# CHAPTER 2

## EXPRESSION OF PHOSPHOPROTEIN PHOSPHATASES IN PROLIFERATING AND DIFFERENTIATING HL-60 CELLS

### SUMMARY

This chapter describes a study of the rhythmic pattern of expression of phosphoprotein phosphatases in acute promyelocytic leukaemic HL-60 cells. The effects of differentiating agents, dimethyl sulfoxide, phorbol-12-myristate-13-acetate, all-trans retinoic acid and 9-cis retinoic acid, on the expression of phosphoprotein phosphatase 1, phosphoprotein phosphatase 2A and protein tyrosine phosphatase-1B during cell proliferation and differentiation was studied. All agents induced differentiation in HL-60 cells; however the exact mechanisms used are still unknown.

Granulocytic differentiation was induced using dimethyl sulfoxide (DMSO), all-trans retinoic acid (ATRA), or 9-cis retinoic acid (9-cis RA) and macrophage differentiation using phorbol-12-myristate-13-acetate (PMA). Expression of the enzyme proteins in cellular extracts was determined by SDS-PAGE and western immunoblotting analysis using specific primary antibodies.

A single immunospecific band, presumably corresponding to the catalytic subunit of PP1, with a molecular mass 38 kDa was detected on Western blots. Differentiation along both the granulocytic and macrophage pathways was associated with variations in the patterns. With PMA, there was also a marked change in the mean expression of PP1.

Three immunospecific bands, of mass 34, 37 and 46 kDa, were detected for PP2A. The 34 and 37 kDa forms were predominant; while the 46 kDa band represented a minor form. Variations in expression of all three subunits of PP2A were seen after using ATRA or PMA. The 34 kDa catalytic subunit showed a marked dampening in its rhythm after ATRA treatment. After PMA treatment, the expression of all three PP2A subunits showed a similar trend, with intensity of the bands falling over the first two days and increasing thereafter.

Two immunospecific bands of PTP-1B, of masses 42 and 46 kDa, were seen on Western blots. There were variations in intensity of both bands, although the 42 kDa band was usually a minor form. In cells treated with ATRA, there was an overall increase in PTP-1B expression and a dampening effect on the oscillation. Of particular note, was the effect seen after 48 hours treatment with PMA, when there was a change to predominance of the 42 kDa subunit. For both the 42 and 46 kDa bands, PMA appeared to increase the magnitude of the oscillation. The phosphatase inhibitor, okadaic acid, increased the expression of

the 46 kDa PTP-1B catalytic subunit; whereas genistein, a kinase inhibitor, increased the expression of the 42 kDa subunit and decreased the expression of the catalytic subunit.

# MATERIALS AND METHODS

## HL-60 CELL LINE

The HL-60 cell culture (HL-60 CCL 240) was purchased from the American Type Culture Collection (ATCC) with a full history, description and characteristic profile (Appendix A) to facilitate data comparison with other published results.

### Maintenance of the HL-60 cell culture

HL-60 cells were seeded at approximately 1 x $10^6$ cells/ml and grown in Roswell Park Memorial Institute-1640 medium (RPMI-1640) supplemented with 10% v/v heat inactivated fetal calf serum, 0.52g/l penicillin and 0.86g/l streptomycin at 37˚C in a humidified 5% $CO_2$ atmosphere. Established cultures were fed every second day and subcultured (or "passaged") every fourth day. Details of cell culture medium and reagents are given in Appendix B.

Cells were fed by allowing the cell suspension to stand in an upright position for one hour, thereby allowing the cells to settle at the bottom of the flask. Approximately 50% of the old medium was poured out and replaced with fresh medium. Subculturing was performed by pouring out 80% of the cell culture

and replacing it with fresh medium. All biological waste products were disposed using universal protocols.

**Differentiation of HL-60 cell cultures**

HL-60 cells were seeded at a cell density of 1 x $10^6$ cells/ml and divided into control and stimulated groups. Various concentrations and combinations of differentiating agents (DMSO, PMA, ATRA and 9-cis RA) were used to induce differentiation in HL-60 cells over time.

**Assay for cellular differentiation**

Cellular differentiation was monitored by using the nitroblue tetrazolium technique described by Kamijo and co-workers in 1990. Cells were incubated 1:1 (v/v) with 1.25 mg/ml nitroblue tetrazolium made up with RPMI for 30 minutes at 37$^o$C and then examined with a hemocytometer to count the percentage of cells containing intracellular formazan deposits as an indirect reflection of cellular differentiation.

**Determination of cell concentration and viability**

Cell concentration and viability assays were carried out at regular intervals using the trypan blue cell viability assay. Only cultures with a viability greater than 90% and concentrations greater then 1 x $10^6$ cells/ml were used. A 1:1 (v/v) mixture of 1% trypan-blue dye and cell suspension was incubated at 37$^o$C for 15 minutes and then transferred to a haemocytometer chamber and the percentage of blue stained (non-viable) cells used to calculate cell viability.

**Microbial Contamination**

Contamination by micro-organisms is the most frequent problem encountered in cell culture, therefore strict aseptic techniques were used. Sterility of medium and cultures were routinely monitored. Antibiotics such as penicillin and streptomycin were used in the preparation of the medium to eliminate bacterial infections. Cultures were regularly inspected visually and microscopically for signs of microbial and fungal contamination.

**Verification of cell line**

When many cell lines are grown in the same incubator spontaneous transformation may occur between cells. Cell populations may express passage-

dependent differences such as a change in cell membrane morphology, or alteration in cell behaviour and growth potential. Other known changes of HL-60 cells include drug resistance, loss of specific lineages markers, alteration of cell growth parameters and karyotypic drift (Parmley et al., 1987). It is a well known fact that transformed cells behave in a very unusual manner. Lactate dehydrogenase electrophoretic profiles were examined to ensure cells showed characteristic HL-60 cell pattern and confirmed species of origin.

**Morphological Evaluation**

Changes in cellular morphology characteristics are the first indication that cell culture conditions are deteriorating. Routine microscopic examination of the HL-60 cell cultures were carried out to detect any abnormalities in morphology such as nuclear granularity, cytoplasmic vacuolation and cellular clumping. These changes indicated that either the culture medium needed to be changed or a more serious problem such as bacterial infection may be present.

**Cryopreservation of cell cultures**

*Preparation and freezing*

Aliquots of HL-60 cells were regularly cryopreserved to provide backup should the cells become infected by bacteria. Cultures were grown to a logarithmic phase or $10^6$-$10^7$ cells/ml and cryopreserved in liquid nitrogen to yield best results.

Twenty milliliters of $10^6$-$10^7$ cells/ml cell culture suspension was centrifuged at 1500 g for 10 minutes at 4˚C with no brake. The supernatant was discarded and the pellet resuspended in 1ml of cryopreservation buffer (20% v/v fresh RPMI medium, 30% v/v glycerol and 50% v/v heat inactivated fetal calf serum) and stored in liquid nitrogen until required.

*Reconstitution of cryopreserved HL-60 cells*

Cryopreserved cells were rapidly thawed in a 37$^o$C water bath over one minute for good cellular recovery. Cells were centrifuged at 1500g for 5 minutes at 4˚C with no brake and resuspended in freshly prepared RPMI-1640 medium containing 10% heat inactivated fetal calf serum, transferred to a culture flask and incubated at 37˚C in a humidified 5% $CO_2$ atmosphere.

## PREPARATION OF CELL EXTRACTS

### Extraction of total cellular protein

Control and differentiated cells were harvested at various time intervals after stimulation. Fifty milliliters of cell culture was centrifuged at 1500g for 10 minutes at 4˚C. The cellular pellet washed three times in phosphate buffered saline (PBS) and resuspended with 1ml lysing buffer (20mM Tris-HCl, pH 7.5, 250mM sucrose and 1% Triton X-100, Appendix C). Cell homogenates were lysed by rapid freezing and thawing in liquid nitrogen. The cellular homogenate was then centrifuged at 15000g for 20 minutes at 4˚C and the supernatant containing total cellular proteins transferred to a new tube and stored at -20˚C until required.

### Total protein concentration determination

Protein concentrations in cell extracts were determined using the Lowry method (Lowry et al., 1951, described in Appendix D). Standard protein dilutions ranging from 25-150 μg/ml were prepared from a 0.5 mg/ml stock solution of bovine serum albumin. Five milliliters of freshly prepared Reagent A (2% w/v $Na_2CO_3$, 0.1% w/v $CuSO_4$ and 0.2% w/v sodium potassium tartrate in 0.1M NaOH) was added to one milliliter of each sample, mixed by vortexing for 5 seconds and allowed to stand at room temperature for 15 minutes. Five hundred

microliters of Folin reagent (diluted 1:1 with sterile distilled water) was added to each sample. Samples were left in a dark cupboard for 30 minutes for colour development. A Beckman DU-65 spectrophotometer was used to read the absorbance at 750nm in each sample.

## PROTEIN ELECTROPHORESIS AND WESTERN IMMUNOBLOTTING

### Preparation of polyacrylamide gels

A 4% stacking and 10% resolving polyacrylamide gel was used to separate total cellular proteins electrophoretically (described in Appendix E).

