Anti-cancer-prostaglandin-induced cell-cycle arrest and its modulation by an inhibitor of the ATP-dependent glutathione S-conjugate export pump (*GS-X* pump)

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The A and J series of prostaglandins (PGs) accumulate in the nuclei to suppress the proliferation of cancer cells. Here we report that Δ^7 -PGA₁ methyl ester, a synthetic anti-cancer PG, increased the level of mRNA for the cyclin-dependent kinase inhibitor p21 in human leukaemia HL-60 cells. The induction of p21 was associated with the accumulation of hypophosphorylated retinoblastoma protein (pRB) and the suppression of *c-myc* gene expression. Since the *p53* gene is deleted in HL-60 cells, the anti-cancer PG is suggested to inhibit cancer cell growth by inducing p21 via a p53-independent pathway. Unlike HL-60 cells, cisplatin-resistant HL-60/R-CP cells were insensitive to Δ^7 -PGA₁ methyl ester. While *c-myc* expression was transiently suppressed,

neither G₁ arrest nor hypophosphorylation of pRB was observed with the anti-cancer PG. Plasma membrane vesicles from HL-60/R-CP cells showed an enhanced level of *GS-X* pump (ATP-dependent glutathione S-conjugate export pump) activity towards the glutathione S-conjugate of Δ^7 -PGA₁ methyl ester (*K_m* 110 nM). GIF-0019 {*N*-carbomethoxy-S-[5-(4-benzoylphenyl)pentyl]glutathione dimethyl ester}, a specific inhibitor of the *GS-X* pump, dose-dependently enhanced the cellular sensitivity of HL-60/R-CP cells to Δ^7 -PGA₁ methyl ester and induced G₁ arrest. The *GS-X* pump is suggested to play a pivotal role in modulating the biological action of the anti-cancer PG.

INTRODUCTION

Arachidonic acid and its metabolites constitute a novel class of intracellular second messengers. Prostaglandins (PGs) are involved in a variety of physiological phenomena, such as sleep/wake regulation, inflammation, the immune response, tissue repair, anti-viral protection and, importantly, inhibition of cancer cell growth [1–4]. Of the different types of PGs, the A and J series of PGs are unique in exerting their biological activity via direct interaction with putative nuclear proteins, rather than with plasma membrane receptors. These PGs suppress the proliferation of cancer cells without affecting intracellular cAMP levels, and their nuclear accumulation is correlated closely with cell-cycle arrest and growth inhibition of cancer cells [5–7]. The significance of the anti-cancer PGs as novel biochemical probes is being strengthened by a newly developed method called ‘three-component coupling synthesis’, which has made it possible to generate large quantities of structurally modified PGs [8,9]. Here we demonstrate that Δ^7 -PGA₁ methyl ester (Figure 1, compound 1), an anti-cancer PG synthesized by this method, inhibits cell growth of human leukaemia HL-60 cells by specifically inducing the cyclin-dependent kinase (Cdk) inhibitor p21^{Sdi1/CIP1/WAF1} (p21) via a p53-independent pathway.

Cisplatin [*cis*-diamminedichloroplatinum(II)]-resistant HL-

60/R-CP cells are resistant to this anti-cancer PG [10]. In the present work, we studied the molecular mechanism underlying cellular resistance to this anti-cancer PG and present a clue to the modulation of the resistance phenotype. Compared with sensitive cells, HL-60/R-CP cells have several-fold higher cellular GSH levels and *GS-X* pump (ATP-dependent glutathione S-conjugate export pump) activity [11,12]. We have claimed that anti-cancer PGs are conjugated with GSH in cells and that the resulting conjugates are subsequently exported from cells by the *GS-X* pump [13,14]. The α,β -unsaturated carbonyl moiety in the cyclopentenone ring of the anti-cancer PGs is a prerequisite for their anti-proliferative activity [15], whereas the enone group readily undergoes Michael addition of thiol groups of cellular constituents, such as GSH, cysteine and proteins. We have recently demonstrated that the reaction of Δ^7 -PGA₁ methyl ester with GSH is reversible and that the anti-cancer PG exists in both glutathione-conjugated and non-conjugated forms in the cell [14]. Excretion of the glutathione conjugate from the cell is considered to be an important mechanism that decreases the cellular accumulation of the anti-cancer PG. The *GS-X* pump is an ATP-dependent export pump for organic anions, such as cysteinyl leukotrienes, GSSG, glutathione conjugates, GSH-metal complexes and glucuronide conjugates. One of the human *GS-X* pumps is encoded by the *MRP1* gene (where MRP stands

