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Enhancement of peripheral benzodiazepine receptor ligand-induced apoptosis and cell cycle arrest of esophageal cancer cells by simultaneous inhibition of MAPK/ERK kinase

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Abstract

Specific ligands of the peripheral benzodiazepine receptor (PBR) activate pro-apoptotic and anti-proliferative signaling pathways. Previously, we found that PBR ligands activated the p38 mitogen-activated protein kinase (MAPK) pathway in esophageal cancer cells, and that the activation of p38MAPK contributed to tumor cell apoptosis and cell cycle arrest. Here, we report that PBR ligands also activate the pro-survival MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway in esophageal cancer cells, which might compromise the efficacy of PBR ligands. Hence, a combination treatment of PBR ligands and MEK inhibitors, which are emerging as promising anticancer agents, was pursued to determine whether this treatment could lead to enhanced apoptosis and cell cycle arrest. Using Western blotting we demonstrated a time- and dose-dependent phosphorylation of ERK1/2 in response to PBR ligands. Apoptosis was investigated by assessment of mitochondrial alterations and caspase-3 activity. Cell cycle arrest was measured by flow cytometric analysis of stained isolated nuclei. The inhibition of MEK/ERK with a pharmacologic inhibitor, 2′-amino-3′-methoxyflavone (PD 98059), resulted in a synergistic enhancement of PBR-ligand-induced growth inhibition, apoptosis and cell cycle arrest. Specifity of the pharmacologic inhibitor was confirmed by the use of 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U 0126), a second MEK/ERK inhibitor, and 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U 0124), a structural analogue of it which does not display any affinity to MEK. Enhanced pro-apoptotic and anti-proliferative effects were observed both in KYSE-140 esophageal squamous cancer and OE-33 adenocarcinoma cells, suggesting that this effect was not cell-type specific. In addition, the PBR-mediated overexpression of the stress response gene (growth arrest and DNA-damage-inducible gene *gadd153*) was synergistically enhanced by MEK inhibition. This is the first report of enhanced PBR-ligand-mediated apoptosis and cell cycle arrest by simultaneous MEK inhibition, suggesting a new anticancer strategy.
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Keywords: Peripheral benzodiazepine receptor; ERK1/2; Apoptosis; Cell cycle; Gene expression; Esophageal cancer

1. Introduction

Adenocarcinoma of the esophagus is a dramatically increasing tumor entity in Western countries. Due to advanced tumor stages at diagnosis and the lack of curative treatment modalities in advanced disease, 5-year survival is below 10% in Western countries [1]. Therefore, innovative approaches are needed for the treatment of esophageal cancer. A promising novel approach is the use of ligands of the peripheral benzodiazepine receptor (PBR) which have been shown to induce both apoptosis and G1/S cell cycle arrest in esophageal cancer cells. Mitochondrial alterations [2] and activation of the p38 mitogen-activated protein kinase (MAPK) pathway [3] are involved in PBR-ligand-induced apoptosis and cell cycle arrest. The target of these drugs, the PBR, was discovered as a binding site for benzodiazepines in peripheral tissues. It is structurally and pharmacologically distinct from the central-type benzodiazepine binding site which is associated with the $GABA_A$ receptor [4].

Abbreviations: BrdU, bromodeoxyuridine; ERK, extracellular signal-regulated kinase; FGIN-1–27, *N*,*N*-di-*n*-hexyl-2-(4-fluorophenyl)indole-3-acetamide; FGIN-1–52, *N*,*N*-di-*n*-octyl-2-phenylindole-3-acetamide; *gadd*, growth arrest and DNA-damage-inducible gene; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PBR, peripheral benzodiazepine receptor; PD 98059, 2′-amino-3′-methoxyflavone; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; RT–PCR, reverse transcriptase–polymerase chain reaction; U 0124, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; U 0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene.

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Drug resistance is one of the major problems of chemotherapy, which causes chemotherapeutic failure leading to progressive disease. Potential mechanisms of resistance include the overexpression of the anti-apoptotic protein bcl-2 [5]. As PBR ligands are known to overcome bcl-2-mediated chemoresistance, they have been successfully applied for combination therapy with several cytotoxic drugs in vitro and in vivo [6–8]. In addition, the epidermal growth factor receptor (EGFR) which is frequently overexpressed in human esophageal cancer [9] is known to confer resistance to treatment with cytotoxic drugs [10]. EGFR signaling usually involves participation of Ras and Raf oncoproteins and activation of MAPK/ERK kinase (MEK), a dual-specificity kinase that phosphorylates extracellular signal-regulated kinase (ERK) [11]. Activation of ERK1/2 via MEK is known to contribute to cell proliferation and survival [12]. A number of pharmacological inhibitors that specifically block the signaling of several members of the ERK1/2 cascade have been identified [13]. In a phase I clinical trial with the MEK inhibitor PD 184352, a patient with pancreatic cancer achieved a partial response lasting more than 6 months, and stable disease was observed in 30% of patients [14,15]. Interestingly, the Ras/Raf/MEK/ERK signal transduction cascade is known to compromise the efficacy of chemotherapy [16]. ERK1/2 may serve as an opposing force to p38MAPK that was shown to mediate PBR-ligand-induced apoptosis and cell cycle arrest in esophageal cancer cells [3]. Thus, the ERK1/2 pathway is a rational molecular target for a combination chemotherapy of esophageal cancer. However, the role of ERK1/2 in PBR-ligand-mediated apoptosis and cell cycle arrest has not yet been studied.

In the present study, we show that PBR ligands induce a MEK-dependent activation of ERK1/2 in esophageal cancer cells. We next tested the effects of PBR ligands combined with specific inhibitors of MEK1/2 kinase on esophageal carcinoma cell proliferation and apoptosis. The combined treatment with PBR ligands plus MEK1/2 inhibitors led to enhanced cell death and cell cycle arrest. These observations indicate that the induction of ERK1/2 compromises the antineoplastic effects of PBR ligands, thus having a protective function. Moreover, these data suggest that the inhibition of ERK signaling coupled with PBR ligand therapy is a promising combination that warrants further evaluation.