### Electrophoresis (PAGE)

Sixty milligrams of total protein made up in 30µl sample loading buffer was boiled for 3 minutes. Samples were loaded onto a 4% polyacrylamide stacking gel and electrophoresed at 10mA/gel until the samples reached the 10% polyacrylamide resolving gel interface, thereafter samples were electrophoresed at 20mA/gel until the dye front reached the bottom of the midget electrophoresis system (Laemmli, 1970). Rainbow molecular weight markers were run on each gel to allow the molecular masses of proteins to be determined. Electrophoresed gels were either stained with Coomassie Brilliant Blue solution (0.05% Coomassie Brilliant Blue-R; 50% methanol; 40% water; 10% acetic acid) and

destained with destaining solution (80% water, 10% acetic acid and 10% methanol) until all background stain was removed, or electrotransferred onto nitrocellulose membrane for specific immunodetection with primary antibodies for PP1, PP2A and PTP-1B.

**Electrotransfer of proteins to nitrocellulose membrane**

Proteins separated by SDS-PAGE were electrotransfered to nitrocellulose membrane as described by Towbin and co-workers (1979). A TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA) containing cold transblot buffer (25mM Tris, pH 8.0, 150mM glycine, 20% methanol) was used to transfer proteins from the polyacrylamide gels to the nitrocellulose membranes.

A piece of Whatmann 3MM filter paper, cut slightly larger than the resolving gel, was presoaked with transblot buffer, and placed on a foam pad. The resolving gel was presoaked in transblot buffer for 20 minutes to allow for size equilibration. The gel was then placed on the filter paper, ensuring that no air bubbles were trapped between it and the filter paper. A piece of nitrocellulose membrane (Hybond C) was cut slightly larger than the resolving gel, dipped in the transblot buffer, and carefully overlaid on the resolving gel, again ensuring that no air bubbles were trapped between the gel and the membrane. A second

piece of filter paper, presoaked in transblot buffer, was placed over the membrane, followed by a second foam pad.

The sandwich was placed in a cassette and inserted into a transblotting chamber filled with cold transblot buffer. A current of 200mA applied across the system allowed the transfer of proteins to take place over 2 hours.

**Immunodetection of proteins**

*Blocking of non-specific sites on the nitrocellulose membrane*

The nitrocellulose membranes were removed from the electrotransfer chamber and incubated overnight at 4$^{o}$C with phosphate-buffered saline (PBS) containing 3% non-fat milk powder to block non-specific binding sites on the membrane.

*Probing of electroblotted proteins with primary antibody*

Nitrocellulose membranes were rinsed gently in PBS to remove excess blocking solution and incubated with primary antibody (PP1/ PP2A/ PTP-1B, described in table 5) diluted in 1% non-fat milk powder. Incubation was done overnight at 4$^{o}$C on a shaking platform. Membranes were gently washed 3 times with PBS to remove unbound and non-specifically bound primary antibody.

*Incubation with secondary antibody*

The nitrocellulose membranes were incubated with 5ml PBS, containing 1% non-fat milk powder and secondary antibody (donkey anti-rabbit Ig horseradish peroxidase-linked whole antibody, described in table 5) at room temperature on a shaking platform. Membranes were washed 3 times with PBS for 10 minutes each and developed either chromogenically or using enhanced chemiluminescence (ECL, Amersham).

**Table 5: Concentrations of primary and secondary antibody** (see Appendix F for full description of antibodies)

| | **Primary antibody** | **Secondary antibody dilution factor (Donkey anti-rabbit horseradish peroxidase)** |
|---|---|---|
| **Phosphoprotein phosphatase 1 (PP1)** | 5μg/ ml | 1: 3000 |
| **Phosphoprotein phosphatase 2A (PP2A)** | 3μg/ ml | 1:5000 |
| **Protein tyrosine phosphatase-1B (PTP-1B)** | 1μg/ ml | 1:3000 |

*Chromogenic detection of antibody complexes*

Immunoreactive bands on the nitrocellulose were visualised by incubating blots with 4-chloro-1-naphthol, a chromogenic substrate, according to the method published by Hawkes and co-workers (1982). Colour development was initiated by adding 20 μl of peroxide to the chromogenic solution (30 mg 4-chloro-1-naphthol dissolved in 10 ml methanol and 40 ml of PBS) and stopped once bands are clearly visualized by adding distilled water to the blots.

*ECL™ detection of antibody complexes*

Nitrocellulose membranes rinsed with PBS were placed on plastic wrap protein side up. Two milliliters of ECL™ (made up of 1ml solution 1 and 1ml of solution 2 mixed together immediately before use) solution was placed on top of the nitrocellulose membrane for 1 minute at room temperature and excess reagent removed by gentle blotting. Membranes were then wrapped in plastic wrap and placed in a film cassette containing intensifying screens and X-ray film (Hyperfilm ECL™, Amersham International) for 1 minute. The Hyperfilm was developed until the protein bands were sufficiently intense.

*Estimation of the molecular mass of proteins*

The molecular mass of immunospecific protein bands was estimated by comparing with Rainbow molecular mass markers (Amersham).

**QUANTITATION AND DATA ANALYSIS**

Blots were scanned densitometrically using a Hitachi light camera and Biomed scanner software. Protein expression is given in terms of peak area (arbitrary units) with a fourth order polynomial fit to the data points.

# RESULTS

## HL-60 cell lactate dehydrogenase electrophoresis

The commercially available Beckman Paragon® lactate dehydrogenase (LDH) agarose gel electrophoresis system was used to check the LDH profile of the HL-60 cell line used in this study. Arbitrary amounts of protein from various tissues were loaded (5µl of sample /lane) and the LDH profiles compared with each other. Results indicated that the HL-60 cell line available in our laboratory before the arrival of the new batch from the ATCC was not suitable for the study since they had transformed. Differences between human and mouse cell lines (lanes 4 and 8) were also noted.

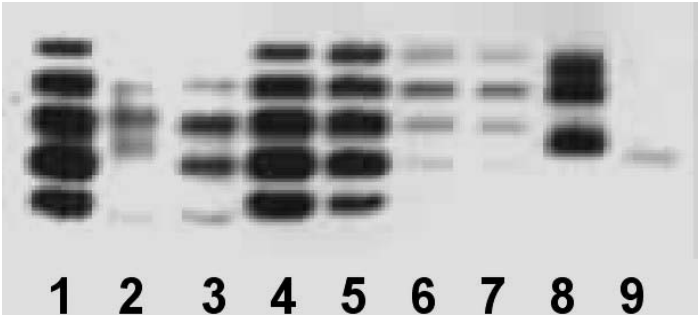


**Figure 19: Beckman Paragon agarose gel lactate dehydrogenase analysis.**

Lanes 1: HL-60 cells obtained from the ATCC used in this thesis; Lane 2 and 3: HL-60 cells available in our lab before the arrival of the new batch of cells from the ATCC (showing transformation); Lane 4 and 5: JURKAT cells (human cell line); Lane 6 and 7: human serum; Lane 8: MEL cells (mouse cell line); Lane 9: transformed MEL cell line

**The effect of differentiating agents on HL-60 cell concentration**

HL-60 cells were seeded at a concentration of $1x10^6$ cells /ml. Granulocytic differentiation induced with either all-trans retinoic acid (ATRA, $1 \times 10^{-6}$ M), 9-cis retinoic acid (9-cis RA, $1 \times 10^{-6}$ M) or dimethyl sulfoxide (DMSO, 1.3%), and macrophage-like differentiation with phorbol-12-myristate-13-acetate (PMA, $1 \times 10^{-9}$ M). Cell concentrations were measured at 24 hour intervals for each of the cell cultures over 120 hours. All differentiating agents demonstrated varying degrees of cellular growth inhibition over 24 hours; with PMA demonstrating the greatest effect and ATRA the least.

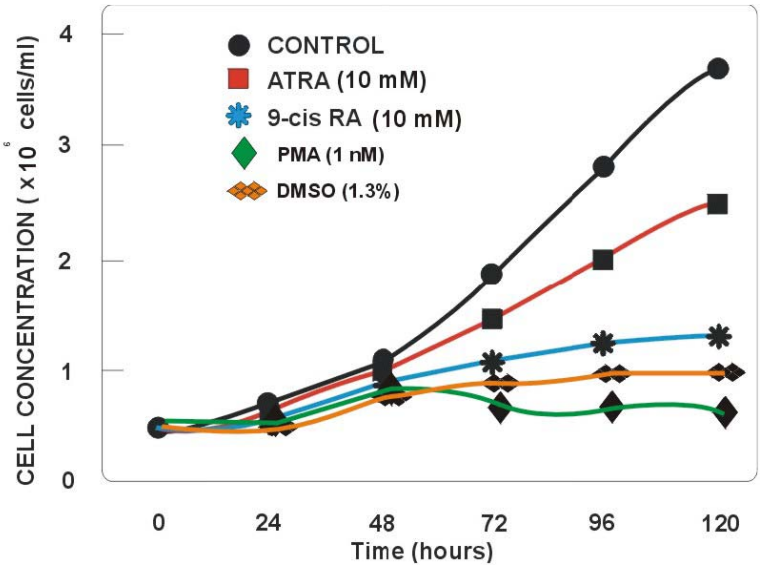


**Figure 20: The effect of differentiating agents on HL-60 cell concentration over 120 hours**

$20x10^6$ HL-60 cells were treated with different inducing agents as described above. A single experiment was done.

**The effect of differentiating agents on HL-60 cell differentiation**

Cellular differentiation was monitored using the nitroblue tetrazolium technique at 24 hour intervals over 120 hours (figure 21). The greatest effect on HL-60 cell differentiation was seen with PMA, which demonstrated more then 80% of the cells being fully differentiated after 120 hours.