Abbreviations used: PG, prostaglandin; *GS-X* pump, ATP-dependent glutathione S-conjugate export pump; Cdk, cyclin-dependent kinase; *GS*-PG, glutathione-(Δ^7 -PGA₁ methyl ester) conjugate; pRB, retinoblastoma protein; MRP, multidrug-resistance-associated protein; GIF-0017, S-[5-(4-benzoylphenyl)pentyl]glutathione; GIF-0019, *N*-carbomethoxy-S-[5-(4-benzoylphenyl)pentyl]glutathione dimethyl ester; cisplatin, *cis*-diamminedichloroplatinum(II); BAPTA/AM, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis-(acetoxymethyl)ester.

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Figure 1 Molecular structures of Δ^7 -PGA₁ methyl ester (1), its GS conjugate (2a), ³H-labelled GS conjugate (2b), GIF-0017 (3) and GIF-0019 (4)

for multidrug-resistance-associated protein) [16,17], and its biological function appears to be closely linked with cellular GSH metabolism [12,18,19]. Although the *MRP1* gene product has been implicated to play a role in cellular resistance to anti-cancer drugs, the actual mechanism is not well understood. In the present study, we have chemically synthesized a potent inhibitor of the *GS-X* pump and examined a potential role of this export pump in the cellular resistance of HL-60/R-CP cells to the anti-cancer PG. Evidence is presented to show that the *GS-X* pump is an important factor in cellular resistance to the anti-cancer PG Δ^7 -PGA₁ methyl ester.

Part of this study, describing the induction of the cyclin-dependent kinase inhibitor p21 by Δ^7 -PGA₁ methyl ester, was presented at the 4th International Conference on Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Radiation Injury, held in Hong Kong in October 1995.

MATERIALS AND METHODS

Biochemicals and enzymes

GSH, GSSG, ATP, phosphocreatine, creatine kinase, PMSF, glutathione reductase and a random-primed labelling kit were purchased from Boehringer Mannheim (Mannheim, Germany). [³H]GSH was obtained from Du Pont–New England Nuclear. [³²P]dCTP was from Amersham Corp. (Arlington Heights, IL, U.S.A.). RPMI 1640 medium, fetal calf serum and gentamicin were from Sigma (St. Louis, MO, U.S.A.).

Δ^7 -PGA₁ methyl ester, GIF-0017 {S-[5-(4-benzoylphenyl)pentyl]-glutathione} and GIF-0019 {N-carbomethoxy-S-[5-(4-benzoylphenyl)pentyl]glutathione dimethyl ester}

Δ^7 -PGA₁ methyl ester (Figure 1, compound 1) was synthesized by the three-component coupling process described previously [8,9]. The chemical synthesis and physical properties of GIF-0017 and GIF-0019 (Figure 1, compounds 3 and 4) will be described separately (K. Furuta, K. Tomokiyo, T. Ishikawa and M. Suzuki, unpublished work; details available on application to the authors). The structures of these GSH adducts were confirmed

definitively by analysis based on H,H-COSY and fast-atom bombardment MS.

Cell culture

Human myelocytic leukaemia HL-60 cells (ATCC no. CCL240), obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were maintained in a humidified chamber (37 °C, 5% CO₂) in RPMI 1640 medium supplemented with glutamine (2 mM), 10% (v/v) heat-inactivated fetal calf serum and gentamicin (50 µg/ml). Cisplatin-resistant HL-60/R-CP cells were established by maintaining HL-60 cells in the presence of cisplatin (Bristol-Myers Squibb, Seattle, WA, U.S.A.), as described previously [11].

