PPAR LIGANDS AFFECT GROWTH-RELATED GENE EXPRESSION IN HUMAN LEUKEMIC CELLS

STEFANO LAURORA, STEFANIA PIZZIMENTI, FEDERICA BRIATORE, ALESSANDRA FRAIOLI, MADDALENA MAGGIO, PATRIZIA REFFO, CARLO FERRETTI, MARIO UMBERTO DIANZANI and GIUSEPPINA BARRERA.

Department of Medicine and Experimental Oncology, University of Turin, Turin, Italy (S.L., S.P., F.B., A.F., M.M., P.R., M.U.D., G.B.) and Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, Turin, Italy (C.F.).

RUNNING TITLE: PPAR LIGANDS EFFECTS ON GROWTH-RELATED GENE EXPRESSION

Correspondence should be addressed to:

Prof. Giuseppina Barrera

Dipartimento di Medicina e Oncologia Sperimentale, Sezione di Patologia Generale

Corso Raffaello, 30; 10125 Torino, Italy.

Tel: +39-011-6707772; Fax: +39-011-6707753

e-mail: giuseppina.barrera@unito.it

Number of text pages: 32

Number of tables: 1

Number of figures: 11

Number of references: 40

Number of words in the *Abstract*: 223

Number of words in the *Introduction*: 692

Number of words in the *Discussion*: 1152

ABBREVIATIONS: PPARs, peroxisome proliferator-activated receptors; PPRE, peroxisome proliferation responsive element; 15d-PGJ2, 15 deoxy-prostaglandin J2; FCS, foetal calf serum; PBS, phosphate buffered saline; CDK, cyclin-dependent kinase; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); EDTA, etylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

SECTION: Cellular and Molecular

JPET Fast Forward. Published on March 20, 2003 as DOI: 10.1124/jpet.103.049098
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

PPARs are ligand-activated nuclear receptors. Three subtypes of PPARs (alpha, beta and gamma) have been identified in different tissues. PPAR alpha and PPAR gamma ligands inhibit cell proliferation and induce differentiation in several human cell models. We demonstrated that both PPAR alpha (clofibrate and ciprofibrate) and PPAR gamma ligands (troglitazone and 15d-PGJ2) inhibited growth, induced the onset of monocytic-like differentiation and increased the proportion of G0/G1 cells in the HL-60 leukemic cell line. Moreover, 3 days after the treatment with 2.5 μM 15d-PGJ2 an increase in sub G0/G1 population occurred, compatible with an induction of programmed cell death. To clarify the mechanisms involved in HL-60 growth inhibition due to the effects of PPAR ligands, we investigated their action on the expression of some genes involved in the control of cell proliferation, differentiation and cell cycle progression such as c-myc, c-myb, cyclin D1 and D2. Clofibrate (50 μM), ciprofibrate (50 μM) and 15d-PG J2 (2.5 μM) inhibited c-myb and cyclin D2 expression, while they did not affect c-myc and cyclin D1 expression. Only troglitazone (5μM) decreased c-myc mRNA and protein levels, besides decreasing c-myb and cyclin D2.

The down-regulations of c-myb and cyclin D2 expression represent the first evidence of the inhibitory effect exerted by PPAR ligands on these genes. Moreover, the inhibition of c-myc expression by troglitazone may depend on a PPAR-independent mechanism.

PPARs (Peroxisome Proliferator-Activated Receptors) are members of the steroid hormone receptor superfamily which act by altering the transcription of PPAR-regulated genes by means of a recognition sequence known as a peroxisome proliferation responsive element (PPRE). Although the nuclear localization is independent of the ligand, PPARs modulate gene expression only when the ligand is bound (Berger and Moller, 2002). Compounds that activate PPARs are known as peroxisome proliferators and comprise a heterogeneous group that includes fatty acids and prostaglandins, plasticizers and anti-diabetic drugs (Willson and Wahli, 1997). At least three subtypes of PPARs have been identified: PPAR alpha, PPAR beta and PPAR gamma (Berger and Moller, 2002). Activating ligands for PPARs are semiselective for the subtype and selectivity is ligand concentration and cell type dependent. PPAR alpha and PPAR gamma ligands can inhibit cell proliferation with varying effectiveness, and can induce differentiation in several cell models (Demetri et al., 1999; Moore et al., 2001). On the contrary, PPAR beta seems to exert opposite actions in the tumorigenesis process. In fact, PPAR beta transcriptional activation enhances hepatic stellate cell proliferation (Hellemans et al., 2003) and promotes the mitotic clonal expansion of 3T3-L1 cells (Hansen et al., 2001). Moreover the suppression of PPAR beta expression contributes to the growth-inhibitory effects of the adenomatosus polyposis coli (APC) tumor suppressor (Park et al., 2001). Recently, we demonstrated that both PPAR alpha ligands (clofibrate and ciprofibrate) and PPAR gamma ligands (troglitazone and 15 deoxy-prostaglandin J2, 15d-PGJ2) inhibit growth of HL-60 human leukemic cells and induce the onset of monocytic like differentiation (Pizzimenti et al., 2002). Moreover PPAR ligands, when added in association with 4-hydroxynonenal (HNE), a product of lipid peroxidation having antiproliferative and differentiative abilities, induced HL-60 cell differentiation towards the monocytic lineage, whereas HNE alone induced a granulocytic-like differentiation (Pizzimenti et al., 2002). In another leukemic cell line, U937 cells, PPAR ligands inhibited proliferation but did not

induce differentiation (except the higher doses of 15d-PG J2 which induced a little monocytic differentiation) (Pizzimenti et al., 2002). Our results and other reports (Pizzimenti et al., 2002; Berger and Moller, 2002), indicate that the differentiative effect displayed by PPAR ligands is cell type specific.

Although the ability of PPAR ligands to inhibit cell growth and to induce cell differentiation has been demonstrated in several cell lines (Demetri et al., 1999; Moore et al., 2001), neither the mechanism by which PPAR ligands inhibit cell growth nor the mechanism involved in differentiation induction has been established conclusively. In particular the effect displayed by PPAR ligands on c-myc expression was controversial. Troglitazone, a synthetic ligand of PPAR gamma, inhibits c-myc expression in myeloid leukaemia cells (Yamakawa-Karakida et al., 2002), and 15-deoxy- prostaglandin J2 inhibits N-myc expression in neuroblastoma cells (Marui et al., 1990) while it does not decrease c-myc expression in vascular smooth muscle cells (Okura et al., 2000). No literature data exists regarding the effect of PPAR alpha or PPAR gamma ligands on the expression of c-myb, another important transcription factor involved in the control of proliferation and differentiation (Oh and Reddy, 1999). Moreover, the effect of these substances in inhibiting cell cycle progression has been documented (Scatena et al., 1999; Kawakami et al., 2002). Fibrates, in dose dependent manner, significantly alter the cell cycle distribution, mainly leading to G0/G1 phase increment and G2/M phase reduction in human leukemic cell lines (Scatena et al., 1999). Troglitazone arrests U937 cells in the G1 phase of the cell cycle (Asou et al., 1999) and inhibits cyclin D1 expression in MCF7 cells (Yin et al., 2001). However, recent findings demonstrate that some mechanisms in cell growth regulation are affected by PPAR ligands through a PPAR-independent action (Palakurthi et al., 2001; Lennon et al., 2002).

To clarify the mechanisms involved in PPAR-induced HL-60 growth inhibition due to the effects of PPAR ligands, we investigated the action of two PPAR alpha ligands (clofibrate

and ciprofibrate) and two PPAR gamma ligands (troglitazone and 15d-PGJ2) on the expression of some genes involved in the control of cell proliferation, differentiation and cell cycle progression such as c-myc, c-myb cyclin D1 and D2. Since PPAR beta demonstrated opposite action on cell proliferation, it has not been investigated in this study.

METHODS

Cells and culture conditions.

HL-60 cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 -air using RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics and 10% foetal calf serum (FCS) (Biochrom AG Seromed, Berlin, Germany). Growth rate and cell viability were monitored daily by the trypan blue exclusion test (Sigma, Milano, Italy).

PPAR ligand treatments.

Clofibrate (Sigma, Milano, Italy), ciprofibrate (Sigma, Milano, Italy), troglitazone (generous gift from Dr. Fabio Marra, University of Florence, Italy) and 15-deoxy-prostaglandin J2 (Calbiochem, La Jolla, CA, USA) were prepared in stock solutions 100 x in ethanol (final concentration of ethanol in flask 0.8%) and added at different concentrations to cell suspension (200.000 cells/ml). Control cells were treated with the vehicle alone (0.8% ethanol).

Detection of differentiation-associated surface antigens

Expression of the cell surface antigen CD14 was tested by immunofluorescence and detected by fluorescence microscopy. Cells were washed twice with PBS, then incubated with mouse monoclonal FITC-conjugated antibody (Sigma, Milano, Italy) directed against CD14 (clone UCHM-1). After incubation with the antibodies, 3 x 106 cells per sample were pelleted, resuspended in 1 ml of 0.1% sodium azide in PBS, layered onto a slide, covered with a coverslip, and scored for fluorescence in microscopy (Leitz, Dialux 20). At least 100 cells were counted for each experiment (3 separate experiments from 3 different preparations for each condition).

Flow Cytometric Analysis

HL-60 ($10x10^6$ cells) were centrifuged at 1,000 rpm for 10 minutes at 4°C and the cell pellets were fixed in 70% ice-cold ethanol for 1 h at 4 °C. The supernatant was centrifuged at 3,000 rpm for 10 minutes at 4°C and fixed in 70% ice-cold ethanol for 1 h at 4 °C. After centrifugation, both pellets were washed once with PBS, collected in one tube, and then treated with 0.4 mg/ml RNase (type 1-A, Sigma, Milano, Italy) for 30 minutes at 37°C. Propidium iodide (Sigma, Milano, Italy) was added to a final concentration of 18 µg/ml and incubated for at least 5 min at room temperature before analyzing in a FACScan cytometer (Becton Dickinson, San Jose, CA), equipped with an argon ion laser tuned at 488 nm (Software: ModFit LT 3.0).

RNA isolation and semi-quantitative RT-PCR analysis

RNA analyses were performed by a semi-quantitative PCR method as previously described (Pizzimenti et al., 1999). Briefly, the experimental strategy included the following precautions: (I) the number of PCR cycles was kept low in order to obtain an exponential amplification of PCR products; (II) all results were standardised using the signal obtained with L7 (large ribosomal subunit protein L7); (III) all experiments were performed with at least three independent cDNA preparations; (IV) to control for DNA contamination, primers were designed to span at least one exon-intron boundary. Total RNA was isolated using the Trizol Kit (Life Technologies, INC. Milano, Italy). cDNA synthesis was performed with 4 µg of total RNA in a reaction volume of 40 µl containing 1.25 µg of oligonucleotide (dT) primer, l mM of dATP, dGTP, dCTP and dTTP (Amersham Biosciences Italia, Cologno Monzese, Italia), 66 units of RNAsin (Promega Italia s.r.l., Milano, Italy), 8 µl of 5x first strand buffer, 10 mM DTT, 300 units of MMLV reverse transcriptase (Gibco BRL, Milano, Italy). Samples

were incubated for l h at 37 °C and the reaction was stopped by heating for l0 min. at 95°C. PCR reactions were performed in a GeneAmp PCR System 9600 (Perkin Elmer), with 1 µl of cDNA reaction mixture in a volume of 50 µl containing 200 µM of dATP, dTTP, dGTP and dCTP, 1 µM of 5'- and 3 '-primer and 1.25 units of TAQ DNA polymerase (Polymed, Firenze, Italy). Samples were subjected to denaturation at 94°C for 30 sec, annealing for 30 sec (the annealing temperature was 60 °C for L7, D2 and c-myc primers, 63 °C for c-myb and 70 °C for D1 primers) and extension at 72°C for 30 sec, followed by a final extension at 72°C for 10 min. Negative controls contained water instead of cDNA. The primer pair sequences used for PCR amplification and the numbers of PCR cycles done are indicated as follow:

c-myc - 20 cycles

(forward primer): 5'- GAGACAACGACGGCGGTG -3'

(reverse primer): 5'-GCTCGTTCCTCCTCTGGC -3'

amplifying a 788-bp fragment.

c-myb - 18 cycles

(forward primer): 5'-TGGACAGAAGAGGAAGACAGAA-3'

(reverse primer): 5'-GCAGAGATGGAGTGGAGTGG-3'

amplifying a 633-bp fragment.

cyclin D1 - 28 cycles

(forward primer): 5'-GCCAACCTCCTCAACGACCGG-3'

(reverse primer): 5'-GTCCATGTTCTGCTGGGCCTG-3'

amplifying a 743-bp fragment.

cyclin D2 - 24 cycles

(forward primer): 5’-CCGCCGGGCTTGGCCAT-3’

(reverse primer): 5’-CTTTCGGCCCAACTGGCATCC-3’

amplifying a 905-bp fragment.