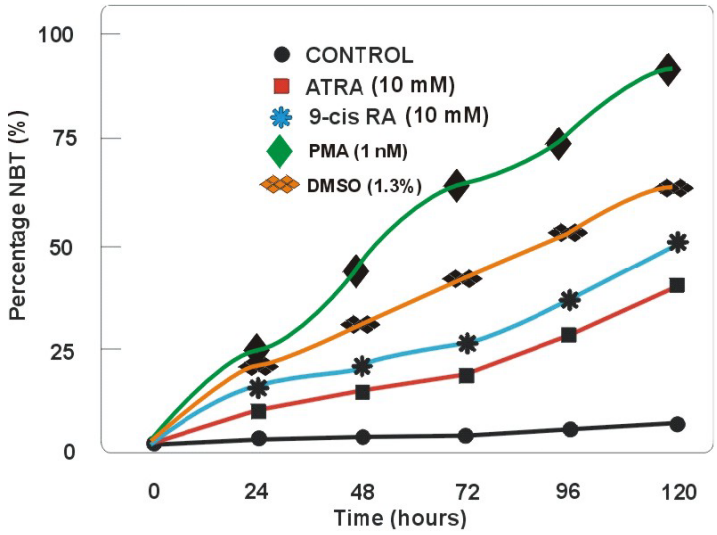


**Figure 21: Effect of differentiating agents on the percentage of HL-60 cells stained with nitroblue tetrazolium over 120 hours**

$20x10^6$ HL-60 cells were treated with different inducing agents as described above. A single experiment was done.

**The effect of differentiating agents on cell viability**

HL-60 cell viability was monitored by the trypan blue method over 120 hours. The decreased cell viability in the control group is a result of the culture medium (RPMI-1640) not being changed for 120 hours. PMA had the most marked effect in decreasing cell viability (figure 22) over 120 hours, since it also had the greatest effect on cellular differentiation.

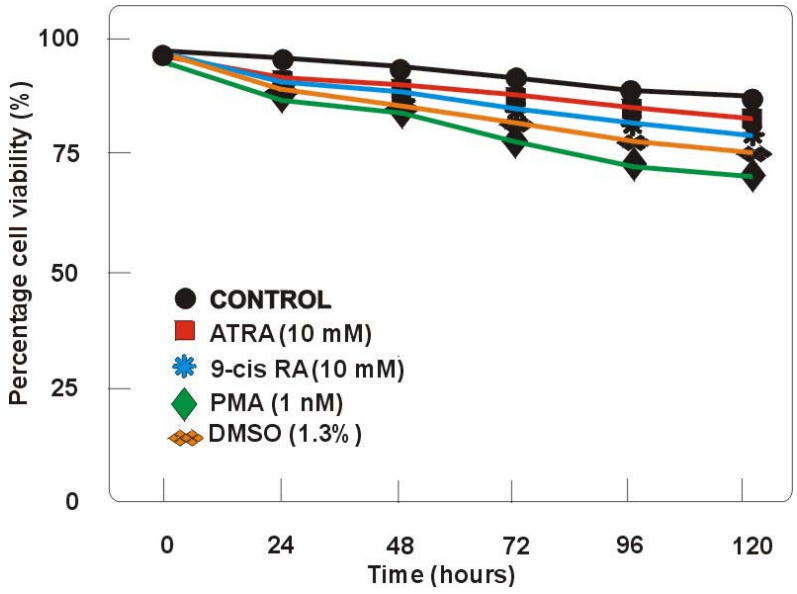


**Figure 22: Effect of various differentiating agents on HL-60 cell viability**

$20x10^6$ HL-60 cells were treated with different inducing agents as described above. A single experiment was done.

## EXPRESSION OF PHOSPHOPROTEIN PHOSPHATASES

A single immunospecific band, corresponding to the catalytic subunit of PP1, with a molecular mass 38 kDa was detected. Three immunospecific bands, of mass 34, 37 and 46 kDa, were detected for PP2A. The 34 and 37 kDa forms were the major bands and the 46 kDa band a minor form. Two immunospecific bands of PTP-1B, of masses 42 and 46 kDa, were seen, the 46 kDa band was the major band.

### The effect of DMSO on the expression of phosphoprotein phosphatases

The expression of phosphoprotein phosphatase 1, phosphoprotein phosphatase 2A and protein tyrosine phosphatase-1B in HL-60 cells was analyzed at 24 hour intervals after induction of granulocytic differentiation by DMSO (figure 23). Results indicate that DMSO had a dampening effect on the oscillatory expression of PP1, PP2A and PTP-1B when compared to the respective untreated controls.

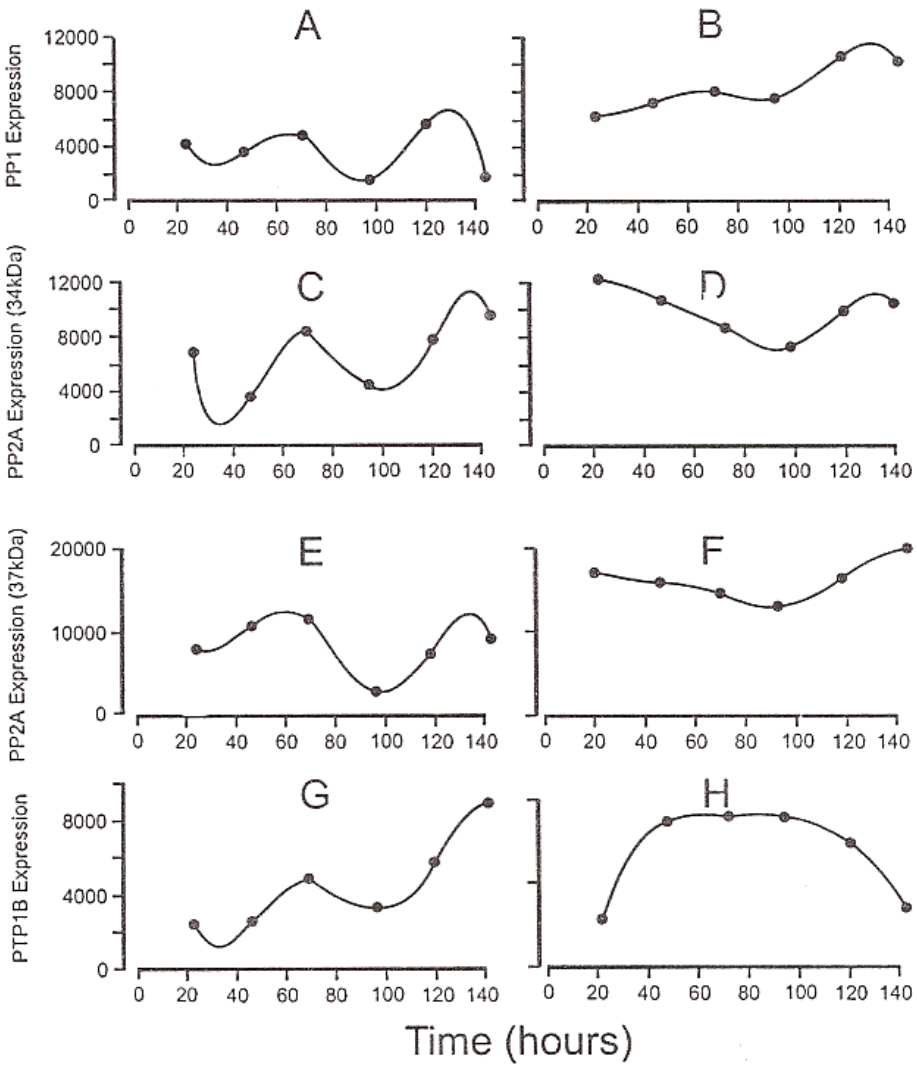


**Figure 23: Temporal changes in the expression of protein phosphatases in HL-60 cells; effect of dimethyl sulfoxide (DMSO).**

Cells were analyzed at 24 hour intervals with antibodies specific for PP1, PP2A and PTP-1B. Control cells are represented by A, C, E and G and the corresponding DMSO-treated cells by B, D, F and H, respectively (Hammond, Bhoola, Bodalina and Gilbert, 1998).

**Effect of ATRA and PMA on the expression of PP1**

A single immunospecific band, with a molecular mass of 38 kDa was detected on Western blots. Figure 24 shows the effect of differentiating agents, ATRA and PMA, on PP1 protein expression over 120 hours. Differentiation along the granulocytic and macrophage-like pathways was associated with dynamic variations in the expression of PP1.