Analyses of cell proliferation and cell cycle

The cells (2.5×10^5 cells/ml) were incubated with 5 µM Δ^7 -PGA₁ methyl ester (1) in RPMI-1640 medium containing 10% (v/v) heat-inactivated fetal calf serum and gentamicin (2 µg/ml). The number of cells was determined in a haemocytometer by Trypan Blue dye exclusion. For cell-cycle analysis, HL-60 cells were incubated with 5 µM Δ^7 -PGA₁ methyl ester for 12 or 24 h. Then, 2×10^6 cells were withdrawn from the incubation medium, washed with ice-cold PBS and fixed with 70% (v/v) ethanol. After another washing with PBS, the cell pellets were treated with RNase (1 mg/ml) at 37 °C for 30 min and subsequently stained with propidium iodide (50 µg/ml). The DNA content was determined with an EPICS flow cytometer (Coulter Corp., Hialeah, FL, U.S.A.).

Northern blot hybridization

Total cellular RNA was extracted by using the RNA STAT-60 kit (TEL-TEST B, Friendswood, TX, U.S.A.). RNA samples (10 µg each) were separated by electrophoresis in 1.2% (w/v) agarose/formaldehyde gels and subsequently transferred to Nytran membranes (Schleicher & Schuell Inc., Keene, NH, U.S.A.). cDNA probes for p21, p27^{Kip1}, c-myc (exon 3) and β -actin (Oncor, Gaithersburg, MD, U.S.A.) were labelled with [³²P]dCTP by random-primed labelling (Boehringer Mannheim, Indianapolis, IN, U.S.A.), and Northern blot hybridization was performed as described previously [11].

Immunoblotting to determine the phosphorylation state of retinoblastoma protein (pRB)

HL-60 and HL-60/R-CP cells were incubated with 5 µM Δ^7 -PGA₁ methyl ester (1) for the time periods specified (0, 0.5, 1, 2, 4, 6, 12, 24 and 36 h). The cells (5×10^6) were then withdrawn from the medium, washed with ice-cold PBS and lysed with ice-cold RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 4 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM Na₃VO₄, 1 mM dithiothreitol, 1 mM PMSF, 50 µg/ml aprotinin and 5 µg/ml leupeptin). The cell lysate was centrifuged at 16000 g at 4 °C for 20 min. Protein samples from the supernatant (10 µg each) were separated by SDS/7%-PAGE. After electrophoretic transfer of proteins to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.), the hypophosphorylated and phosphorylated forms of pRB were detected immunologically using the anti-pRB polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and the ECL detection system (Amersham, South Clearbrook, IL, U.S.A.).

Synthesis of [^3H]glutathione-(Δ^7 -PGA $_1$ methyl ester) conjugate (GS-PG)

Dithiothreitol was first removed from the [^3H]glycine-labelled GSH sample (Du Pont-New England Nuclear) by extraction with ethyl acetate under acidic conditions (10 mM HCl, pH 2.2) according to [20]. A 50 μl sample of [^3H]GSH (50 μCi) was mixed with 50 μl of 20 mM non-radiolabelled GSH. The resulting [^3H]GSH was treated with 10 mM Δ^7 -PGA $_1$ methyl ester in 100 μl of 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM EDTA at 37 °C for 6 h. The reaction solution was spotted on to reverse-phase silica gel plates (RP-18 F $_{254\text{S}}$; 0.25 mm; Merck 15685) and developed in a TLC chamber containing ethanol/water/acetic acid (60:40:0.05, by vol.). [^3H]GS-PG (Figure 1, compound **2b**) (R_f = 0.60) was visualized by UV light and extracted from the silica gel in ethanol/water/acetic acid (60:40:0.05, by vol.). The [^3H]GS-PG extract was dried in a Speed-Vac, dissolved in 100 μl of 5 mM acetic acid and stored at -20 °C. The overall yield was 46 %.

