36 h (Fig. 1C). Co-administration of MS-275 did not interfere with the AZD6244-induced inhibition of ERK1/2 phosphorylation, and co-administration of AZD6244 did not interfere with the MS-275-induced increase in the acetylation of histone H3 (Supplementary Fig. 1).

TUNEL staining of tumor sections for cells undergoing apoptotic death revealed that AZD6244 treatment alone did not increase the number of apoptotic cells (Fig. 1D), consistent with results obtained in vitro [18]. MS-275 treatment alone slightly increased the number of TUNEL-positive cells in H1650 xenografts by 24 h, and this effect was markedly enhanced by co-administration of

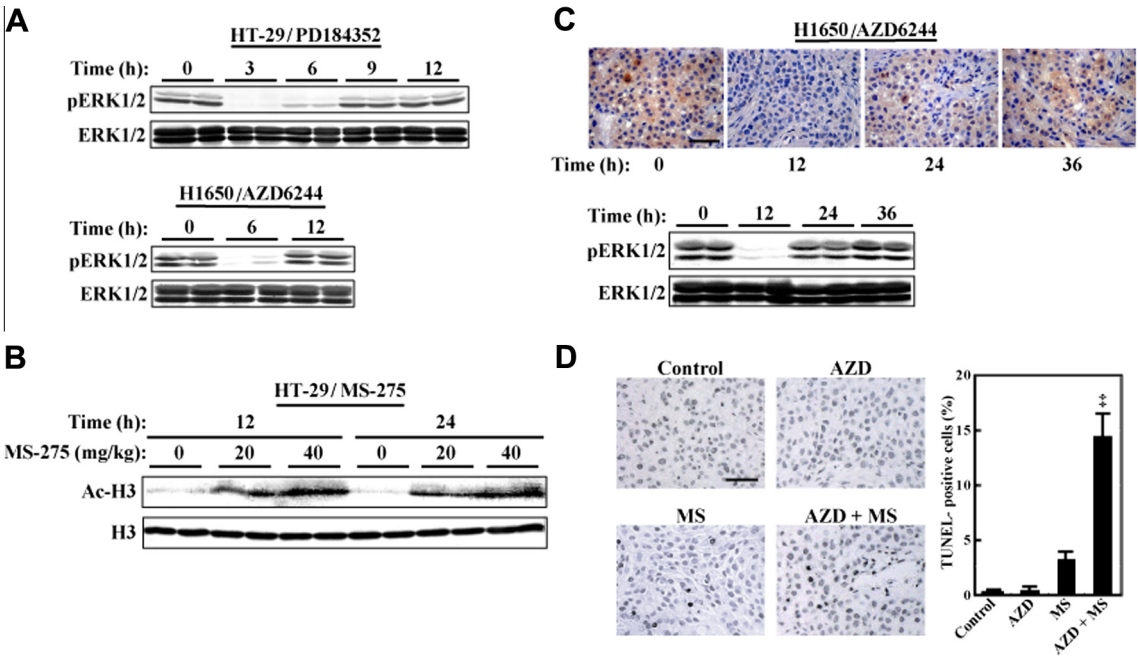

Fig. 1. Potentiation of the apoptosis-inducing effect of an HDAC inhibitor by a MEK inhibitor in tumor xenografts. (A) Mice harboring subcutaneous HT-29 or H1650 tumors (~200 mm^3) were dosed orally with PD184352 (200 mg/kg) or AZD6244 (50 mg/kg), respectively, and the tumors were excised at the indicated times thereafter. Tumor extracts (15 μg of protein) were subjected to immunoblot analysis with antibodies to total or diphosphorylated (p) ERK1/2. (B) Mice bearing HT-29 xenografts were treated with MS-275 (20 or 40 mg/kg), and the tumors were excised at the indicated times thereafter. Tumor extracts (15 μg of protein) were subjected to immunoblot analysis with antibodies to total or acetylated (Ac, Lys^9) histone H3. (C) Mice bearing H1650 xenografts were treated with AZD6244 (50 mg/kg) twice with an interval of 6 h between administrations. Tumors excised 0, 12, 24, or 36 h after the first AZD6244 treatment were subjected to immunohistochemical analysis with antibodies to diphosphorylated ERK1/2 (upper panels; scale bar, 50 μm), or were lysed and subjected to immunoblot analysis (15 μg of protein) with antibodies to total or diphosphorylated ERK1/2 (lower panels). (D) Mice with H1650 xenografts were treated with AZD6244 (50 mg/kg) or vehicle twice, with an interval of 6 h between dosings, as well as with MS-275 (40 mg/kg) or vehicle, administered 1 h after the first AZD6244 administration. Tumors were excised 24 h after the administration of MS-275 and subjected to the TUNEL assay (left panels); scale bar, 50 μm. The percentage of TUNEL-positive cells was determined for five randomly selected microscopic fields (right panel); data are means ± SD from two tumors for each condition, each assayed in triplicate. Two-way ANOVA revealed a significant synergistic interaction between the two drugs ($^{‡}P < 0.01$).

