Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 1611–1622
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Resistance to phorbol 12-myristate 13-acetate-induced cell growth arrest in an HL60 cell line chronically exposed to a glutathione *S*-transferase π inhibitor

Laurent Gaté, Alexandra Lunk, Kenneth D. Tew*

Department of Pharmacology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA

Received 16 May 2002; accepted 7 November 2002

Abstract

Glutathione *S*-transferase π (GSTπ; EC 2.5.1.18) has been shown recently to be a regulator of mitogen-activated protein kinases (MAPK). We have developed, by chronic exposure of HL60 cells to increasing concentrations of a peptidomimetic GSTπ inhibitor TLK199, a 10-fold resistant cell line (HL60/TLK199). Among the cellular adaptations observed in this cell line was an increase in extracellular signal-regulated kinase (ERK) activity without modification of basal expression levels. Phorbol 12-myristate 13-acetate (PMA) induced monocyte/macrophage cytodifferentiation in both HL60 wild-type (WT) and HL60/TLK199 cells. In contrast, PMA induced a pronounced cell growth inhibition and G_0/G_1 cell cycle arrest in HL60 WT cells, while this differentiating agent had only a mild effect on cell growth without G_0/G_1 cell cycle arrest in HL60/TLK199. This effect was associated with a rapid and sustained activation of ERK (up to 6 hr) in HL60 WT cells but only a transient induction of these kinases (between 30 and 60 min) in HL60/TLK199. Furthermore, treatment of both cell lines with PMA in combination with the protein tyrosine phosphatase inhibitors sodium orthovanadate (OV) or 3,4-dephostatin (DPN) circumvented the resistance to cell growth arrest and potentiated differentiation in HL60/TLK199 but had no effect on HL60 WT cells. The circumvention of the resistance to PMA was associated with a sustained activation of ERK. These data suggest that chronic exposure of HL60 cells to TLK199 alters cellular ERK activation by PMA, which may contribute to the differential response of the WT and resistant cells to PMA.

Keywords: Glutathione *S*-transferase π; Inhibitor; Resistance; Extracellular signal-regulated kinase; Phorbol 12-myristate 13-acetate; Differentiation

1. Introduction

GSTs represent a superfamily of detoxifying enzymes involved in the conjugation of glutathione to electrophilic compounds in order to increase their hydrophilicity and subsequently their cellular export. In mammals, at least six isozyme families α, μ, π, θ, ζ, and ω have been described. Because of their high expression in many tumors as compared to the surrounding normal tissues and their overexpression in a large number of cancer cell lines resistant to chemotherapeutic agents, GSTs have been considered as cancer markers and associated with anticancer drug resistance [1]. However, there is growing evidence that GSTs are also involved in the regulation of stress signaling and resistance to apoptosis by mechanisms independent of their catalytic activity. For example, it has been observed recently that GSTμ interacts with and inhibits ASK1 (apoptosis signaling kinase 1) and consequently prevents apoptosis mediated by this kinase [2]. GSTπ inhibits JNK [3] through ligand binding with the N-terminal fragment of the kinase [4]. In addition, GSTπ may prevent apoptosis induced by H_2O_2 by inhibiting the JNK pathway and stimulating ERK and p38 kinase pathways [5]. TLK117 (Fig. 1; [6]), which was originally synthesized to inhibit GSTπ activity, can destabilize the interaction between GSTπ and JNK. It has also been shown that this drug has an unanticipated myeloproliferative effect dependent upon GSTπ expression. Indeed, treatment of mice wild type for GSTπ expression induced myeloproliferation, while no effect was seen with GSTπ knock-out mice [7]. To gain insight into the mechanism of action of this drug, an HL60

* Corresponding author. Tel.: +1-215-728-3137; fax: +1-215-728-4333.
E-mail address: KD_Tew@fccc.edu (K.D. Tew).

Abbreviations: DPN, 3,4-dephostatin; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; OV, sodium orthovanadate; PMA, phorbol 12-myristate 13-acetate; TBS, Tris-buffered saline; WT, wild type.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(03)00152-7

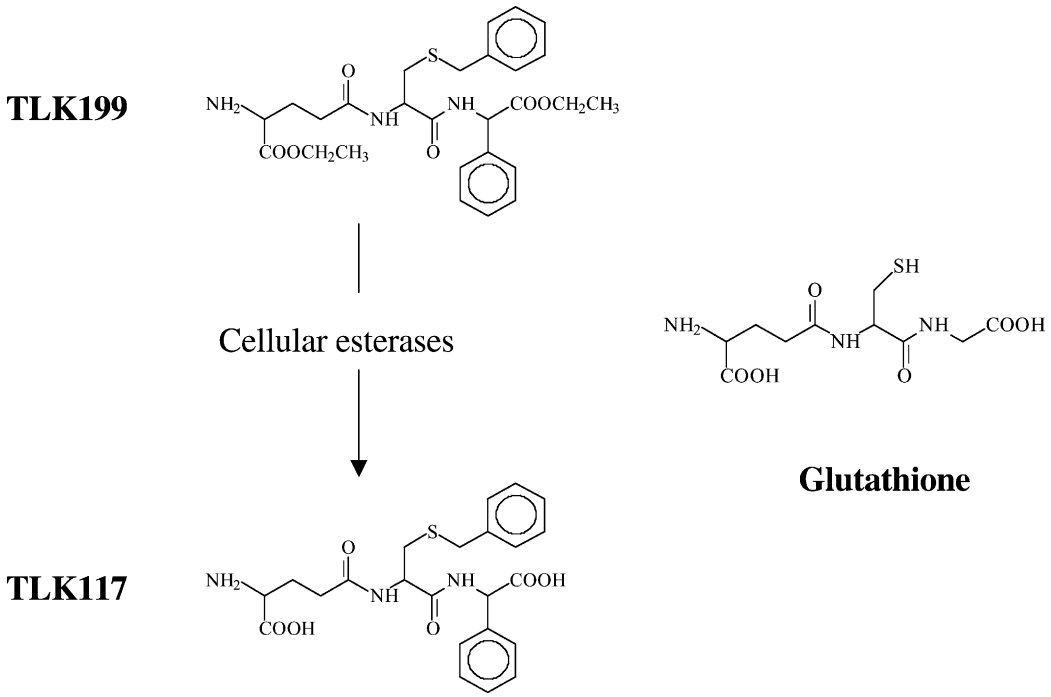

Fig. 1. Structures of TLK199, TLK117, and glutathione.

cell line resistant to TLK199, a cell permeable form of TLK117, was developed by using chronic exposure to increasing concentrations of this drug. Among the various cellular adaptations observed, a higher expression of JNK1 and a higher basal activity of ERK were found [7], suggesting a possible link between drug action (and consequently GSTπ) and the regulation of MAPK pathways.

The activation of MAPK pathways in response to various physiological stimuli as well as environmental stress can lead to multiple cellular responses, which may include apoptosis, cell proliferation, and differentiation [8]. The differentiation of promyelocytic HL60 cells into monocytes/macrophages is characterized by specific morphological changes and by the expression of specific markers including CD11b and CD14 [9,10] and by the activation of protein kinase C β-isoforms [11]. This process is also associated with a G_0/G_1 cell cycle arrest induced by the overexpression of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$ [12]. The activation of ERK, but not JNK and p38 kinase, pathways has also been observed during PMA-induced macrophage-like differentiation of HL60 cells [12]. By using a specific inhibitor of MEK1, an upstream activator of ERK, it has been shown that ERK pathway activation is required in this process [12].

The ability of HL60 to differentiate in response to PMA has provided a widely used model for studying cytodifferentiation. Because of the ongoing development of TLK199 as a myeloproliferative agent, the study of chemically induced differentiation of HL60/TLK199 cells provides a valuable model for gaining a better understanding of the chronic effect of this drug during myeloid proliferation and differentiation. In addition, resistant cell lines have been used extensively to better characterize the mechanism(s) of action of drugs. The implication that pharmacological manipulation of GSTπ might influence critical signaling pathways involved in the regulation of proliferation and differentiation provided the framework for the present study. In addition, the capacity of HL60/TLK199 cells to proliferate after PMA-induced monocyte/macrophage-like differentiation suggests that cell differentiation and cell growth arrest may occur through independent mechanisms. This cell line provides an interesting model to distinguish mechanisms specific for differentiation and those particular to cell growth arrest during chemically induced cytodifferentiation of HL60.