2. Material and methods

2.1. Cell lines and drugs

The human esophageal squamous carcinoma cell line KYSE-140 [17] was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The human esophageal adenocarcinoma cell line OE-33 [18] was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cell lines were cultured in a humidified atmosphere (5% CO_2) at 37 °C. To evaluate the effects of PBR-specific ligands and their analogues, cells were incubated with either culture medium without drug, or culture medium containing micromolar concentrations of *N*,*N*-di-*n*-hexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1–27), 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) (Tocris), clonazepam (Sigma), or *N*,*N*-di-*n*-octyl-2-phenylindole-3-acetamide (FGIN-1–52) [19]. 2′-Amino-3′-methoxyflavone (PD 98059, 10–50 μM, Calbiochem) and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U 0126, 1–5 μM, Calbiochem) were used for MEK inhibition. 1,4-Diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U 0124, Calbiochem) is an ineffective analogue of U 0126 and was therefore used for control of specificity. For combination treatment, cells were pretreated with MEK inhibitor for 1 h and subsequently with a combination of PBR ligand and MEK inhibitor for the indicated periods of time.

2.2. Western blotting

1×10^6 cells were seeded in culture medium on 6-well plates for preparation of protein lysates. After 24 h, cells were incubated with the compounds and for the times indicated. Subsequently, whole-cell extracts were prepared by lysing cells with lysis buffer (sodium dodecyl sulfate 0.1%, sodium deoxycholic acid 0.5%, Nonidet P-40 1%, PMSF 0.1 mM, aprotinin 1 μg/ml, Pepstatin A 1 μg/ml). The protein content of the lysate was determined using the BCA protein assay kit (Pierce). Lysates (20 μg protein) were subjected to gel electrophoresis. Proteins were then transferred to PVDF membranes by electroblotting for 2 h. Blots were blocked in 1.5% bovine serum albumin for 1 h, and then incubated at 4 °C overnight with anti-human ERK1/2 (1:500, clone K-23, Santa Cruz Biotechnology), or phospho-ERK1/2 (1:500, clone E-4, Santa Cruz Biotechnology), or β-actin (1:5000, Sigma). After incubation with horseradish peroxidase-coupled anti-IgG antibody (1:10,000, Amersham) at room temperature for 1 h, the blot was developed using enhanced chemiluminescent detection (Amersham). Blots were exposed to Hyperfilm ECL film (Amersham) for 0.5–5 min.

2.3. RNA extraction and semi-quantitative reverse transcriptase–polymerase chain reaction (RT–PCR)

1×10^6 cells were seeded in culture medium on 6-well plates for preparation of RNA. After 24 h, cells were incubated with the compounds and for the times indicated. Subsequently, total RNA was extracted with RNAClean following the recommendation of the manufacturer (Hybaid).

Semi-quantitative analysis of mRNA expression of the genes coding for growth arrest and DNA-damage-inducible gene *gadd45*, *gadd153* and for the housekeeping gene

β-actin was carried out by RT–PCR with the number of cycles at which the band intensity increased linearly with the amount of mRNA used. PCR was performed with the primers and at the conditions previously described [3].

2.4. Determination of proliferation

Cell proliferation was measured by bromodeoxyuridine (BrdU) incorporation during DNA synthesis using the Cell Proliferation ELISA (Roche). In brief, 1×10^4 cells/well were seeded in culture medium on 96-well plates. After 24 h, cells were incubated with the compounds indicated for 24 h in a final volume of 200 μl/well. Subsequently, 20 μl of BrdU-labeling solution was added and the cells were reincubated for 4 h. Then, cells were fixed and denatured with FixDenat solution for 30 min at room temperature. Samples were incubated for 90 min with peroxidase-conjugated anti-BrdU antibody (anti-BrdU-POD). After washing, the substrate solution was added. Light extinction was determined at 450 nm (reference value 630 nm) after 5–10 min using a microplate reader [20].

2.5. Determination of cell number

PBR-ligand-induced growth inhibition of esophageal cancer cells was evaluated by crystal violet staining, as described [21]. In brief, 1×10^3 cells/well were seeded in culture medium on 96-well plates. After 72 h, cells were incubated with the compounds indicated for 0–96 h in a final volume of 200 μl/well. Cells were fixed with 1% glutaraldehyde. Then, cells were stained with 0.1% crystal violet. The unbound dye was removed by washing. Crystal violet which had absorbed onto the cells was solubilized with 0.2% Triton X-100. Light extinction was analyzed at 570 nm using an ELISA-Reader.

2.6. Cell cycle analysis

5×10^5 cells/well were seeded in culture medium on 12-well plates and treated after 24 h for further 24 h for cell cycle analysis. Cell cycle analysis was performed by the method of Vindelov and Christensen [22]. Cells were trypsinized, washed, and the nuclei were isolated using CycleTest PLUS DNA Reagent Kit (Becton Dickinson). DNA was stained with propidium iodide according to the manufacturers' instructions. The DNA content of the nuclei was detected by flow cytometry and analyzed using CellFit software (Becton Dickinson).

2.7. Detection of apoptosis

5×10^5 cells/well were seeded in culture medium on 12-well plates for the assessment of mitochondrial alterations and caspase-3 activation and were then treated after 24 h for the indicated times. Changes in mitochondrial membrane potential ($\Delta\Psi_M$) and mitochondrial mass were assessed as described [23]. Cells were stained using the fluorogenic lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1, 1 μg/ml, Molecular Probes) for 15 min at 37 °C in the dark, prior to analysis by flow cytometry [24].

Caspase-3 activity was assessed as described [2]. 1×10^6 cells were lysed with lysis buffer (Tris–HCl 10 mM, NaH_2PO_4/Na_2HPO_4 10 mM, NaCl 130 mM, Triton X-100 1%, $NaPP_i$ 10 mM, pH 7.5), and total protein content was quantified using the BCA protein assay kit (Pierce). The activity of caspase-3 was calculated from the cleavage of the fluorogenic substrate DEVD-AMC (Calbiochem-Novabiochem).