L7 - 18 cycles

(forward primer): 5’-ATGGAGGGTGTAGAAGAGAA-3’

(reverse primer 3’): 5'-AATCATGGTAGACACCTTAG-3'

amplifying a 764-bp fragment.

A l0 µl sample of the PCR reaction mixture was separated on a 1% agarose gel and amplification products were stained with GelStar nucleic acid gel staining (FMC BioProducts, Rockland, ME USA). Densitometric analysis was perfomed by using a software program (Multi-Analyst, version 1.1, BioRad Laboratories, Segrate, Italy).

Preparation of total extracts and Western blot analysis

10 x 106 cells were washed twice in cold PBS, pH 7.4. Total extracts were prepared by lysis in a buffer containing Tris-HCl buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, and 0.05% aprotinin. Insoluble proteins were discarded by high-speed centrifugation at 4°C. Protein concentration in the supernatant was measured in triplicate using a commercially available assay (Bio-Rad Laboratories, Segrate, Italy).

All proteins were separated by SDS-PAGE and electroblotted on nitrocellulose membrane (Bio Rad Laboratories, Segrate, Italy). Membranes were blocked overnight at 4°C in Tris buffered saline (TBS) containing 5% milk plus 0.5% Tween 20 and then incubated at room

temperature with primary (anti-c-myc clone 9E10, anti-cyclin D1 clone HD11, anti-cyclin D2 clone C-17 from Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA; anti c-myb, clone 1-1 from Upstate Biotechnology, Lake Placid, NY, USA; anti beta-actin, clone AC-15 from Sigma, Milano, Italy) and horseradish peroxidase-conjugated secondary antibodies (Bio Rad Laboratories, Segrate, Italy). Detection was carried out by enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham-Pharmacia Biotech. Italia, Cologno Monzese, Italy). Densitometric analysis was performed by using a software program (Multi-Analyst, version 1.1, Bio Rad Laboratories, Segrate, Italy). All results were standardised using the signal obtained with beta-actin.

RESULTS

PPAR alpha and PPAR gamma ligands inhibit HL-60 cell growth and induced CD14 expression.

The growth of HL-60 cells treated with clofibrate, ciprofibrate, troglitazone and 15d-PGJ2 is shown in Fig.1. The effect on cell growth was dose-dependent for all the substances used and the effectiveness in inhibiting growth was higher for the PPAR gamma ligands (in particular for the 15d-PG J2) than for PPAR alpha ligands. According to previous results (Pizzimenti et al., 2002), the highest doses of PPAR ligands induced the onset of CD14 expression starting from day 4 to day 6 after the treatment. In fact, after clofibrate and ciprofibrate 50 μM, troglitazone 5 μM and 15d-PG J2 2.5 μM treatments, the values of CD14 positive-cells were between 28% to 42.5% at day 4. These values increased in the following days, except after ciprofibrate 50 mM treatment, where at days 5 and 6 the number of CD14 positive-cells decreased (Table 1).

The reduction of cell growth by PPAR ligand treatment may depend on growth-related gene modulation or cell death induction. Necrosis has been excluded by the Trypan blue exclusion test which indicated similar number of Trypan blue-positive cells in control and treated cell populations. Previous results demonstrated that high doses of clofibrate (100 μM), troglitazone (50 μM) and prostaglandin J2 (10 μM) induced apoptosis in 15-20% of the HL-60 cell population at day one after the treatment (Pizzimenti et al., 2002). To investigate the possibility that lower PPAR ligand concentrations, although able to inhibit cell growth, may induce programmed cell death in the days following the treatment, we performed a cell cycle analysis with particular regard to the individuation of sub G0/G1 population.

Effect of PPAR alpha and gamma ligands on cell cycle distribution of HL-60 cells.

Cell cycle analysis demonstrated that both PPAR alpha and PPAR gamma ligands induced an increase of cells in the G0/G1 phase of cell cycle (Fig.2). This phenomenon was more evident at day 3 where the percentage of G0/G1 cells was 41% in the control cells, 61% in cells treated with 50 µM clofibrate, 55% in cells treated with 50µM ciprofibrate, 60% in cells treated with 5µM troglitazone and 70% in cells treated with 2.5 µM 15d- PG J2.

Fig. 3 shows that the sub-G0/G1 population is three-fold increased in 15d-PG J2- treated cells 3 days after the treatment, whereas other PPAR ligands did not increase the sub-G0/G1 population. This action of 15d-PGJ2 is already evident yet at days 1 and 2 (data not shown), where the sub G0/G1 population was 27% and 33%, respectively, whereas the control values were similar to those detected at day 3.

Effect of PPAR ligands on oncogene expression

The effect of 50 µM clofibrate on c-myc, c-myb and cyclin D1 and D2 mRNA levels is shown in Fig. 4. Clofibrate inhibited c-myb and cyclin D2 expression starting from 8 hours after its addition, while it did not affect c-myc and cyclin D1 expression. A similar effect was displayed by 50 µM ciprofibrate (Fig. 5).

PPAR gamma ligands (troglitazone and 15d- PG J2) displayed different patterns in the modulation of mRNA levels. Troglitazone (5µM) transiently inhibited both c-myc and c-myb oncogene expression, mainly at 8-24 hours after the treatments, and cyclin D2 until 48 hours after the treatment (Fig. 6). On the contrary, 2.5 µM 15d-PG J2 did not inhibit c-myc expression. This substance, similarly to PPAR alpha ligands, affected c-myb and cyclin D2 expression (Fig. 7). The inhibition of c-myb expression was transient (the nadir was observed after 8 hours from the treatment), such as observed after troglitazone treatment. In all cases cyclin D1 expression was not affected.

The analysis of the protein content after PPAR ligands treatment of HL-60 cells, was performed by western blot, at the same times of mRNA content analysis. Clofibrate (50 μM) induced a complete disappearance of the c-myb protein 8-24 hours after addition, as well as strong inhibition of cyclin D2 expression starting from 8 to 48 hours (Fig. 8). Similar effects were displayed by 50 μM ciprofibrate, except that cyclin D2 inhibition was transient (Fig. 9). Troglitazone (5 μM) transiently decreased the protein concentration of c-myc and c-myb (from 8 to 24 hours for c-myc and only at 8 hours for c-myb), and progressively decreased, starting from 24 hours, the level of cyclin D2 protein (Fig. 10). Results obtained in protein extracts derived from 15d-PG J2-treated cells, confirmed the patterns obtained by PCR. 15d-PG J2 (2.5 μM) transiently reduced the c-myb protein level (from 8 to 24 hours) and induced a progressive reduction of cyclin D2 protein (Fig.11).

DISCUSSION

Among the gene expressions tested in this study, only c-myb and cyclin D2 gene expression were inhibited by both PPAR alpha and PPAR gamma ligands. However, the effective concentrations are higher for PPAR alpha ligands with respect to PPAR gamma ligands, according to that observed on cell growth inhibition and cell differentiation induction.

Cell cycle analysis indicates that an increase of G0/G1 cells occurs in the culture treated with PPAR ligands, and in particular with 15d-PG J2, according to data reported by others (Scatena et al., 1999; Kawakami et al., 2002). 15d-PG J2 also increases the sub G0/G1 population 2 and 3 days after the treatment.

The down-regulations of c-myb and cyclin D2 expression represent the first evidence of the inhibitory effect exerted by PPAR ligands on these genes. The myb gene family (whose members are A-myb, B-myb and c-myb) encodes nuclear protein that functions as a transcriptional transactivator (Oh and Reddy, 1999). Expression of these genes is cell cycle-regulated and inhibition of their expression with antisense oligonucleotides has been found to affect cell cycle-progression, cell division and/or differentiation (Raschella et al., 1992). Inhibition of c-myb expression by compounds inducing differentiation has been widely studied in leukemic cells (Kuehl et al., 1988) and c-myb down- regulation accompanied the cessation of growth and the onset of differentiation markers (Yen et al., 1992). Our results also indicate that PPAR ligands induce the monocytic differentiation of HL-60 cells, as measured by CD14 expression, at the same dose effective in decreasing c-myb mRNA and protein, suggesting that these two phenomena may be linked.

Cyclin D2 expression is also inhibited by the PPAR ligands. According to previous observation in the HL-60 cell model, cyclin D1 expression was not affected by PPAR ligand treatment, in contrast to that observed in pancreatic (Toyota et al., 2002) and in ras-

transformed rat intestinal epithelial cells (Kitamura et al., 2001). In cell culture D-type cyclins which show tissue specific expression, do not seem to functionally overlap (Sherr, 1995). In HL-60 cells cyclin D1 and D2 are down-regulated during differentiation, while cyclin D3 is upregulated (Bartkova et al., 1998). We restricted our observation to the D1 and D2 cyclins, since their role in differentiation is better defined. Despite their importance in the control of growth, cell cycle progression and development, the exact role played by each cyclin D-type is not yet understood. Individual knockout of D1 or D2 genes in mice does not affect the overall development of the animal but rather affects the development of specialized tissues and cell lineages (Fantl et al., 1995; Sicinski et al., 1995).

According to previous observations in the HL-60 cell model (Pizzimenti et al., 1999), our results suggest that the inhibition of cyclin D2 expression induced by PPAR ligands, contributes to the cessation of proliferation and to the onset of differentiation.

The major part of PPAR actions in stimulating gene expression depends on the binding between PPAR (after dimerization with retinoic X receptor alpha) and the PPAR response element (PPRE) sequences located on the promoter of target genes. Agonists stimulate binding of PPAR to PPRE (Schlezinger et al., 2002). Some PPRE sequences are identical for PPAR alpha and PPAR gamma (i.e. the UDP-glucuronosyltransferase 1A9 enzyme) (Barbier et al., 2003), others are differentially regulated by PPAR alpha and PPAR gamma ligands (i.e. the expression of Uncoupling Proteins, UCP 1)(Teruel et al., 2000). A PPAR- indirectly dependent mechanism has been postulated for the FAT/CD36 which is activated by PPAR alpha and PPAR gamma ligands in absence of PPRE in the responding upstream promoter region (Sato et al., 2002).