2. Materials and methods

2.1. Cell lines

HL60 cells are currently grown in RPMI 1640 containing 10% fetal bovine serum, 4 mM glutamine, and 10 μg streptomycin and 10 IU penicillin, all obtained from Mediatech. HL60 cells that are 10-fold resistant to the GST inhibitor TLK199 (HL60/TLK199) have been established by chronic exposure of HL60 WT cells to increasing concentrations of this drug.

2.2. Chemicals and antibodies

The GSTπ inhibitor TLK199 was supplied by Telik; PMA and OV were obtained from Sigma; DPN was from Calbiochem. ERK2, p-ERK1/2, MKP-1, MKP-3, and p21 antibodies were obtained from Santa Cruz Biotechnologies;

the actin antibody was purchased from Sigma, and secondary antibodies conjugated to horseradish peroxidase were obtained from Amersham Pharmacia Biotech.

2.3. Cell growth assays

To determine cell growth inhibition induced by PMA, 5×10^4/mL of either HL60 WT or HL60/TLK199 cells were plated in complete medium with or without PMA in the presence or absence of the different inhibitors and incubated at 37° for 48 hr. After treatment, cells were scraped to detach those HL60 cells that had taken on a macrophage-like phenotype and were counted using a Coulter Counter (Coultronics Corp.).

To analyze the rate of cell growth following PMA treatment, 5×10^4 cells/mL of both cell lines were plated in complete medium with or without 10 nM PMA in the presence or absence of 10 μM TLK199. Cells were counted every 24 hr as described above.

2.4. Differentiation marker expression

Expression of CD11b and CD14 surface markers was analyzed by flow cytometry. HL60 WT and HL60/TLK199 cells (5×10^4/mL) were incubated for 48 hr with 10 nM PMA in the presence or absence of TLK199, OV, or DPN. Cells were then collected and washed twice in ice-cold PBS containing 1% bovine serum albumin and 0.1% sodium azide (FACS buffer). After suspending the final pellets in 100 μL of FACS buffer and 20 μL of mouse anti-human CD11b PE-conjugate antibody and 20 μL of mouse anti-human CD14 FITC-conjugate antibody (BD Immunocytometry Systems), cells were incubated for 30 min at 4°. Cells were washed and finally resuspended in 250 μL of FACS buffer. Samples were sorted by flow cytometry using a FACScan flow cytometer (Beckman), and data were analyzed using CellQuest software. Cell populations were gated using isotyping antibodies (Sigma) as negative controls.

2.5. Cell sorting and proliferation of HL60/TLK199

HL60/TLK199 cells were exposed to 10 nM PMA for 48 hr. Cells were marked with CD11b-PE and CD14-FITC antibodies as described above. Then, double positive (DP) cells expressing both CD11b and CD14 and double negative cells (DN), which did not express either of these markers, were sorted using a FACS-vantage flow cytometer (Beckman) and collected in sterile Eppendorf tubes. Cells were counted, replated at 5×10^4/mL in complete medium, and then counted 48 hr later using a Coulter Counter.

2.6. Cell cycle analysis

Cells were treated as described earlier, collected, and washed twice with ice-cold PBS. The resulting pellets were resuspended in 70% ethanol and incubated at −20° overnight. Cells were centrifuged for 10 min at 400 *g* at 4° and pellets were resuspended in PBS containing propidium iodide and RNase A and incubated at room temperature for 30 min in the dark. Finally, flow cytometry was used to estimate the percentage of cells in each phase of the cycle by using Mac Cycle software (Phoenix Flow System).

2.7. Cellular pharmacokinetics of PMA

2.7.1. Cell accumulation

HL60 WT and HL60/TLK199 cells were exposed to 10 nM PMA containing 0.1 μCi [^{3}H]PMA (ICN Pharmaceuticals). At various time points, cells were collected at 4° and washed twice in ice-cold PBS, and the intracellular [^{3}H]PMA was quantified using a scintillation counter (Beckman).

2.7.2. Cell efflux

HL60 WT and HL60/TLK199 cells were exposed for 2 hr to 10 nM PMA containing 0.1 μCi [^{3}H]PMA. Cells were washed twice with ice-cold PBS and reincubated in drug-free medium at 37°. Cells were collected at various time points and washed twice with ice-cold PBS; the intracellular [^{3}H]PMA was quantified using a scintillation counter.

2.8. Immunoblotting

Following treatments, cells were collected and washed twice in ice-cold PBS. The final pellets were resuspended in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM OV] and incubated for 30 min on ice. Lysates were sonicated three times for 10 sec and centrifuged for 20 min at 10,000 *g* at 4°. The resulting supernatants were considered as whole cell extracts. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad). Equal amounts of protein were separated on SDS–polyacrylamide gels and transferred overnight on polyvinylidene fluoride (PVDF) membranes (NEN). Protein expression was determined by using specific primary and secondary antibodies. Briefly, after transfer, membranes were incubated in TBS-Tween containing 5% skim milk, washed with TBS-Tween, and incubated with primary antibody in TBS-Tween containing 5% skim milk for 1 hr. Membranes were washed twice with TBS-Tween, incubated with secondary antibody in TBS-Tween containing 5% skim milk for 1 hr, and washed three times with TBS-Tween. Specific proteins were revealed by chemiluminescence using ECL western blotting reagents from Amersham Pharmacia Biotech.

2.9. Reverse transcription and polymerase chain reaction (RT–PCR)

Following PMA treatment, cells were collected and washed twice with ice-cold PBS; then total RNA was

extracted with an RNeasy mini kit (Qiagen), as described by the manufacturer. RNA were reverse transcribed as follows: 1 μg of RNA was mixed with 1 μL of 0.5 μg/μL of Random Hexamer and incubated for 10 min at 70°. The sample was mixed with 4 μL of first strand buffer, 2 μL of 0.1 M dithiothreitol, 1 μL of dNTP mix, and 1 μL of Superscript II (Invitrogen) and was incubated for 1 hr at 42° and for 15 min at 70°. cDNA (50 ng) was amplified by PCR using a cDNA Advantage Taq polymerase kit (BD Clontech). PCR products were separated on 1% agarose gels containing ethidium bromide, and bands were visualized by UV illumination. The primers used for these experiments were: 5′-CCATGGTCATGGAAGTGG-CAC-3′ and 5′-GGGCAGGAAGCCGAAAACGC-3′ for MKP-1; 5′-GATAGATACGCTCAGACCCG-3′ and 5′-CCGAGGAAGAGTCGGAGCTGATCC-3′ for MKP-3; and 5′-GGATAGCAACGTACATGGCTGG-3′ and 5′-GGACGACATGGAGAAAATCTGG-3′ for actin.

3. Results

3.1. PMA-induced cell growth inhibition in HL60 WT and HL60/TLK199 cells

Cell growth curves in the presence or absence of PMA + TLK199 are shown in Fig. 2. A concentration–response curve showed that in either the presence or absence of 10 μM TLK199, HL60 WT cells were markedly more sensitive to PMA (Fig. 2A). A time-course experiment (Fig. 2B) confirmed that TLK199 was not cytotoxic at 10 μM in either HL60 WT or HL60/TLK199 cells.