2.8. Statistical analysis

If not stated otherwise, means of four independent experiments ±S.E.M. are shown. Individual drug therapy was compared by the unpaired, two-tailed Mann–Whitney *U*-test. *P* values were considered to be significant at <0.05.

3. Results

3.1. Activation of ERK1/2 by PBR ligands

Phosphorylation-mediated activation of ERK1/2 by PBR-specific ligands was determined by Western blotting (Fig. 1). Both PBR-specific ligands FGIN-1–27 and PK 11195 induced a time- and dose-dependent phosphorylation of ERK1/2. The kinetics and extents of induction, however, differed for the two cell lines: ERK1/2 activation by FGIN-1–27 occurred 5 min (KYSE-140) or 1 h (OE-33) after treatment already. In contrast, PK 11195-induced ERK1/2 activation was maximal after 1 h (KYSE-140) or 4 h (OE-33) of treatment. The response to FGIN-1–27 was stronger in KYSE-140 than in OE-33 cells, whereas PK 11195 elicited a pronounced ERK1/2 activation both in KYSE-140 and OE-33 cells. Longer exposure (4–8 h) to PBR-specific ligands resulted in a down-regulation of active ERK1/2. ERK1/2 expression was unaltered except for OE-33 cells treated with PK 11195. Despite the benzodiazepine structure of clonazepam or the indoleacetamide structure of FGIN-1–52 being similar to FGIN-1–27, respectively, both agents display almost no affinity to PBR [19,25]. Surprisingly, both clonazepam and FGIN-1–52 induced a transient ERK1/2 activation (Fig. 2), indicating that ERK1/2 activation was not PBR-mediated.

3.2. Inhibition of PBR-ligand-induced ERK1/2 activation by MEK inhibitors

For combination experiments with MEK inhibitors, we chose a concentration of 50 μM FGIN-1–27 or PK 11195, being sufficient to induce ERK1/2 activation (Fig. 1),

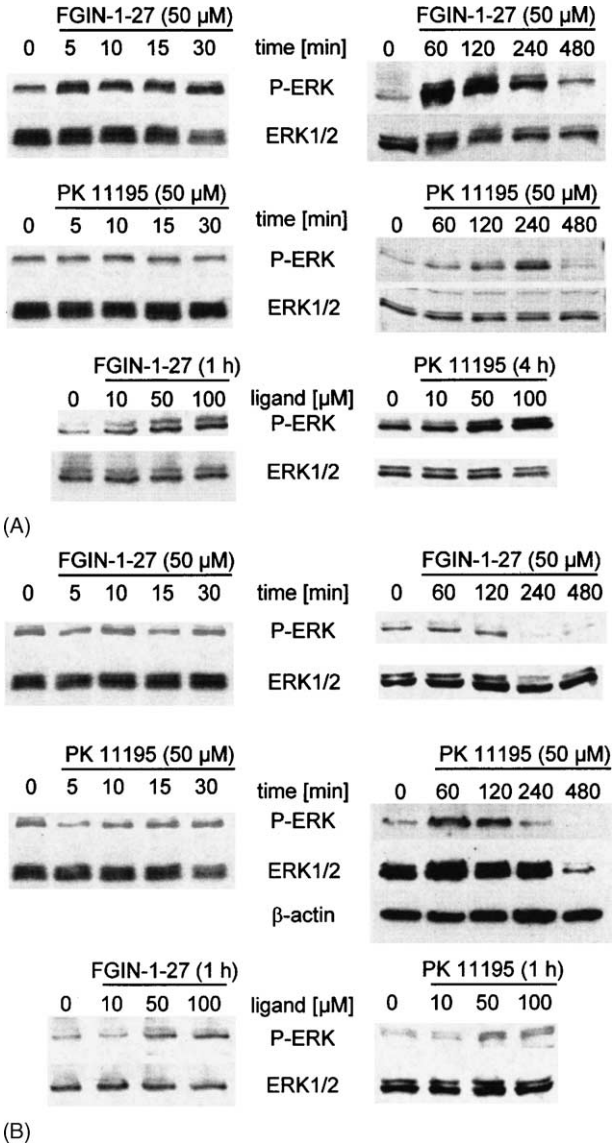

Fig. 1. Effects of PBR ligands on ERK1/2 activity. Phosphorylation of ERK1/2 was analyzed by Western blotting using antibodies against the active form, P-ERK1/2, and total ERK1/2. The PBR ligands FGIN-1–27 or PK 11195 induced a time- and dose-dependent activation of ERK1/2 in human esophageal squamous, KYSE-140, (A) and adenocarcinoma, OE-33, (B) cell lines. Dose-dependency of PBR-ligand-mediated ERK1/2 phosphorylation was analyzed at the respective time points, at which a maximal ERK1/2 activation was observed.

apoptosis and cell cycle arrest [2,26,27]. The effects of exposing human esophageal cancer cells to both PBR ligands and MEK inhibitors were first examined in relation to ERK1/2 activation. PD 98059 [28], and U 0126, whose affinity to MEK is significantly greater than that of PD 98059 [29], were used for MEK inhibition. Cells were pretreated with MEK inhibitors for 1 h, and subsequently FGIN-1–27 (1 h) or PK 11195 (KYSE-140, 4 h; OE-33, 1 h) were added. The MEK inhibitors PD 98059 or U 0126 dose-dependently blocked PBR-ligand-induced ERK1/2 activation (Fig. 3). In contrast, U 0124 (5 μM), a structural analogue of U 0126 which displays no affinity to MEK, did