PPAR- indirectly dependent mechanisms have also been demonstrated for the inhibitory action displayed by PPAR on some growth-regulatory genes: i.e. cyclin D1 repression by PPAR gamma involved competition for limiting the abundance of p300 through a c-Fos

binding site of the cyclin D1 promoter; 15d-PG J2 enhanced recruitment of p300 to PPAR gamma but reduced the binding to c-Fos (Wang et al., 2001). Other authors reported that PPAR gamma ligands attenuated the mitogen-induced degradation of p21 and p27, two important cyclin/CDK inhibitory proteins (Wakino et al, 2000). In spite of the amount of evidences accumulated in these last years about the PPAR antiproliferative action, the mechanism whereby PPAR mediates growth inhibition and, in particular, growth-related gene expression inhibition, has yet to be elucidated. Certainly, it appears to be different in relation to cell type (Berger and Moller, 2002). Our results demonstrated that both PPAR alpha and gamma ligands inhibited c-myb and cyclin D2 expression in human leukemic cells. Since no PPRE sequences have been found on the promoter of c-myb and cyclin D2 gene, we can hypothesize a PPAR- indirectly dependent mechanism which involved the modulation of transcription factor activity. Recently, it has been demonstrated that STAT5 activation is sufficient to drive transcriptional induction of the cyclin D2 gene (Friedrichsen et al., 2003) and PPAR gamma ligands suppress JAK-STAT signalling (Park et al. 2003). Likewise, PPAR alpha (Pahan et al., 2002) and PPAR gamma (Straus et al., 2000) ligands inhibited activation of NF-kappa B and AP-1, two transcription factor involved in the regulation of c-myb expression (Suhasini et al., 1997).

Among the PPAR ligands tested, only troglitazone affects c-myc mRNA and protein levels. The inhibition of c-myc expression, observed after troglitazone treatment, has been also confirmed by other works (Yamakawa-Karakida et al., 2002; Shimada et al., 2002). Some authors (Yamakawa-Karakida et al., 2002) suggest that the down-regulation of c-myc expression by this ligand can be linked to apoptosis induction. However, our data suggest that the inhibition of c-myc expression by this ligand can contribute to growth inhibition and differentiation induction rather than apoptosis induction, since troglitazone was used at non-apoptotic doses. Interestingly, from our results it arises that neither the PPAR alpha ligands

(clofibrate and ciprofibrate) nor the natural PPAR gamma ligand, 15d-PG J2, inhibited c-myc expression. Thus, it is possible that the inhibition of c-myc mRNA and protein expression, in troglitazone treated cells, may depend on a PPAR-independent mechanism, through the recruitment of free Tcf-4 and thus the inhibition of Tcf-4 binding to c-myc promoter, as suggested by Yamakawa-Karakida et al. (2002). On the other hand a PPAR-independent mechanism has been demonstrated for other important cell functions modulated by troglitazone, such as the activation of MAP kinase cascade (Lennon et al., 2002) and the inhibition of translation initiation (Palakurthi et al., 2001).

In conclusion, our results demonstrate that PPAR ligands inhibit HL-60 cell proliferation and induce differentiation through the down-modulation of nuclear transcription factors (c-myc and c-myb) and cyclin D2 expression. The greater effect on cell growth inhibition, displayed by 15d-PG J2, can be also ascribed to the induction of programmed cell death, as indicated by the increase in sub G0/G1 cell population. Moreover, we cannot exclude that PPAR gamma ligands, which affect cell growth and gene expression at lower doses, may also affect other growth-regulatory gene expressions and thus inhibit the cell growth with higher effectiveness.

ACKNOWLEDGMENTS

We would like to thank Ronald Medina for the careful revision of the paper.

REFERENCES

1. Asou H, Verbeek W, Williamson E, Elstner E, Kubota T, Kamada N and Koeffler HP (1999) Growth inhibition of myeloid leukaemia cells by troglitazone, a ligand for peroxisome proliferator activated receptor gamma, and retinoids. *Int J Oncol* **15**:1027-1031.

2. Barbier O, Villeneuve L, Bocher V, Fontaine C, Pineda Torra I, Duhem C, Kosykh V, Fruchart JC, Guillemette C and Stael B (2003) The UDP-glucoronosyltransferase 1A9 enzyme is a peroxisome proliferator-activated receptor alpha and gamma target gene. *J Biol Chem.* (in press).

3. Bartkova J, Lukas J, Strauss M and Bartek J (1998) Cyclin D3: requirement for G1/S transition and high abundance in quiescent tissues suggest a dual role in proliferation and differentiation. *Oncogene* **17**:1027-1037.

4. Berger J and Moller DE (2002) The mechanisms of action of PPARs. *Annu Rev Med* **53**: 409-435.

5. Demetri GD, Fletcher CD, Mueller E, Sarraf P, Naujoks R, Campbell N, Spiegelman BM and Singer S (1999) Induction of solid tumor differentiation by the peroxisome proliferator-activated receptor gamma ligand troglitazone in patients with liposarcoma. *Proc Natl Acad Sci USA* **96**:3951-3956.

6. Fantl V, Stamp G, Andrews A, Rosewell I and Dickson C (1995) Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev* **9**:2364-2372.

7. Friedrichsen BN, Richter HE, Hansen JA, Rhodes CJ, Nielsen JH, Billestrup N and Moldrup A (2003) STAT5 activation is sufficient to drive trascriptional induction of cyclin D2 gene and proliferation of rat pancreatic beta-cells. *Molecular Endocrinology* (in press).

8. Hansen JB, Zhang H, Rasmussen TH, Petersen RK, Flindt EN and Kristiansen K (2001) Peroxisome proliferator-activated receptor delta (PPARdelta)-mediated regulation of preadipocyte proliferation and gene expression is dependent on cAMP signaling. *J Biol Chem* **276**:3175-3182.

9. Hellemans K, Michalik L, Dittie A, Knorr A, Rombouts K, De Jong J, Heirman C, Quartier E, Schuit F, Wahli W and Geerts A (2003) Peroxisome proliferator-activated receptor-beta signaling contributes to enhanced proliferation of hepatic stellate cells. *Gastroenterology* **124**:184-201.

10. Kawakami S, Arai G, Hayashi T, Fujii Y, Xia G, Kageyama Y and Kihara K (2002) PPAR gamma ligands suppress proliferation of human urothelial basal cells in vitro. *J Cell Physiol* **191**:310-319.

11. Kitamura S, Miyazaki Y, Hiraoka S, Nagasawa Y, Toyota M, Takakura R, Kiyohara T, Shinomura Y and Matsuzawa Y (2001) PPARgamma agonists inhibit cell growth

and suppress the expression of cyclin D1 and EGF-like growth factors in ras-transformed rat intestinal epithelial cells. *Int J Cancer* **94**:335-342.

12. Kuehl WM, Bender TP, Stafford J, McClinton D, Segal S and Dmitrovsky E (1988) Expression and function of the c-myb oncogene during hematopoietic differentiation. *Curr Top Microbiol Immunol* **141**:318-323.

13. Lennon AM, Ramauge M, Dessouroux A and Pierre M (2002) MAP kinase cascade are activat ed in astrocytes and preadipocytes by 15-deoxy-prostaglandin J2 and the thiazolidinedione ciglitazone through peroxisome proliferator activated receptor gamma-independent mechanism involving reactive oxygenated species. *J Biol Chem* **277**:29681-29685.

14. Marui N, Sakai T, Hosokawa N, Yoshida M, Aoike A, Kawai K, Nishino H and Fukushima M (1990) N-myc suppression and cell cycle arrest at G1 phase by prostaglandins. *FEBS Lett* **270**:15-18.

15. Moore KJ, Rosen ED, Fitzgerald ML, Rondow F, Andersson LP, AltshulerD, Milstone DS, Mortensen RM, Spiegelman BM and Freeman MW (2001) The role of PPAR gamma in macrophage differentiation and cholesterol uptake. *Nature Med* **7**:41-47.

16. Oh IH and Reddy EP (1999) The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* **18**:3017-3033.

17. Okura T, Nakamura M, Takata Y, Watanabe S, Kitami Y and Hiwada K (2000) Troglitazone induces apoptosis via the p53 and Gadd45 pathway in vascular smooth muscle cells. *Eur J Pharmacol* **407**:227-235.

18. Pahan K Jana M, Liu X, Taylor BS, Wood C and Fischer SM (2002) Gemfibrozil, a lipid-lowering drug, inhibits the induction of nitric-oxide synthase in human astrocytes. *J Biol Chem* **277**:45984-45991.

19. Palakurthi SS, Aktas H, Grubissich LM, Mortensen RM and Halperin JA (2001) Anticancer effects of thiazolidinediones are independent of peroxisome proliferator-activated receptor gamma and mediated by inhibition of translation initiation. *Cancer Res* **61**:6213-6218.

20. Park BH, Volgestein B and Kinzler KW (2001) Genetic disruption of PPARdelta decreases the tumorigenety of human colon cancer. *Proc Natl Acad Sci USA* **98**:2598-2603.

21. Park EJ, Park SY, Joe EH and Jou I (2003) 15d-PGJ2 and rosiglitazone suppress JAK-STAT inflammatory signaling through induction of SOCS1 and SOCS3 in glia. *J Biol Chem* (in press).

22. Pizzimenti S, Barrera G, Dianzani MU, Brusselbach S (1999) Inhibition of D1, D2, and A-cyclin expression in HL-60 cells by the lipid peroxydation product 4-hydroxynonenal. *Free Rad Biol Med* **26**:1578-1586.

23. Pizzimenti S, Laurora S, Briatore F, Ferretti C, Dianzani MU and Barrera G (2002) Synergistic effect of 4-hydroxynonenal and PPAR ligands in controlling human leukemic cell growth and differentiation. *Free Rad Biol Med* **3**:233-245.

24. Raschella G, Negroni A, Skorski T, Pucci S, Nieborowska-Skorska M, Romeo A and Calabretta B (1992) Inhibition of proliferation by c-myb antisense RNA and oligodeoxynucleotides in transformed neuroectodermal cell lines. *Cancer Res* **52**:4221-4226.

25. Sato O, Kuriki C, Fukui Y and Motojima K (2002) Dual promoter structure of mouse and human fatty acid translocase/CD36 genes and unique transcriptional activation by peroxisome proliferator-activated receptor alpha and gamma ligands. *J Biol Chem* **277:**15703-15711.

26. Scatena R, Nocca G, Sole PD, Rumi C, Puggioni P, Remiddi F, Bottoni P, Ficarra S and Giardina B (1999) Benzafibrate as differentiating factor of human myeloid leukemia cells. *Cell Death Diff* **6**:781-787.

27. Schlezinger JJ, Jensen BA, Mann KK, Ryu HY and Scherr DH (2002) Peroxisome proliferator-activated receptor gamma-mediated NF-kappa B activation and apoptosis in pre-B cells. *J immunol* **169**:6831-6841.

28. Sherr CJ (1995) D-type cyclins. *Trends Biochem Sci* **20**:187-190.

29. Shimada T, Kojima K, Yoshiura K, Hiraishi H and Terano A (2002) Characteristics of the peroxisome proliferator activated receptor gamma (PPARgamma) ligand induced apoptosis in colon cancer cells. *Gut* **50**:658-664.

30. Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, Haslam SZ, Bronson RT, Elledge SJ and Weinberg RA (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* **82**:621-630.

31. Strauss DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G and Glass CK (2000) 15-deoxy-delta 1,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. *Proc Natl Acad Sci USA* **97**:4844-4849.

32. Suhasini M, Reddy CD, Reddy EP, DiDonato JA, Pilz RB (1997) cAMP-induced NF-kappaB (p50/relB) binding to a c-myb intronic enhancer correlates with c-myb up-regulation and inhibition of erythroleukemia cell differentiation. *Oncogene* **15**:1859-1870.

33. Teruel T, Smith SA, Peterson J and Clapham JC (2000) Synergistic activation of UCP-3 expression in cultured fetal rat brown adipocytes by PPARalpha and PPARgamma ligands. *Biochem Biophys Res Commun* **273**:560-564.

34. Toyota M, Miyazaki Y, Kitamura S, Nagasawa Y, Kiyohara T, Shinomura Y and Matsuzawa Y (2002) Peroxisome proliferator-activated receptor gamma reduces the

growth rate of pancreatic cancer cells through the reduction of cyclin D1. *Life Sci* **70**:1565-1575.

35. Wang C, Fu M, D'Amico M, Albanese C, Zhou JN, Brownlee M, Lisanti MP, Chatterjee VK, Lazar MA, Pestell RG (2001) Inhibition of cellular proliferation through IkappaB kinase-independent and peroxisome proliferator-activated receptor gamma-dependent repression of cyclin D1. *Mol Cell Biol* **21**:3057-3070.

