Potent Prostaglandin A_1 Analogs That Suppress Tumor Cell Growth through Induction of p21 and Reduction of Cyclin E*

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Although the cyclopentenone prostaglandin A_1 (PGA_1) is known to arrest the cell cycle at the G_1 phase in vitro and to suppress tumor growth in vivo, its relatively weak activity limits its usefulness in cancer chemotherapy. In an attempt to develop antitumor drugs of greater potency and conspicuous biological specificity, we synthesized novel analogs based on the structure of PGA_1. Of the newly synthesized analogs, 15-epi-Δ^2-PGA_1 methyl ester (NAG-0092), 12-iso-Δ^2-PGA_1 methyl ester (NAG-0093), and ent-Δ^2-PGA_1 methyl ester (NAG-0022) possess a cross-conjugated dienone structure around the five-member ring with unnatural configurations at C(12) and/or C(15) and were found to be far more potent than native PGA_1 in inhibiting cell growth and causing G_1 arrest in A172 human glioma cells. These three analogs induced the expression of p21 at both RNA and protein levels in a time- and dose-dependent fashion. Kinase assays with A172 cells treated with these analogs revealed that both cyclin A- and E-dependent kinase activities were markedly reduced, although cyclin D1-dependent kinase activity was unaffected. Immunoprecipitation-Western blot analysis showed that the decrease in cyclin A-dependent kinase activity was due to an increased association of p21 with cyclin A-cyclin-dependent kinase 2 complexes, whereas the decrease in cyclin E-dependent activity was due to a combined mechanism involving reduction in cyclin E protein itself and increased association of p21. Thus, these newly synthesized PGA_1 analogs may prove to be powerful tools in cancer chemotherapy as well as in investigations of the structural basis of the antiproliferative activity of A series prostaglandins.

Prostaglandins of the A series (PGAs) contain an α,β-un saturated carbonyl group at the five-member ring and are derived from E type prostaglandins by dehydration in plasma or aqueous solution (1). PGAs have been reported to have antiproliferative activity in vitro (2–8) as well as antitumor activity in vivo (2, 7, 9–11), and although these properties suggest that PGAs would be potentially useful in chemotherapy of malignant tumors, their relatively weak activity and extreme instability in plasma limit their clinical application.

In order to develop more stable and potent PGA analogs, it appeared to be important to decipher the structural features contributing to the stability and antiproliferative activity of A type PGs. A type PGs are conjugated with glutathione in the cells, and the resultant PG-glutathiones are immediately exported from the cells through the ATP-dependent glutathione S-conjugate export pump (MRP/GS-X pump) (12), thereby reducing the intracellular accumulation of PGs. The GS-X pump was first reported to eliminate cytotoxic drugs from tumor cells and to play an important role in developing drug resistance in tumor cells (13). Thus, it is necessary to develop PGA analogs that are not readily conjugated with glutathione.

We previously demonstrated that the cross-conjugated dione PGs, including native Δ^12-PGJ_2 and an artificial analog Δ^1-PGA_1 methyl ester, reveal much more potent antiproliferative effects than simple enone-type PGs, such as PGA_1 (12). Although PGs containing a cross-conjugated dienone structure still react reversibly with glutathione to form equilibrium mixtures of free PG and PG-glutathione conjugate in the cell, this reaction biases the equilibrium reaction with intracellular glutathione to the formation of enone-conjugated forms, accumulating free PGs in the cell (12). Thus, the eminent potency of dione PGs over simple enone PGs may be explained by the difference in the extrusion mechanism. In accordance with this concept, we have demonstrated that the antiproliferative effect of Δ^1-PGA_1 methyl ester is significantly enhanced in the presence of an MRP/GS-X pump inhibitor.

Cell cycle progression in eukaryotes is controlled by specific activation and subsequent inactivation of cyclin-dependent kinases (Cdks) (14). Two distinct families of Cdk inhibitors (CKIs) have been described in mammalian cells (15). One of these is the Cip/Kip family, which includes the structurally related proteins p21, p27, and p57. Members of this family inhibit a variety of Cdk activities in vitro (16–24). The other family of CKIs is the INK4 family, the members of which specifically inhibit cyclin D-dependent kinases in vitro (25–29). It has recently been reported that treatment of breast carcinoma cells with PGA_1 leads to G_1 arrest in association with increased expression of p21 (30), suggesting that PGA_1 inhibits tumor cell growth through the induction of p21. We also found that Δ^1-PGA_1 methyl ester induces p21 via a p53-independent

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The abbreviations used are: PGA, prostaglandin A; PG, prostaglandin; Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor; IP, immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
pathway causing cell cycle arrest at G1 phase in HL-60 cells (31). p21 CKI has been implicated in cell cycle arrest associated with a variety of cellular events, such as cellular senescence (32), quiescence (33), differentiation (34–37), and DNA damage (38). Although the expression of p21 is largely dependent on the presence of functional p53, it is also regulated by p53-independent mechanisms (39–41).

