Peripheral Benzodiazepine Receptor Ligands Reverse Apoptosis Resistance of Cancer Cells in Vitro and in Vivo

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ABSTRACT

The mitochondrial peripheral benzodiazepine receptor (mPBR) is involved in a functional structure designated as the permeability transition pore, which controls apoptosis. Binding of Fas/APO-1/CD95 triggers a prototypic apoptosis-inducing pathway. Using four different human tumor cell lines (T-cell Jurkat, neuroblastoma SHEP, osteosarcoma 143N2, and glioblastoma SNB79 cell lines), all of which express CD95 and mPBR, we investigated the potential role of mPBR ligands in CD95-induced apoptosis. We show that, in vitro, the three mPBR ligands tested (RO5-4864, PK11195, and diazepam) enhanced apoptosis induced by anti-CD95 antibody in Jurkat cells, as demonstrated by mitochondrial transmembrane potential drop and DNA fragmentation. In contrast, RO5-4864, but not PK11195 or diazepam, enhanced anti-CD95 apoptosis in all other cell lines. These effects were obtained in Bcl-2-overexpressing SHEP cell lines, but not in Bcl-XI SHEP cell lines. Enhancement of anti-CD95 antibody-induced apoptosis by RO5-4864 was characterized by an increased mitochondrial release of cytochrome c and Smac/DIABLO proteins and an enhanced activation of caspases 9 and 3, suggesting a mitochondrion-dependent mechanism. Preincubation of cells with the different mPBR ligands or anti-CD95 did not affect the levels of expression of either mPBR or CD95. In vivo, we found that the RO5-4864 mPBR ligand significantly increased the growth inhibition induced by two chemotherapeutic agents, etoposide and ifosfamide, using human small cell lung cancers xenografted into nude mice. Peripheral benzodiazepine receptor ligands may therefore act as chemosensitizing agents for the treatment of human neoplasms.

INTRODUCTION

Failure of cell death induction leads to resistance of cancer cells to antimtumor therapies. Reversion of this apoptosis resistance and the consequent potentiation of cytotoxic effects constitute a challenge for the improvement of chemotherapy. The apoptotic process is marked by a series of morphological and molecular alterations, including disruption of mitochondrial membrane integrity, caspase activation, and DNA fragmentation. Over recent years, it has been widely accepted that apoptosis is under the control of mitochondria and that the PT pore plays a key role in this regulation. The PT pore is a permeability transition pore complex located at the contact site between the mitochondrial inner and outer membranes. Several proteins contribute to PT pore formation, including mitochondrial hexokinase, the PBR, and the voltage-dependent anion channel in the outer membrane, creatine kinase in the intermembrane space, adenine nucleotide translocator in the inner membrane, and cyclophilin D in the matrix. Opening of the PT pore induces apoptosis, whereas pharmacological inhibition of this pore prevents cell death. Concurrently, the oncoprotein Bcl-2, which is particularly abundant at the location of the PT pore (4), prevents apoptosis induction (5).

The mPBR belongs to the PT pore complex (6) and is up-regulated in several tumors such as glioblastoma and ovarian and hepatocellular carcinomas (7–9). We reported recently that PK11195, a ligand of the mPBR, enhanced apoptosis induced by a number of different agents, such as etoposide, doxorubicin, dexamethasone, γ-irradiation (10), lonidamine (11), and the proapoptotic second messenger ceramide (10). When added to isolated mitochondria, PK11195 facilitated dissipation of the Δψm and release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor. Moreover, PK11195 abolished apoptosis inhibition by Bcl-2 via a direct effect on mitochondria (10). Altogether, these findings suggest that mPBR ligands act on the PT pore to facilitate apoptosis induction and that they appear to be pharmacological targets for apoptosis modulation.