36. Wakino S, Kintscher U, Kim S, Yin F, Hsueh WA, Law RE (2000) Peroxisome proliferator-activated receptor gamma ligands inhibit retinoblastoma phosphorylation and G1 → S transition in vascular smooth muscle cells. *J Biol Chem* **275**:22435-22441.

37. Willson TM and Wahli W (1997) Peroxisome proliferators-activated receptor agonists. *Curr Opin Chem Biol* **1**:235-241

38. Yamakawa-Karakida N, Sugita K, Inukai T, Goi K, Nakamura M, Uno K, Sato H, Kagami K, Barker N and Nakazawa S (2002) Ligand activation of peroxisome proliferator-activated receptor gamma induces apoptosis of leukemia cells by down-regulating the c-myc gene expression via blockade of the Tcf-4 activity. *Cell Death Diff* **9**:513-526.

39. Yen A, Samuel V and Forbes M (1992) Regulation of cell proliferation: late down-regulation of c-myb preceding myelo-monocytic cell differentiation. *J Cell Physiol* **153**:147-156.

40. Yin F, Wakino S, Liu Z, Kim S, Hsueh WA, Collins AR, Van Herle AJ and Law RE (2001) Troglitazone inhibits growth of MCF-7 breast carcinoma cells by targeting G1 cell cycle regulators. *Biochem Biophys Res Commun* **286**:916-922.

FOOTNOTES

This work was supported by MURST (Cofin. 1999) and Turin University (EX-60% 2002).

Reprint requests should be addressed to: Prof. Giuseppina Barrera, Dipartimento di Medicina e Oncologia Sperimentale, Sezione di Patologia Generale, Corso Raffaello 30 – 10125 Torino, Italy. E-mail: giuseppina.barrera@unito.it

LEGENDS FOR FIGURES

Figure 1. **Panel A**: Growth of HL-60 cells treated with clofibrate at the indicated concentrations (10 and 50 µM). **Panel B:** Growth of HL-60 cells treated with ciprofibrate at the indicated concentrations (10 and 50 µM). **Panel C**: Growth of HL-60 cells treated with troglitazone at the indicated concentrations (2.5 and 5 µM). **Panel D:** Growth of HL-60 cells treated with 15-deoxy-prostaglandin J2 (15d-PG J2) at the indicated concentrations (1 and 2.5 µM). C: control cultures; C+EtOH: cultures treated with 0.8% ethanol. Data are the mean ± S.D. of five separate experiments. Variance analysis: * : $p<0.05$, ** : $p<0.01$, vs. C+EtOH.

Fig. 2. Effect of 50 µM clofibrate (CLOF), 50µM ciprofibrate (CIPROF), 5 µM troglitazone (TG) and 2.5 µM 15 deoxy-prostaglandin J2 (15d-PG J2) on cell cycle distribution at different time points (8, 24, 48 and 72 hours), compared to untreated control cells (C). HL-60 cells were stained with propidium iodide as described in Meterials and Methods and analyzed by flow cytometry. Values are the mean ± SD three different cell preparations.

Fig. 3 FACScan analysis of cell cycle and apoptosis. **A**: Cytofluorimetric histograms of cells collected at 72 hours, representative of different cell preparations, are shown. Cells were trated with 50 µM clofibrate, 50µM ciprofibrate, 5 µM troglitazone and 2.5 µM 15d-PG J2 or untreated (control). **B**: Analysis of hypo-diploid cell population. Data represent the sub-G0/G1 population identified on the basis of fluorescence intensity and are the mean ± SD of three different cell preparations.

Fig. 4. Panel A: c-myc, c-myb, cyclin D1 (D1) and cyclin D2 (D2) mRNA levels were determined by RT-PCR in HL-60 cells treated with 50 µM clofibrate and collected at the indicated times after the beginning of treatment; **Panel B:** quantification of RT-PCR products

was performed by densitometric scanning. Data are normalized using the L7 (Large ribosomal subunit protein 7) signal and represent the mean ± SD from three independent experiments. Values are expressed as percent of control value.

Fig. 5. Panel A: c-myc, c-myb, cyclin D1 (D1) and cyclin D2 (D2) mRNA levels were determined by RT-PCR in HL-60 cells treated with 50 μM ciprofibrate and collected at the indicated times after the beginning of treatment; **Panel B:** quantification of RT-PCR products was performed by densitometric scanning. Data are normalized using the L7 (Large ribosomal subunit protein 7) signal and represent the mean ± SD from three independent experiments. Values are expressed as percent of control value.

Fig. 6. Panel A: c-myc, c-myb, cyclin D1 (D1) and cyclin D2 (D2) mRNA levels were determined by RT-PCR in HL-60 cells treated with 5 μM troglitazone and collected at the indicated times after the beginning of treatment; **Panel B:** quantification of RT-PCR products was performed by densitometric scanning. Data are normalized using the L7 (Large ribosomal subunit protein 7) signal and represent the mean ± SD from three independent experiments. Values are expressed as percent of control value.

Fig. 7 Panel A: c-myc, c-myb, cyclin D1 (D1) and cyclin D2 (D2) mRNA levels were determined by RT-PCR in HL-60 cells treated with 2.5 μM 15d-PG J2 and collected at the indicated times after the beginning of treatment; **Panel B:** quantification of RT-PCR products was performed by densitometric scanning. Data are normalized using the L7 (Large ribosomal subunit protein 7) signal and represent the mean ± SD from three independent experiments. Values are expressed as percent of control value.

Fig. 8. Panel A: Western blot analysis of c-MYC, c-MYB, cyclin D1 (D1) and cyclin D2 (D2) protein levels in HL-60 cells treated with 50 μM clofibrate and collected at the indicated times after the beginning of treatment. Equal protein loading was confirmed by exposure of the membranes to the anti-beta-actin antibody. **Panel B:** : Relative densitometric values of c-MYC, c-MYB, cyclin D1 and cyclin D2 protein levels. Quantification of protein products was performed by densitometric scanning. Data are normalized using the beta-actin signal and are indicated as mean ± S.D. from three independent experiments and are expressed as percent of control value.

Fig. 9. Panel A: Western blot analysis of c-MYC, c-MYB, cyclin D1 (D1) and cyclin D2 (D2) protein levels in HL-60 cells treated with 50 μM ciprofibrate and collected at the indicated times after the beginning of treatment. Equal protein loading was confirmed by exposure of the membranes to the anti-beta-actin antibody. **Panel B:** : Relative densitometric values of c-MYC, c-MYB, cyclin D1 and cyclin D2 protein levels. Quantification of protein products was performed by densitometric scanning. Data are normalized using the beta-actin signal and are indicated as mean ± S.D. from three independent experiments and are expressed as percent of control value.

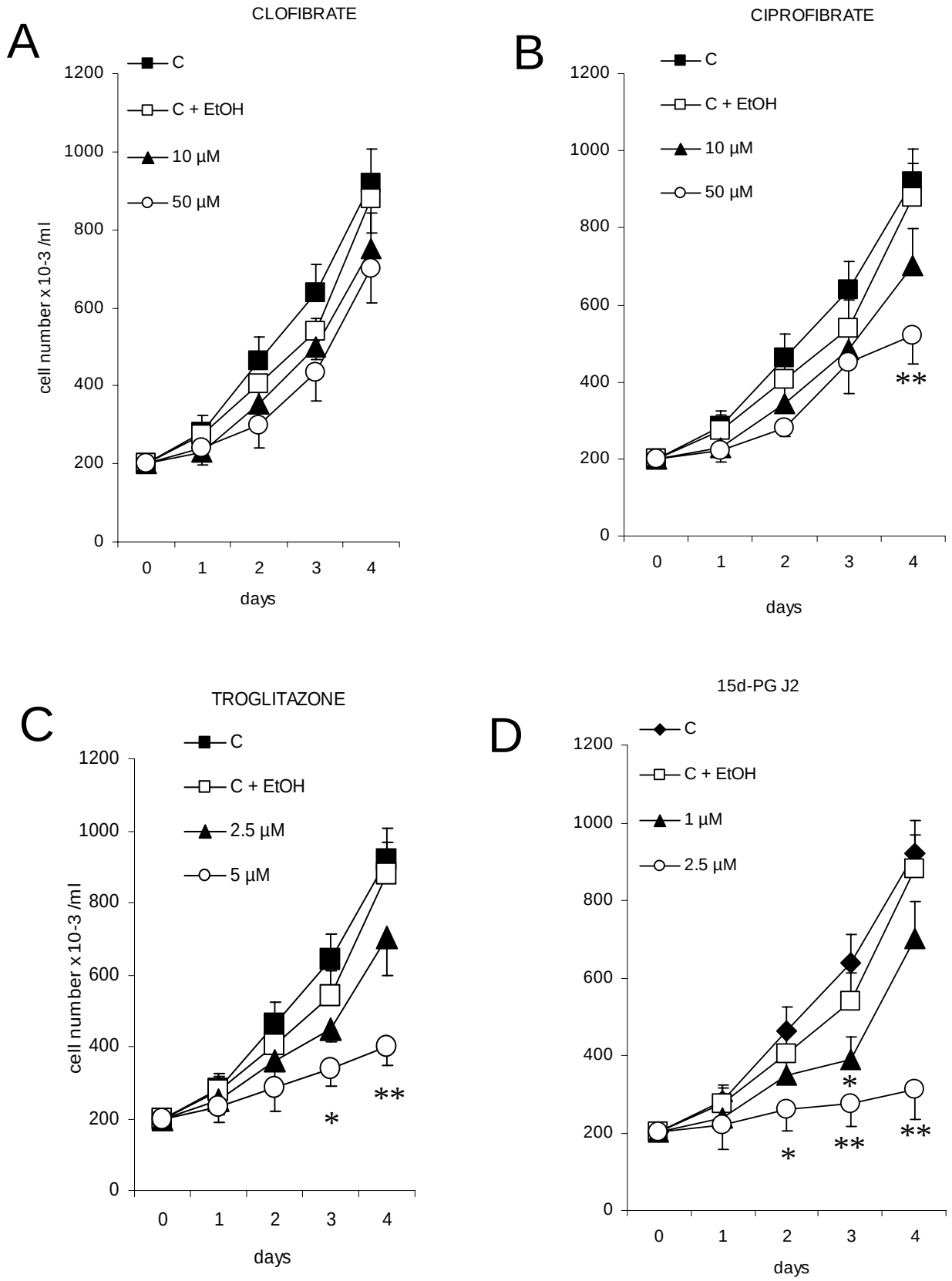
Fig. 10. Panel A: Western blot analysis of c-MYC, c-MYB, cyclin D1 (D1) and cyclin D2 (D2) protein levels in HL-60 cells treated with 5 μM troglitazone and collected at the indicated times after the beginning of treatment. Equal protein loading was confirmed by exposure of the membranes to the anti-beta-actin antibody. **Panel B:** : Relative densitometric values of c-MYC, c-MYB, cyclin D1 and cyclin D2 protein levels. Quantification of protein products was performed by densitometric scanning. Data are normalized using the beta-actin

signal and are indicated as mean ± S.D. from three independent experiments and are expressed as percent of control value.

Fig. 11. Panel A: Western blot analysis of c-MYC, c-MYB, cyclin D1 (D1) and cyclin D2 (D2) protein levels in HL-60 cells treated with 2.5 μM 15d-prostaglandin J2 and collected at the indicated times after the beginning of treatment. Equal protein loading was confirmed by exposure of the membranes to the anti-beta-actin antibody. **Panel B:** : Relative densitometric values of c-MYC, c-MYB, cyclin D1 and cyclin D2 protein levels. Quantification of protein products was performed by densitometric scanning. Data are normalized using the beta-actin signal and are indicated as mean ± S.D. from three independent experiments and are expressed as percent of control value.