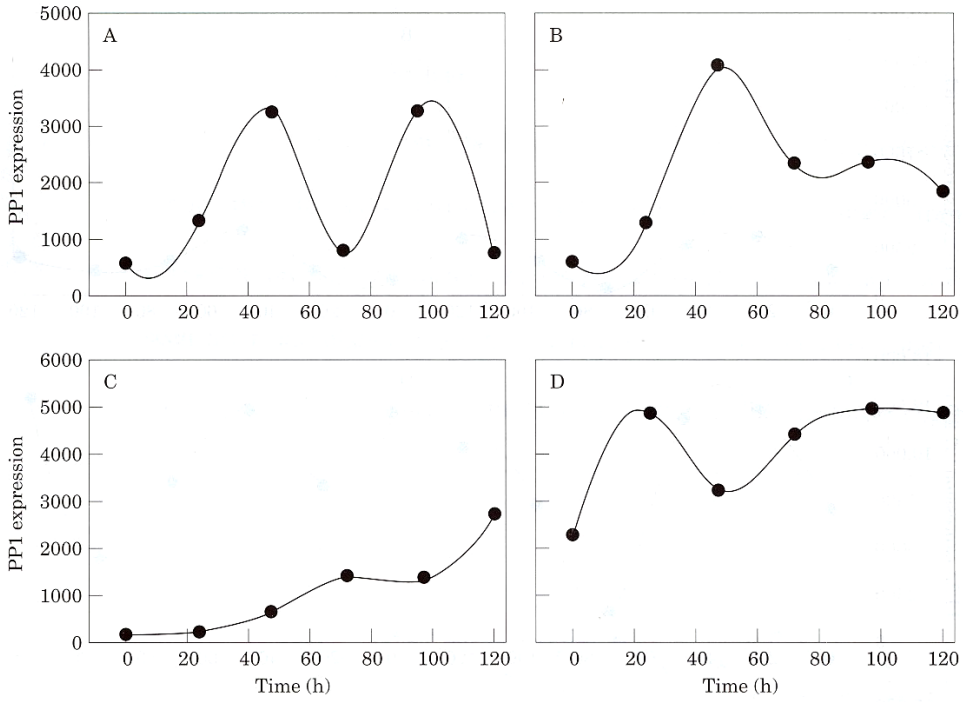


**Figure 24: Temporal changes in the expression of protein phosphatase 1 in HL-60 cells treated with ATRA or PMA.**

Cell lysates were analysed at 24 hour intervals over a period of 120 hours, using antibody specific for PP1. Control cells and their corresponding ATRA treated cells are represented by A and B, respectively. Control cells and their corresponding PMA treated cells are represented by C and D, respectively (Bhoola and Hammond, 2000).

**Effect of ATRA on the expression of PP2A**

Three immunospecific bands, of masses 34, 37 and 46 kDa, were detected for PP2A protein expression. Variations in the expression of all three subunits of PP2A were seen after stimulation with ATRA (figure 25). The 34 kDa catalytic subunit showed a marked dampening in its rhythm after ATRA treatment (figure.25.F).

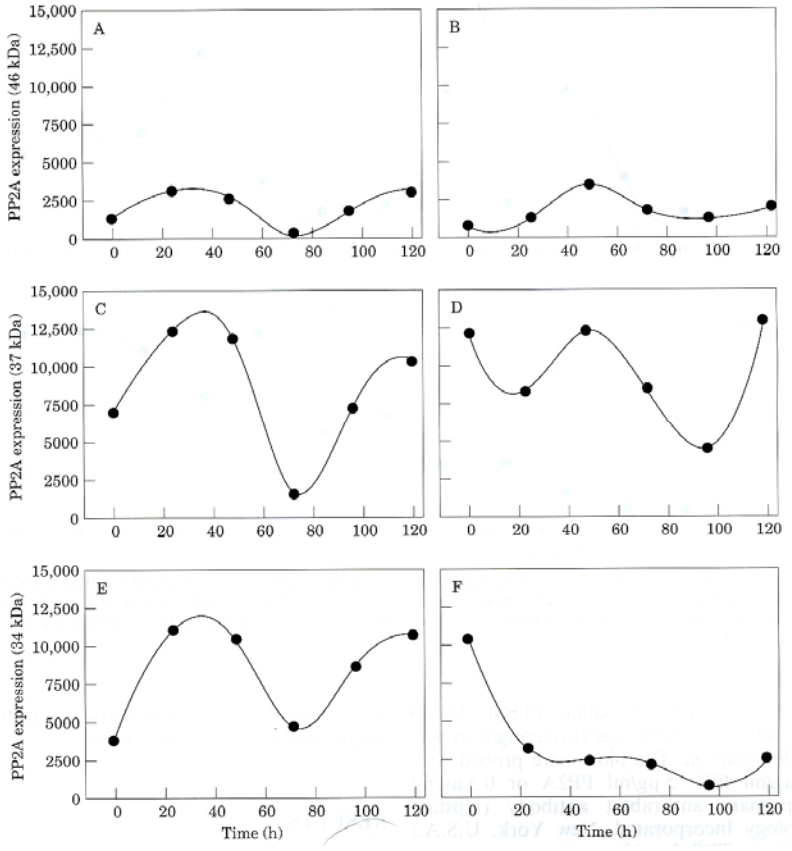


**Figure 25: Temporal changes in the expression of protein phosphatase 2A in HL-60 cells treated with ATRA.**

Cell lysates were analysed at 24 hour intervals over a period of 120 hours, using an antibody specific for PP2A. Three immunospecific bands of 46 kDa (A and B), 37 kDa (C and D) and 34 kDa (E and F) were observed. Control cells are represented by A, C and E; their corresponding ATRA treated cells are shown in B, D and F, respectively (Bhoola and Hammond, 2000).

**Effect of PMA on the expression of PP2A**

Variations in the expression of all three subunits of PP2A were seen after stimulation with PMA (figure 26). After PMA treatment, the expression of all three PP2A subunits showed a similar trend, with intensity of the bands falling over the first two days and increasing thereafter (Figure 26B, 26D and 26F).

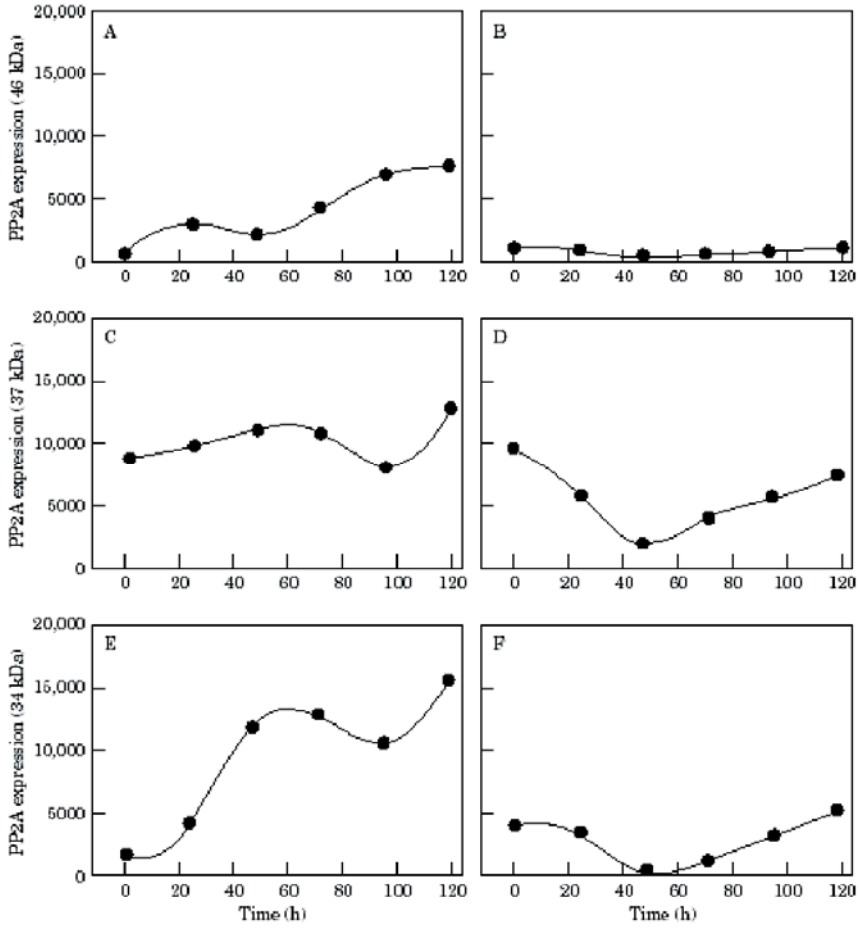


**Figure 26: Temporal changes in the expression of phosphoprotein phosphatase 2A in HL-60 cells treated with PMA.**

Cell lysates were analysed at 24 hour intervals over a period of 120 hours, using an antibody specific for PP2A. Three immunospecific bands of 46 kDa (A and B), 37 kDa (C and D) and 34 kDa (E and F) were observed. Control cells are represented by A, C and E; their corresponding PMA treated cells are shown in B, D and F, respectively (Bhoola and Hammond, 2000).

**The effect of ATRA and PMA on the expression of PTP-1B**

An example of a Western blot for PTP-1B is shown in figure 27. There were variations in intensities of the major form and in some instances there was a reversal of pattern.

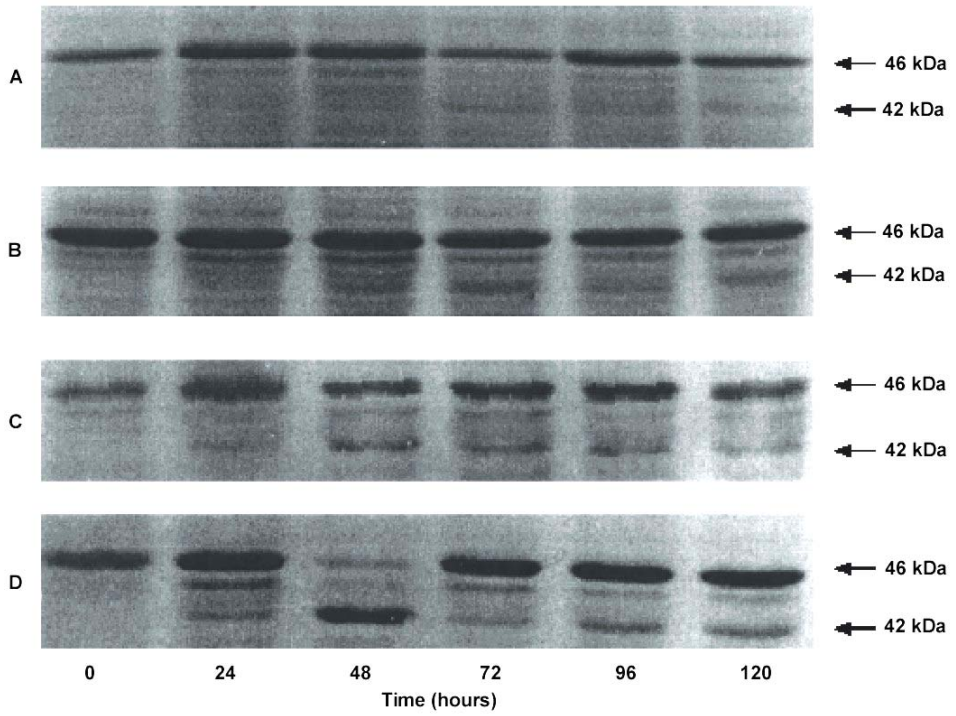


**Figure 27: An example of a Western blot showing PTP-1B expression in HL-60 cells; effect of ATRA and PMA.**

Two immunospecific bands of molecular mass 46 kDa and 42 kDa can be seen. Control cells are represented by A and C and the corresponding ATRA and PMA treated cells are represented in B and D, respectively (Bhoola and Hammond, 2000).