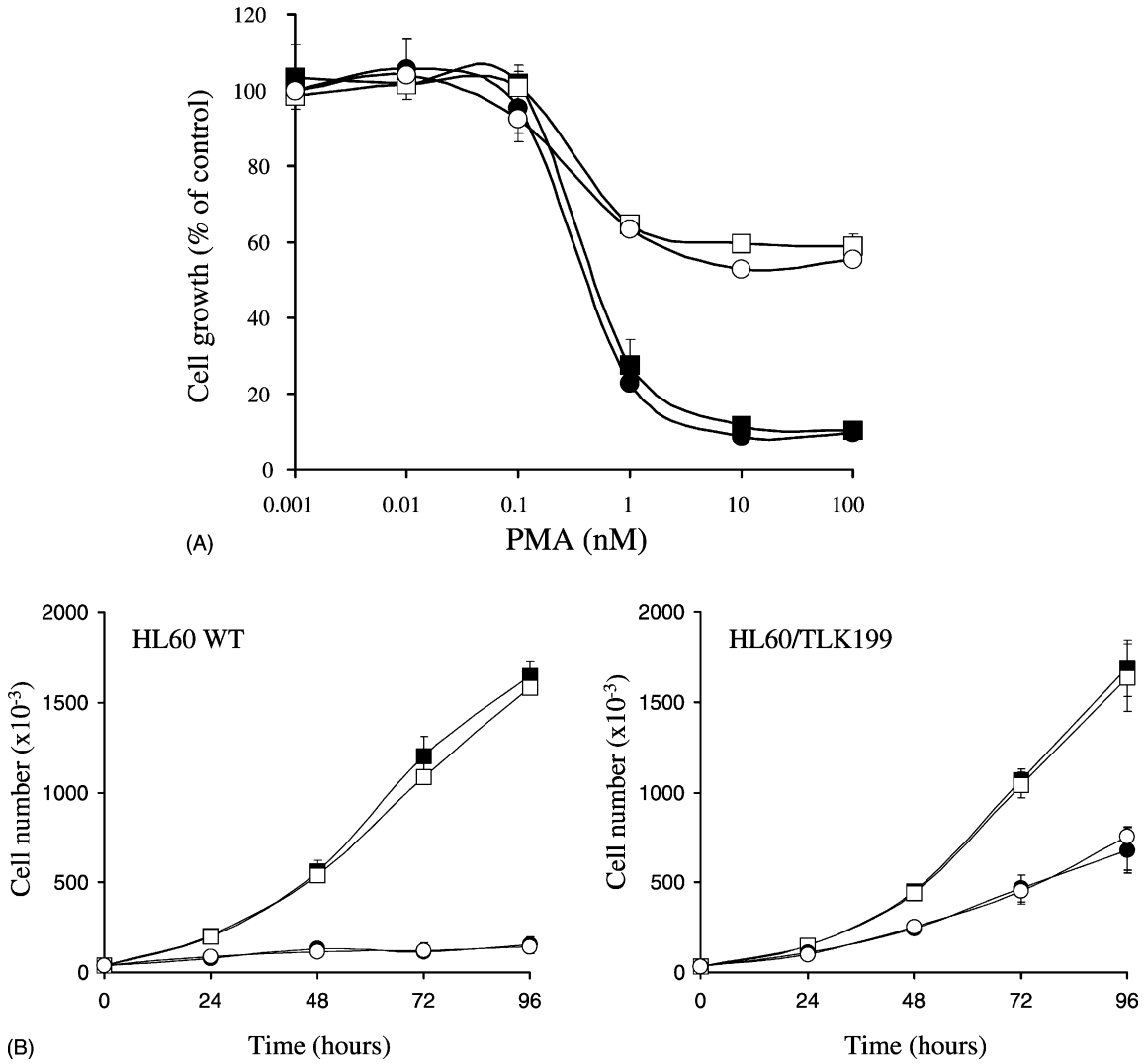

Fig. 2. (A) Effect of increasing concentrations of PMA alone or in combination with 10 μM TLK199 on HL60 WT and HL60/TLK199 cell growth. Key: (■) HL60 WT; (●) HL60 WT + TLK199; (□) HL60/TLK199; and (○) HL60/TLK199 + TLK199. (B) Cell proliferation of HL60 WT and HL60/TLK199 treated with PMA (10 nM) in the presence or absence of TLK199 (10 μM). Key: (■) untreated; (●) PMA; (□) TLK199; and (○) PMA + TLK199. Cells (5×10^4/mL) were exposed to the various agents for 48 hr and counted using a Coulter Counter. Values are means ± SD, N = 3. These graphs are representative of 3 independent experiments.

Table 1
Influence of TLK199, OV, and DPN on PMA-induced cell growth inhibition and cell cycle profile modification in the HL60WT and HL60/TLK199 cell lines

Treatment	Cell cycle (% of total cells)		
	G_0/G_1	S	G_2/M
HL60 WT			
Untreated	41.0 ± 0.6	48.3 ± 1.0	10.6 ± 1.4
PMA (10 nM)	86.5 ± 0.6	6.2 ± 0.4	7.2 ± 1.7
TLK199 (10 μM)	34.9 ± 1.1	51.8 ± 0.8	13.1 ± 1.6
TLK199 + PMA	70.7 ± 1.7	14.3 ± 0.7	14.9 ± 0.9
OV (10 μM)	40.6 ± 0.4	47.0 ± 0.8	12.3 ± 0.7
OV + PMA	79.4 ± 0.3	4.6 ± 0.6	15.9 ± 0.9
DPN (10 μM)	33.9 ± 0.4	51.2 ± 1.3	14.9 ± 1.1
DPN + PMA	74.7 ± 0.6	6.1 ± 0.2	19.1 ± 0.4
HL60/TLK199			
Untreated	33.7 ± 0.9	54.0 ± 0.8	12.0 ± 0.2
PMA (10 nM)	44.0 ± 1.1	45.7 ± 1.4	10.4 ± 1.1
TLK199 (10 μM)	38.0 ± 0.4	52.3 ± 1.1	10.6 ± 1.4
TLK199 + PMA	37.6 ± 0.8	51.3 ± 1.3	11.5 ± 0.5
OV (10 μM)	37.0 ± 5.4	51.4 ± 3.2	11.6 ± 2.7
OV + PMA	70.0 ± 1.3	19.0 ± 0.9	15.8 ± 0.4
DPN (10 μM)	36.3 ± 5.6	51.1 ± 1.6	12.4 ± 4.2
DPN + PMA	68.4 ± 1.7	18.2 ± 0.9	13.3 ± 1.0

Values represent means ± SD, N = 3, and are representative of 3 independent experiments.

Cell growth in HL60 WT was suppressed completely by 10 nM PMA treatment, while a degree of resistance was shown in HL60/TLK199 cells (Fig. 2B, right panel). PMA-induced inhibition of cell growth has been correlated with a G_0/G_1 cell cycle arrest. As shown in Table 1, in untreated HL60 WT and HL60/TLK199 cells, the majority of cells were in the S phase (48.3 ± 1.0 and 54.0 ± 0.8%, respectively). Following PMA treatment, most of the HL60 WT cells were blocked in G_0/G_1 (86.5 ± 0.6%). Under the same conditions, only a slight increase in the number of HL60/TLK199 cells in G_0/G_1 was observed. Co-treatment of cells with TLK199 did not influence these profiles.

3.2. PMA-induced expression of CD11b and CD14 surface markers

The effect of drugs on differentiation status was assessed by flow cytometry measurements of the expression of monocyte/macrophage specific markers CD11b and CD14 (Fig. 3). The percentage of cells expressing these membrane proteins was low in both untreated HL60 WT and HL60/TLK199 cells. In contrast, after 48 hr of treatment with PMA, nearly 40% of both HL60 WT and HL60/TLK199 cells expressed CD11b and CD14. Co-treatment with TLK199 did not alter the PMA effect.

3.3. Proliferative capabilities of HL60/TLK199 CD11b/CD14 double positive cells following PMA treatment

Forty-eight hours after PMA exposure, double positive (DP) and double negative (DN) HL60/TLK199 cells were sorted out to assess their proliferative capabilities. As shown in Fig. 4, DP cells proliferated more slowly than the entire population exposed to PMA or the DN cells. In contrast, DN cells, which do not express any differentiation markers, proliferated faster than the whole population of