Surprisingly, apoptosis enhancement by PK11195 is only observed at a concentration 1000-fold higher than that required for its specific binding to the high-affinity mPBR, thus questioning the relationship between the apoptosis-sensitizing effect of PK11195 and its mitochondrial binding. To confirm the therapeutic potential of mPBR ligands and to elucidate the mechanisms by which they enhance apoptosis, we studied the in vitro and in vivo antitumor activities of several apoptotic inducers in combination with various mPBR ligands, namely, RO5-4864, PK11195, and diazepam (6, 12). We focused our investigations on the Fas/APO-1/CD95 pathway, using the human anti-CD95 Mab CH-11, and looked for enhancement of the in vitro cytotoxic effect of CH-11 by several mPBR ligands. Finally, we showed in vivo enhancement of chemotherapy-induced apoptosis by RO5-4864 in two human small cell lung cancers xenografted into nude mice. These results support the hypothesis that mPBR ligands could be used for chemosensitization of solid cancers.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Apoptosis Induction. The human Fas-resistant T-cell line Jurkat (kindly provided by J. Wijdenes; Diaclone, Besançon, France); the human neuroblastoma cell line SHEP stably transfected with either Bcl-2 (SHEP-Bcl-2), Bcl-XI (SHEP-Bcl-XI), or control vector (SHEP-control; a gift from Klaus-Michael Debatin, University Hospital, Ulm, Germany); the human osteosarcoma cell line 143N2 (UMR 147 CNRS); and the human glioblastoma cell line SNB79 (UMR 147 CNRS) were cultured in DMEM or RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (Dutsch, Brunmath, France), penicillin G (10,000 U/ml) + streptomycin (50 μg/ml; Sigma Chemical Co.), and L-glutamine (2 mM; Sigma Chemical Co.). Cells were cultured in the presence of the CD95 cross-linking IgM Mab CH-11 (1 μg/ml; Immunotech, Marseille, France), during or after exposure to RO5-4864 (Sigma Chemical Co.), PK11195 (Sigma Chemical Co.), or diazepam (Roche, Neuilly/Seine, France) at different concentrations, as indicated in the figures. After the indicated intervals, cells were studied for apoptosis quantification or viability.

Quantification of Apoptosis. DiOC(3), a cationic lipophilic fluorochrome (Molecular Probes, Eugene, OR), was used to measure the Δψm. Briefly, cells were incubated at 37°C for 15 min in the presence of DiOC(3) (40 nM), followed by immediate analysis of fluorochrome incorporation in an
caspase-3 induction was determined as a percentage of total cells. PCR-based method with total RNA isolated from living cells using Trizol expressions of CD95 and mPBR were measured by a reverse transcription-

H9004 dissipation of the nuclear dye Hoescht 33342 (2 sec) secondary antibodies conjugated to ALEXA 568 and counterstained with the 1067; Calbiochem; dilution, 1:50) and cleaved caspase 9 and cleaved caspase Probes). Cells were also stained for the detection of Smac/DIABLO (Mab SHEP-control cells were cultured on coverglasses for 24 h in the presence or absence of ROS-4864 (100 μM) and/or anti-CD95 antibody (1 μg/ml), washed once with PBS, and fixed with paraformaldehyde (4% w/v) and picric acid fixative solution. A Mab specific for cytotoxicity c (Mab 6H2.B4; BD PharMingen; dilution, 1:250) was used and revealed by a goat antinouse IgG ALEXA 488 conjugate (Molecular Probes). Cells were also stained for the detection of Smac/DIABLO (Mab 1067; Calbiochem; dilution, 1:50) and cleaved caspase 9 and cleaved caspase 3 (Cell Signaling Technology; dilution, 1:50), all revealed by appropriate secondary antibodies conjugated to ALEXA 568 and counterstained with the nuclear dye Hoescht 33342 (2 μM; Molecular Probes). Quantification of mitochondrial cytotoxicity c and Smac/DIABLO release and of caspase-9 and caspase-3 induction was determined as a percentage of total cells.