Plots illustrating time-dependent changes in expression of the 42 and 46 kDa forms, in the absence or presence of inducer, are shown in figure 28. In cells treated with ATRA, there was an overall increase in PTP-1B expression and a dampening effect on the oscillations (figure 28. B). Of particular note, was the effect seen after 48 hours of treatment with PMA, when there was a change to predominance of the 42 kDa subunit (figure 27.D, 28.C and 28.D). For both the 46 and 42 kDa bands, PMA appeared to increase the magnitude of the oscillation.

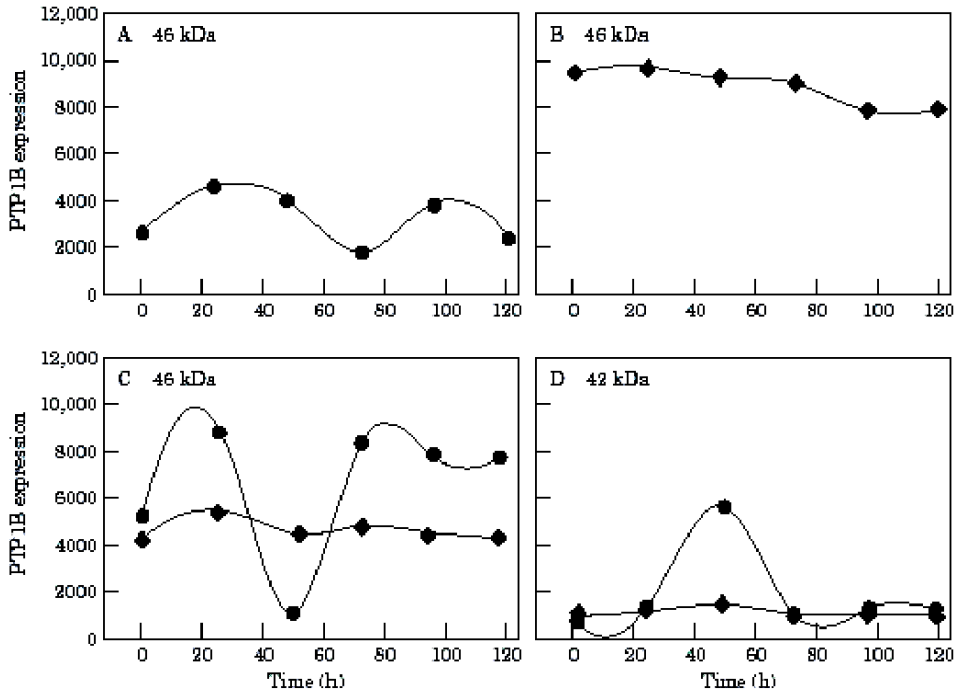


**Figure 28: Temporal changes in the expression of PTP-1B in HL-60 cells; effect of ATRA and PMA.**

Cell lysates were analysed at 24 hour intervals over a period of 120 hours, using an antibody specific for PTP-1B. Results for the 46 kDa immunospecific band in control and ATRA treated cells are represented in A and B, respectively. Results for the 46 kDa and 42 kDa immunospecific bands in control and PMA treated cells are shown in C and D, respectively. Control cells ♦, treated cells ● (Bhoola and Hammond, 2000).

**The effect of different concentrations of ATRA on PTP-1B expression**

The effect of ATRA on the 46 kDa band of PTP-1B is shown in figure 29. Results indicate the expression of PTP-1B protein increases in a concentration dependent manner (figure 30).

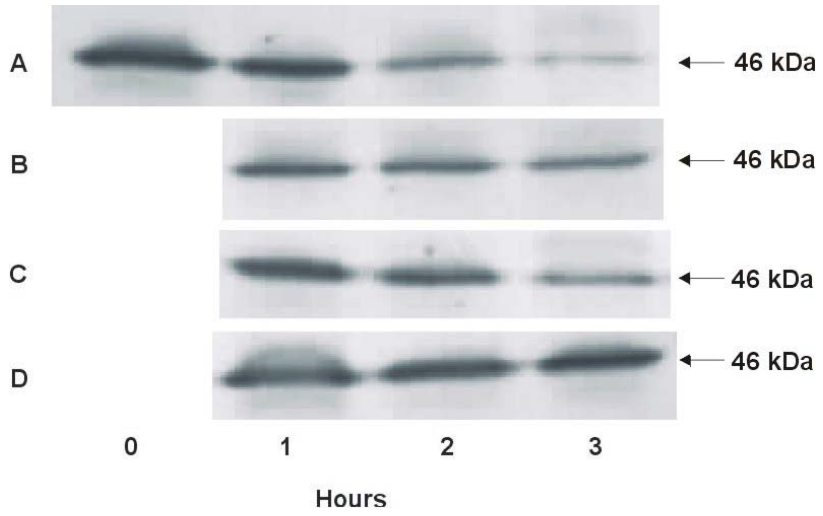


**Figure 29: Western blot showing the effect of different concentrations of ATRA on PTP-1B expression over 3 hours.**

Control represented in lane A, lane B cells were stimulated with 10nM ATRA, lane C with 10μM ATRA and lane D with 10mM ATRA. Samples were taken at 0, 1, 2 and 3 hours for each concentration of ATRA. 100μg of total protein was loaded per lane.

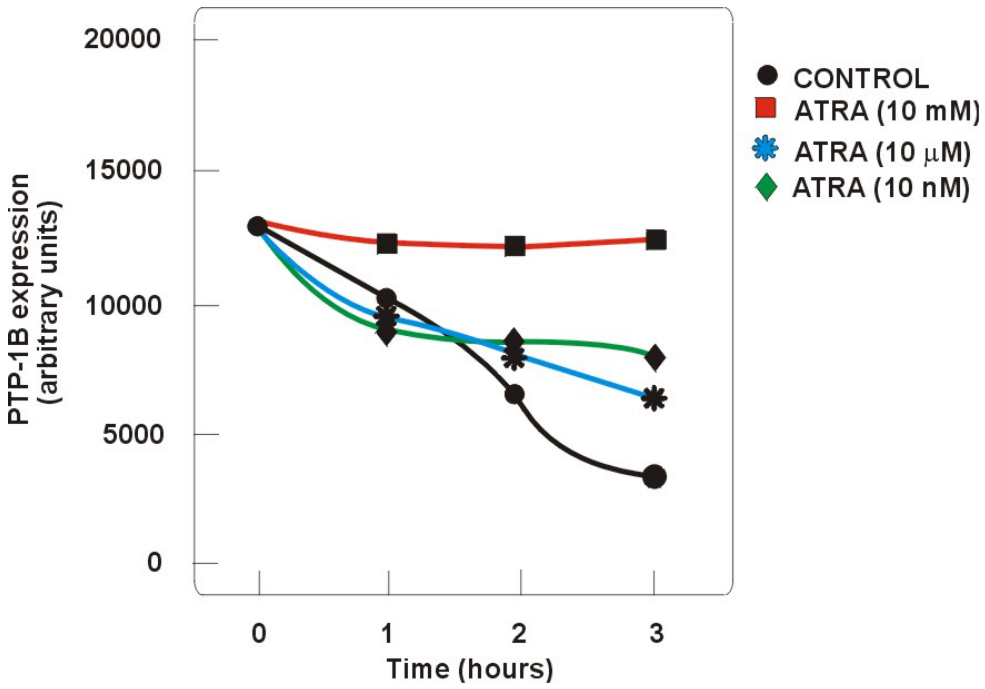


**Figure 30:** **Graphical representation of figure 29 demonstrating the effect of different concentrations of ATRA on PTP-1B 46 kDa protein expression in HL-60 cells over 3 hours.**

**The effect of different concentrations of 9-cis RA on PTP-1B expression**

Effects of 9-cis RA on the 46 kDa PTP-1B subunit are shown in figure 31. An inhibitory effect was noted when 10nM 9-cis RA was used and no change when using 10 mM 9-cis RA. When using 10μM 9-cis RA a third immunospecific band of molecular mass 38kDa was noted.