In this study, we examined the ability of PGA1 analogs that contain a cross-conjugated dienone unit to inhibit the proliferation of A172 human glioma cells at molecular level. The results indicate that three analogs, 15-epi-Δ7-PGA1 methyl ester (NAG-0092), 12-iso-Δ7-PGA1 methyl ester (NAG-0093), and ent-Δ7-PGA1 methyl ester (NAG-0022), are far more potent than native PGA1 in arresting cells at the G1 phase through a combined mechanism involving the induction of p21 and the suppression of cyclin E.

MATERIALS AND METHODS

PGs and Their Analogs—PGA1, PGA2 (Sigma), PGJ2, 15-deoxy-Δ12,14-PGJ2 (Cayman Chemical Company), and PGA1 analogs were dissolved in absolute ethanol, and dilutions were made in Dulbecco’s modified essential medium. The final concentration of ethanol did not exceed 0.1% or affect cell growth. All manipulations of these compounds were performed under low lighting conditions. The code names and chemical structures of these compounds are shown in Fig. 1. Detailed methods for the design and synthesis of PGA1 analogs are described elsewhere.2

Cell Culture—Human glioma A172 cells were cultured in Dulbecco’s modified essential medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and penicillin-streptomycin (Life Technologies, Inc.) under standard tissue culture conditions. Cell numbers were counted with a cell counter (Coulter).

Cell Cycle Analysis—Cell cycle distribution was analyzed by flow cytometry. Briefly, 2 × 106 cells were harvested in PBS containing 2 mM EDTA, washed once with PBS, and fixed in PBS containing 0.2% paraformaldehyde for 30 min. Fixed cells were washed once in PBS and permeabilized for 30 min with 0.2% Tween 20 and 1 mg/ml RNase A in PBS. Cells were then washed once in PBS and stained with 50 μg/ml of propidium iodide (Boehringer Mannheim). The stained cells were analyzed by flow cytometry (Coulter).

Northern and Western Blot Analysis—Total cellular RNA was extracted from A172 cells using RNA isolation reagent (Isogen, Nippon Gene Co., Tokyo, Japan) according to the manufacturer’s instructions. RNA (20 μg of each) was denatured, separated by electrophoresis on 1.0% agarose-formaldehyde gels, and transferred onto nylon membranes (Micron Separations, MA). p21 or cyclin E cDNA was labeled with α-l[32P]dCTP using a random primer labeling kit (Amersham Pharmacia Biotech), and the hybridization signal was quantified by means of a Fuji Imaging Analyzer BAS-1500. GAPDH mRNA was used as an internal control for variations in loading and transfer efficiency among samples.

For Western analysis, cells were lysed in cell lysis buffer (20 mM Tris, pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 2 μg/ml aprotinin, 20 μg/ml soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate (Na3VO4), and 1 mM sodium fluoride (NaF)) at 16 h after treatment with the PGA1 analogs. Total cell lysates (100 μg each) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. p21, cyclin A, and cyclin E proteins were detected with anti-p21, anti-cyclin A, and anti-cyclin E antibodies (Santa Cruz Biotechnology; H-164 for p21, H-432 for cyclin A, and C-19 for cyclin E), respectively, and protein signals were enhanced by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Immunoprecipitation and Kinase Assay—Twenty-four hours after treatment with PGA1 analogs, cells were lysed in IP-kinase buffer (HEPES, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Nonidet P-40, 2 μg/ml aprotinin, 0.1 μM phenylmethylsulfonyl fluoride, 1 mM NaF, 0.1 mM Na3VO4, and 10 μM β-glycerophosphate). Lysates were mixed with 10 μl of antibodies against cyclin A (Santa Cruz Biotechnology, H-432), cyclin E (Santa Cruz Biotechnology, C-19), and cyclin D1 (Santa Cruz Biotechnology, H-295). After 1 h of rotation at 4 °C, the immune complexes were precipitated with protein A beads (Amersham Pharmacia Biotech). The precipitated complexes were analyzed by Western blotting or assayed for kinase activity at 30 °C for 30 min in a 30-μl reaction mixture containing 50 mM HEPES, pH 8.0, 10 mM MgCl2, 2.5 mM EGTA, 1 mM DTT, 10 μM β-glycerophosphate, 1 mM NaF, 0.1 mM Na3VO4, 0.1 μM phenylmethylsulfonyl fluoride, 10 μM ATP, and 185 kBq of [γ-32P]ATP (222 TBq/mmol; NEN Life Science Products). One micromgram of histone H1 (for cyclin A- or E-dependent kinases) or 0.2 μg of GST-pRb (for cyclin D1-dependent kinases) was used as a substrate. The reaction products were separated on SDS-polyacrylamide gel electrophoresis, and phosphorylated proteins were detected by autoradiography and quantitated using a Fuji Image Analyzer BAS-1500.