Determination of *GS-X* pump activity in plasma membrane vesicles

Plasma membrane vesicles were prepared from HL-60 and HL-60/R-CP cells as described previously [11,12] and stored at -85 °C until use. Frozen stock membrane vesicles were thawed

quickly at 37 °C and stored on ice until used. Of the total membrane vesicles, 42–46 % were inside-out as assessed by the sialidase accessibility assay [21]. The standard incubation medium for the assay of *GS-X* pump activity in the preparation contained plasma membrane vesicles (50 μg of protein), 1 μM [^3H]GS-PG (Figure 1, compound **2b**), 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM MgCl $_2$, 1 mM ATP, 10 mM phosphocreatine and 100 $\mu\text{g}/\text{ml}$ creatine kinase in a final volume of 110 μl . The reaction was started by adding [^3H]GS-PG to the incubation medium. The reaction was carried out at 37 °C, and the amount of [^3H]GS-PG incorporated into the vesicles was measured by the rapid filtration technique, as described previously [21].

Figure 2 Inhibitory effects of Δ^7 -PGA $_1$ methyl ester on cell proliferation and cell-cycle progression

Upper panel: time course of proliferation of human leukaemia HL-60 (●) and HL-60/R-CP (○) cells incubated with 5 μM Δ^7 -PGA $_1$ methyl ester. Data are expressed as means $\pm$ S. D. of triplicate experiments. Lower panel: cell-cycle analysis of HL-60 cells incubated with Δ^7 -PGA $_1$ methyl ester. At time 0, the cell-cycle distribution was 45% (G $_1$ phase), 42% (S phase) and 13% (G $_2$ /M phase). After 12 and 24 h, the cell populations in G $_1$ phase were 53% and 66% respectively.

Figure 3 Induction of p21, hypophosphorylation of pRB and suppression of *c-myc* expression by Δ^7 -PGA $_1$ methyl ester

Upper panels: effects of Δ^7 -PGA $_1$ methyl ester on the expression of p21 and p27 Kip1 in HL-60 cells. HL-60 cells were treated with 5 μM Δ^7 -PGA $_1$ methyl ester for the specified time periods (0, 1, 3, 6, 9, 12 and 24 h). The transcripts of p21 and p27 Kip1 were detected by Northern blot hybridization at 2.1 kb and 2.5 kb respectively. Lower panels: effects of actinomycin D, cycloheximide and BAPTA/AM on p21 induction by Δ^7 -PGA $_1$ methyl ester in HL-60 cells. HL-60 cells were preincubated with actinomycin D (1 $\mu\text{g}/\text{ml}$; Calbiochem) for 30 min, cycloheximide (10 $\mu\text{g}/\text{ml}$; Sigma) for 30 min or BAPTA/AM (20 μM ; Calbiochem) for 3 h. The cells were then incubated with (+) or without (-) 5 μM Δ^7 -PGA $_1$ methyl ester (PG) for 6 h, as indicated. Extraction of total cellular RNA and Northern hybridization for p21 were performed as described above.

Figure 4 Effects of Δ^7 -PGA₁ methyl ester on the phosphorylation state of pRB (upper panel) and on *c-myc* and β -actin mRNA levels (lower panel)

HL-60 and HL-60/R-CP cells were incubated with 5 μ M Δ^7 -PGA₁ methyl ester for the specified time periods. pRB (hypophosphorylated state) and ppRB (phosphorylated state) were detected immunologically as described in the Materials and methods section. Northern blot hybridization was performed using cDNA probes for *c-myc* (exon 3) and β -actin.

RESULTS

Effects of Δ^7 -PGA₁ methyl ester on cell proliferation and the cell cycle

Δ^7 -PGA₁ methyl ester (Figure 1, compound 1) exhibited remarkable biological properties: at a concentration of 1.5 μ M, it suppressed the proliferation of human leukaemia HL-60 cells by 50%, i.e. 3–5-fold more effectively than PGA₂, a natural anti-cancer PG. Figure 2 shows that 5 μ M Δ^7 -PGA₁ methyl ester suppressed the proliferation of HL-60 cells for more than 100 h by blocking them in G₁ phase of the cell cycle. As judged by Wright's staining, no cell differentiation was induced by the synthetic anti-cancer PG (results not shown). On the other hand, HL-60/R-CP cells, which overexpress cellular GSH and the GS-X pump, were resistant to Δ^7 -PGA₁ methyl ester (Figure 2, upper panel).