AZD6244. Similar results were obtained with PD184352 and MS-275 in the HT-29 xenograft model (Supplementary Fig. 2).

3.2. PD184352 potentiates the therapeutic efficacy of MS-275 in an HT-29 tumor xenograft model

Mice bearing subcutaneous HT-29 tumors (~200 mm^3) were treated every 3 or 4 days (twice per week) with MS-275 (20 or 40 mg/kg) for a total of six dosings. Under these conditions, MS-275 inhibited the growth of HT-29 xenografts in a dose-dependent manner (Fig. 2A). To evaluate the effect of blockade of the ERK pathway on the therapeutic efficacy of MS-275, we administered PD184352 (200 mg/kg) twice (with an interval of 6 h) on each day that MS-275 was given. Such dosing of the MEK inhibitor alone inhibited the growth of HT-29 xenografts only slightly. However, PD184352 greatly enhanced the therapeutic efficacy of MS-275 (Fig. 2A). Furthermore, hematoxylin–eosin (H&E) staining of tumors revealed that the drug combination induced the disappearance of tumor cells as well as a spongelike appearance of the interstitium associated with residual mucin, characteristics that were much less prominent in tumors treated with either drug alone (Fig. 2B). None of the treated mice showed weight loss or other overt clinical signs of toxicity, including gastrointestinal toxicity.

3.3. AZD6244 potentiates the therapeutic efficacy of MS-275 in an H1650 tumor xenograft model

We next examined whether the combination of AZD6244 and MS-275 would also exhibit enhanced therapeutic efficacy in H1650 tumor xenografts, which manifest resistance to EGFR tyrosine kinase inhibitors [22]. Mice bearing subcutaneous H1650 tumors (~200 mm^3) were dosed every 3 or 4 days (twice per week) with MS-275 (10, 20, or 40 mg/kg), AZD6244 (50 mg/kg, twice with an interval of 6 h), or combinations thereof. Seven such dosings of MS-275 alone inhibited the growth of H1650 xenografts in a dose-dependent manner, whereas treatment with AZD6244 had no such effect (Fig. 3A). Co-administration of AZD6244, however, markedly enhanced the therapeutic efficacy of MS-275 at each dose examined. Indeed, the combination of AZD6244 and MS-275 at 40 mg/kg induced essentially complete suppression of tumor growth. Histopathologic analysis of such treated tumors revealed a marked reduction in cellularity (Fig. 3B). None of the mice treated with the combination of AZD6244 and MS-275 showed substantial signs of drug toxicity such as weight loss or gastrointestinal toxicity.

3.4. The combination of AZD6244 and MS-275 induces oxidative stress in an H1650 xenograft model

The combination of a MEK inhibitor and an HDAC inhibitor synergistically induced the accumulation of ROS in tumor cells in culture, and this increased oxidative stress appeared to contribute to the synergistic induction of tumor cell death [18]. To examine whether such a drug combination might induce similar effects in vivo, we subjected H1650 xenografts isolated from mice treated with AZD6244 (50 mg/kg), MS-275 (20 mg/kg), or the combination thereof for a total of seven cycles to immunostaining with antibodies to 8-OHdG, a product of oxidative DNA damage and a sensitive marker of oxidative stress [23]. The drug combination, but neither AZD6244 nor MS-275 alone, greatly increased the number of