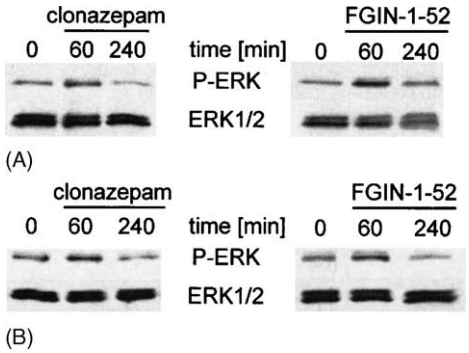

Fig. 2. ERK1/2 activation by structure analogues of PBR ligands. Phosphorylation of ERK1/2 was analyzed by Western blotting using antibodies against the active form, P-ERK1/2, and total ERK1/2. 50 μM clonazepam and FGIN-1–52, neither of which displaying any affinity to PBR, induced a transient activation of ERK1/2 in human esophageal squamous, KYSE-140, (A) and adenocarcinoma, OE-33, (B) cell lines.

not inhibit PBR-ligand-induced ERK1/2 phosphorylation (data not shown). These results demonstrate that two structurally divergent MEK inhibitors are capable of substantially inhibiting PBR-ligand-induced ERK1/2 activation due to their affinity to MEK, conferring specificity of inhibition. For functional experiments, MEK inhibitors were then applied at concentrations that potently inhibited ERK1/2 activation (PD 98059: 10–50 μM; U 0126: 1–5 μM).

3.3. Enhancement of PBR-ligand-mediated gadd153 induction by MEK inhibition

We recently demonstrated that PBR-specific ligands induced a p38MAPK-dependent overexpression of the *gadd45* and *gadd153* genes during apoptosis and cell cycle arrest of esophageal cancer cells [3]. We now determined whether PBR-ligand-induced ERK1/2 activation was directly associated with the induction of *gadd45* and *gadd153*. Preincubation of KYSE-140 cells with PD 98059 (50 μM) for 1 h synergistically enhanced the FGIN-1–27- or PK 11195-mediated induction of *gadd153* transcripts (Fig. 4). PD 98059 alone had no effect on *gadd153* expression. In contrast, the expression of *gadd45* was induced by PD 98059 alone, whereas the combination with PBR ligands did not significantly lead to a further increase. These data suggest that ERK1/2 activation negatively regulates the induction of *gadd153* by PBR-specific ligands.

3.4. Enhanced sensitivity of esophageal cancer cells to PBR ligands by MEK inhibition

BrdU incorporation of esophageal cancer cells exposed to either PBR ligand or MEK inhibitor alone or in combination was monitored to determine what impact the inhibition of PBR-ligand-induced ERK1/2 activation had on cell proliferation. Pretreatment of KYSE-140 or OE-33 cells

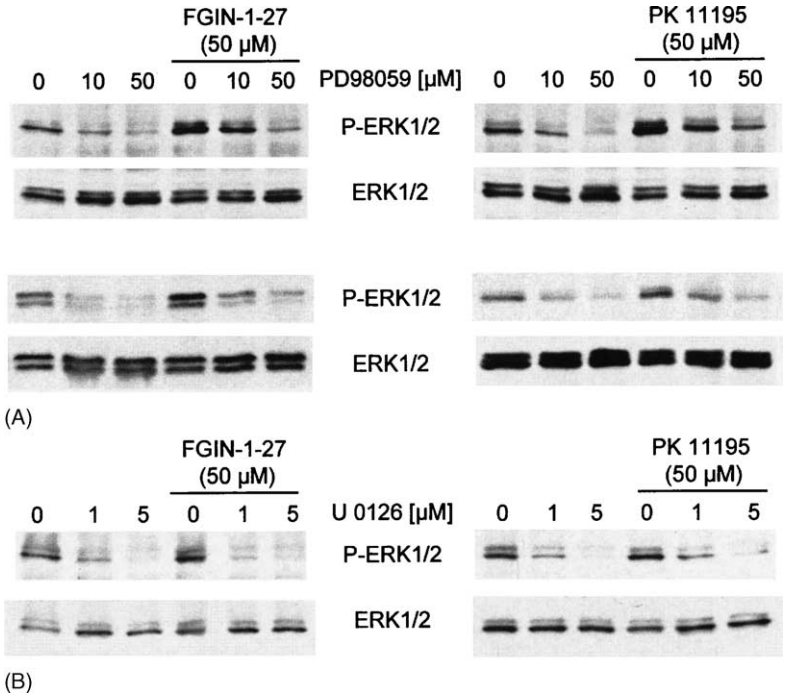

Fig. 3. Effects of MEK inhibition on PBR-ligand-mediated ERK1/2 activation. Esophageal cancer cells were treated with either vehicle, MEK inhibitor alone, FGIN-1–27 (1 h), PK 11195 (KYSE-140: 4 h; OE-33: 1 h), or pretreated with MEK inhibitor for 1 h and subsequently treated with FGIN-1–27 (1 h) or PK 11195 (KYSE-140: 4 h; OE-33: 1 h) in combination with a MEK inhibitor. (A) PBR-ligand-induced ERK1/2 activation was antagonized by the MEK inhibitor PD 98059 in KYSE-140 (upper panel) and OE-33 cells (lower panel). (B) U 0126 similarly prevented ERK1/2 phosphorylation in KYSE-140 cells.

with PD 98059 (50 μM) 1 h prior to application of PBR ligands resulted in an enhanced growth inhibition after 24 h of treatment compared to treatment with PBR ligands alone (Fig. 5A). Synergistic effects with PBR ligands were observed only at concentrations above 10 μM PD 98059, reflecting the ligand concentrations needed for ERK1/2 activation (see Fig. 1). Long-term combination treatment (96 h) strikingly decreased the number of KYSE-140 and OE-33 esophageal cancer cells as assessed by crystal violet staining (Fig. 5B). Similar to BrdU incorporation, a significant potentiation occurred only above 10 μM PD 98059, suggesting that a distinct MEK inhibition is required for the synergistic effects (see Fig. 3).