JPET Fast Forward. Published on March 20, 2003 as DOI: 10.1124/jpet.103.049098
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 1



JPET Fast Forward. Published on March 20, 2003 as DOI: 10.1124/jpet.103.049098
This article has not been copyedited and formatted. The final version may differ from this version.

CD 14 expression in PPAR ligands treated HL-60 cells.

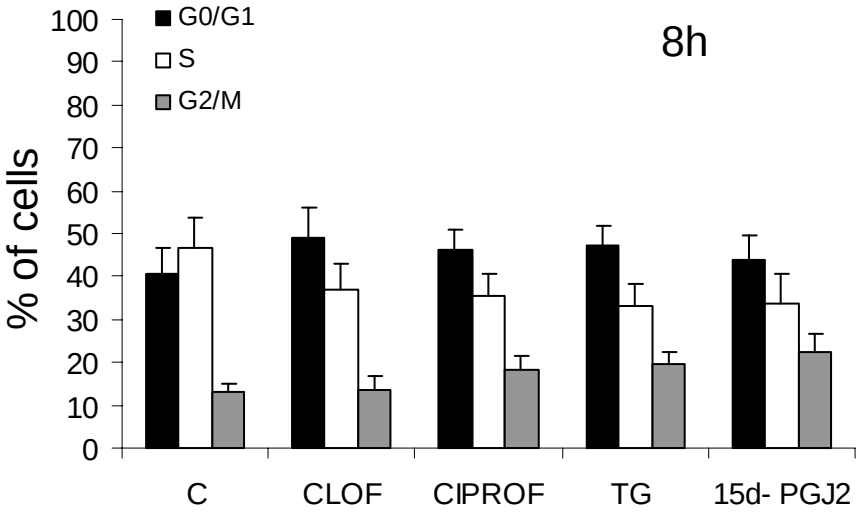
	Days [a]		
Drug treatment	4	5	6
C [b]	4.1 ± 1.5	2.1 ± 1.3	5.3 ± 1.5
C + EtOH [c]	4.2 ± 1.7	2.6 ± 11	5.1 ± 2.6
CLOFIBRATE 10 µM	9.2 ± 3.1	13.1± 4.2	10.0 ± 3.5
CLOFIBRATE 50 µM	28.0 ± 7.2 **	29.1 ± 8.0 **	29.5 ± 7.5 **
CIPROFIBRATE 10 µM	20.2 ± 7.1 *	24.3 ± 6.5 **	22.1 ± 8.3 *
CIPROFIBRATE 50 µM	42.5 ± 8.9 **	32.6 ± 8.4 **	30.3 ± 9.1 **
TROGLITAZONE 2.5 µM	9.6 ± 2.2	12.8 ± 3.1	13.2 ± 5.1
TROGLITAZONE 5 µM	17.4 ± 7.0 *	24.0 ± 8.5 **	25.1 ± 8.8 *
15d PG J2 1 µM	9.0 ± 3.2	22.1 ± 5.4 *	23.2 ± 7.1 *
15d PG J2 2.5 µM	33.3 ± 9.0 **	38.1 ± 15.3 **	50.2 ± 15.0 **

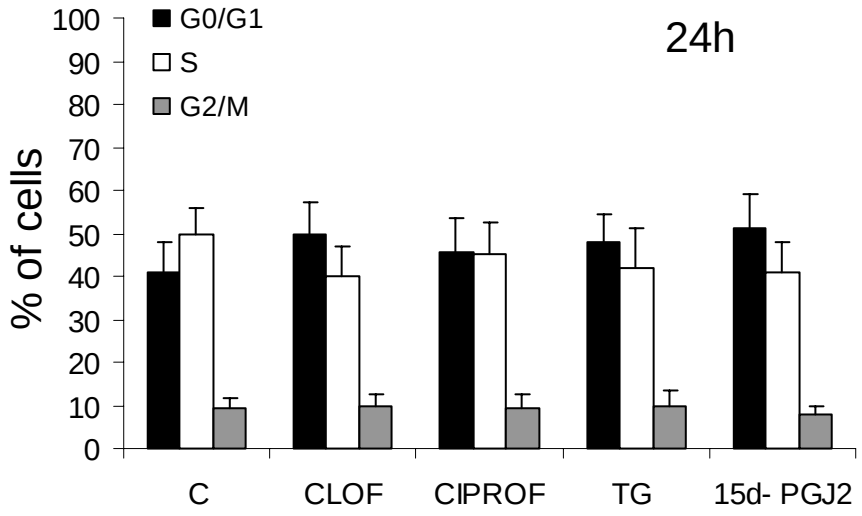
[a] CD14 expression was detected by fluorescence microscopy. Results are expressed as percentage of fluorescent cells and are the mean ± S.D. of three separate experiments from three different preparations for each condition. Immunofluorescence was performed at days 4, 5 and 6.

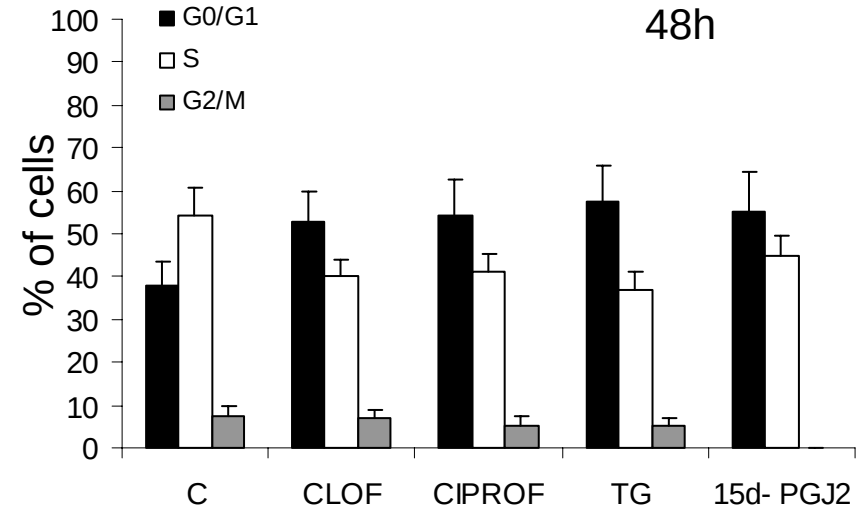
[b] C: control cultures.

[c] C+EtOH: cultures treated with 0.8% ethanol.

Variance analysis: *: $p<0.05$, ** : $p<0.01$, vs. C+EtOH.







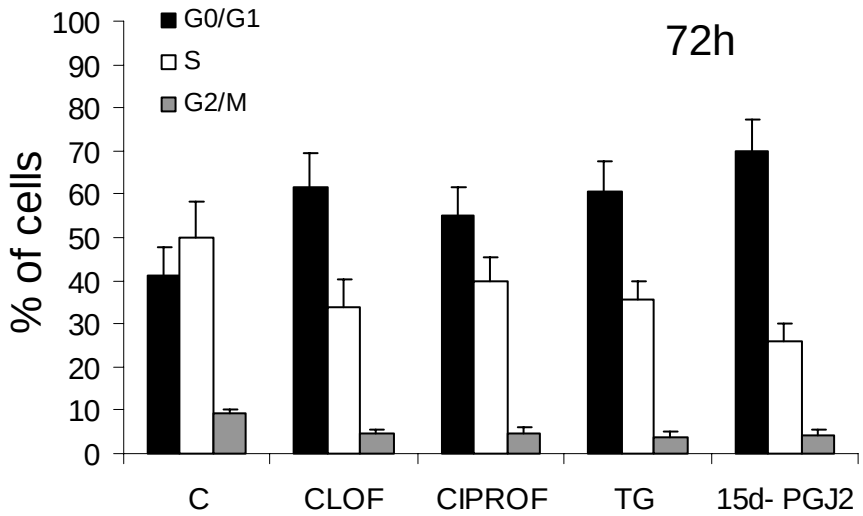
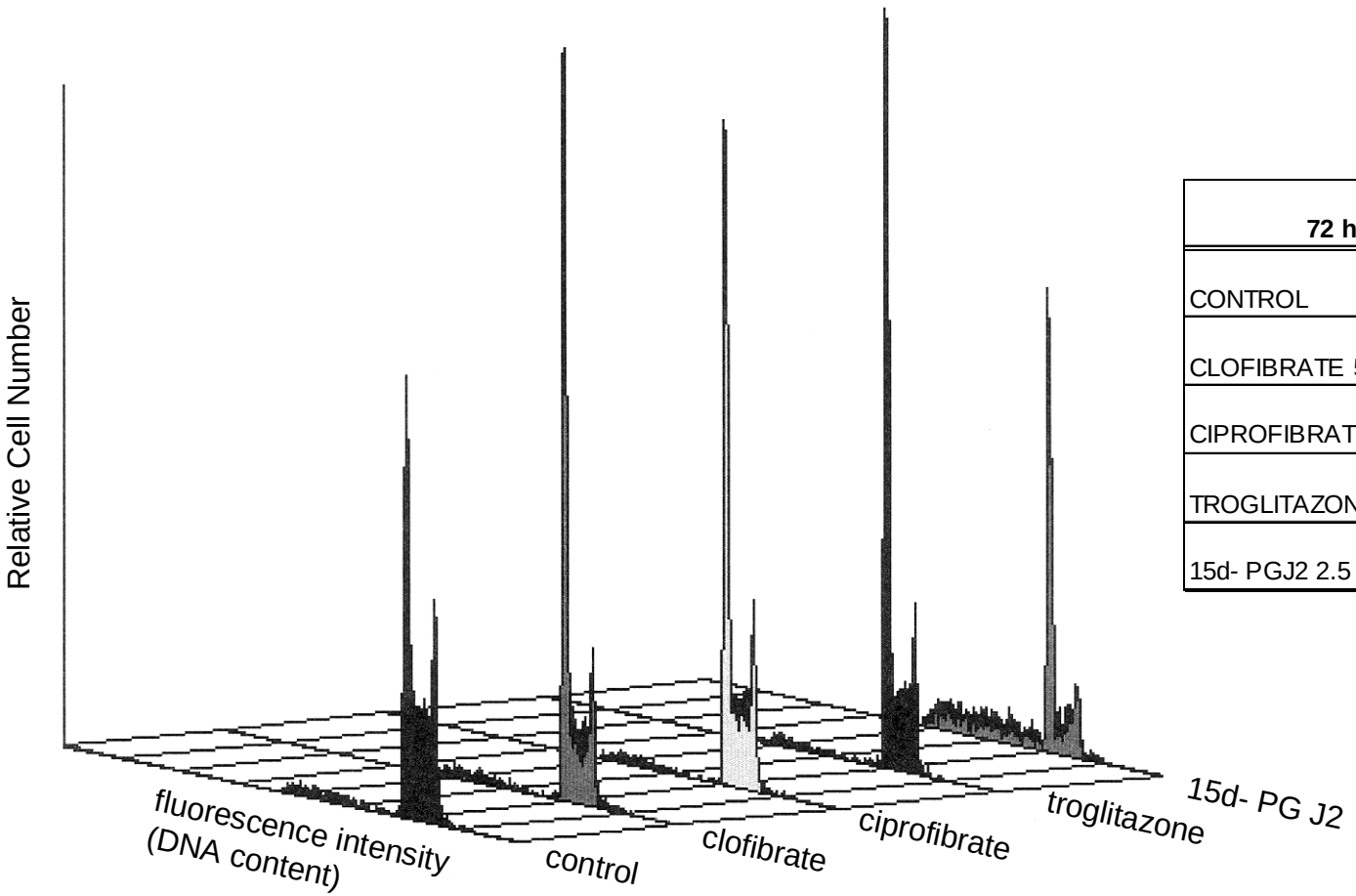