Statistical Analysis—Statistical significance was determined by the analysis of variance. Scheffe’s F test procedure for multiple pairwise post-hoc comparisons of means was implemented, and a p value of less than 0.05 was considered statistically significant.

RESULTS

Chemical Structure of the Synthetic PGA1 Analogs—Fig. 1 shows the chemical structures of the synthetic PGA1 analogs identified in this study as more potent antitumor compounds than native PGs. All of the analogs possess a cross-conjugated dienone unit and a methyl ester form at C(1), whereas native PGA1 and PGA2 are simple enone compounds and contain no methyl ester. Some of the synthetic PGs have unnatural configurations at C(12) and C(15).

Potent Antiproliferative Activity of PGA1 Analogs—The synthetic PGA1 analogs were first tested for their ability to inhibit cell proliferation, using PGA1, PGA2, and PGJ2 as references. A172 cells (from a glioblastoma cell line) were treated with each of the test compounds at a 5 μM concentration, and...
changes in the cell number were examined over a period of 72 h. As shown in Fig. 2, NAG-0022, NAG-0092, and NAG-0093 were most potent in suppressing the proliferation of A172 cells at 48 and 72 h \((p < 0.05)\). NAG-0026 also displayed a substantial antiproliferative activity \((p < 0.05)\). PGA1, PGA2, and PGJ2 had almost no effect on the cell growth at 5 \(\mu M\) (Fig. 2 and data not shown).

**G1 Arrest by PGA1 Analogs**—In order to examine the mechanism(s) of growth suppression by PGA1 analogs and also to rule out the possibility of nonspecific cytotoxicity, we next determined the cell cycle distribution of A172 cells after treatment of these cells with the various PGA1 analogs, using a fluorescence-activated cell sorter (Fig. 3). At a concentration of 5 \(\mu M\), NAG-0022, NAG-0092, and NAG-0093 were most potent in arresting cells at the G1 phase (66.7, 74.9, and 71.0%, respectively) as compared with vehicle-treated cells (42.2%), whereas the other compounds showed only a modest effect on cell cycle distribution (Fig. 3 and data not shown). The growth inhibitory activity of the PGA1 analogs (Fig. 2) correlated well with their ability to cause G1 arrest (Fig. 3), suggesting that most of these analogs inhibit cell growth in this manner.

**Induction of p21 Expression by PGA1 Analogs**—It has been reported that PGA2 arrests the cell cycle at the G1 phase through the induction of p21 CKI at high concentrations (25–36 \(\mu M\)) (30, 42). To determine whether this is also the case with PGA1 analogs, we examined changes in p21 expression after using the analogs to treat the A172 cells. As shown in Fig. 4, the baseline level of p21 mRNA expression of these cells was low, and NAG-0022, NAG-0092, and NAG-0093 caused a dose-dependent increase in the steady-state mRNA level, with a clear-cut effect being observed at 1.0 \(\mu M\) and a maximal response at 5.0–10 \(\mu M\) (approximately 7-fold as compared with vehicle-treated cells). Only a modest increase in p21 mRNA was observed with NAG-0026, and PGA1, PGA2, and 15-PGJ2 had little or no effect at 5 \(\mu M\) (Fig. 4).