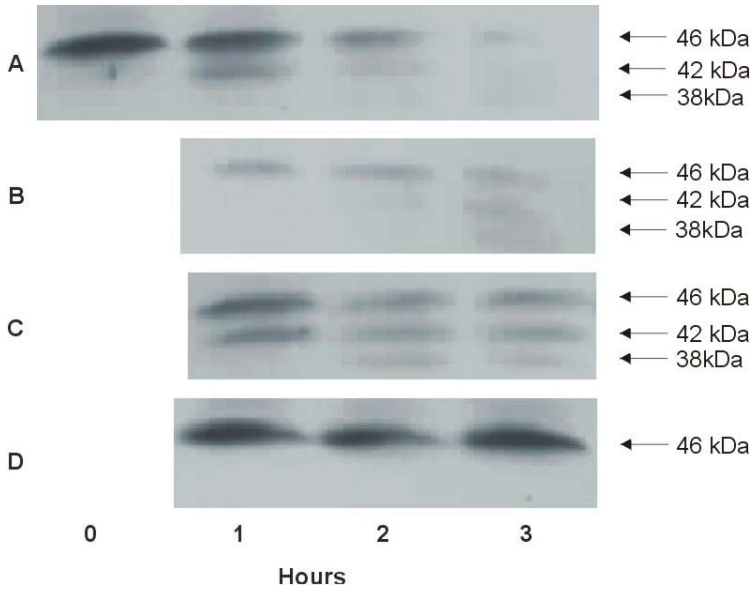


**Figure 31: Western blot showing the effect of different concentrations of 9-cis RA on PTP-1B expression over 3 hours.**

Control represented in lane A, lane B cells were stimulated with 10nM 9-cis RA, lane C with 10μM 9-cis RA and lane D with 10mM 9-cis RA. Samples were taken at 0, 1, 2 and 3 hours for each concentration of 9-cis RA. 100μg of total protein was loaded per lane.

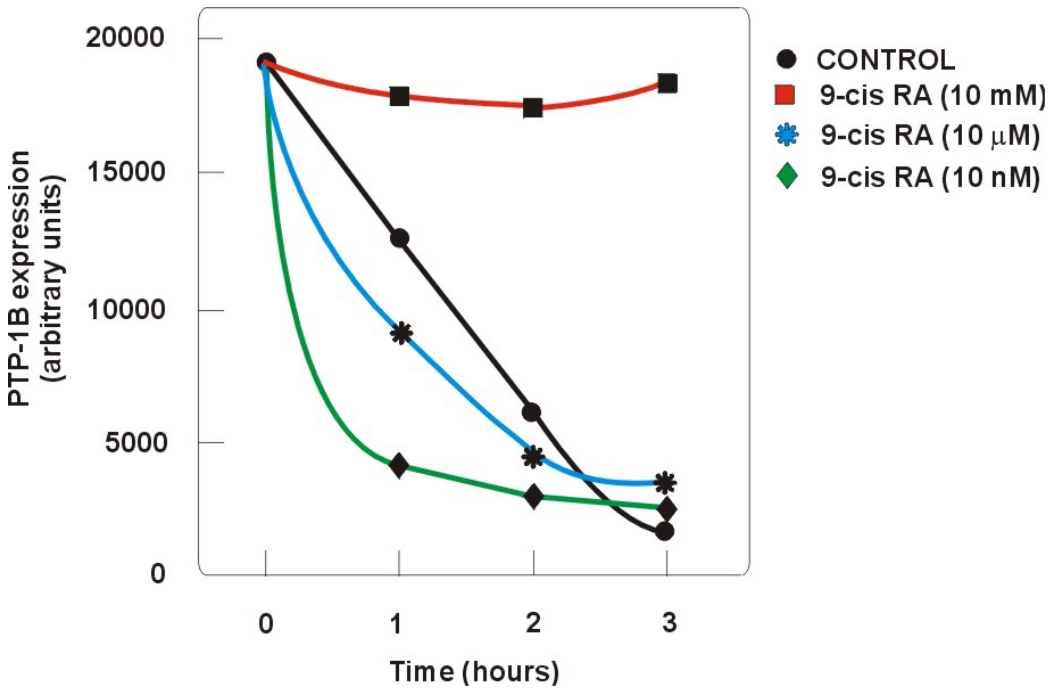


**Figure 32:** **Graphical representation of figure 31 demonstrating the effect of different concentrations of 9-cis RA on PTP-1B 46 kDa protein expression in HL-60 cells over 3 hours.**

**Comparison of the effects of ATRA and 9-cis RA on PTP-1B expression**

The effects of 10mM ATRA and 10mM 9-cis RA on PTP-1B 46 kDa protein expression over 3 hours were compared. Cells treated with ATRA and 9-cis RA showed an increase in PTP-1B expression over 3 hours, with the increase greatest in 9-cis RA treated cells (figure 34).

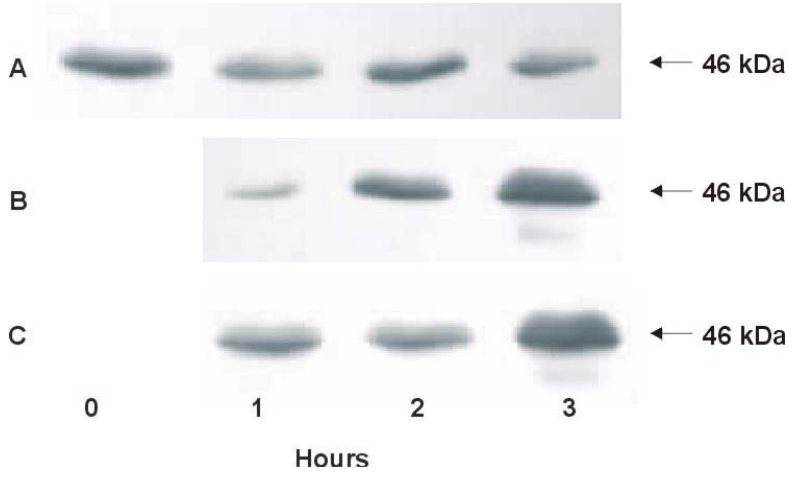


**Figure 33: Western blot showing the effect of 10mM 9-cis RA and 10mM ATRA on PTP-1B expression over 3 hours.**

Lane A: control, lane B: 10mM ATRA, lane C: 10mM 9-cis RA, samples were taken at 0, 1, 2 and 3 hours respectively. 100µg of total protein was loaded per lane.

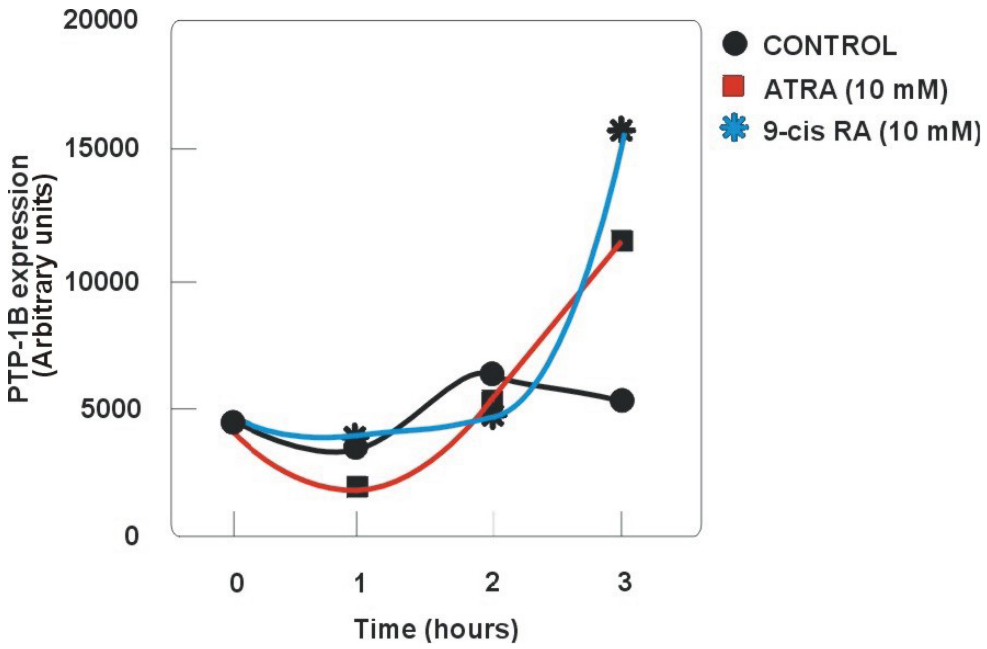


**Figure 34:** **Graphical representation of figure 33 demonstrating the effect of 10mM ATRA and 10mM 9-cis RA on PTP-1B 46 kDa protein expression in HL-60 cells over 3 hours.**

**The effect of phosphorylation and dephosphorylation inhibitors on PTP-1B protein expression**

Okadaic acid is a known phosphatase inhibitor that increases total cellular phosphorylated proteins, whereas genistein is a kinase inhibitor that decreases total protein phosphorylation. The effect of these two inhibitors on HL-60 cell PTP-1B 46 kDa protein expression was studied. Below is a western blot (figure 35) of PTP-1B expression after stimulation with genistein and okadaic acid for one hour. Results indicate that there is an increase in expression after okadaic acid (lane 3) treatment and a decrease in expression after stimulation with genistein (lane 2, figure 35), confirming the hypothesis that these inhibitors could play a role in influencing protein phosphorylation.

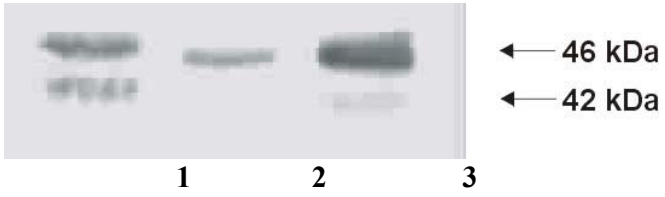


**Figure 35: Western blot of PTP-1B protein expression after incubation with either okadaic acid or genistein for one hour.**

Lane 1: control, Lane 2: genistein, Lane 3: okadaic acid. 100µg of total protein was loaded per lane.