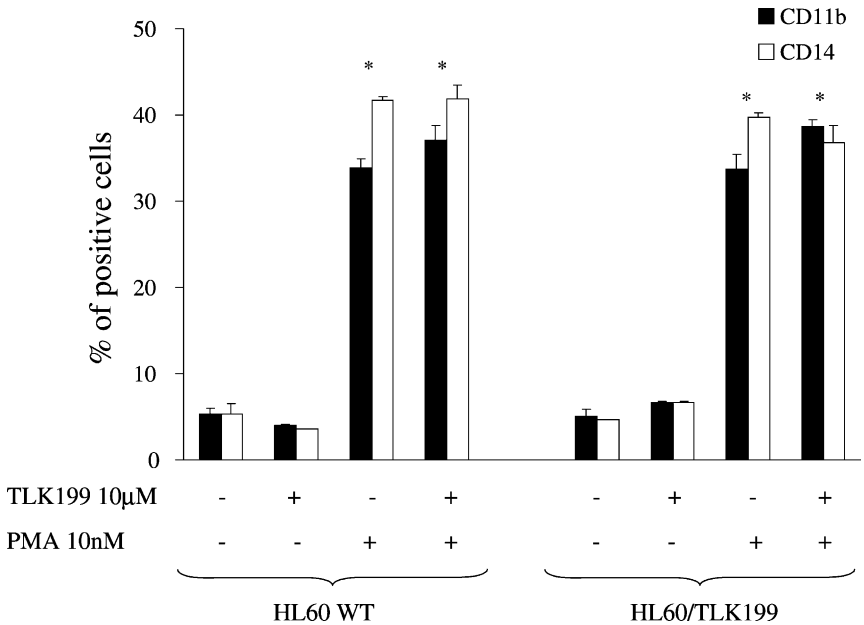

Fig. 3. Effect of PMA alone or in combination with TLK199 on the expression of the differentiation markers CD11b and CD14 in HL60 WT and HL60/TLK199 cells. Cells were exposed for 48 hr to the various agents, and the expression of the differentiation markers was analyzed by flow cytometry using specific antibodies as described in "Section 2." Values represent means ± SD, N = 3. This histogram is representative of 3 independent experiments. Key: (*) significantly different from the untreated control, $P < 0.01$.

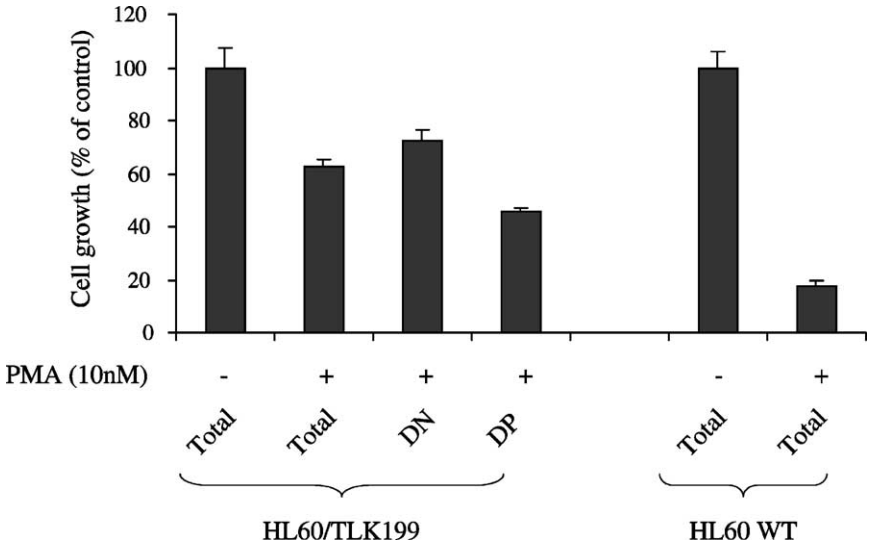

Fig. 4. Proliferation of untreated HL60/TLK199 and PMA-treated CD11b/CD14 double positive (DP) and double negative (DN) HL60/TLK199 cells. Following PMA treatment, DP and DN cells were sorted by flow cytometry and analyzed for their capacity to proliferate in culture. Cells (5×10^4/mL) were exposed to 10 nM PMA for 48 hr and counted using a Coulter Counter. Values are means ± SD, N = 3. This histogram is representative of 3 independent experiments.

treated cells. However, DP cells still grew faster than HL60 WT cells exposed to similar doses of PMA. These results indicate that despite the appearance of markers of terminal differentiation, DP HL60/TLK199 cells can still proliferate.

3.4. PMA-induced $p21^{WAF1}$ and $p27^{KIP1}$ expression

The G_0/G_1 cell cycle arrest induced by PMA generally correlates with increased expression of the cyclin kinase-dependent inhibitors $p21^{WAF1}$ and $p27^{KIP1}$. The basal expression of these two proteins was almost undetectable in untreated cells; however, exposure to PMA for 24 hr resulted in an enhanced expression that was higher in HL60 WT than in HL60/TLK199 cells (Fig. 5). TLK199 as a single agent had no effect on the basal or PMA-induced expression of these two proteins (Fig. 5).

3.5. PMA cellular pharmacokinetics in HL60 cell lines

Because of the altered expression of certain ABC transporters in HL60/TLK199 cells [7,13], we analyzed the transport of radiolabeled PMA in both cell lines. Panels A and B of Fig. 6 showed that PMA was incorporated and effluxed at equivalent rates in wild-type and resistant cells.

3.6. PMA-induced activation of ERK in wild-type and resistant HL60

Induction of monocyte/macrophage differentiation by PMA requires activation of the ERK pathway. The HL60/TLK199 cells presented a higher ERK enzyme activity (phosphorylation of ERK or ELK1) compared to HL60 WT cells without altered expression of the basal protein levels

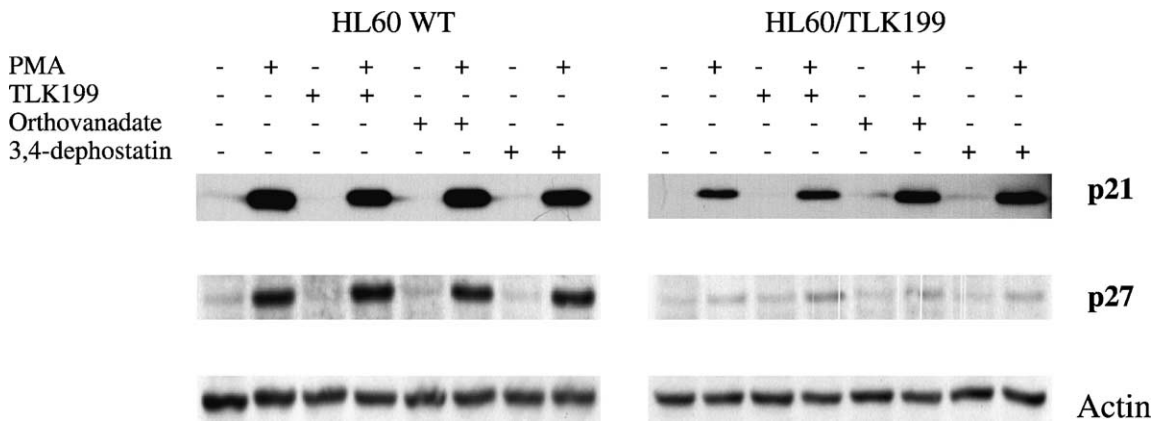

Fig. 5. Expression of p21 and p27 in HL60 WT and HL60/TLK199 cells treated with or without PMA in the presence or absence of TLK199, OV, or DPN. Cells were exposed for 24 hr to PMA in the presence or absence of the different inhibitors. They were then collected, and protein samples were submitted to electrophoresis and immunoblotting as described in "Section 2." Actin was used as a loading control.