Fig. 3

A

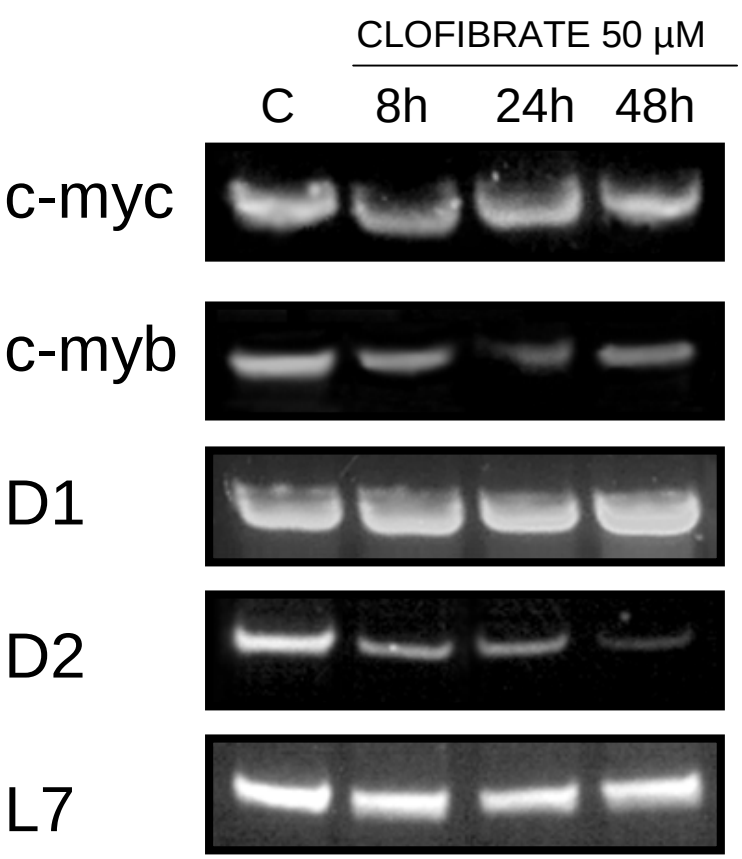


B

72 h	Debris	Hypo-diploid	Diploid
CONTROL	6.7 ± 0.8	11.4 ± 1.6	81.9 ± 2.4
CLOFIBRATE 50 µM	7.7 ± 0.7	11.6 ± 1.6	80.8 ± 0.7
CIPROFIBRATE 50 µM	6.4 ± 0.9	9.0 ± 1.5	84.4 ± 2.4
TROGLITAZONE 5 µM	5.7 ± 0.8	9.5 ± 1.6	84,9 ± 2.0
15d- PGJ2 2.5 µM	14.6 ± 1.4	35.7 ± 2.3	49,8 ± 2.9

Fig. 4

A



B

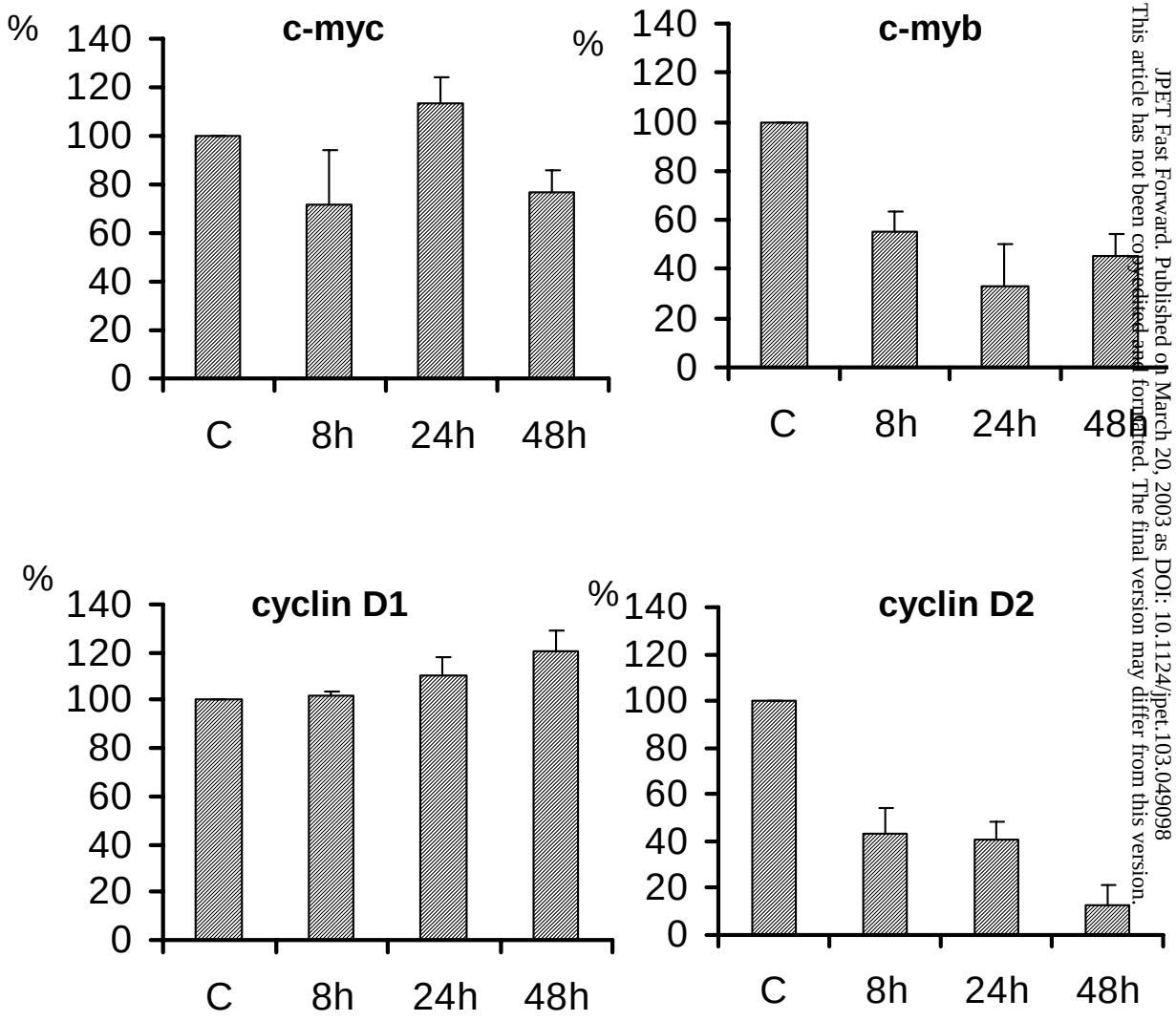
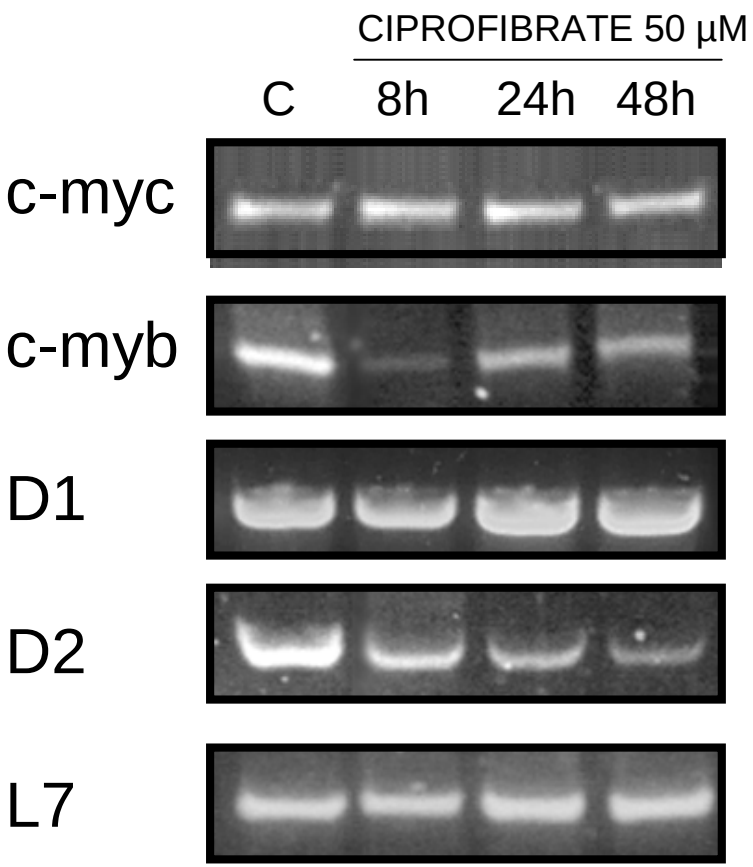


Fig. 5

A



B

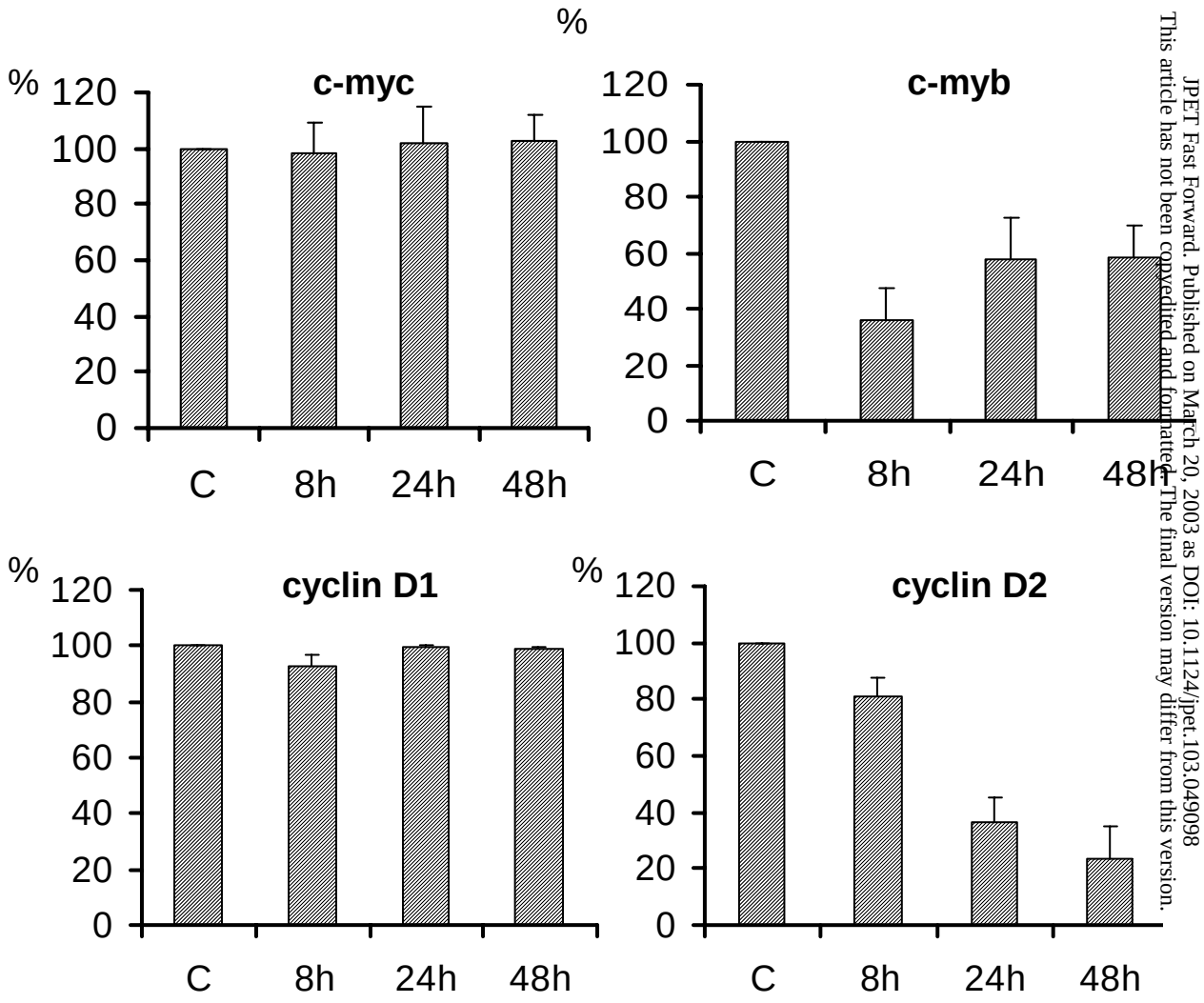
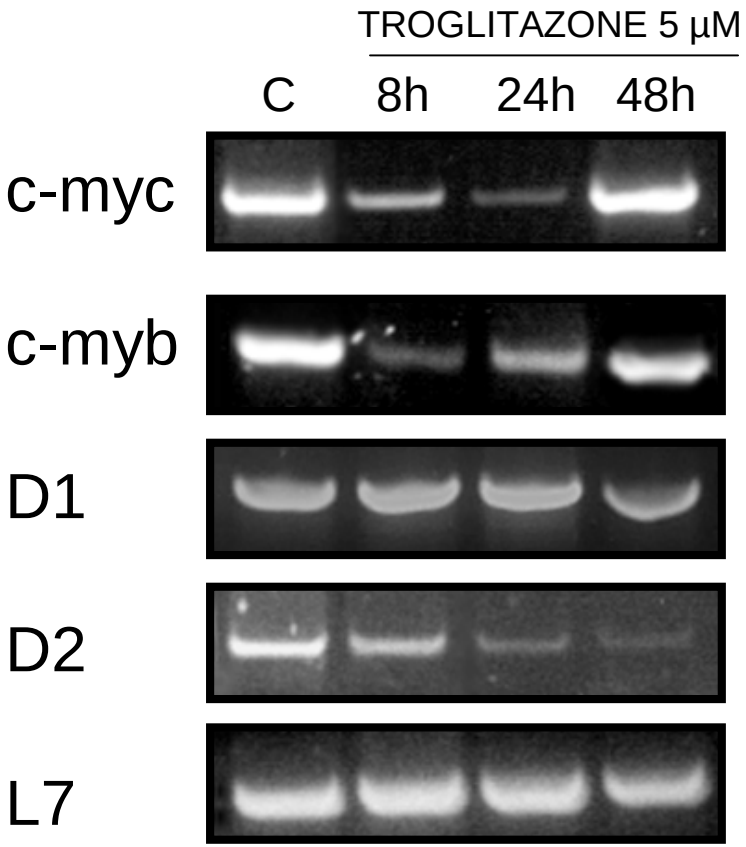