Effect of Δ^7 -PGA₁ methyl ester on p21 mRNA level

Figure 3 (upper panel) shows that Δ^7 -PGA₁ methyl ester induced p21 expression in HL-60 cells. p21 mRNA was not detectable in HL-60 cells lacking the synthetic anti-cancer PG; however, it increased significantly during incubation (for up to 24 h) with Δ^7 -PGA₁ methyl ester. The induction of p21 by Δ^7 -PGA₁ methyl ester was abolished by actinomycin D (Figure 3, lower panel), but was significantly enhanced by cycloheximide. BAPTA/AM [1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis-(acetoxymethyl)ester], a calcium-chelating agent, had no effect on the mRNA level. Expression of p27^{Kip1}, which is related structurally to p21 and is induced by transforming growth factor- β [22] or cAMP [23], was not affected by Δ^7 -PGA₁ methyl ester in HL-60 cells (Figure 3, upper panel). In the light of these data, the induction of p21 by the synthetic anti-cancer PG seems to require neither cAMP nor cellular calcium. Moreover, levels

Figure 5 ATP-dependent transport of Δ^7 -PGA₁ methyl ester in plasma membrane vesicles derived from HL-60 and HL-60/R-CP cells

Upper panel: time courses of uptake of ³H-labelled GS-PG by plasma membrane vesicles prepared from HL-60 (triangles) and HL-60/R-CP (circles) cells in the absence (closed symbols) or presence (open symbols) of 1 mM ATP. Data are expressed as means $\pm$ S.D. (*n* = 3). Lower panel: Lineweaver-Burk plot of ATP-dependent transport of ³H-labelled GS-PG in plasma membrane vesicles from HL-60/R-CP cells.

of p16^{INK4A} and p18^{INK4C} mRNAs remained unchanged (results not shown), and no p15^{INK4B} expression was detected in the presence or the absence of Δ^7 -PGA₁ methyl ester in HL-60 cells.

Effects of Δ^7 -PGA₁ methyl ester on the phosphorylation state of pRB and expression of the *c-myc* gene

The induction of p21 by the anti-cancer PG correlated with cell-cycle arrest (Figure 2, lower panel) and, importantly, the accumulation of hypophosphorylated pRB in HL-60 cells (Figure 4, upper panel). In HL-60 cells treated with 5 μ M of the synthetic PG, the *c-myc* mRNA level decreased within 2 h, and the suppression of *c-myc* expression lasted for more than 36 h (Figure 4, lower panel). The level of β -actin mRNA remained unchanged (Figure 4, lower panel), suggesting that the effect of Δ^7 -PGA₁ methyl ester is specific for *c-myc* expression. By contrast, in HL-60/R-CP cells resistant to the anti-cancer PG, no hypophosphorylation of pRB was detected (Figure 4, upper panel), whereas *c-myc* expression was suppressed for a short period (< 6 h) and recovered to normal levels after 12 h (Figure 4, lower panel).

Figure 6 Inhibition of ATP-dependent transport of [^3H]GS-PG by GIF-0017 in plasma membrane vesicles prepared from HL-60/R-CP cells

Plasma membrane vesicles were incubated with $1 \mu\text{M}$ [^3H]GS-PG in the absence or presence of GIF-0017 (0.1, 0.3, 1, 3, 10 or $30 \mu\text{M}$) for 10 min, as described in the Materials and methods section. ATP-dependent transport was calculated from the difference between amounts of radioactivity incorporated into membrane vesicles incubated with and without ATP. Data are expressed as means $\pm$ S.D. ($n = 3$).

ATP-dependent transport of GS-PG and its inhibition by GIF-0017

We have recently reported that $\Delta^7\text{-PGA}_1$ methyl ester (Figure 1, compound 1) reacts readily with GSH to form GS-PG (Figure 1, compound 2a) [14]. Figure 5 (upper panel) shows the ATP-dependent transport of ^3H -labelled GS-PG conjugate 2b in plasma membrane vesicles prepared from HL-60 and HL-60/R-CP cells. Uptake of GS-PG by plasma membrane vesicles from HL-60/R-CP cells was remarkably enhanced by ATP, whereas ATP-dependent uptake was less affected in plasma membrane vesicles from HL-60 cells. The initial rate of ATP-dependent transport was 9-fold greater in membrane vesicles from HL-60/R-CP cells than in those from HL-60 cells. The apparent K_m value for the GS-PG conjugate was 110 nM in the plasma membrane vesicles from HL-60/R-CP cells (Figure 5, lower panel). GIF-0017 (Figure 1, compound 3), a potent inhibitor of the *GS-X* pump, dose-dependently inhibited the ATP-dependent transport of GS-PG, with an IC_{50} value of $0.52 \mu\text{M}$, in plasma membrane vesicles from HL-60/R-CP cells (Figure 6).