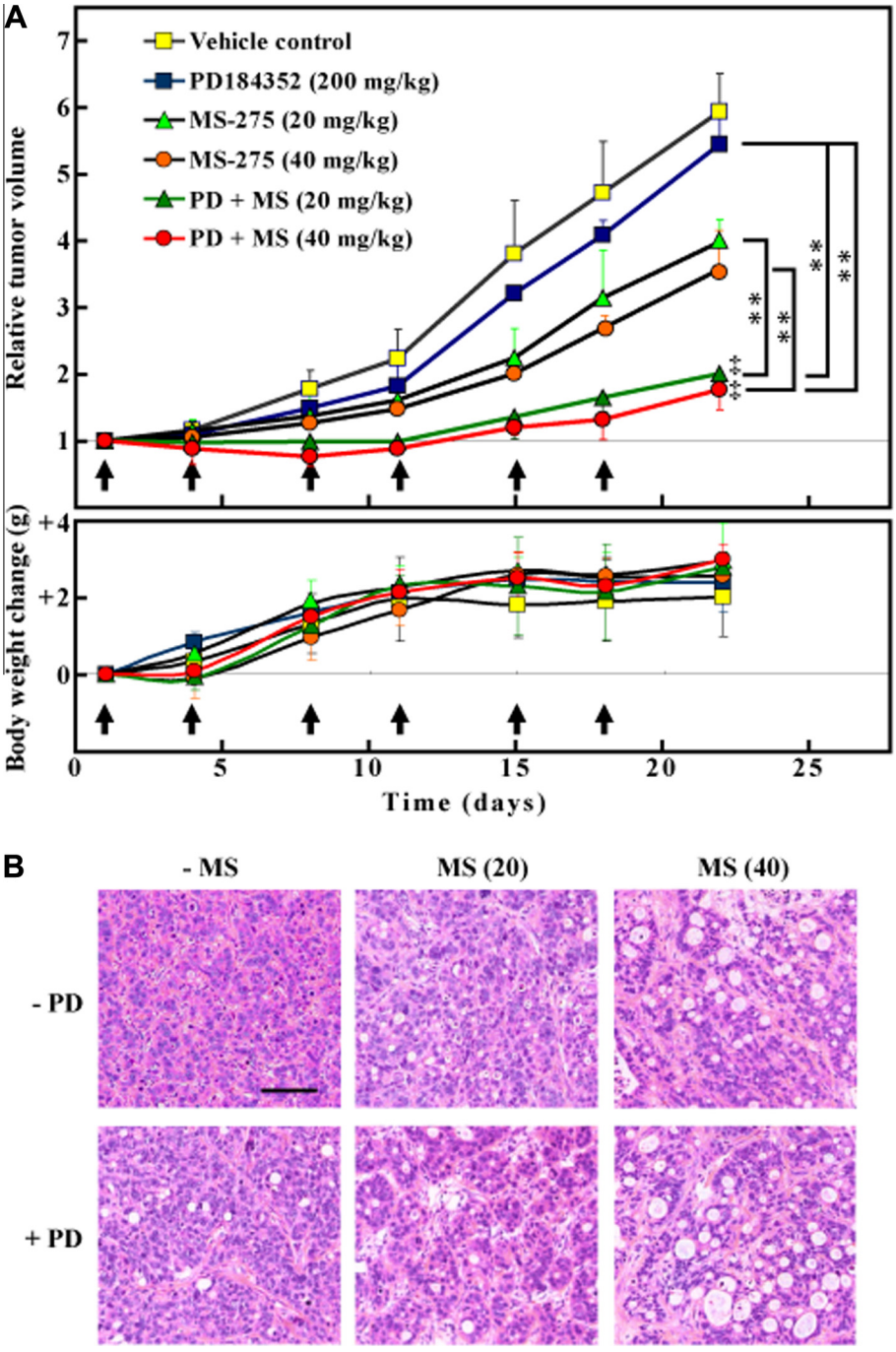

Fig. 2. Potentiation of the therapeutic efficacy of MS-275 by PD184352 in an HT-29 xenograft model. (A) Mice bearing HT-29 xenografts (200 ± 20 mm^3) were treated every 3 or 4 days (arrows) with PD184352 (200 mg/kg, twice with an interval of 6 h) or vehicle as well as with MS-275 (20 or 40 mg/kg, at 1 h after the first dosing of PD184352) or vehicle, as indicated. Tumor volume and body weight were measured every 3 or 4 days. Data are means ± SD from five mice for each condition. Two-way ANOVA revealed a significant synergistic interaction between the two drugs at 22 days ($^{‡}P < 0.01$); $^{**}P < 0.01$ for the indicated comparisons. (B) Sections of the tumors excised at 22 days after the onset of treatment as in (A) were subjected to H&E staining. Scale bar, 50 μm.

tumor cells positive for nuclear staining with antibodies to 8-OHdG (Fig. 4). In contrast, even the drug combination did not induce the appearance of liver parenchymal cells positive for such staining, suggesting that the induction of oxidative stress was specific to the tumor xenografts.