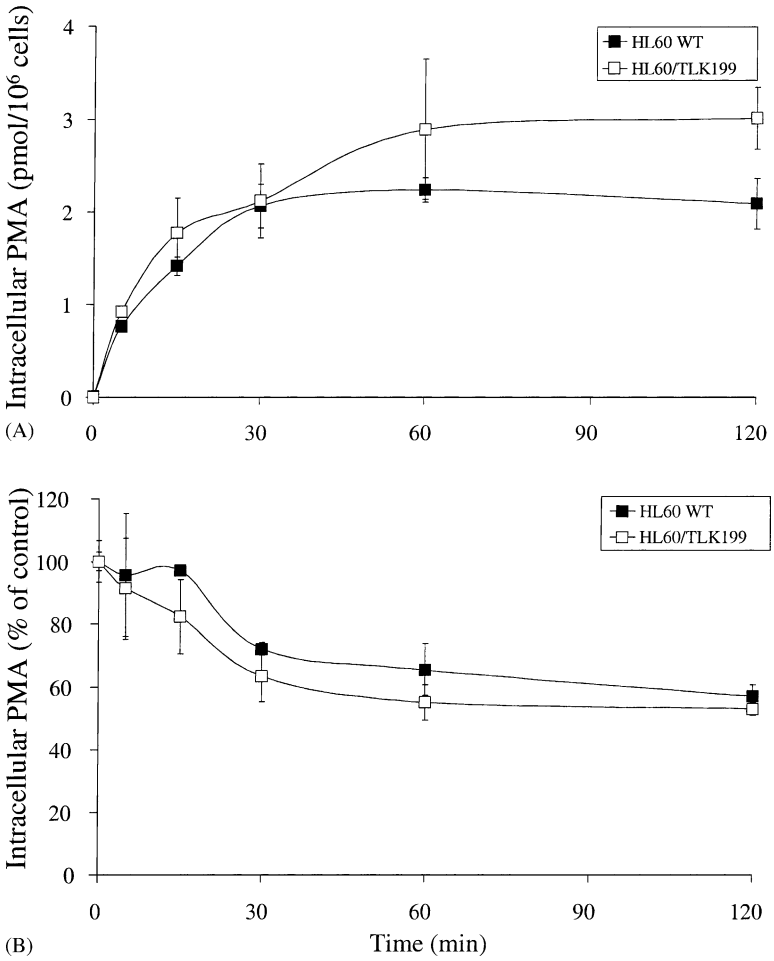

Fig. 6. (A) Accumulation of PMA in HL60 WT and HL60/TLK199 cells. (B) Efflux of PMA in wild-type and resistant cells. Values are means ± SD, N = 3. These graphs are representative of 3 independent experiments.

of these kinases (Fig. 7A). Treatment of HL60 WT with PMA induced a rapid (after 5 min) and sustained (up to 360 min) activation of the enzymatic activity of these kinases (Fig. 7B). In contrast, PMA induced only a transient and lower activation of ERK (between 30 and 60 min) in HL60/TLK199 cells. PMA did not alter the basal expression of ERK1/2 in either cell line (Fig. 7, lower panels). Similar time courses of ERK activation were observed when cells were co-treated with PMA and TLK199 (Fig. 7, right panels).

3.7. Effect of OV and DPN on PMA-induced cell growth inhibition

The ERK pathway is tightly regulated by various tyrosine phosphatases that may be inhibited by either OV or DPN. HL60 WT and HL60/TLK199 cells were pretreated for 4 hr with a 10 μM concentration of either OV or DPN and then incubated with PMA (Fig. 8). The drug combinations did not change the growth inhibitory effects of PMA in the WT cells. In contrast, in HL60/TLK199 cells co-treatment with the phosphatase inhibitors potentiated cell growth inhibition induced by PMA (48.2 ± 2.4% without phosphatase inhibitors versus 12.1 ± 0.8 or 8.5 ± 0.9 with OV or DPN, respectively). The phosphatase inhibitors had no effect on the G_0/G_1 cell cycle arrest induced by PMA in HL60 WT. However, in HL60/TLK199, while PMA alone induced only a slight increase in the percentage of cells in G_0/G_1, co-treatment with OV or DPN induced a pronounced G_0/G_1 cell cycle arrest (Table 1). Neither OV nor DPN modified the enhanced expression of p21 and p27 caused by PMA treatment in HL60 WT. In contrast, in HL60/TLK199, these phosphatase inhibitors increased the expression of p21 induced by PMA (Fig. 5).

3.8. Influence of OV and DPN on PMA-induced CD11b and CD14 expression

The influence of these phosphatase inhibitors on the expression of differentiation markers CD11b and CD14 was also assessed (Fig. 9). In untreated HL60 cells, either OV or DPN slightly decreased the expression of CD11b and CD14. Following PMA treatment, the increase of CD14 but not CD11b positive cells observed in HL60 WT was potentiated significantly by either OV or DPN.

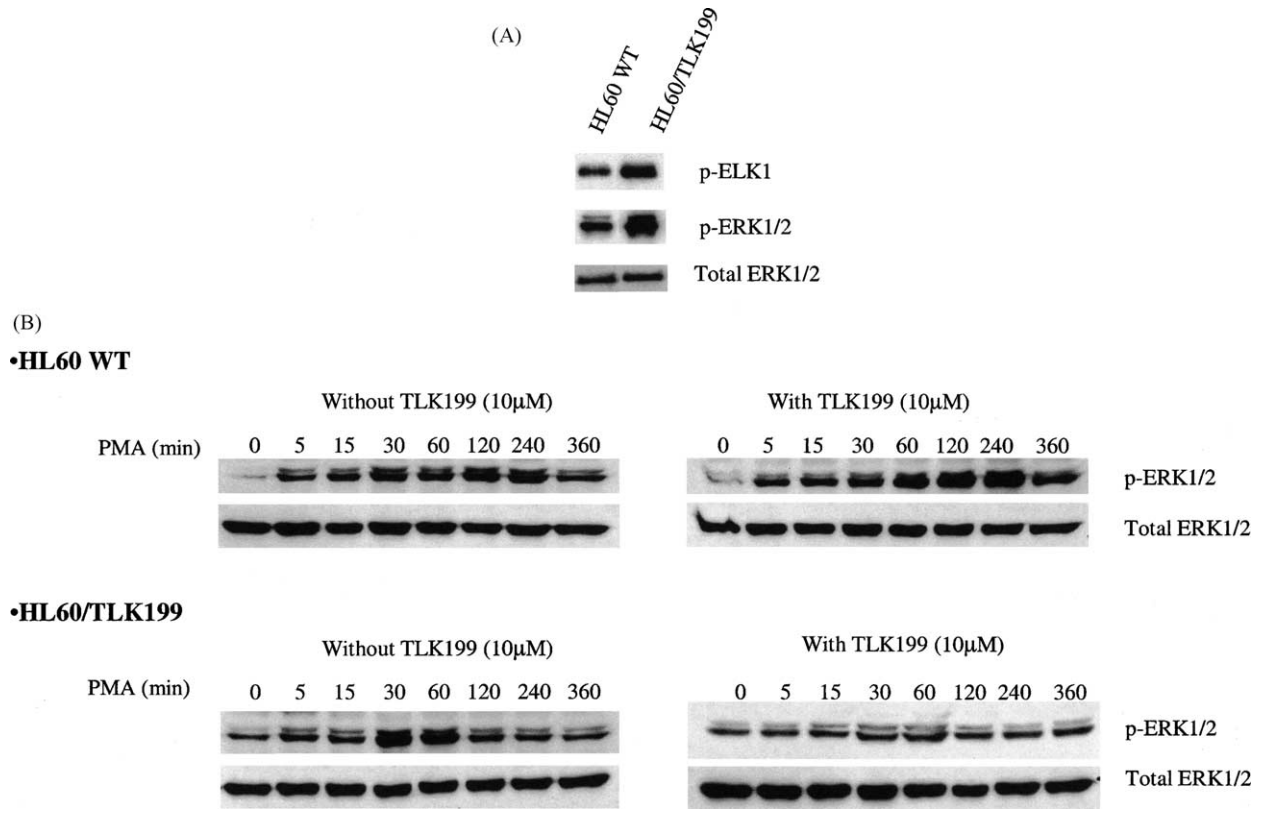

Fig. 7. (A) Basal expression and activity of ERK in HL60 WT and HL60/TLK199 cells. (B) Effect of PMA on ERK phosphorylation and expression in HL60 WT and HL60/TLK199 cells in the presence or absence of TLK199. Cells were pretreated with TLK199 and then were exposed to PMA and collected at various time points; the phosphorylation and expression of ERK were evaluated by immunoblotting.

In HL60/TLK199 cells, treatment with OV or DPN, as a single agent, induced an increase of CD11b and CD14 positive cells. Also in HL60/TLK199, with concomitant PMA treatment, OV or DPN potentiated the expression of CD11b and CD14.