Fig. 6

A



B

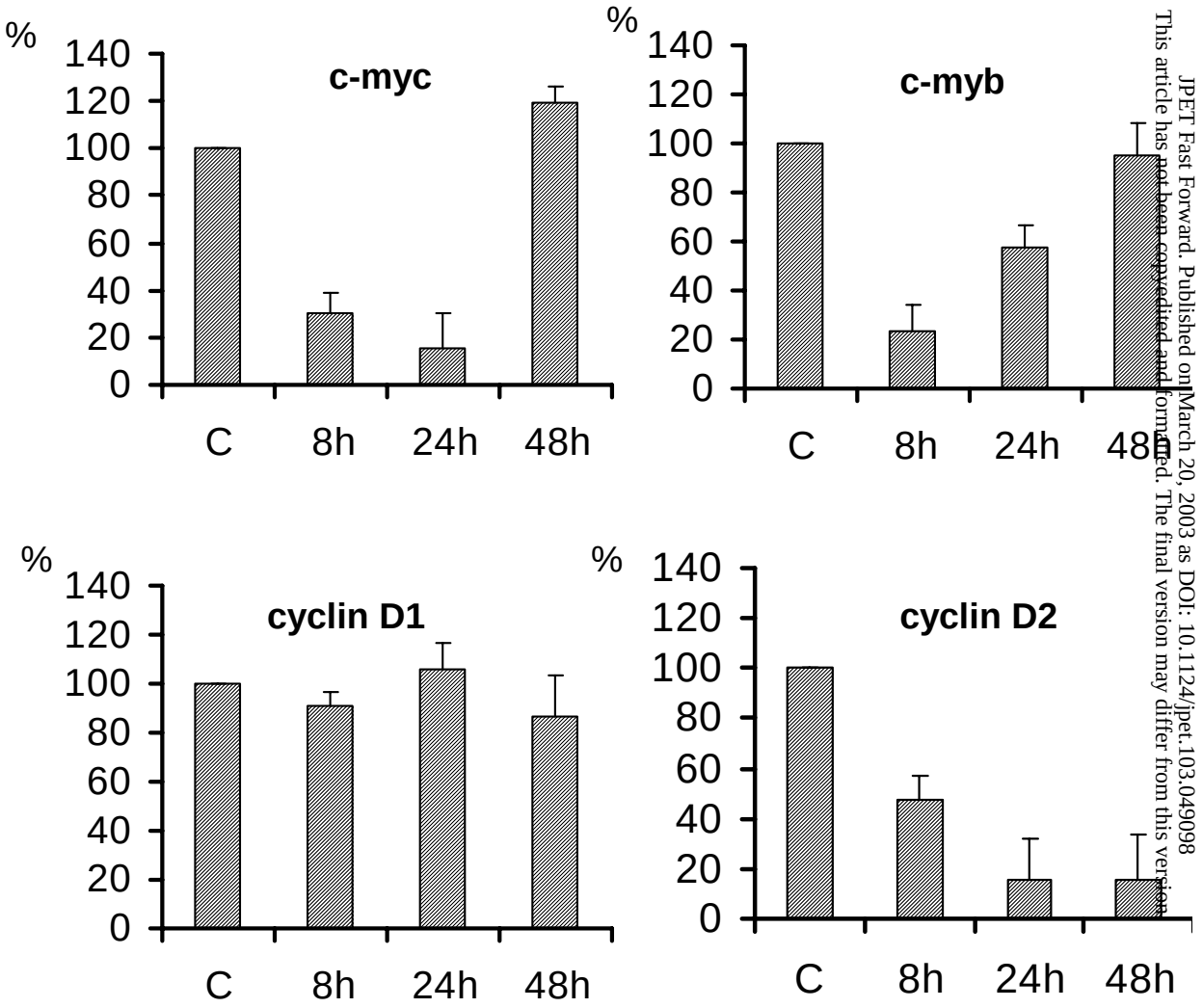
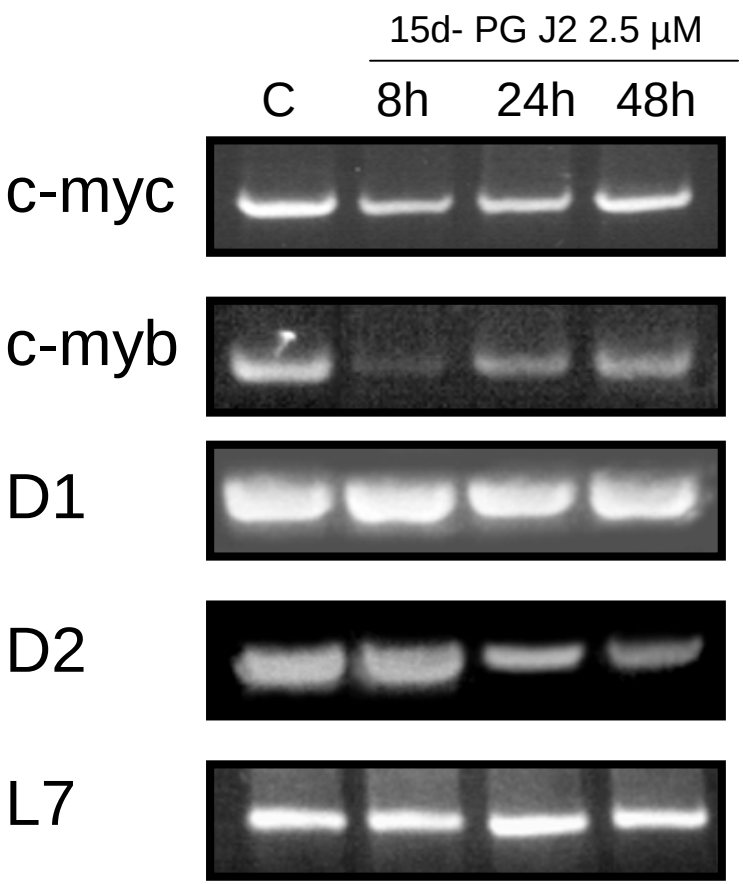