4. Discussion

Blockade of the ERK pathway by a MEK inhibitor markedly sensitized HT-29 and H1650 tumor xenografts to HDAC inhibitor cytotoxicity. Doses of MS-275 that alone showed only moderate cytotoxicity were thus able to markedly suppress the growth of these tumor xenografts when combined with PD184352 or AZD6244, with the inhibitory effects of the drug combinations being synergistic. Moreover, these drug combinations induced a marked loss of tumor cells in the remaining tumor tissue in both xenograft models. These results suggest that the therapeutic efficacy of the drug combinations is even greater than that suggested by their effects on tumor volume. Furthermore, the prominent therapeutic efficacy of the combination of AZD6244 and MS-275 was apparent

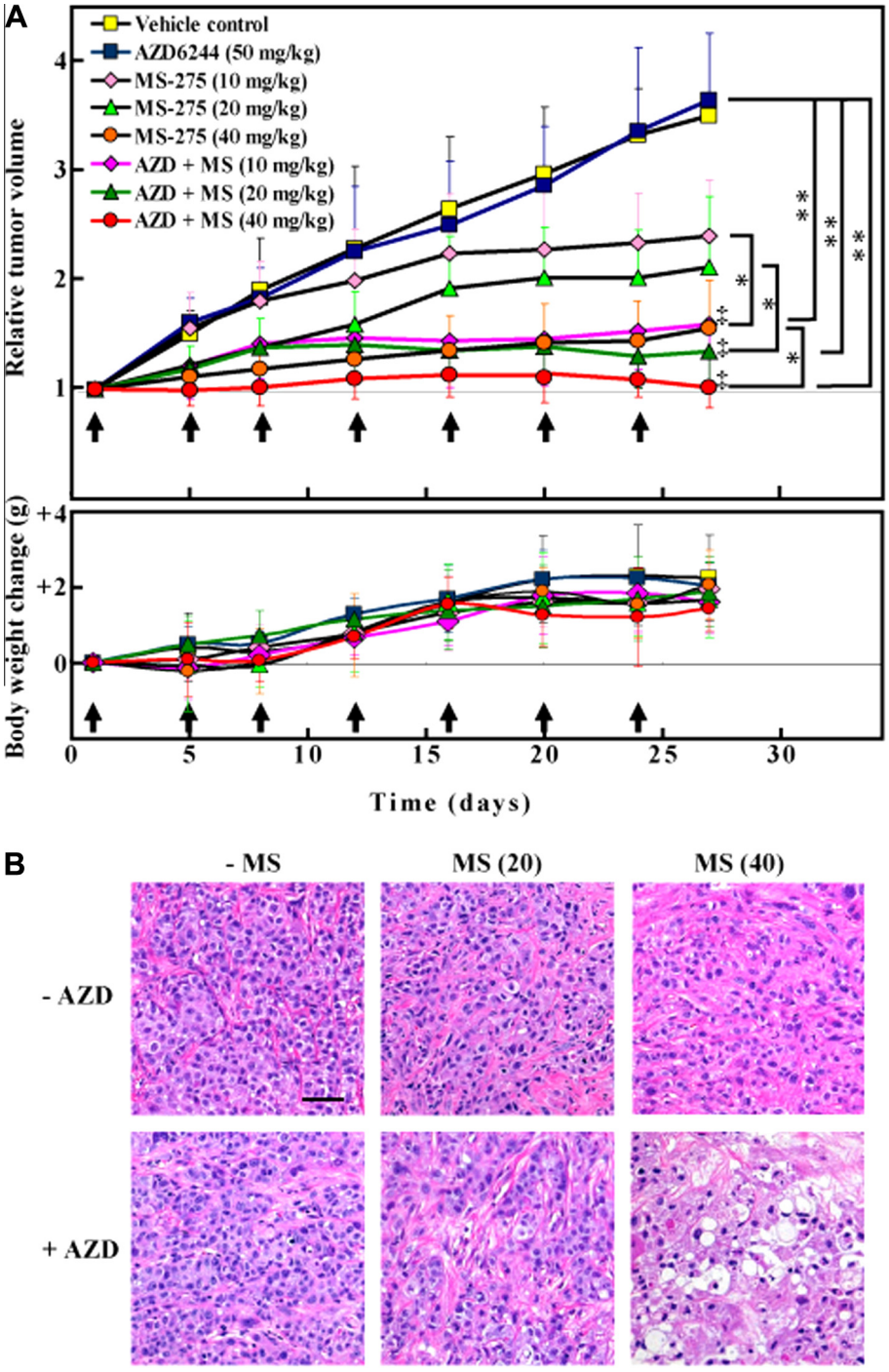

Fig. 3. Potentiation of the therapeutic efficacy of MS-275 by AZD6244 in H1650 xenografts. (A) Mice bearing H1650 xenografts (200 ± 30 mm^3) were treated every 3 or 4 days (arrows) with AZD6244 (50 mg/kg, twice with an interval of 6 h) or vehicle as well as with the indicated doses of MS-275 (at 1 h after the first dosing of AZD6244) or vehicle. Tumor volume and body weight were measured every 3 or 4 days. Data are means ± SD from six mice for each condition. Two-way ANOVA revealed a significant synergistic interaction between the two drugs at 27 days ($^{\ddagger}P < 0.01$); $^{*}P < 0.05$, $^{**}P < 0.01$ for the indicated comparisons. (B) Sections of the tumors excised at 27 days after the onset of treatment as in (A) were subjected to H&E staining. Scale bar, 50 μm.

in the H1650 tumor xenograft model that is resistant to EGFR tyrosine kinase inhibitors. Co-administration of a MEK inhibitor might therefore be expected to contribute to the development of safer anticancer strategies with high therapeutic efficacy by lowering the required dose of a cytotoxic HDAC inhibitor for the treatment of various cancers with constitutive ERK pathway activation.

We found that a relatively transient blockade of the ERK pathway efficiently enhanced the therapeutic efficacy of MS-275. In our xenograft experiments, we aimed to suppress the phosphorylation of ERK1/2 in tumor cells for the initial ~12 h after the administration of MS-275. Mice were thus dosed with PD184352 or AZD6244 only on the day that they received the cytotoxic drug. Given the relative metabolic instability of these MEK inhibitors [24], mice were treated with PD184352 or AZD6244 twice with an interval of 6 h between administrations in order to ensure suppression of ERK1/2 phosphorylation for at least 12 h. Under such conditions, although phosphorylation of ERK1/2 remained totally suppressed for at least 12 h, it had returned to control levels by