Evaluation of FasR and mPBR mRNA and CD95 Expression. mRNA expressions of CD95 and mPBR were measured by a reverse transcription-PCR-based method with total RNA isolated from living cells using Trizol reagent (Life Technologies, Inc., Cergy Pontoise, France) according to the manufacturer’s instructions, as described previously (16). Briefly, cDNA was prepared in an oligodeoxythymidylic acid-primed reverse transcription reaction consisting of 2 μg of total RNA; a total volume of 20 μl of cDNA was then used for PCR amplification. The final PCR reaction volume was 50 μl:1 μl of the cDNA solution, and the following mixture containing 5 μl of Taq buffer (Appligene-Oncor, Illkirch, France), 1 μl of deoxynucleotide triphosphate (final concentration, 0.25 mM each), 1 μl of each of the 5’ and 3’ primers (100 ng/μl), 40.5 μl of water, and 0.5 μl (2.5 units) of Taq polymerase (Appligene-Oncor) was added. Primers used were as follows: (α) for CD95, 5’-CAA-GTC-CTT-GAG-ACT-3’ (sense) and 5’-TGT-CAG-TTT-3’ (antisense); (β) for Bcl-2, 5’-CGT-GCT-GAG-GCA-3’ (sense) and 5’-CCT-TTT-TCA-TTC-3’ (antisense); (γ) for Bcl-XL, 5’-AGG-AGA-AGG-AGC-3’ (sense) and 5’-CTT-GTG-TAT-GCG-3’ (antisense); (δ) for neomycin resistance gene (sense) (control) were concomitantly cultured for 24 h with (square) or without (circle) 1 μg/ml CH-11 and ROS-4864. Determination of cell viability was performed using a methylene blue assay.
Swiss mice (Ligands. anti-IgG secondary antibody (PharMingen). control was performed using isotype control IgG followed by FITC-conjugated as soon as the xenografted tumors reached a 5-mm diameter (or a tumor investigators. For therapeutic trials, the tumor-bearing mice were randomly handled of the mice were performed in accordance with the recommendations specific pathogen-free conditions with artificial lighting (12-h light/12-h dark °C for 1 min, 25 cycles for CD95 PCR; and (b) denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, 35 and 40 cycles for mPBR PCR. PCR products (7 µl) were separated by electrophoresis in a 1.8% agarose gel and visualized by ethidium bromide (0.5 µg/ml) staining under UV exposure for 6–30 s. The image was then processed by software, analyzed using the NIH version 1.60 Image program, and photographed.

To determine cell surface CD95 expression, cells were incubated with a murine antihuman IgG1 anti-Fas Mab (dilution, 1:800; Immunotech) for 30 min on ice in 1% FCS-PBS, washed twice, and then incubated for 30 min on ice with FITC-conjugated goat antimouse IgG1 Mab (Coulter, Coultronics, Marseille, France). After additional washing with PBS, cells were analyzed using an Epics Profile II cytofluorometer. Fluorescence data were collected in 10^4 viable cells, as determined by forward light scatter intensity. Negative control was performed using isotype control IgG followed by FITC-conjugated anti-IgG secondary antibody (PharMingen).

In Vivo Enhancement of Chemotherapy-induced Apoptosis by mPBR Ligands. Swiss mice (nu/nu; female, 6–8 weeks old) weighing 30 g were bred in the animal facilities (Institut Curie, Paris, France), maintained under specific pathogen-free conditions with artificial lighting (12-h light/12-h dark cycle), and fed a regular diet and water ad libitum. The care, housing, and handling of the mice were performed in accordance with the recommendations of the French Ethics Committee and under the supervision of authorized investigators. For therapeutic trials, the tumor-bearing mice were randomly divided into equivalent groups of four to eight animals, and mice were treated as soon as the xenografted tumors reached a 5-mm diameter (or a tumor volume of approximately 60 mm³).