3.9. Effect of OV and DPN on PMA-induced activation of ERK

Analysis of ERK activation showed that pretreatment of HL60 cells with either OV or DPN did not alter the time

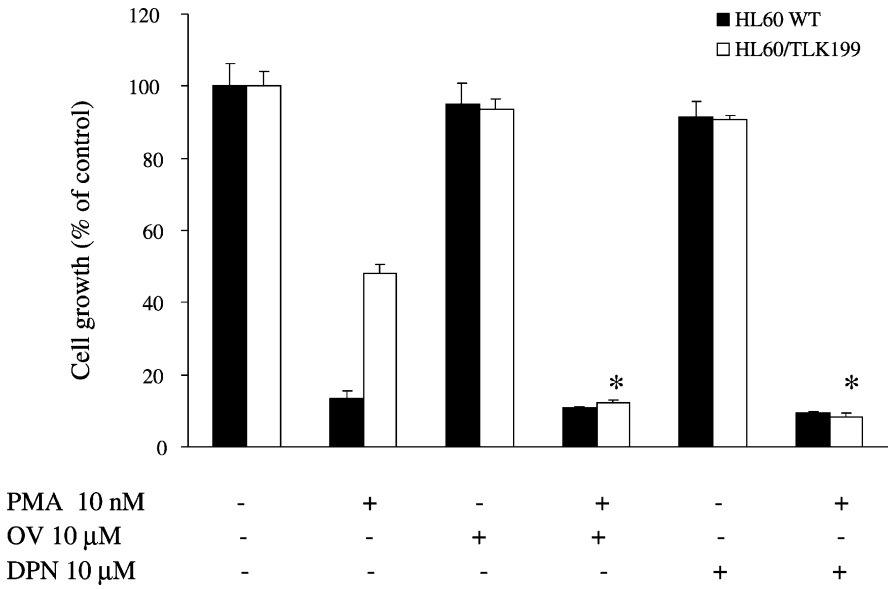

Fig. 8. Cell growth inhibition induced by PMA in the HL60 WT and HL60/TLK199 cell lines in the presence or absence of OV or DPN. Cells (5×10^4/mL) were pretreated for 4 hr with OV or DPN and then were exposed to PMA for 48 hr. Cells were counted using a Coulter Counter. Values are means ± SD, N = 3. These graphs are representative of 3 independent experiments. Key: (*) significantly different from PMA alone, $P < 0.01$.

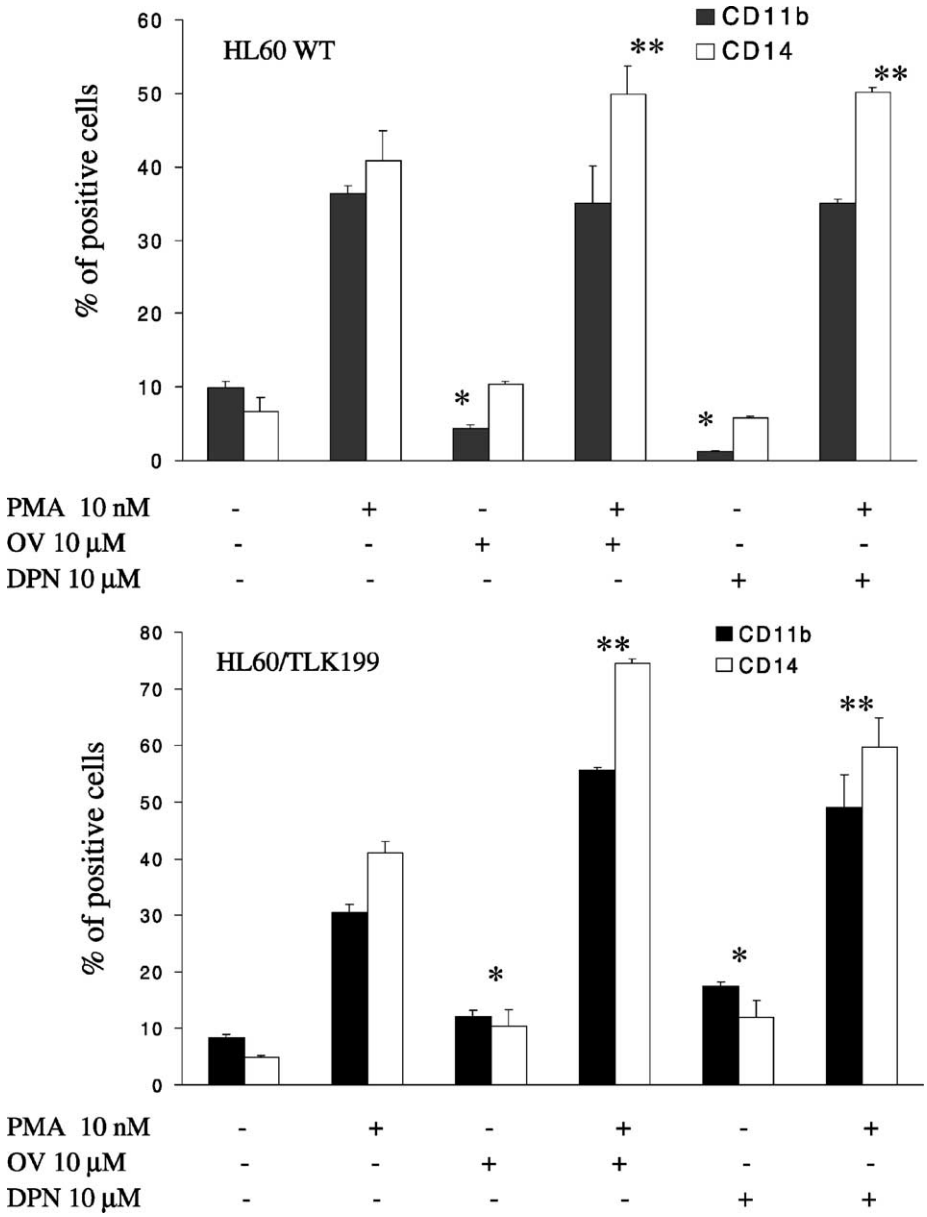

Fig. 9. Modulation of CD11b and CD14 expression by OV and DPN in HL60 WT and HL60/TLK199 cells with or without PMA. Cells were pretreated with OV or DPN for 4 hr and exposed for 48 hr to PMA. Expression of the differentiation markers was analyzed by flow cytometry using specific antibodies as described in "Section 2." Values are means ± SD, N = 3. These histograms are representative of 3 independent experiments. Key: (*) significantly different from the untreated control, $P < 0.05$; and (**) significantly different from PMA alone, $P < 0.01$.

course of sustained activation of ERK following PMA (Fig. 10). In contrast, in resistant cells, while PMA alone induced only a transient activation of ERKs (see Fig. 7), after the pretreatment of the HL60/TLK199 with either OV or DPN, PMA induced a sustained activation of these kinases similar to the one observed in HL60 WT (Fig. 10).

3.10. Expression of MKP-1 and MKP-3 in HL60 WT and HL60/TLK199 cells

The dual specific phosphatases MKP-1 and MKP-3 are involved in the deactivation of ERK and are possible targets for inhibition by OV and DPN. Therefore, due to the transient nature of ERK phosphorylation in HL60/TLK199 cells, we examined the expression levels of these two phosphatases in HL60 WT and HL60/TLK199 cells, and found a 2-fold elevated expression of the MKP-1 in the HL60/TLK199 resistant cell line (Fig. 11A). In addition, the exposure of HL60 WT cells to PMA induced a rapid increase of MKP-1 RNA and protein levels, while in resistant cells similar treatment did not modulate the expression of this phosphatase (Fig. 11A and B). In contrast, the expression of MKP-3, which is similar in both cell lines, was not changed following PMA treatment (Fig. 11A and B).