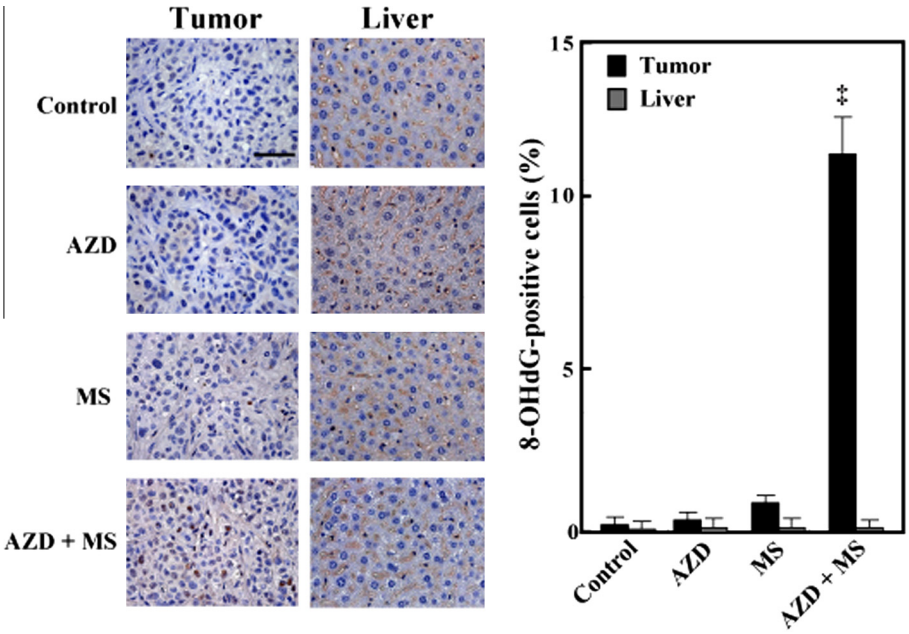

Fig. 4. The combination of AZD6244 and MS-275 induces oxidative stress in H1650 xenografts. Mice harboring H1650 xenografts (200 ± 30 mm^3) were treated with the combination of AZD6244 (50 mg/kg) and MS-275 (20 mg/kg) as in Fig. 3. Sections of tumor or liver tissue excised 24 h after the last drug dosing were subjected to immunohistochemical analysis with antibodies to 8-OHdG (left panels); scale bar, 50 μm. The percentage of 8-OHdG-positive cells was determined for five randomly selected microscopic fields (right panel); data are means ± SD from two mice for each condition, each assayed in triplicate. Two-way ANOVA revealed a significant synergistic interaction between the two drugs ($^{‡}P < 0.01$).

24–36 h. Recent clinical trials of MEK inhibitors in patients with advanced cancers have found 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 [11,12]. However, given the essential role of the ERK pathway in regulation of a wide range of cellular processes including the immune response [25], 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. We recently showed that suppression of ERK1/2 phosphorylation for the initial 24 h of each treatment cycle (once a week) was sufficient to enhance the therapeutic efficacy of microtubule-destabilizing agents such as TZT-1027 and vinorelbine in nude mice harboring HT-29 or HT1080 xenografts [17]. In contrast to our findings, continuous blockade of the ERK pathway by administration of PD184352 or AZD6244 two or three times a day has been shown to be required for enhancement of the therapeutic efficacy of paclitaxel or docetaxel in animal models [15,16].

HDAC inhibitors have emerged as a promising new class of anticancer drug [26]. They reactivate the transcription of tumor suppressor genes, an effect that is thought to contribute to their anticancer activity. Several HDAC inhibitors are in various stages of development, including clinical trials either as monotherapy or in combination with other anticancer drugs. It has been proposed that HDAC inhibitors will require combination with other agents to fulfill their therapeutic potential [27]. Our results indicate that MEK inhibitors are promising candidates for such combination with HDAC inhibitors. The combination of a MEK inhibitor and an HDAC inhibitor induced marked oxidative stress that appeared to result in DNA damage and the eventual induction of massive cell death preferentially in tumor xenografts. The cytotoxicity of HDAC inhibitors has been associated with the generation of ROS [28], whereas the ERK pathway is thought to protect against oxidative stress-induced cell death [29]. The precise mechanism by which the combination of an HDAC inhibitor and a MEK inhibitor induces a synergistic increase in intracellular ROS levels in tumor cells remains to be elucidated.

In summary, we have shown that blockade of the ERK pathway by PD184352 or AZD6244 markedly and synergistically enhanced the therapeutic efficacy of the HDAC inhibitor MS-275 in nude mice bearing HT-29 or H1650 xenografts by sensitizing the tumor cells to the cytotoxicity of the latter drug. Combination of a MEK inhibitor with an HDAC inhibitor may provide a basis for the development of safer anticancer chemotherapies with enhanced efficacy for the treatment of a wide variety of cancers in which the ERK pathway is constitutively activated.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.009.

References

[1] H. Oka, Y. Chatani, R. Hoshino, et al., Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma, Cancer Res. 55 (1995) 4182–4187.
[2] R. Hoshino, Y. Chatani, T. Yamori, et al., Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors, Oncogene 18 (1999) 813–822.
[3] N.E. Hynes, H.A. Lane, ERBB receptors and cancer: the complexity of targeted inhibitors, Nat. Rev. Cancer 5 (2005) 341–354.
[4] J. Downward, Targeting RAS signaling pathways in cancer therapy, Nat. Rev. Cancer 3 (2003) 11–22.
[5] C. Wellbrock, M. Karasarides, R. Marais, The RAF proteins take centre stage, Nat. Rev. Mol. Cell Biol. 5 (2004) 875–885.