Two different human tumors, small cell lung cancers SCLC6 and SCLC61, were used and treated by a combination of etoposide (Pierre Fabre, Boulogne, France) and ifosfamide (Asta Medica, Mérignac, France) or etoposide alone, respectively, with or without RO5-4864. RO5-4864 was diluted in 100 µl of excipient solution containing ethanol and Tween 80 and was injected i.p. Etoposide and ifosfamide were diluted in 200 µl of 0.9% sodium chloride and injected i.p. The control group received injections according to the same schedule as experimentally treated mice.

All mice were weighed once weekly. Tumor growth was monitored by measuring two perpendicular diameters with calipers. Mice were sacrificed when the tumor volume reached 2500 mm³ in the control group.

Statistical Analysis. We used Student’s t test to assess the in vivo enhancement of chemotherapy-induced apoptosis by RO5-4864 on growth of xenografted tumors in nude mice. The t values were two-sided and were considered significant when they were less than or equal to 0.05.

RESULTS

In Vitro Studies of Anti-FasR Antibody and mPBR Ligands. The T-cell Jurkat cell line was found to be relatively resistant to the human anti-CD95 Mab, which only induced apoptosis in 9–24% of cells (Fig. 1A). RO5-4864, PK11195, or diazepam used at concentrations that were not cytotoxic enhanced the incidence of nuclear apoptosis (measured as subdiploidy) induced by the anti-CD95 antibody (Fig. 1A). The induction of apoptosis depended on the concentrations of the mPBR ligands, RO5-4864, PK11195, or diazepam. A cooperative apoptosis-inducing effect between CD95 and PBR ligands was confirmed when, instead of DNA loss, other features of apoptosis, namely, the drop in ΔΨm and production of anion superoxide, were assessed (Fig. 1B).

Distinctive Efficacy of Various mPBR Ligands to Reverse CD95-dependent Apoptosis Resistance. Combined with anti-CD95 antibody, RO5-4864 induced apoptosis in SHEP-control, 143N2, and SNB79 cells (Figs. 2, 3A, and 4A). In contrast, PK11195 and diaze-
Pam sensitized only SHEP-control cells (data not shown), but not 143N2 (Fig. 3A) or SNB79 cells, to CD95-induced apoptosis (data not shown). RO5-4864, but not PK11195 or diazepam, sensitized SHEP-Bcl-2 (Fig. 2) but not SHEP.Bcl-XL cells (data not shown). The RO5-4864-mediated sensitization to anti-CD95 was observed over a wide range of anti-CD95 antibody concentrations (0.25–2 μg/ml; Fig. 3B). When cells were cultured for 24 h in the presence of different mPBR ligands before exposure to anti-CD95, no apoptogenic activity was detected in any of the cell lines tested (Fig. 3B). To test the dose dependency of apoptosis enhancement, human osteosarcoma (143N2) and neuroblastoma (SHEP-control or SHEP-Bcl-2) cell lines were cultured for 24 h concomitantly with anti-CD95 and low doses of RO5-4864 (1–100 nM); no potentiation of the cytotoxic effect of the anti-CD95 Mab was observed, showing that doses of up to 30 μM of the various mPBR ligands were necessary to induce apoptosis (data not shown).