3.5. Modulation of PBR-ligand-induced cell cycle arrest by MEK inhibition

To test whether an alteration of cell cycle regulation contributed to the synergistic anti-proliferative effects of PBR ligands and MEK inhibitors, we performed cell cycle analyses. Administration of MEK inhibitors by themselves had no significant effect on the cell cycle of KYSE-140 cells, whereas PBR ligands significantly increased the G0/G1 population. Pretreatment of KYSE-140 cells with the MEK inhibitors PD 98059 (50 μM, Fig. 6A, left panel) or U 0126 (5 μM, Fig. 6B) for 1 h and subsequent addition of PBR ligands for further 24 h caused a significant and synergistic enhancement of cell cycle arrest, suggesting that ERK1/2 activation attenuates the PBR-ligand-induced G1/S arrest. Conversely, U 0124 did not have any impact

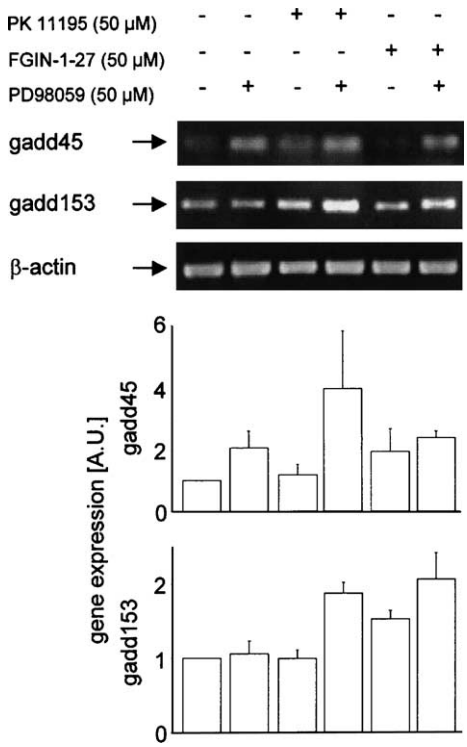

Fig. 4. Enhanced *gadd* gene expression by combination treatment with PBR ligands and PD 98059. mRNA expression of *gadd45* and *gadd153* in KYSE-140 cells was assessed using semi-quantitative RT–PCR. Cells were treated with PBR ligands for 4 h either alone or in combination with PD 98059. Pretreatment with PD 98059 synergistically enhanced *gadd153* but not *gadd45* induction in response to PBR ligands. The upper panel shows a representative result, whereas in the lower panel data are given as the mean relative gene expression ±S.E.M. out of three independent experiments.

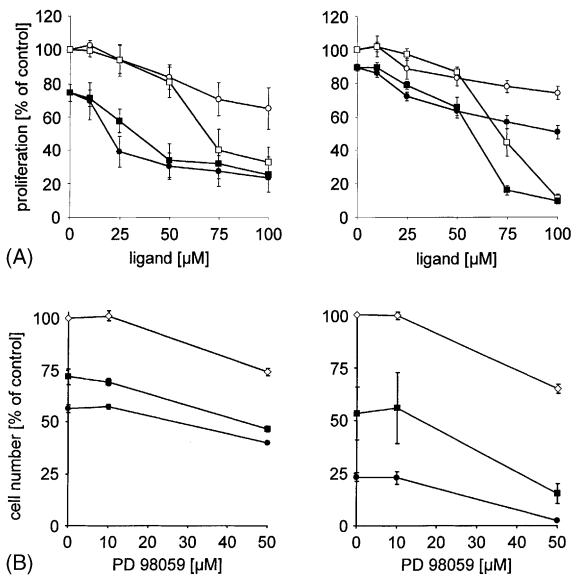

Fig. 5. Effects of PBR ligands and MEK inhibitors on proliferation in esophageal cancer cells. (A) The influence of a treatment with PD 98059 (50 μM) on PBR-ligand-induced growth inhibition of KYSE-140 (left panel) and OE-33 cells (right panel) was investigated using the BrdU assay. Cells were treated with PBR ligands for 24 h either alone (FGIN-1–27 (○); PK 11195 (□)) or in combination with PD 98059 (FGIN-1–27 (●); PK 11195 (■)). (B) The long-term effects of combination treatment were investigated by crystal violet staining. KYSE-140 (left panel) and OE-33 cells (right panel) were treated for 96 h with 0–50 μM PD 98059 alone (◇) or in combination with PBR ligands (50 μM FGIN-1-27 (●); 50 μM PK 11195 (■)). Both short- and long-term combination treatment with PD 98059 caused a dramatic enhancement of PBR-ligand-induced growth inhibition. Data are given as percentage of untreated control (means ± S.E.M. of four independent experiments).