[6] M. Kohno, J. Pouyssegur, Targeting the ERK pathway in cancer therapy, Ann. Med. 38 (2006) 200–211.
[7] R. Hoshino, S. Tanimura, K. Watanabe, et al., Blockade of the extracellular signal-regulated kinase pathway induces marked G1 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.
[8] S. Tanimura, K. Asato, S. Fujishiro, et al., 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.
[9] S. Tanimura, A. Uchiyama, K. Watanabe, et al., Blockade of constitutively activated ERK signaling enhances cytotoxicity of microtubule-destabilizing agents in tumor cells, Biochem. Biophys. Res. Commun. 378 (2009) 650–655.
[10] T.C. Yeh, V. Marsh, B.A. Bernat, et al., Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor, Clin. Cancer Res. 13 (2007) 1576–1583.
[11] J. Rinehart, A.A. Adjei, P.M. LoRusso, et al., Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non–small-cell lung, breast, colon, and pancreatic cancer, J. Clin. Oncol. 22 (2004) 4456–4462.
[12] A.A. Adjei, R.B. Cohen, W. Franklin, et al., Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers, J. Clin. Oncol. 26 (2008) 2139–2146.
[13] M. Kohno, S. Tanimura, K. Ozaki, Targeting the extracellular signal-regulated kinase pathway in cancer therapy, Biol. Pharm. Bull. 34 (2011) 1781–1784.
[14] 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.
[15] H.M. McDaid, L. Lopez-Barcons, A. Grossman, et al., Enhancement of the therapeutic efficacy of Taxol by the mitogen-activated protein kinase kinase inhibitor CI-1040 in nude mice bearing human heterotransplants, Cancer Res. 62 (2005) 2854–2860.
[16] N.K. Haass, K. Sproesser, T.K. Nguyen, et al., The mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor AZD6244 (ARRY-142886) induced growth arrest in melanoma cells and tumor regression when combined with docetaxel, Clin. Cancer Res. 14 (2008) 230–239.
[17] K. Watanabe, S. Tanimura, A. Uchiyama, et al., Blockade of the extracellular signal-regulated kinase pathway enhances the therapeutic efficacy of microtubule-destabilizing agents in human tumor xenograft models, Clin. Cancer Res. 16 (2010) 1170–1178.
[18] 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.
[19] K. Ozaki, M. Kosugi, N. Baba, et al., Blockade of the ERK or PI3K-Akt signaling pathway enhances the cytotoxicity of histone deacetylase inhibitors in tumor cells resistant to gefitinib or imatinib, Biochem. Biophys. Res. Commun. 391 (2010) 1610–1615.
[20] T. Suzuki, T. Ando, K. Tsuchiya, et al., Synthesis and histone deacetylase inhibitory activity of new benzamide derivatives, J. Med. Chem. 42 (1999) 3001–3003.
[21] T. Koji, Y. Hishikawa, H. Ando, et al., Expression of Fas and Fas ligand in normal and ischemia-reperfusion testes: involvement of the Fas system in the induction of germ cell apoptosis, Biol. Reprod. 64 (2001) 946–954.
[22] M.L. Sos, M. Koker, B.A. Weir, et al., PTEN loss contributes to erlotinib resistance in EGFR-mutant lung cancer by activation of Akt and EGFR, Cancer Res. 69 (2009) 3256–3261.
[23] S. Toyokuni, Reactive oxygen species-induced molecular damage and its application in pathology, Pathol. Int. 49 (1999) 91–102.
[24] J.S. Sebolt-Leopold, D.H. Dudley, R. Herrera, et al., Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo, Nat. Med. 5 (1999) 810–816.
[25] C. Dong, R.J. Davis, R.A. Flavell, MAP kinases in the immune response, Annu. Rev. Immunol. 20 (2002) 55–72.
[26] S. Minucci, P.G. Pelicci, Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer, Nat. Rev. Cancer 6 (2006) 38–51.
[27] L. Nolan, P.W.M. Johnson, A. Ganesan, et al., Will histone deacetylase inhibitors require combination with other agents to fulfil their therapeutic potential?, Br J. Cancer 99 (2008) 689–694.
[28] R.R. Rosato, J.A. Almenara, S. Grant, The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of $p21^{CIP1/WAF1}$, Cancer Res. 63 (2003) 3637–3645.
[29] X. Wang, J.L. Martindale, Y. Liu, et al., The cellular response to oxidative stress: influences of mitogen-activated protein kinase signaling pathways on cell survival, Biochem. J. 333 (1998) 291–300.