The time course of the effect of NAG-0092 on p21 mRNA is shown in Fig. 5. Induction of p21 mRNA expression occurred as early as 2 h and reached a maximal level at 8 h after treatment with 5 \(\mu M\) NAG-0092. The induction of p21 protein was also examined by Western blot analysis. As shown in Fig. 6, NAG-0092 and NAG-0093 at 5 \(\mu M\) markedly increased the protein level of p21, whereas PGA1 and PGA2 had only a modest effect. These results correlated well with those of Northern blot analysis (Fig. 4). Taken together with the findings that PGA1 analogs did not induce the expression of other known CKIs, including p27, p57, p15, and p16 (data not shown), it is suggested that PGA1 analogs arrest the cell cycle at the G1 phase, at least in part through the induction of p21 CKI.

**PGA1 Analogs Suppress Cyclin A- and Cyclin E- but not Cyclin D1-dependent Kinase Activities**—To further elucidate the mechanism(s) by which PGA1 analogs arrested the cell cycle at G1, we examined cyclin E-, A- and D1-dependent kinase activities, all of which are necessary for G1 progression and G1/S transition. As shown in Fig. 7A, cyclin A- and E-dependent kinase activities were almost completely inhibited at 24 h after treatment with 5 \(\mu M\) NAG-0092, whereas cyclin...
D1-dependent kinase activity was not affected (data not shown).

To determine whether the suppression of cyclin A- and E-dependent kinase activities by the PGA1 analog was due to the induction of p21 and its association with the Cdk complexes, we analyzed cyclin A-Cdk2 and cyclin E-Cdk2 complexes by IP-Western analysis. As shown in Fig. 7B, the amount of Cdk2 complexed with cyclin A did not change after treatment with PGA1 or NAG-0092. In contrast, the amount of p21 in the cyclin A-Cdk2 complex was markedly increased after treatment with NAG-0092, but only modest increases were noted with PGA1 (Fig. 7B). Taken together, the increased association of p21 with cyclin A-Cdk2 complexes appeared to be important for the inhibition of kinase activity. On the other hand, analysis of cyclin E-Cdk2 complexes revealed that the amount of Cdk2 associated with cyclin E decreased after PGA1 treatment whereas that of p21 in the complexes remained constant (Fig. 7B). It is likely, therefore, that the decrease in cyclin E-dependent kinase activity is due to a combined mechanism involving decline in cyclin E protein itself and increased p21 level in cyclin E complexes.

Unexpectedly, cyclin E- but not cyclin A-dependent kinase activity was enhanced rather than suppressed by PGA1 treatment (Fig. 7A). Taken together with our observations that the amount of Cdk2 associated with cyclin E decreased after PGA1 treatment whereas that of p21 in the complexes remained constant (Fig. 7B), it is conceivable that the relative amount of cyclin E-Cdk2-p21 ternary complexes was increased by PGA1. In light of the reported findings that p21 can promote the association of Cdk with cyclins and thus stabilize cyclin-Cdk complexes (43), the apparent stimulation of cyclin E-dependent kinase activity by PGA1 may be explained by this novel function of p21 as an assembly factor rather than as a Cdk inhibitor.

Interestingly, although cyclin E mRNA did not change after treatment with PGA1 (Fig. 7C), the level of cyclin E protein was significantly reduced (Fig. 7D), which may also be related to enhanced cyclin E-dependent kinase activity after PGA1 treatment. Recently, it was reported that cyclin E could be phosphorylated by Cdk2 and that phosphorylated cyclin E is readily degraded by ubiquitine-proteosome pathway (44). Therefore, enhanced cyclin E-Cdk2 activity may result in phosphorylation of cyclin E and its rapid degradation, leading to the reduction of cyclin E protein without changes in its mRNA level.
DISCUSSION

In the present study, we tested a series of PGA analogs for their activities in inhibiting the growth of A172 glioblastoma cells and found that three of these were far more potent than native PGA1, PGA2, and PGJ2. Although it has been reported that some PGs of the A and J series are capable of suppressing tumor cell growth both in vitro (2–8) and in vivo (2, 7, 9–11), their activities are rather modest, presumably due to their intracellular instability, necessitating the use of high doses to obtain substantial antitumor effects. We set out to develop PG analogs of the A and J series that show increased stability, because we considered that some of these compounds might prove to be more potent in inhibiting tumor cell growth than the native compounds. In parallel with this task, we attempted to elucidate the molecular mechanism(s) by which our novel analogs inhibit cell growth.