**DISCUSSION**

The study of oscillatory behaviour in cells is fascinating and of particular interest are the phosphatases, because of their possible role in cell differentiation and reversal of malignant transformation. For quite some time, the kinase-mediated phosphorylation process was avidly pursued as a central theme of many cellular processes, while dephosphorylation and the action of phosphatases were seemingly neglected. Over the past years, it has become clear that the protein products of certain cellular oncogenes are protein kinases, which are elements in intracellular signal networks controlling growth factors specific responses, such as mitosis, differentiation, migration, survival, transformation and death (Hunter, 1991).

The phosphoprotein phosphatases must also be critical regulators of these pathways, and recent evidence suggests that some, at least, may function as tumour suppressor proteins by antagonizing the action of kinases; they may also regulate tumour cell invasion and metastasis through interactions via focal adhesions. Strategies aimed at inhibiting protein kinase activity are being developed for the treatment of cancer, whereas an alternative, and as yet unexplored, therapeutic approach may be to modulate the activities of the protein phosphatases (Wagner and Flanagan, 1997).

The expression of PP1, PP2A and PTP-1B was assessed in HL-60 cells. In the preliminary studies (figure 23), analyses were carried out over a period of several days and oscillations were apparent for each of the proteins. The major immunoreactive PP1 protein in these cells had a molecular mass of 38kDa and this was assumed to be the catalytic subunit. Again, two major bands were detected for PP2A, a form of mass 37kDa, probably the catalytic subunit. The predominant PTP-1B band had a molecular mass of 46kDa. When differentiation was induced along the granulocytic pathway using DMSO, the effects on expression of PP1, the two forms of PP2A and PTP-1B were dramatic, the oscillations apparently being suppressed as compared with the respective untreated controls (Hammond et al., 1998).

The importance of protein phosphorylation and dephosphorylation in the control of cell proliferation, differentiation and the development of cancer is well known (see, for example, Feuerstein and Cooper, 1984; Morita et al., 1992; Tawara et al., 1993; Pennisi, 1997). Relatively little attention, however, has been paid to the temporal aspects of these processes and the possible operation of dynamic control mechanisms. The involvement of reversible phosphorylation during HL-60 cell differentiation was demonstrated earlier by Feuerstein and Cooper (1984) and Morita et al. (1992). In the work reported here, we investigate temporal variations in phosphoprotein phosphatase expression during induced differentiation along the granulocytic and monocytic pathways in these cells.

In this study, results from individual experiments are considered rather than statistical means (Hammond et al., 1998). Although certain trends can often be identified, variations in temporal behaviour are generally seen from one experiment to another. This is to be expected, since disturbances to the cells in any way may cause alterations in metabolism, a factor which is not always appreciated (Ferreira et al., 1994). A range of results may be possible due to periodic behaviour, without the oscillation being evident. Once again, in this study, we highlight the complexity of such systems and the need for time analyses. In experiments where only single time-points are used, standard deviations are often high, as would be expected in an oscillating system, and the information obtained may be incomplete and possibly misleading (Hammond et al., 1998). The fluctuations seen in this and in our earlier studies (Hammond et al., 1998) are in many instances considerable, for PP1 as much as 7–8-fold and for PP2A and PTP1B up to 10–12-fold, and cannot be explained in terms of experimental error. The latter may, however, be a factor where differences between time-points are very small.

The PP1 protein is a multimeric structure comprising a catalytic subunit which interacts with a variety of regulatory subunits, each modifying the catalytic subunit for performing a specific function in a particular region of the cell (Egloff. et al., 1997). The molecular mass of 38 kDa for PP1 obtained in our study corresponds closely to that given by Mumby and Walter (1993) for the

catalytic subunit of the protein; Tawara and co-workers (1993), however, using the same cell line, reported a molecular mass of 32 kDa for this subunit.

PP2A is a structurally complex protein in which a single catalytic subunit, of mass 32–38 kDa, can associate with a wide array of regulatory and targeting subunits in various combinations to influence a wide variety of cellular processes, including protein kinase cascades in the various signal transducing networks (Baharians and Schonthal, 1998; Millward et al., 1999). Tawara et al. (1993) detected a single PP2A band, corresponding to the catalytic subunit, of mass 33 kDa in HL60 cells. Phosphoprotein phosphatase 2A subunit C appears as a doublet of masses 37 and 35 kDa when run on SDS-PAGE due to intramolecular disulfide bond formation (Walter et al., 1990). Carboxymethylation of proteins is a highly conserved means of regulation in eukaryotic cells. The protein phosphatase 2A catalytic subunit C is reversibly methylated at its carboxyl terminus by specific methyltransferase and methylesterase enzymes. One such protein is protein phosphatase methylesterase-1 (PME-1) which is conserved from yeast to human and contains a motif found in lipases having a catalytic triad-activated serine as their active site nucleophile. Bacterially expressed PME-1 demethylated PP2A C subunit in vitro, and okadaic acid, a known inhibitor of the PP2A methylesterase, inhibited this reaction. Also the doublet that was noted on SDS-PAGE of the C subunit was no longer a doublet, suggesting that carboxymethylation of protein phosphatases may result in subunit doublet formation (Ogris et al., 1999).

Using an antibody specific for the catalytic subunit of PP2A, however, we detected two bands corresponding to masses of 34 and 37 kDa. Phosphorylation of the PP2A catalytic subunit is known to occur in vitro (Mumby and Walter, 1993), and it is possible that the 2 bands may represent the different phosphorylation states. The presence of a doublet in this region has previously been reported (Walter et al., 1990; Turowski et al., 1995; Ogris et al., 1999). It was ascribed by Walter et al. (1990) to oxidation of disulphide bonds, whereas Ogris et al. (1999) indicated that its appearance was variable, sometimes only a single band was seen, and that it did not seem to be the result of degradation. The nature and origin of the minor 46 kDa form of PP2A, also recognized by our antibody, is not yet known. During ATRA-induced differentiation, our results show different trends in the patterns of expression of the 34 and 37 kDa subunits, suggesting that their expression may be modulated independently of each other. With PMA, the patterns are modified and the trends become similar, and in phase, for all three of the subunits.

A possible role for PP1 and PP2A in regulating granulocytic differentiation of HL-60 cells was suggested by Morita et al. (1992), who assessed the enzyme activity, and Tawara et al. (1993), who investigated protein expression as well as activity. Tawara's group showed a decrease in phosphatase activity, as measured using myosin light chain substrate, over a period of four days after exposure of cells to ATRA. They found that, while there was little change in expression of a 33 kDa PP1 immunoreactive protein, the expression of the

PP2A catalytic subunit, measured over several days, was decreased during ATRA-induced differentiation. These workers, however, analysed only treated cells over the time period studied; there were no corresponding control cells and the possibility of time dependent changes in patterns of expression, as observed in the present study, had not been considered. In our study, the fluctuations seen for PP1 were not as great as those seen for the PP2A 34 and 37 kDa bands, but clear differences between control and treated cells were apparent. Our results for PP2A expression were consistent with those of Tawara et al. (1993) in that the expression of the protein decreased during differentiation. We showed further that this represented a dampening effect, of possible regulatory significance, as compared with untreated proliferating cells. In contrast to our findings, Tawara et al. (1993) showed little change in the expression of the PP2A protein during the course of phorbol ester-induced differentiation.

The protein tyrosine phosphatases comprise a group of enzymes of which approximately one third are trans-membrane receptor-like proteins and the remainder are intracellular; PTP1B is a cytoplasmic phosphatase first isolated from human placenta (Tonks and Charbonneau, 1989; Walton and Dixon, 1993; Hunter, 1995). For PTP1B, we found that the 46 kDa band was predominant in control and ATRA-treated cells and, again, there was temporal variation in expression. The effect seen with PMA was different and is remarkable, with a complete change in distribution of the two forms in a time-dependent manner. The findings for PTP1B, as for PP1 and PP2A, indicate that the dynamic control

mechanisms operating during induced differentiation along the granulocytic and monocytic pathways are different.

The change to predominance of the 42 kDa form of PTP1B seen with PMA could be the result of dephosphorylation or, alternatively, calpain catalysed cleavage of the protein may be causing the mobility shift. Studies indicate that HeLa cells undergo phosphorylation of PTP1B during mitosis when the slower moving band is seen; in asynchronous cells, the faster band predominates. In a study of the effects of different lysing buffers on PTP-1B expression, we showed that when fluoride and phosphate were included, the protein was detected exclusively as the higher molecular mass form; in buffers from which these substances were omitted only the lower band was seen (results not shown). Proteolytic cleavage of PTP1B has been demonstrated in human platelets; the cleavage of a 50 kDa protein to produce a 42 kDa form is catalysed by the calcium-dependent neutral protease, calpain, and is a general feature of platelet agonist-induced aggregation (Frangioni et al., 1993). Similarly, the 46 kDa form of PTP1B, observed in the present study, may be susceptible to calpain-mediated proteolysis, generating a 42 kDa protein. Interestingly, platelet aggregation, induced by PMA, resulted in cleavage of more than 70% of PTP1B to the 42 kDa species (Frangioni et al., 1993). Calpain-induced cleavage of PTP1B has important biochemical consequences in that it is associated with relocation of the enzyme from membranes to cytosol and with activation of tyrosine phosphatase activity (Frangioni et al., 1993). This may represent a

novel mechanism for altering tyrosine phosphorylation and studies of the effects of calpain in our system may shed further light on the control of cell differentiation.