Sensitization of HL-60/R-CP cells by GIF-0019

GIF-0019 (Figure 1, compound 4), a cell-membrane-permeable derivative of the *GS-X* pump inhibitor GIF-0017, suppressed the proliferation (Figure 7, top panel) and cell-cycle progression (Figure 7, middle panel) of HL-60/R-CP cells during incubation with $\Delta^7\text{-PGA}_1$ methyl ester. When cells were incubated with the PG in the presence of the inhibitor at a concentration of $10 \mu\text{M}$, cell proliferation was suppressed completely over 96 h (Figure 7, top panel) and the cell cycle was concomitantly arrested at G_1 phase (Figure 7, middle panel). The inhibitory effects were detected only in the presence of $\Delta^7\text{-PGA}_1$ methyl ester, but not with the inhibitor alone, suggesting that GIF-0019 is active in sensitizing HL-60/R-CP cells to the anti-cancer PG. In accordance with these observations, GIF-0019 at $10 \mu\text{M}$ enhanced

Figure 7 Effects of GIF-0019 on proliferation and cell cycle of HL-60/R-CP cells

Top panel: time course of proliferation of HL-60/R-CP cells incubated with $5 \mu\text{M}$ $\Delta^7\text{-PGA}_1$ methyl ester (○), $10 \mu\text{M}$ GIF-0019 (▲), $5 \mu\text{M}$ $\Delta^7\text{-PGA}_1$ methyl ester plus $2 \mu\text{M}$ GIF-0019 (△) or $5 \mu\text{M}$ $\Delta^7\text{-PGA}_1$ methyl ester plus $10 \mu\text{M}$ GIF-0019 (□). In the control, cells were incubated in the absence of $\Delta^7\text{-PGA}_1$ methyl ester and GIF-0019 (●). Data are expressed as means $\pm$ S.D. of triplicate experiments. Middle panel: time courses of cell cycles of HL-60/R-CP cells incubated with $5 \mu\text{M}$ $\Delta^7\text{-PGA}_1$ methyl ester plus $10 \mu\text{M}$ GIF-0019. Bottom panel: p21 mRNA levels in HL-60/R-CP cells incubated with $5 \mu\text{M}$ $\Delta^7\text{-PGA}_1$ methyl ester in the absence (left) or presence (right) of $10 \mu\text{M}$ GIF-0019 for different time periods (0, 3, 6, 12, 24 or 48 h).

p21 mRNA levels in HL-60/R-CP cells during incubation with the anti-cancer PG (Figure 7, bottom panel).

DISCUSSION

Biological actions of the synthetic anti-cancer PG

The present study demonstrates that the synthetic anti-cancer PG induced p21 in a p53-independent manner to lead to cell-cycle arrest of cancer cells, and that a *GS-X* pump inhibitor exerted cellular sensitivity to the anti-cancer PG. Evidence is accumulating to show that Cdk inhibitors play a crucial role in regulation of the cell cycle [24–26], and at least seven different Cdk inhibitors have hitherto been identified. The p21 protein reportedly binds both cyclins and Cdks to inhibit phosphorylation of pRB by cyclin–Cdk complexes (i.e. cyclin A–Cdk2, cyclin E–Cdk2, cyclin D1–Cdk4 and cyclin D1–Cdk6) [27–29], and overexpression of this gene causes cell-cycle arrest at G₁ phase [30,31]. The accumulation of hypophosphorylated pRB is an important event in cell-cycle arrest, by virtue of the potent inhibition by this protein of the activity of the E2F family of transcription factors [32,33]. Hypophosphorylated pRB associates with and inactivates E2F-1, resulting in suppression of *c-myc* gene expression [34,35]. Since *c-myc* expression is known to be required for cell proliferation, and especially for cell-cycle transition from G₁ to S phase [36], we considered the long-term suppression of *c-myc* expression to be one of the major mechanisms of the anti-proliferatory actions of Δ^7 -PGA₁ methyl ester in HL-60 cells (Figure 4, lower panel).