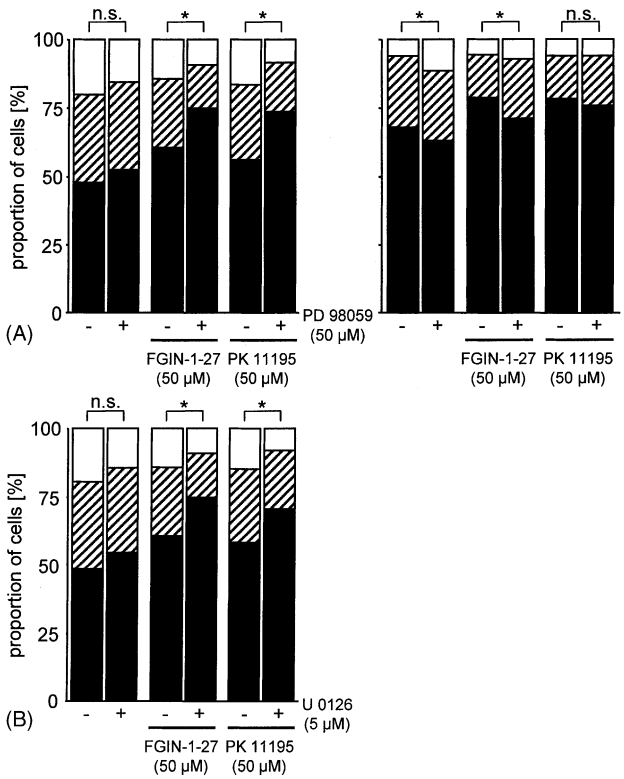

Fig. 6. Effects of PBR ligands and MEK inhibitors on cell cycle regulation in esophageal cancer cells. The influence of the MEK inhibitors PD 98059 (50 μM) (A) or U 0126 (5 μM) (B) on PBR-ligand-mediated cell cycle arrest was determined by flow cytometry. KYSE-140 (A, left panel; B) and OE-33 cells (A, right panel) were treated either with PBR ligands alone for 24 h (−), or with MEK inhibitor for 1 h followed by PBR ligand plus MEK inhibitor for 24 h (+). Treatment with PBR ligands increased the proportion of cells in the G0/G1 phase (black columns), whereas the proportion of cells in the S (hatched columns) and G2/M phase (white columns) decreased. In KYSE-140 cells, MEK inhibition alone induced a slight albeit not significant ($P > 0.05$) G1/S arrest. The addition of a MEK inhibitor caused an enhancement of PBR-ligand-mediated G1/S arrest of the cell cycle. In contrast, treatment with PD 98059 alone caused a G2/M arrest in OE-33 cells. Thus, no synergistic effects on the cell cycle of OE-33 cells were observed. Means of four to six independent experiments are shown.

on PBR-ligand-mediated cell cycle arrest (data not shown). In OE-33 cells, however, PD 98059 significantly increased the G2/M population, whereas PBR ligands induced a G1/S arrest. As PBR ligands and PD 98059 had opposing effects on the cell cycle of OE-33 cells, no synergistic effects on the cell cycle were observed. In contrast, FGIN-1–27-induced G1/S arrest was attenuated, whereas PK 11195-induced G1/S arrest was not significantly affected by coadministration of PD 98059 (Fig. 6A, right panel).

3.6. Mitochondria-independent enhancement of PBR-ligand-induced apoptosis by MEK inhibition

PBR-ligand-mediated apoptosis involves mitochondrial alterations, caspase-3 activation and p38MAPK activation, leading to DNA fragmentation and cell death [2,3]. We now investigated whether the induction of apoptosis by PBR ligands was also affected by ERK1/2 activation.

First, the influence of ERK1/2 activation on PBR-ligand-induced mitochondrial alterations was studied by preincubation of KYSE-140 cells with PD 98059 (50 μM) for 1 h and subsequent treatment with FGIN-1–27 for 6 h. Neither FGIN-1–27-induced $\Delta\Psi_M$ disruption nor mitochondrial swelling were significantly affected by MEK inhibition (Fig. 7A). Conversingly, combination treatment with PD 98059 (50 μM) significantly potentiated PBR-ligand-induced caspase-3 activation in KYSE-140 and OE-33 cells after 8 h (Fig. 7B). Synergistic induction of caspase-3 activity was also achieved by U 0126 (5 μM, Fig. 7C), but not U 0124 (data not shown), indicating MEK specifity of apoptosis induction. These results suggest that inhibition of MEK potentiates PBR-ligand-mediated apoptosis by elevating caspase-3 activity independently of pro-apoptotic mitochondrial alterations. Furthermore, ERK1/2 activation compromises PBR-ligand-mediated apoptosis.

4. Discussion

In this study, we demonstrate a novel approach leading to the enhancement of PBR-mediated anti-proliferative