**Combined Ligation of mPBR and CD95 Results in Enhanced Mitochondrial Release of Cytochrome c and Smac/DIABLO.** Immunofluorescence detection of cytochrome c (Fig. 5A, green fluorescence), Smac/DIABLO (B, red), caspase 9 (C, red), and caspase 3 (D, red) in SHEP-control cells counterstained with the nuclear dye Hoechst 33342 (blue fluorescence). Cells were cultured for 24 h in the presence or absence of RO5-4864 (100 μM) and anti-CD95 antibody (1 μg/ml), as indicated. Representative cells in the presence of RO5-4864 alone (left panels) or RO5-4864 + anti-CD95 (middle panels) are shown. Note the punctate mitochondrial staining pattern of cytochrome c and Smac/DIABLO (left panels) and the diffuse pattern (middle panels) indicative of mitochondrial protein release. Quantification of mitochondrial cytochrome c and Smac/DIABLO release and of caspase-9 and caspase-3 induction was determined as a percentage of total cells (right panels).
SNB79, SHEP, SNB19, Jurkat, and 143N2 cells. Positive control was ensured by mPBR mRNA expression was measured by a reverse transcription-PCR-based method in and SHEP-control cell lines and after RO5-4864 or CH-11 pre-exposure. Data are shown for 143N2 stained with FITC-conjugated goat antimouse IgG1 Mab (H9252/microglobulin (H2m)). CD95 expression was low for the SNB19 cell line and high (Fig. 6A) or presence of RO5-4864 (C), PK11195, diazepam, or CH-11 (D). Cells were stained with a murine antihuman IgG1 antibody expression was studied in SNB19, 143N2, SHEP-control, SHEP-Bcl-2, and SHEP-Bcl-XL cell lines cultured for 12 h in the absence (B) or presence of RO5-4864 (C), PK11195, diazepam, or CH-11 (D). Cells were stained with a murine antihuman IgG1 anti-FasR Mab and revealed with a FITC-conjugated goat antihuman IgG1 Mab. Cell staining was quantified by flow cytometry on 10^4 viable cells. Negative controls were stained with FITC-conjugated goat antihuman IgG1 Mab (A). Data are shown for 143N2 and SHEP-control cell lines and after RO5-4864 or CH-11 pre-exposure.

using antibodies that recognized cleaved processed caspases. Cleaved caspase 9 (Fig. 5C) and 3 (Fig. 5D) were detected in SHEP-control cells cultured in the simultaneous presence of anti-CD95 antibody and RO5-4864, but not in cells cultured in the presence of CH-11 alone or RO5-4864 alone. Taken together, these data are compatible with the notion that RO5-4864 sensitizes cells to CH-11-induced mitochondrial release of caspase activators, thereby resulting in enhanced caspase activation and apoptosis.

Evaluation of CD95 and mPBR mRNA and CD95 Expression in the Cells Used. All cancer cell lines and tumors used, including SNB19, Jurkat, SHEP-control, SNB79, 143N2, SCLC6, and SCLC61, expressed detectable mRNA transcripts of both CD95 and mPBR (Fig. 6A). CD95 expression was low for the SNB19 cell line and high for all other cell lines. CD95 expression was not affected by a 12-h exposure to anti-CD95 (1 μg/ml) and mPBR ligands (60 μM), as shown in 143N2 and SHEP-control cell lines (Fig. 6B).

In Vivo Enhancement of Chemotherapy-induced Tumor Growth Inhibition by RO5-4864. Two transplantable xenografts of tumors derived from surgical samples of fresh human tumors were used (two small cell lung cancers, SCLC6 and SCLC61). Mice bearing SCLC61 xenografts were treated on days 1–3 by one i.p. injection of etoposide at a dosage of 12 mg/kg, with or without RO5-4864 at a dose of 12 mg/kg. Mice of the two control and etoposide groups received the excipient used to dissolve RO5-4864 according to the same schedule as experimentally treated mice. Tumor growth was not modified by administration of the excipient. We observed that the growth inhibition induced by etoposide was significantly increased by concomitant administration of mPBR ligand (P < 0.05; Fig. 7A). Similar results were obtained when mice bearing SCLC6 xenografts received a combination of etoposide (12 mg/kg) and ifosfamide (90 mg/kg) with or without concomitant administration of 40 mg/kg RO5-4864. RO5-4864 significantly enhanced the growth-inhibitory effect of chemotherapy (P < 0.05; Fig. 7B) but had no effect on tumor growth alone.