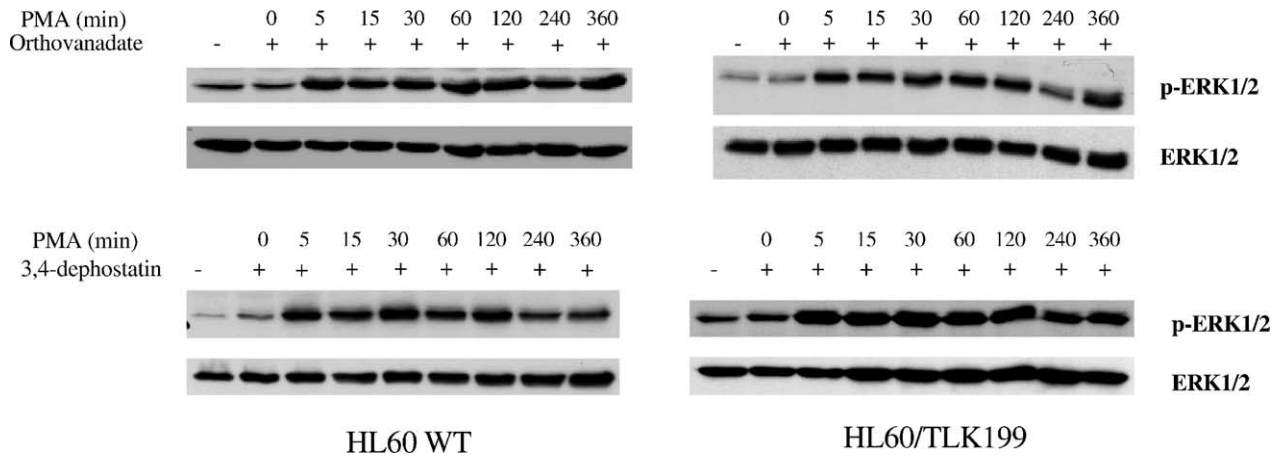

Fig. 10. Modulation by OV or DPN of the expression and activation of ERK in HL60 WT and HL60/TLK199 cells treated with PMA. Cells were pretreated with OV or DPN and then exposed to PMA and collected at various time points; the phosphorylation and expression of ERK were evaluated by immunoblotting.

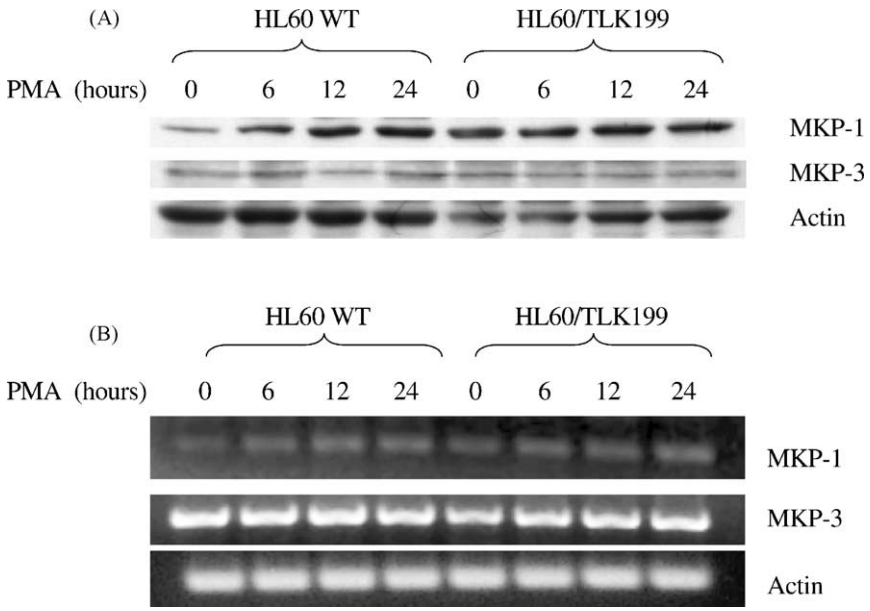

Fig. 11. (A) Basal and PMA-induced protein levels of MKP-1 and MKP-3 in HL60 WT and HL60/TLK199 cells. (B) RNA levels of MKP-1 and MKP-3 in wild-type and resistant HL60 exposed to PMA. Actin was used as a loading control.

4. Discussion

The peptidomimetic TLK199 is a GSTπ inhibitor that is currently under development as a myeloproliferative agent. Administration of this drug to wild-type mice, but not to animals null for GSTπ, increases the number of circulating white blood cells, and can stimulate *ex vivo* myeloproliferation of $GST\pi^{+/+}$ bone marrow cells but not $GST\pi^{-/-}$ [7]. To gain an understanding of the mechanism of action of TLK199, an HL60 cell line has been made resistant to the drug by chronic exposure to escalating concentrations. The resultant phenotype presents multiple alterations including the overexpression of JNK1 [7], which interacts with, and is inhibited by, GSTπ [4]. Additionally, increased ERK activity was observed without modification of basal protein expression (Fig. 7A). These modulations of expression and/or activity of proteins involved in MAPK pathways in HL60/TLK199 cells are consistent with the involvement of GSTπ in the regulation of kinases critical to cell survival pathways. As mentioned above, GSTπ is able to inhibit JNK activation in unstressed cells [4] by interacting with its N-terminal moiety [5]. Because of the critical importance of MAP kinases in the regulation of apoptosis, proliferation, and differentiation, an implied role for GSTπ in these processes is inferred. For example, GSTπ is able to protect mouse fibroblasts (NIH 3T3 cells) against apoptosis by stimulating the activation of ERK and p38 kinase pathways and by inhibiting JNK1 [6].

Drug-induced cell differentiation of myeloid cancer cells represents a potential alternative to conventional chemotherapy. It has been suggested that differentiation of HL60 cells induced by PMA, 1,25-dihydroxyvitamin D_3, or all-*trans* retinoic acid requires ERK activation [10,12]. PMA-induced monocyte/macrophage-like differentiation of HL60 represents an interesting model to analyze the influence of acute and chronic exposure of TLK199 on this process. In addition, chronic exposure to a drug tends to mimic *in vivo* conditions when a therapeutic molecule is administered to animals or patients. Any link between the myeloproliferative effects of TLK199 and differentiation may be dissected through the use of this model system. We observed that HL60/TLK199 cells were resistant to PMA-induced cell growth arrest but not to PMA-induced cell differentiation. In addition, following PMA treatment, the HL60/TLK199 CD11b/CD14 double positive cells, which express markers of terminal differentiation, were still able to proliferate. The cellular pharmacokinetics of PMA showed that there were no differences in uptake or efflux in wild-type and resistant cells. Collectively, our data suggest that cell differentiation and cell growth arrest are two independently regulated mechanisms. Similar findings have been obtained in a K562 erythroleukemia cell line that was resistant to PMA-induced cell growth arrest but not megakaryocytic differentiation [14]. In HL60/TLK199 cells, resistance to PMA-induced cell growth arrest was associated with a lower expression of the CDK inhibitors $p21^{WAF1}$ and $p27^{KIP1}$. These proteins block cells in the G_0/G_1 phase of the cell cycle by inhibiting the phosphorylation of retinoblastoma protein (pRb) and may play a causal role in cell differentiation. Indeed, the forced expression of these proteins accelerates PMA-induced differentiation of HL60 cells [15].

Chronic exposure and selection in TLK199 resulted in a higher activity of ERK and a differential activation of these kinases following PMA treatment. PMA induced a sustained activation of these enzymes in wild-type cells, but only a transient and lower activation in resistant cells. In addition, we observed that the co-treatment of resistant cells with tyrosine phosphatase inhibitors both potentiated cell growth arrest and cell differentiation following PMA treatment and induced a sustained activation of ERK. These data are consistent with the results of Hu and collaborators [16], who observed in a human leukemic cell line, TF-1, that macrophage-like differentiation induced by PMA was associated with a sustained activation of ERK, while proliferation induced by granulocyte macrophage-colony stimulating factor was associated with a transient kinase activation. Our results show that OV and DPN circumvent resistance to PMA and enhance differentiation and the activation of ERK by PMA. The involvement of DPN in the potentiation of differentiation has been inferred previously in pheochromocytoma PC12 cells and was associated with a sustained activation of ERK [17]. These results suggest that tyrosine phosphatases involved in MAPK regulation may be involved in resistance to PMA. Our data also imply that after PMA treatment, these phosphatases may quickly dephosphorylate ERK, leading to a transient activation of these kinases. However, the exact mechanism responsible for this effect remains partially unclear. It has been suggested that various phosphatases may be involved in ERK inactivation and ERK pathway inhibition [18,19]. Dephosphorylation of ERK could be catalyzed by any of the three major groups of phosphatases: serine/threonine phosphatases (such as PP2A), protein tyrosine phosphatases (including HePTP), or dual specific phosphatases (DUS) [19]. However, the two phosphatase inhibitors OV and DPN used in this experiment inhibit more likely the PTPs and the DUS. We focused our attention on the dual specific phosphatases MKP-1 and MKP-3 that are known to be able to dephosphorylate both phosphotyrosine and phosphothreonine residues of the activation loop of ERK [20]. These members of the PTP superfamily have differential subcellular localization; MKP-3 is found preferentially in the cytoplasm, while MKP-1 is in the nucleus. This might infer that these enzymes have different roles in the dephosphorylation and regulation of ERK. Immunoblot and RT–PCR analysis showed that MKP-1 but not MKP-3 was overexpressed in HL60/TLK199 cells. These data imply that MKP-1 may contribute to resistance to PMA-induced cell growth arrest by inhibiting MAPK activity. ERK1/2 are first activated in the cytoplasm by MEK1/2 and then translocated to the nucleus. This nuclear localization is required for the phosphorylation and activation of transcription factors such as ELK-1 [21]. It has been shown that MKP-1 expression is induced quickly following ERK activation in response to various mitogenic stimuli [22]. Shortly after PMA treatment (within 6 hr), MKP-1 expression increased in wild-type but not in resistant cells. The protein levels of MKP-1 following treatment appear to be similar to those observed in untreated resistant cells. These data are in accordance with the findings of Seimiya *et al.* [23], who showed that the up-regulation of various phorbol ester-sensitive PTPs observed in leukemia U937 cells during PMA-induced differentiation was not occurring in U937 cells resistant to PMA. In our experiments, these observations could be the consequence of the transient activation of ERK in HL60/TLK199 cells, which is not sufficient to stimulate the expression of this phosphatase. In addition, the high levels of MKP-1 in resistant cells could be a direct consequence of ERK hyperactivity. Our results suggest that the transient activation of ERK caused by PMA in HL60/TLK199 cells may be a consequence of the increased efficiency of dephosphorylation of the activated protein as a consequence of increased protein tyrosine phosphatase expression and/or activity, and to this end, MKP-1 may represent a plausible candidate. Such a concept would provide an explanation for the resistance of these cells to PMA-induced cell growth arrest.