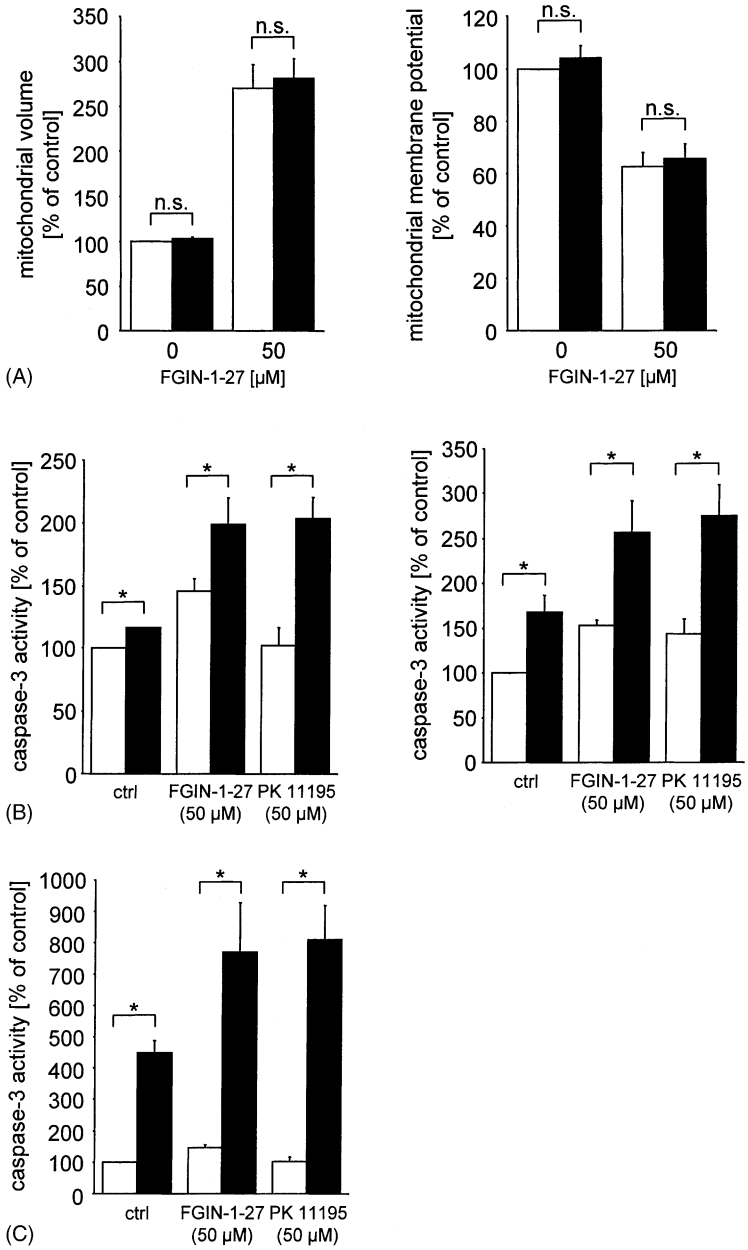

Fig. 7. Enhancement of PBR-ligand-mediated apoptosis by MEK inhibition. MEK inhibition enhances PBR-ligand-mediated caspase-3 activation independently of mitochondrial alterations. Esophageal cancer cells incubated with 50 μM PBR ligand alone (white columns) or in combination with 50 μM PD 98059 (black columns) were analyzed for mitochondrial volume (A, left panel) and mitochondrial membrane potential (A, right panel) after 6 h, and for caspase-3 activity (B,C) after 8 h. Neither FGIN-1–27-induced mitochondrial swelling nor $\Delta\Psi_M$ disruption were affected by PD 98059 in KYSE-140 cells (A). In contrast, PBR ligand-mediated caspase-3 activation was enhanced by combination treatment with PD 98059 in KYSE-140 (B, left panel) and OE-33 cells (B, right panel). Synergistic activation of caspase-3 in KYSE-140 cells was also induced by PBR ligands with 5 μM U 0126, confirming MEK specifity (C). Data are given as percentage of untreated control (means of five independent experiments ±S.E.M.). $^*P < 0.05$, n.s.: not significant.

and pro-apoptotic effects. Moreover, we provide further insight into the signal transduction pathways by which PBR-specific ligands induce cell cycle arrest and apoptosis in esophageal cancer cells. PBR-specific ligands activate the ERK1/2 signaling pathway. Inhibition of this pro-survival pathway leads to synergistic inhibition of proliferation and decrease of cell number, suggesting ERK1/2 to be a negative regulator of PBR ligands' antineoplastic action. The sensitizing effect of the inhibition of the ERK1/2 pathway was mediated by enhancement of both apoptosis and G1/S cell cycle arrest and was found to be associated with enhanced overexpression of *gadd153*.

In our previous report on PBR-mediated signal transduction in esophageal cancer cells, we showed the promoting effect of the p38MAPK pathway on PBR-ligand-mediated apoptosis and cell cycle arrest; the pharmacological inhibition of the p38MAPK pathway resulted in partial protection of the cells from PBR ligands [3]. We now demonstrate that after 1–4 h of incubation PBR ligands activate the ERK1/2 pathway which is known to

promote survival and proliferation. The anti-proliferative effects of PBR ligands were synergistically enhanced by inhibition of the ERK1/2 pathway, qualifying this strategy to be used for the treatment of esophageal cancer. Longer exposure (4–8 h) to PBR-specific ligands resulted in a down-regulation of the level of active ERK1/2 (Fig. 1), whereas p38MAPK remained fully active [3]. The activation of the p38MAPK pathway and the concomitant decrease in the level of active ERK1/2 coincided with a decrease in survival and proliferative capacity of the cells, suggesting that an equilibrium exists between the two pathways in esophageal cancer cells that can be affected by PBR ligands. In agreement with these findings, PK 11195-induced caspase-3 activity did not occur before 16 h of treatment in KYSE-140 cells [2] but as early as after 8 h if cells were treated in combination with MEK inhibitors. The opposing role of MAPKs in apoptosis has been described in breast cancer and other cells [12]. The possibility of the alternative use of these two pathways is further supported by the experiments with specific inhibitors acting on the MAPK pathways. Inhibition of the ERK1/2 pathway by PD 98059 or U 0126 increased the anti-neoplastic effects of PBR ligands, thus confirming that this pathway is essential for the proliferation and survival of esophageal cancer cells. Despite the temporal relationship and contrary to the findings of Ding and Adrian [30], PBR-ligand-induced p38MAPK activation was not further augmented by MEK inhibition in esophageal cancer cells (data not shown). Thus, a direct cross-talk between the ERK1/2 and p38MAPK pathways is unlikely to account for the synergistic effects observed.

To further characterize the anti-proliferative effects observed, we analyzed the impact of MEK inhibition on PBR-ligand-induced apoptosis. KYSE-140 and OE-33 cells showed a substantial increase of the apoptotic response, i.e. caspase-3 activity when treated with PBR ligands in combination with PD 98059 or U 0126. However, enhancement of caspase-3 activation occurred independently of mitochondrial alterations, as coadministration of PD 98059 with FGIN-1–27 failed to potentiate the loss of $\Delta\Psi_M$ nor the increase of mitochondrial volume. Mitochondrial alterations are known to be essential for FGIN-1–27-induced apoptosis [2]. The fact that ERK1/2 activation occurs independently both of PBR and mitochondrial alterations supports the hypothesis that activation of the MEK/ERK pathway is not related to PBR-mediated apoptosis induction and points to mitochondria being the central organelles of PBR-induced cell death. ERK1/2-mediated attenuation of PBR-induced cell death may be achieved by the modulation of the activity and/or expression of caspase inhibitors [31,32].

Furthermore, ERK1/2 activation also proved to negatively regulate PBR-ligand-mediated cell cycle arrest of KYSE-140 cells, counteracting the p38MAPK pathway. Hence, we demonstrate that PBR-ligand-induced ERK1/2 activation is involved both in the regulation of apoptosis and proliferation. ERK1/2 is known to regulate the cell cycle [33–35]. The inhibition of cell cycle promoting events by MEK inhibitors may hold responsible for the enhancement of PBR-ligand-induced cell cycle arrest of KYSE-140 cells. The lack of synergistic effects on the cell cycle of OE-33 cells may be due to opposing effects of MEK inhibitors and PBR ligands on the cell cycle of OE-33 cells. The fact that MEK inhibition causes both a G1/S, like in KYSE-140, and a G2/M arrest, like in OE-33 cells, is well known [36].