DISCUSSION

In this report, we describe that apoptosis induced by anti-CD95 receptor antibody is enhanced by various ligands of the mitochondrial benzodiazepine receptor in human tumor cell lines. This effect has been observed in several histological types of neoplasms, including T-cell lymphoma, neuroblastoma, osteosarcoma, and glioblastoma. This enhancement requires concomitant exposure to mPBR ligand and anti-CD95 Mab. Moreover, all tumor cell lines studied expressed detectable mRNA transcripts of mPBR, suggesting that this expression is a prerequisite of apoptosis facilitation. Note that the Jurkat cell line used in this study was found to express mPBR mRNA, a finding that contrasts with a previous report suggesting that Jurkat cells lack PBRs (12). We found that Bcl-2-mediated cytoprotection could be counteracted by RO5-4864, whereas no such effect of RO5-4864 was observed on Bcl-XL-mediated apoptosis inhibition. There is no explanation for the specific effect of RO5-4864 on Bcl-2, but not Bcl-XL, at the present time. One possibility is that Bcl-2 and Bcl-XL may...
interact in slightly different ways with mitochondrial membranes, including the PBR. Finally, we observed an in vivo enhancement of apoptosis induction by RO5-4864 in human xenografted tumors because we demonstrated significant growth inhibition in two small cell lung cancers. The difference observed between chemotherapy only- and chemotherapy/RO5-4864-treated mice seems to be the consequence of prolongation of chemotherapy-induced cytotoxicity rather than a stronger growth-inhibitory effect.

Enhancement of anti-CD95 antibody-induced apoptosis by RO5-4864 was characterized by numerous mitochondrial alterations, namely, the drop of the ΔΨm and the production of superoxide anion. We also observed enhancement of the mitochondrial release of cytochrome c and Smac/DIABLO regulatory proteins, as well as activation of caspases 9 and 3. Note that quantification of mitochondrial cytochrome c and Smac/DIABLO release and caspase 9 and 3 induction was performed on adherent cells, leading to an underestimation of apoptosis resulting in loss of cellular adhesion. These observations suggest that activation of caspase 3 results from a mitochondrial megachannel disrupter responsible for cytochrome c and Smac/DIABLO release and caspase 9 activation (17, 18) and argue in favor of a mitochondrial-dependent mechanism of anti-CD95 antibody-induced apoptosis by RO5-4864.

Mitochondrial benzodiazepine receptors have already been reported as therapeutic targets using a PBR ligand-melphalan conjugate (19) or porphyrins in photodynamic tumor therapy (20). Similarly, we have previously reported potentiation of lonidamine-induced cytotoxicity by coconcomitant exposure to diazepam in human glioblastoma tumor xenografted into nude mice (21). Bono et al. (22) showed that PK11195 enhanced the sensitivity of cells to tumor necrosis factor α and abolished the apoptosis-inhibitory effect of Bcl-2 via a direct effect on mitochondria. Nevertheless, the same authors reported that at concentrations between 10 and 100 nM, ROS-4864 reduced the proapoptotic effect of tumor necrosis factor α. This observation is not in agreement with our own data showing that at concentrations <1 μM, ROS-4864, PK11195, and diazepam failed to stimulate CD95-induced apoptosis. This apparent discrepancy could be explained by the fact that we used different cell lines. An alternative explanation could be that PK11195 (an isocoumarine carboxamide) and RO5-4864 (a benzodiazepine) exert different conformational and/or physicochemical changes on their mitochondrial binding partners. Indeed, differential effects observed with RO5-4864 and PK11195 supported the assumption that RO5-4864 acts as an agonist and PK11195 as an antagonist of the mPBR (12) and that they interact with two different conformations or domains of the mPBR (23). It could be speculated that the binding of the mPBR ligand to its receptor induces a peculiar conformation of the mitochondrial PT pore, which sensitizes the cell to an apoptotic message. This would emphasize the importance of mPBR-associated proteins modulating the cell death process, such as Bcl-2, Bcl-XL, or Bax (24). All these hypotheses must be explored in additional studies to explain the enhancement of apoptosis by mPBR ligands.

In conclusion, the present data indicate that RO5-4864 mPBR ligand can sensitize a panel of different human tumor cells to apoptosis induction, both in vitro and in vivo. Irrespective of the exact molecular mechanisms accounting for their chemosensitizing effect, mPBR ligands therefore appear to be potential antineoplastic agents.

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