Fig. 7

A



B

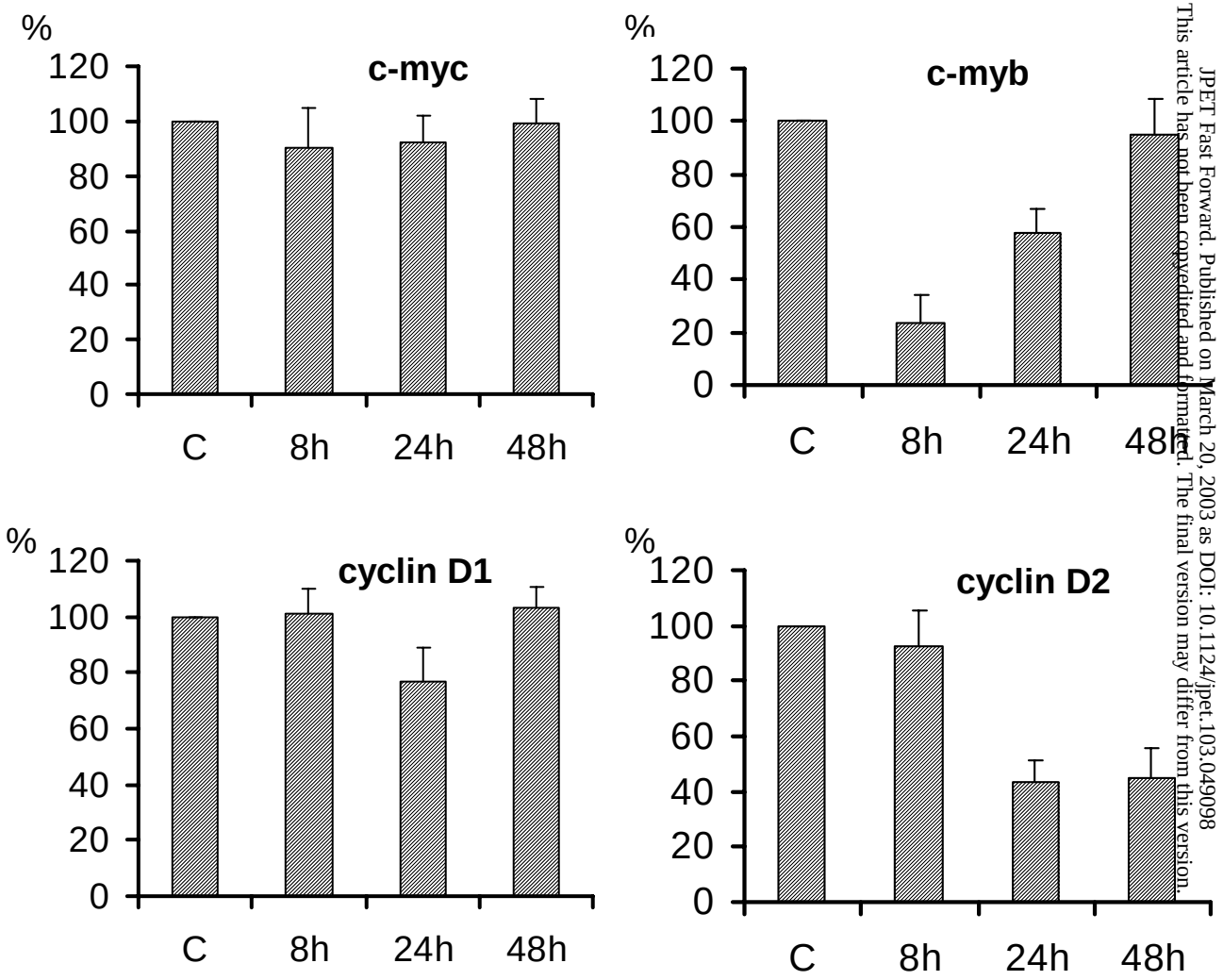


Fig. 8

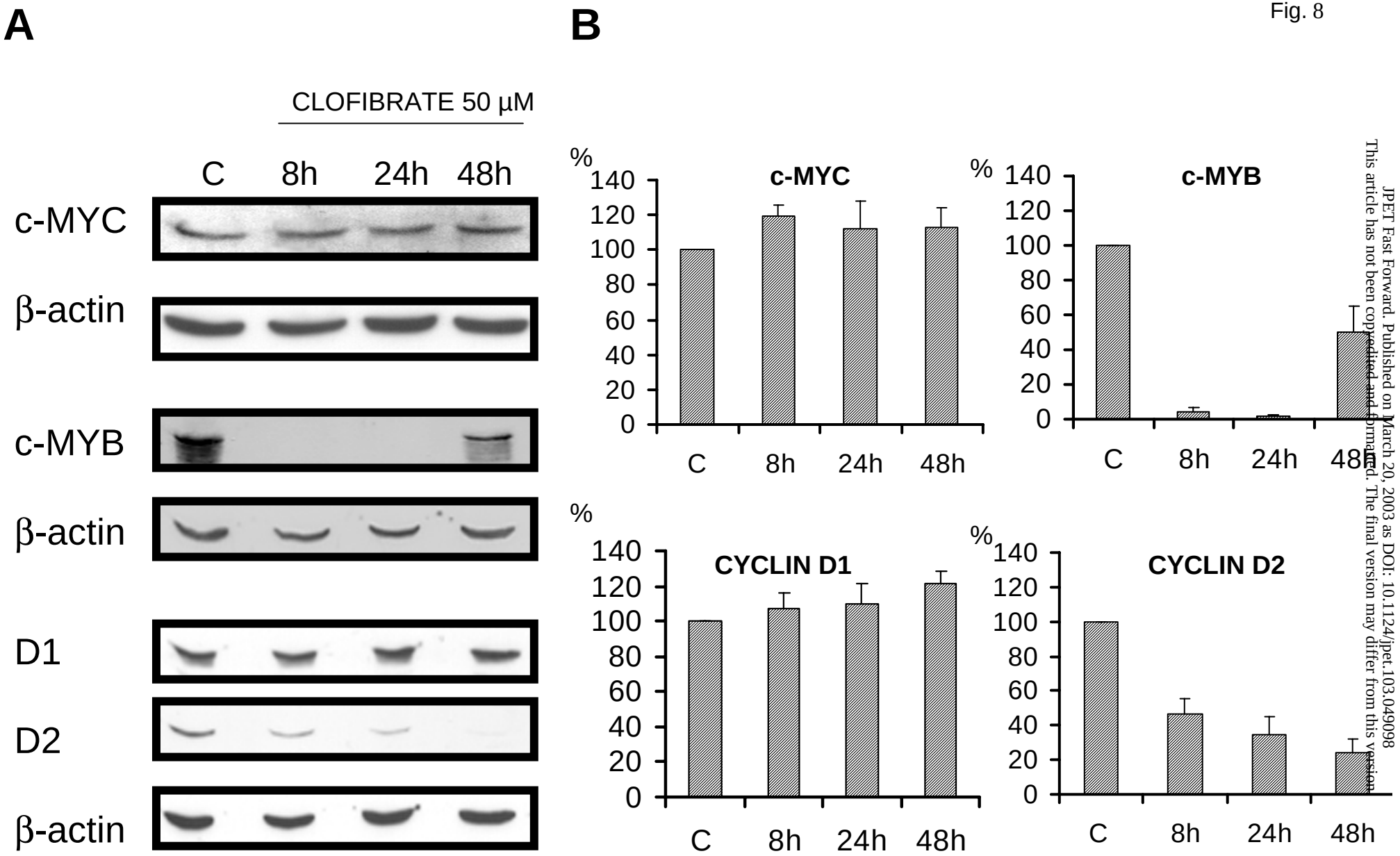


Fig. 9

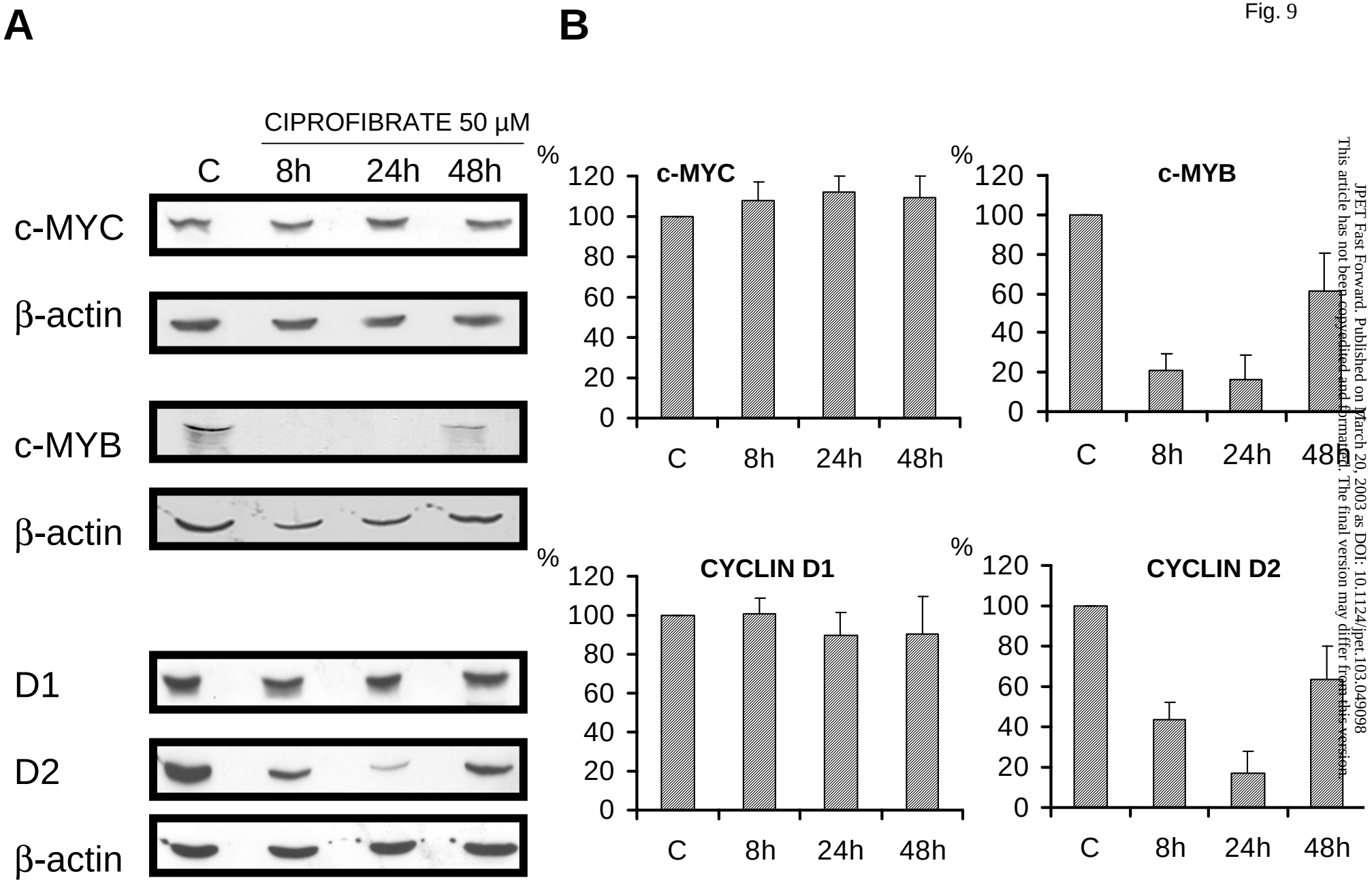


Fig. 10

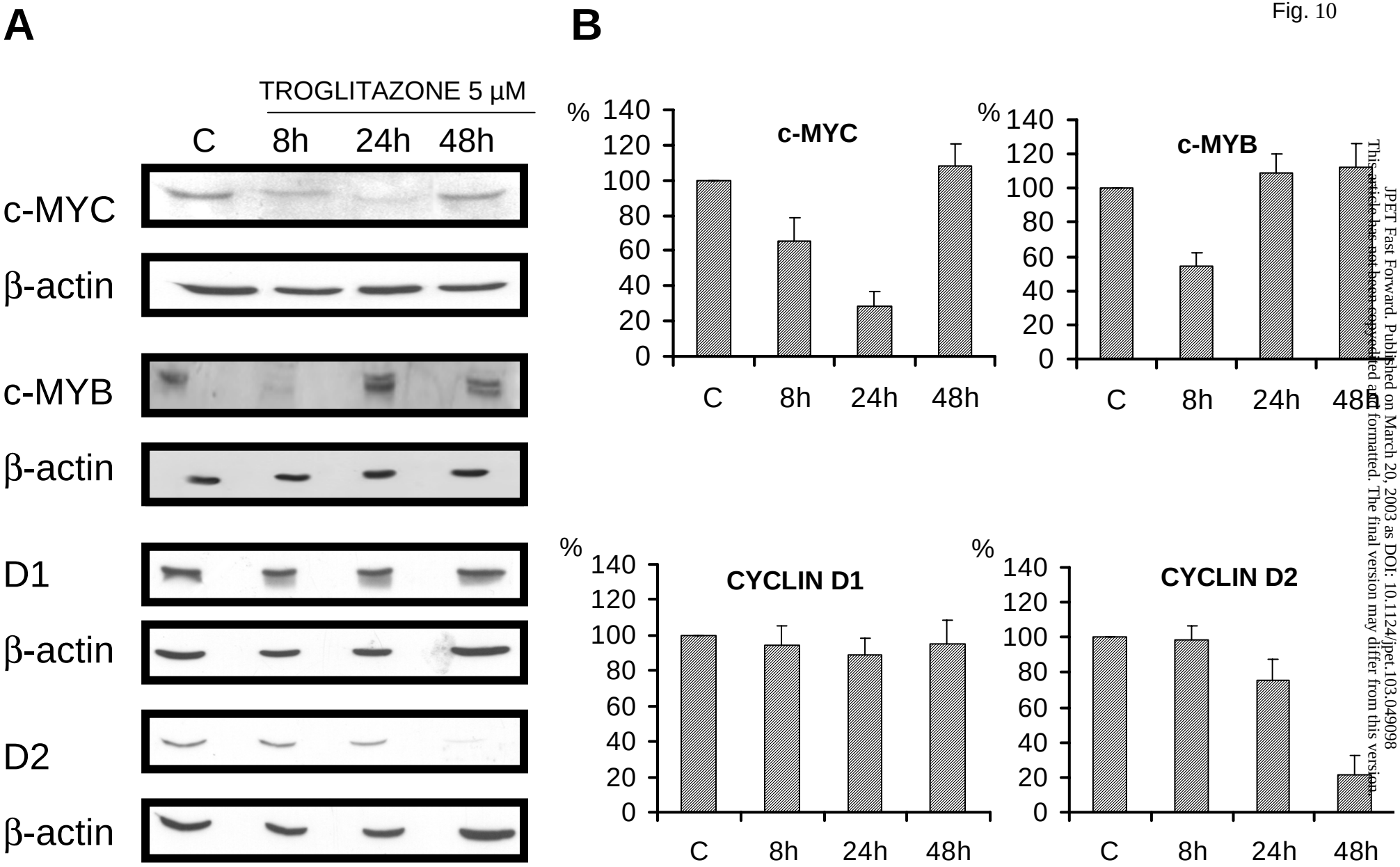


Fig. 11