The effects of two chemically divergent MEK inhibitors, PD 98059 and U 0126, were studied in order to analyze the role of ERK1/2 in the response to PBR ligands. ERK1/2 is phosphorylated by MEK and thus represents a direct measure of MEK inhibition. U 0126 effectively inhibited ERK1/2 activation at lower concentrations (one tenth) than those of PD 98059 due to its higher affinity to MEK [29]. We demonstrated both the inhibition of ERK1/2 activation and the enhanced sensitivity to PBR ligands using two chemically distinct inhibitors. Thus, we provide evidence that the enhancement of apoptosis and cell cycle arrest was due to MEK inhibition or the following downstream effects. Moreover, U 0124, a structural analogue of the MEK inhibitor U 0126 but without any affinity to MEK, did not affect ERK1/2 phosphorylation, nor apoptosis, nor cell cycle arrest induced by PBR ligands (data not shown). ERK1/2 activation has generally been associated with cytoprotective and mitogenic functions [37]. However, it is important to note that in esophageal cancer cells disruption of this pathway did not inhibit growth by itself but did so when combined with PBR ligands.

As the synergism between PBR ligands and MEK inhibitors was observed in two histologically distinct esophageal cancer cell lines, these effects appear not to be limited to a particular cell type. However, the kinetics and extents of ERK1/2 activation by FGIN-1–27 or PK 11195 differed for the two esophageal cancer cell lines. Differences between the effects of these two drugs in a specific cell line may be related to uptake kinetics of these chemically divergent compounds or to differences in the signaling pathways leading to ERK1/2 activation. To investigate the role of PBR in PBR-ligand-induced ERK1/2 activation, we applied the control compounds FGIN-1–52 and clonazepam. Both clonazepam and FGIN-1–52 induced ERK1/2 phosphorylation, indicating that the activation of the ERK1/2 signaling pathway was not PBR specific. In contrast to the mitogenic and anti-apoptotic properties of PBR ligands described in this article, their pro-apoptotic and anti-mitotic effects were clearly shown to be PBR-mediated, as neither PBR-ligand-induced p38MAPK phosphorylation, nor *gadd* expression [3], nor apoptosis, nor cell cycle regulation [2] were modulated by control substances [26]. The fact that only pro-apoptotic and anti-proliferative effects of PBR ligands are receptor-mediated supports our hypothesis that PBR ligands, by interacting with PBR, antagonize the anti-

apoptotic and mitogenic functions ascribed to PBR [38,39]. Nevertheless, inhibiting these unspecific mitogenic effects of PBR ligands is a feasible approach to enhance the antineoplastic efficacy of PBR ligands.

Differences between the responses of the two esophageal cancer cell lines to the same drug, however, may be related to their histological origin, which may also explain differences in the kinetics of apoptosis induction: FGIN-1–27 elicited a fast (8 h) caspase-3 activation in both cell lines, whereas the response to PK 11195 treatment was faster in OE-33 cells (8 h) than in KYSE-140 cells (24 h, [2]). The lack of synergistic effects of combination treatments on the cell cycle of OE-33 cells reflects the lower anti-proliferative synergistic effects on OE-33 cells after 24 h of treatment. Cell-type specifity is also reflected in different transcriptional responses to FGIN-1–27 in KYSE-140 and OE-33 cells [3]. In spite of these cell-type-specific differences in PBR-ligand-induced signaling, the overall functional and molecular effects of FGIN-1–27 and PK 11195 nevertheless show high similarity in both cell lines tested, suggesting that ERK1/2 activation by FGIN-1–27 and PK 11195 could arise from a common mechanism.

MAPK signal transduction pathways are known to regulate the expression of *gadd* genes [40,41]. We have recently shown that PBR ligands induced the p38MAPK-dependent overexpression of *gadd45* and *gadd153* [3]. Our next goal was to elucidate if PBR-ligand-mediated ERK1/2 activation was involved in *gadd* overexpression. In this study, inhibition of ERK1/2 phosphorylation by PD 98059 synergistically enhanced the expression of *gadd153*. Thus, it is intriguing to speculate that *gadd153* plays a major role in PBR-ligand-induced apoptosis and cell cycle arrest based on the following results: (a) PBR-ligand-induced p38MAPK activation leads to *gadd153* overexpression, apoptosis and cell cycle arrest [3] and (b) PBR-ligand-mediated ERK1/2 activation leads to *gadd153* suppression and attenuation of apoptosis and cell cycle arrest (Fig. 4). In addition to our findings, *gadd153* has been reported to be positively regulated by p38MAPK and negatively by ERK1/2 [41]. In contrast, *gadd45* expression was strongly promoted by the MEK inhibitor alone but was not significantly potentiated by combination treatment with PBR ligands. Based on our observations, we suggest that *gadd153* is a more useful surrogate biomarker for future PBR-ligand intervention trials than *gadd45*. Moreover, the enhancement of PBR-ligand-induced *gadd153* overexpression by MEK inhibition may contribute to the increase of cell cycle arrest in KYSE-140 cells, as *gadd* overexpression is known to lead to G1/S arrest [42].

Recently, the MEK inhibitor PD 184352 has been evaluated for the treatment of cancer with promising results [14,15]. However, findings demonstrating enhanced sensitivity to apoptotic signals upon interruption of the ERK pathway have been described [43]. Therefore, it is likely that the design of future clinical trials with MEK inhibitors will attempt to boost therapeutic kill by employing combination regimens [44]. By being considerably less toxic than conventional chemotherapeutic agents, these new drugs may be of special value to esophageal cancer patients, who are commonly in poor health condition. In this context, the dramatic anti-proliferative effect of a 4-day treatment with PBR ligand plus PD 98059 emphasizes the potential clinical relevance of this combinatory approach.

In summary, in this report we demonstrate the induction of ERK1/2 by specific PBR ligands in esophageal cancer cells. Inhibition of this pathway potentiates the apoptotic and cell cycle arresting effects of PBR ligands. This illustrates thereby the power of molecular and rational drug targeting. Hence, enlarging our understanding of the cellular responses to PBR ligands is critical for developing novel potent combination therapies.

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