There is accumulating evidence that active export of anticancer drugs from cells is one of the major mechanisms of drug resistance. Recent studies of the multidrug resistance phenotype of tumor cells have led to the discovery of P-glycoprotein, which mediates the efflux of anticancer drugs, such as doxorubicin, vincristine, and taxol (45, 46). More recently, another type of drug transporter (GS-X/ MRP pump) has been identified as a mediator of glutathione-associated drug resistance. It has been reported that A type PGs are effectively conjugated with glutathione in the cells (12), raising the possibility that GS-X/MRP pump may play an important role in excluding the PGs from the cells. This concept was further supported by the observation that HL-60 cells overexpressing functional GS-X/ MRP pump reveal far more resistance to the antiproliferative effect of Δ2-PGA1 methyl ester than control HL-60 cells.

We have previously shown that the cross-conjugated diene unit, as compared with the simple enone unit in native PGA, protects PGs against removal from the cytoplasm by GS-X/ MRP pump (12) and allows PGs to bind more stably to target proteins in the nucleus or cytosol, suggesting that the cross-conjugated diene unit stabilizes PGs within the cell. However, in terms of clinical application, it was required to develop more stable and potent PGA1 analogs.

Of the newly synthesized analogs that we synthesized, NAG-0092, NAG-0093 and NAG-0022, which showed the higher antiproliferative activity for A172 cells than NAG-0026 (Δ2-PGA1 methyl ester) (Figs. 1 and 2), possess both a hydroxy group at C(15) and a double bond at C(13)–C(14). Thus, the double bond and hydroxy group in the ω-chain are essential for the potent antiproliferative activities of PGs for these cells. The double bond is thought to fix the conformation of PGs, and the hydroxy group constitutes a hydrogen bonding, allowing sufficient interaction with the target molecule. Although native PGA2 has been reported to inhibit cell growth at 25–36 μM (30, 42, 47), almost no growth inhibitory effect was observed at the lower concentrations (up to 10 μM) used in the current study.

FACS analysis revealed that PGA1 analogs arrested the cell cycle at the G1 phase (Fig. 3). Interestingly, although NAG-0026 substantially inhibited cell growth, it did not cause accumulation of cells at G1 (data not shown), raising the possibility that growth inhibition by this particular analog may be due to its nonspecific toxicity.

It has recently been reported that PGA2 inhibits the G1 cyclin-dependent kinase activities through the induction of p21
Although it has been demonstrated that PGA2 can also activate superfamily, is mediated by metabolites of J type PGs (48, 49). Cyclin E are responsible for the growth suppressive function of known Cdk inhibitors or cyclins (data not shown), it is concluded into nuclei without further metabolism (12).

That PGA1 analogs did not induce the expression of other known Cdk inhibitors or cyclins (data not shown), it is concluded that the combined effects on the expression of p21 and cyclin E are responsible for the growth suppressive function of PGA1 analogs. Although the molecular and cellular mechanisms by which PGA1 analogs induce p21 and suppress cyclin E expression remain elusive, the induction of p21 and the suppression of cyclin E by PGA1 analogs may involve a transcriptional mechanism because the PGA1 analogs are incorporated into nuclei without further metabolism (12).

Members of the J series of PGs have been reported to have a unique spectrum of biological effects, including the inhibition of cell cycle progression, the suppression of viral replication, and the stimulation of osteogenesis (11). Results of previous studies have indicated that the activation of peroxisome proliferator-activated receptor γ, a member of the nuclear receptor superfamily distinct from which both PGJ2 and 15-deoxy-D12,14-PGJ2, putative ligands for the former mechanism, whereas suppression of cyclin E-Cdk2 activity appears to involve both increased p21 and reduced cyclin E expressions. Taken together with the findings that PGA1 analogs did not induce the expression of other known Cdk inhibitors or cyclins (data not shown), it is concluded that the combined effects on the expression of p21 and cyclin E are responsible for the growth suppressive function of PGA1 analogs.

In conclusion, we have identified novel PGA1 analogs that strongly suppress cellular proliferation through their combined effects on p21 CKI and cyclin E. Our results may help to decipher the structural features essential to the antiproliferative activity of the A and J series of PGs. Although it remains to be determined whether the increased potency of our analogs reflects an increased stability within cells or an increased binding affinity to proteins, these analogs may prove to be powerful tools for elucidating the molecular functions of the A series of PGs in both in vitro and in vivo studies.