p21 has been shown to be involved in p53-mediated G₁ arrest [37,38], which is related to DNA damage responses. It has also been shown that p21 can be induced via a p53-independent pathway(s). The p53-independent induction of p21 is considered to be an important process in cancer cell growth inhibition induced by anti-cancer agents, such as bleomycin, etoposide and mitomycin C [39]. Since the *p53* gene is completely deleted in the HL-60 cells used in the present study, the induction of p21 by our synthetic anti-cancer PG must be mediated by a process independent of the p53 transcription factor. This is accordance with previous studies with PGA₁ [40–42]. Because mutations and deletions of the *p53* gene are the most frequent genetic alterations detected in human cancers, anti-cancer PGs would be efficacious therapeutic agents for the suppression of cancer cell growth.

Role of GSH and of the *GS-X* pump in modulation of the biological activity of the anti-cancer PG

Cisplatin-resistant HL-60/R-CP cells were cross-resistant to the anti-cancer PG (Figure 2, upper panel). It was first speculated that the cisplatin-resistant cells lack a critical component of signal transduction for the suppression of cell proliferation. However, retinoic acid, DMSO and PMA inhibited *c-myc* expression in both HL-60 and HL-60/R-CP cells, leading to cell differentiation [11]. There was no difference between the sensitive and resistant cell lines with respect to the kinetics of *c-myc* suppression elicited by these cell-differentiation-inducing agents [11], suggesting that HL-60/R-CP cells virtually maintain the cell proliferation and differentiation properties of parent HL-60 cells. Thus the mechanism underlying the cellular resistance of HL-60/R-CP cells to the anti-cancer PG is considered to involve other factors, such as increased metabolism and disposition of the PG. While GSH conjugation of anti-cancer PGs, such as PGA₁, can be catalysed by GSH transferases [43], the non-enzymic reaction of Δ^7 -PGA₁ with GSH occurs rapidly. There was no significant difference in GSH transferase activity between HL-60 and HL-60/R-CP cells, as assessed with 1-chloro-2,4-

dinitrobenzene [11]. GSH transferase activity thus does not appear to be a major factor in the difference in cellular sensitivity to Δ^7 -PGA₁ methyl ester between HL-60 and HL-60/R-CP cells.

We have recently demonstrated that the synthetic anti-cancer PG reacts with different thiol groups [14]. Anti-cancer PGs with an α,β -unsaturated carbonyl group are electrophilic and react readily with the thiol groups of proteins and GSH. The GSH conjugates of anti-cancer PGs have no anti-proliferative activities in cancer cells and cannot be transported into nuclei. When the GSH level was lowered by treating cells with buthionine sulfoximine, cellular sensitivity to anti-cancer PGs was significantly enhanced (results not shown; [44]). Anti-cancer PGs with a simple enone group, such as PGA₂, react with GSH; however, the equilibrium favours GSH adduct formation. The simple enone PGs are therefore expected to be present in the cytoplasm predominantly as biologically inactive GSH adducts. On the other hand, Δ^7 -PGA₁, which possesses a cross-conjugated dienone structure, reacts with GSH in a reversible manner, but the equilibrium is shifted to the free PG to a considerable extent [14]. In such a case, the increased GSH level in HL-60/R-CP cells (6-fold higher than in HL-60 cells [11]) is considered to be a major factor in shifting the equilibrium of the reaction of Δ^7 -PGA₁ with GSH towards adduct formation.