In conclusion, we observed that HL60/TLK199 cells were resistant to PMA-induced cell growth arrest but not differentiation. This resistance was associated with a transient activation of ERK following PMA treatment, while a sustained activation was seen in HL60 WT cells under the same conditions. In addition, resistance to PMA was circumvented by protein tyrosine phosphatase inhibitors and was associated with a sustained activation of ERK. These data suggest that chronic exposure to the GSTπ inhibitor induces various cellular alterations including the modulation of MAPK pathways and leads to the impairment of the HL60 response to the differentiating agent PMA. This serves to emphasize the involvement of GSTπ in the regulation of MAPK pathways and suggests that pharmacological manipulation of GSTπ can lead to altered cellular patterns of growth and differentiation. In addition to providing information pertinent to the mechanism of action of TLK199, these results suggest that, during PMA-induced monocyte/macrophage-like differentiation, the mechanisms involved in differentiation and cell growth arrest appear to be distinct. Thus, HL60/TLK199 cells represent a useful model for distinguishing the cell signaling pathways involved in either differentiation or proliferation.

Acknowledgments

This work was supported, in part, by National Institutes of Health Grants CA06927 and CA85660 to Kenneth D. Tew and by an appropriation from the Commonwealth of Pennsylvania. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute. TLK199 was a gift from Telik Inc.

References

[1] Gate L, Tew KD. Glutathione *S*-transferases as emerging therapeutic targets. Expert Opin Ther Targets 2001;5:477–89.

[2] Cho SG, Lee YH, Park HS, Ryoo K, Kang KW, Park J, Eom SJ, Kim MJ, Chang TS, Choi SY, Shim J, Kim Y, Dong MS, Lee MJ, Kim SG, Ichijo H, Choi EJ. Glutathione *S*-transferase mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. J Biol Chem 2001;276:12749–55.

[3] Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ, Ronai Z. Regulation of JNK signaling by GSTp. EMBO J 1999;18:1321–34.

[4] Wang T, Arifoglu P, Ronai Z, Tew KD. Glutathione *S*-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. J Biol Chem 2001;276:20999–1003.

[5] Yin Z, Ivanov VN, Habelhah H, Tew K, Ronai Z. Glutathione *S*-transferase p elicits protection against H_2O_2-induced cell death via coordinated regulation of stress kinases. Cancer Res 2000;60:4053–7.

[6] Morgan AS, Ciaccio PJ, Tew KD, Kauvar LM. Isozyme-specific glutathione *S*-transferase inhibitors potentiate drug sensitivity in cultured human tumor cell lines. Cancer Chemother Pharmacol 1996;37:363–70.

[7] Ruscoe JE, Rosario LA, Wang T, Gate L, Arifoglu P, Wolf CR, Henderson CJ, Ronai Z, Tew KD. Pharmacologic or genetic manipulation of glutathione *S*-transferase P1-1 (GSTπ) influences cell proliferation pathways. J Pharmacol Exp Ther 2001;298:339–45.

[8] Hazzalin CA, Mahadevan LC. MAPK-regulated transcription: a continuously variable gene switch? Nat Rev Mol Cell Biol 2002;3:30–40.

[9] Danilenko M, Wang X, Studzinski GP. Carnosic acid and promotion of monocytic differentiation of HL60-G cells initiated by other agents. J Natl Cancer Inst 2001;93:1224–33.

[10] Wang X, Studzinski GP. Inhibition of p38MAP kinase potentiates the JNK/SAPK pathway and AP-1 activity in monocytic but not in macrophage or granulocytic differentiation of HL60 cells. J Cell Biochem 2001;82:68–77.

[11] Slosberg ED, Yao Y, Xing F, Ikui A, Jirousek MR, Weinstein IB. The protein kinase C β-specific inhibitor LY379196 blocks TPA-induced monocytic differentiation of HL60 cells. Mol Carcinog 2000;27:166–76.

[12] Das D, Pintucci G, Stern A. MAPK-dependent expression of $p21^{WAF}$ and $p27^{kip1}$ in PMA-induced differentiation of HL60 cells. FEBS Lett 2000;472:50–2.

[13] O'Brien ML, Vulevic B, Freer S, Boyd J, Shen H, Tew KD. Glutathione peptidomimetic drug modulator of multidrug resistance-associated protein. J Pharmacol Exp Ther 1999;291:1348–55.

[14] Shelly C, Petruzzelli L, Herrera R. K562 cells resistant to phorbol 12-myristate 13-acetate-induced growth arrest: dissociation of mitogen-activated protein kinase activation and Egr-1 expression from megakaryocyte differentiation. Cell Growth Differ 2000;11:501–6.

[15] Zhou P, Yao Y, Soh JW, Weinstein IB. Overexpression of $p21^{Cip1}$ or $p27^{Kip1}$ in the promyelocytic leukemia cell line HL60 accelerates its lineage-specific differentiation. Anticancer Res 1999;19:4935–45.

[16] Hu X, Moscinski LC, Valkov NI, Fisher AB, Hill BJ, Zuckerman KS. Prolonged activation of the mitogen-activated protein kinase pathway is required for macrophage-like differentiation of a human myeloid leukemic cell line. Cell Growth Differ 2000;11:191–200.

[17] Fujiwara S, Watanabe T, Nagatsu T, Gohda J, Imoto M, Umezawa K. Enhancement or induction of neurite formation by a protein tyrosine phosphatase inhibitor, 3,4-dephostatin, in growth factor-treated PC12h cells. Biochem Biophys Res Commun 1997;238:213–7.

[18] English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, Cobb MH. New insights into the control of MAP kinase pathways. Exp Cell Res 1999;253:255–70.

[19] Zhou B, Wang ZX, Zhao Y, Brautigan DL, Zhang ZY. The specificity of extracellular signal-regulated kinase 2 dephosphorylation by protein phosphatases. J Biol Chem 2002;277:31818–25.

[20] Camps M, Nichols A, Arkinstall S. Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J 2000;14:6–16.

[21] Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J 1999;18:664–74.

[22] Brondello JM, Brunet A, Pouyssegur J, McKenzie FR. The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the $p42/p44^{MAPK}$ cascade. J Biol Chem 1997;272:1368–76.

[23] Seimiya H, Sawabe T, Toho M, Tsuruo T. Phorbol ester-resistant monoblastoid leukemia cells with a functional mitogen-activated protein kinase cascade but without responsive protein tyrosine phosphatases. Oncogene 1995;11:2047–54.