HL-60/R-CP cells overexpress the *GS-X* pump, encoded by the *MRP1* gene [12]. Once anti-cancer PGs have been conjugated with GSH in the cell, the resultant conjugates are actively exported from cells by the *GS-X* pump. Transport of the GSH conjugates of PGA₁ in *MRP1*-transfected cells has been demonstrated by Evers et al. [45]. Plasma membrane vesicles from HL-60/R-CP cells transported the GSH adduct of Δ^7 -PGA₁ methyl ester with an apparent K_m value of 110 nM (Figure 5), suggesting that the *GS-X* pump has a high affinity for the GS–PG conjugate. Indeed, the K_m value is comparable with that for leukotriene C₄, an endogenous GSH conjugate. GIF-0017, a potent inhibitor of the *GS-X* pump, effectively inhibited the transport of the GS–PG conjugate in plasma membrane vesicles prepared from HL-60/R-CP cells (Figure 6). GIF-0019, a derivative of GIF-0017 modified to penetrate the cell membrane, elicited cellular sensitivity of HL-60/R-CP cells to Δ^7 -PGA₁ methyl ester (Figure 7, top panel). In experiments using monochlorobimane [11], GIF-0019 inhibited the efflux of the GSH conjugate of monochlorobimane from HL-60/R-CP cells with an IC₅₀ value of 3 μ M (results not shown). These results suggest that, in the cell, GIF-0019 readily undergoes hydrolysis catalysed by esterase to form GIF-0017, which blocks the *GS-X* pump responsible for the extrusion of the GS–PG conjugate from the cell. Because of the reversible reaction between GSH and Δ^7 -PGA₁, the free active form of the PG can be formed from its GSH adduct (Scheme 1). The *GS-X* pump is considered to play a significant role in reducing accumulation of Δ^7 -PGA₁ in the cell, thereby modulating the anti-proliferative activity of the anti-cancer PG.

Anti-cancer PGs, such as PGA and PGJ, are generated from PGE and PGD through enzymic dehydration. In addition, anti-cancer PG-like substances are also biosynthesized from allene oxides of fatty acid hydroperoxides in plants, corals and starfish [46]. It is interesting to speculate that such endogenous anti-cancer PGs and related compounds may be involved in the regulation of cell proliferation, and that GSH and the *GS-X* pump could modulate the biological activity of such compounds in the cell. GSH and the *GS-X* pump are widely distributed in plants, yeasts and animals [47]. In mammals, the *GS-X* pump has been shown to be encoded by at least two different genes, i.e. *MRP1* [16,17,48,49] and *cMOAT* (*MRP2*) [50–52]. In a recent study using erythrocytes from *MRP1* gene knockout mice [45], it was

Scheme 1 Putative pathways of hypophosphorylation of pRB and suppression of *c-myc* expression triggered by anti-cancer-PG-mediated induction of p21, as well as GSH conjugation of the anti-cancer PG and its subsequent elimination via the *GS-X* pump

The putative mechanism of Δ^7 -PGA₁-induced cell cycle arrest involves the induction of p21, the inhibition of Cdk 2, 4 and 6 by p21, and the association of hypophosphorylated pRB with E2F-1, a transcription factor of the *c-myc* gene. GIF-0017 inhibits the elimination of the GSH conjugate of Δ^7 -PGA₁ mediated by the *GS-X* pump, resulting in cellular accumulation of Δ^7 -PGA₁, Cyc, cyclin.

suggested that, besides *MRP1* and *cMOAT* (*MRP2*), one more novel *GS-X* pump may be involved in the transport of the GSH conjugate of PGA₁. By screening databases of expressed sequence tags from human cancer cell lines, at least five MRP homologues have been identified [53]. The biological functions of these MRP homologues and their potential contribution to the transport of anti-cancer PGs remain to be elucidated.

Conclusions

Kikuchi et al. [54] have reported that anti-cancer PGs have significant adjuvant effects to cisplatin in nude mice bearing human ovarian cancer cells. The present study demonstrates that Δ^7 -PGA₁ methyl ester, a synthetic anti-cancer PG, induced p21 in a p53-independent manner to lead to cell-cycle arrest in human leukaemia cells, and that a *GS-X* pump inhibitor exerted cellular sensitivity to the anti-cancer PG. Identifying the target molecule(s) of the anti-cancer PG in the induction of p21 through precise molecular investigations based on organic synthesis and cell biology is the next important step. This in turn may open up a new route to the rational creation of selective anti-cancer agents that regulate the expression of specific genes linked to cancer cell growth, in order to arrest the cell cycle at G₁ phase, providing a new methodology for cancer chemotherapy.

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