Elevation of intracellular calcium concentrations induced by ionomycin or adherence to fibronectin, resulted in a calpain mediated cleavage of PTP-1B near the endoplasmic reticulum-targeting sequence and results in the generation of an enzymatically active form of the phosphatase. Both the native and the cleaved forms of PTP-1B interact with $p130^{Cas}$ in T cells. This interaction may serve to relocate PTP-1B to sites of focal contact resulting in potential interactions with substrates previously inaccessible to the endoplasmic reticulum-associated phosphatase. Thus, a novel calcium-dependent signaling pathway in T-cells that may mediate signals generated by β1 integrin adherence to the extracellular matrix (Rock et al., 1997).

At this stage, we have determined the expression of the PP1, PP2A and PTP1B at intervals of 24 h, as in our previous study, which showed temporal changes in the expression of these proteins during HL-60 cell differentiation induced by dimethyl sulfoxide (Hammond et al., 1998). It would be of interest to carry out analyses with different time intervals and to obtain information relating to the periods of the rhythms. From studies with phosphotyrosine phosphatase (Ferreira et al., 1996b), we suggested that the period of the activity oscillations

may be less than 10 min, as found for lactate dehydrogenase activity and isoenzymes (Ferreira et al., 1996a). This could be the case for the expression of the PP1, PP2A and PTP1B proteins, although the dynamics may differ from one protein to another and there may be differences between expression and activity. Studies of the temporal relationships between the activities of these enzymes and their protein expression may shed further light on the dynamic control mechanisms influencing dephosphorylation.

PTP-1B protein expression after stimulation with ATRA over 120 hours indicates that there is a dampening effect on the oscillatory cycling of protein expression. We looked at the effect of different concentrations of ATRA over 3 hours on PTP-1B expression and noticed that there was a concentration dependent effect on expression of PTP-1B. A similar dampening effect seen with ATRA over 120 hours could be reproduced with high concentrations of ATRA over 3 hours, concluding cellular differentiation is dependent on the concentration of inducing agent used in the case of ATRA and not on the time of exposure to the agent (figure 30). A similar concentration dependent effect with the use of 9-cis retinoic in HL-60 cell PTP-1B expression was seen. The effect of ATRA and 9-cis RA on the proliferation and differentiation of primitive hematopoietic cells demonstrated a reversible and dose dependent inhibition of proliferation. The 50% effective dose was 10nM for both ATRA and 9-cis RA in murine $Lin^{-}Sca\text{-}1^{+}$ bone marrow cells (Jacobsen et al., 1994). Three specific PTP-1B bands were noted when using 9-cis retinoic acid,

indicating calpain cleavage of PTP-1B (Frangioni et al., 1993) might be induced by 9-cis retinoic acid. A novel band previously undescribed of molecular mass 38 kDa was formed when inducing HL-60 cells with 9-cis retinoic acid, indicating that this might also be acting via calpain to induce PTP-1B cleavage. The 37kDa PTP-1B is a proteolysed form of PTP-I (placental membrane protein phosphotyrosine phosphatase) and provides evidence, that a larger form of PTP-1B exists in vivo, at least in association with placental membranes (Pallen et al., 1991).

The second messenger hydrogen peroxide is required for optimal activation of numerous signal transduction pathways, particularly those mediated by protein tyrosine kinases. One mechanism by which hydrogen peroxide regulates cellular processes is the transient inhibition of protein tyrosine phosphatases through the reversible oxidation of their catalytic cysteine, which suppresses protein dephosphorylation (Mahadev et al., 2001). When PTP-1B protein was subjected to varying amounts of $H_2O_2$, it was noted that at higher concentrations of $H_2O_2$, PTP-1B was irreversibly oxidized and a shift in relative molecular mass was noted. At lower concentrations of $H_2O_2$, which reversibly oxidized PTP-1B, no formation of Cys-SOH modification was noted (Salmeen et al., 2003). This aspect of PTP-1B was not analysed in this study but may be interesting to study in future experiments.

PTP-1B is a widely expressed non-transmembrane tyrosine-specific phosphatase. Previous studies indicated that, at mitosis, PTP-1B undergoes phosphorylation on two sites, 352 Ser-Pro-Leu and 386 Ser-Pro-Ala. Although the Ser-386 site can be phosphorylated by Cyclin B/Cdc2 in vitro, the kinase for the Ser-352 site is still unknown. Phosphorylation events are not unique in mitosis and treatment with many, but not all, three stimuli, in particular osmotic shock and certain phosphatase and protein synthesis inhibitors, lead to phosphorylation of PTP-1B (Shifrin et al., 1997). It can therefore be deduced that if mitosis induced stress leads to phosphorylation of PTP-1B, then differentiation leads to dephosphorylation of PTP-1B and activation of this protein.

The work presented here demonstrates that biochemical processes underlying cell function and behaviour can be time dependent and should be regarded as such when designing experiments and interpreting results. Temporal aspects relating to PP1, PP2A and PTP1B have been considered in HL60 cells and it is possible that modulation of the oscillatory characteristics of key enzymes such as these by potential therapeutic agents may be crucial to the control of cell differentiation and transformation and treatment of diseases. The effect on proliferation and differentiation of individual drugs was reversible, while the proliferation arrest and differentiation induced by the combination of ATRA and 9-cis RA persisted and even progressed after withdrawal of the drugs (Verstuyf et al., 1995).

The acyclic retinoid (9-cis RA), a synthetic retinoid X receptor α (RXRα)-ligand, suppresses the development of hepatocellular carcinoma (HCC) in patients with chronic liver disease. The 9-cis RA ligand restores the function of RXRα in human HCC-derived HuH7 cells by inactivating the Ras-Erk½ signaling system, thereby dephosphorylating RXRα. In contrast, 9-cis RA failed to suppress phosphoErk ½ levels and subsequently RXRα phosphorylation. Although 9-cis RA also suppressed Ras activity, it simultaneously down-regulated mitogen-activated protein kinase phosphatase-1, an enzyme that inactivates Erk, thereby leaving the phosphorylation status of Erk unchanged providing a novel molecular basis for the tumour activity of 9-cis RA against HCC (Matsushima-Nishiwaki et al., 2003). This model could also be used in future studies with HL-60 cells to see if promyelocytic leukaemic cells use similar mechanisms when stimulated with 9-cis retinoic acid.

It has been shown that 9-cis RA is a potent inhibitor of mammary carcinogenesis induced by N-nitroso-N-methylurea in Spargue-Dawley rats. The combination of 9-cis RA and low levels of tamoxifen was effective in doubling the number of rats that were tumour-free. For suppression of carcinogenesis in vivo, 9-cis RA was much more potent then ATRA, both as a single agent or in combination with tamoxifen (Anzano et al., 1994). It has been shown that 9-cis-β-carotene can be a source of 9-cis RA in humans. This conversion has been shown to occur in human intestinal mucosa in vitro and may have significance in the anticarcinogenic action of β-carotene (Wang et al., 1994). Damage to the

intestinal mucosa therefore must make people more susceptible to malignancies of the mammary glands when suffering from crohns disease and ulcerative colitis.

Endogenous retinoids are potential regulators of vertebrate embryogenesis that have been implicated in early anterior-posterior patterning and limb-bud development. 9-cis Retinoic acid was first detected after the midblastula transition and by the end of gastrulation is localized primarily within the anterior and posterior dorsal regions of the embryo. Since 9-cis-retinoic acid is a 6-fold more potent dysmorphogen than trans-retinoic acid, it was suggested that it is involved in the early specification of the Xenopus anterior-posterior axis (Kraft et al., 1994). These results suggest that 9-cis RA could be involved in embryogenesis control and disruption. However, since it has such a potent effect it needs to be used with care if it is to be used as a chemotherapeutic agent since it may itself induce transformation of normal cells.

Treatment of HL-60 cells with PMA results in arrest of growth and terminal differentiation of the cells into macrophages. Within minutes of exposure of PMA to HL-60 cells there is a severalfold increase in phosphorylation of two cytosolic protein pp17 and pp27 (Feuerstein and Cooper, 1984). A similar increase after PMA stimulation was noted with PP1, PP2A and PTP-1B in HL-60 cells (Bhoola and Hammond, 2000). PMA caused a ten fold increase in ten

phosphorylated proteins when stimulated for 30 minutes (Anderson et al., 1985). PTP-1B is phosphorylated on seryl residues in vivo. Increased phosphorylation of PTP-1B is seen to accompany the transition from G2 to M phase of the cell cycle. Stimulation of HeLa cells with phorbol esters (TPA) enhances phosphorylation of PTP-1B. TPA-stimulated phosphorylation of PTP-1B in vivo appears to result directly from phosphorylation of PKC (Flint et al., 1993).

The inhibition of acetaldehyde-induced paracellular permeability by the tyrosine kinase inhibitor, genistein, demonstrated that acetaldehyde reduced PTPase activity in plasma membrane and soluble fractions, whereas tyrosine kinase activity remained unaffected. Treatment with acetaldehyde resulted in a 97% loss of protein tyrosine phosphatase (PTP-1B) activity and a partial reduction of PTP-1C and PTP-1D activities. Strongly suggesting that acetaldehyde inhibits PTPases to increase protein tyrosine phosphorylation, which may result in disruption of the tight junctions between cells (Atkinson and Rao, 2001). Genistein is known to inhibit both tyrosine protein kinases and DNA topoisomerase II. Genistein concentrations greater then 185 μM were cytotoxic to HL-60 cells following exposure for 24 hours, however nuclear fragmentation from apoptosis was observed within 8 hours. Short-term exposure (4-8 hrs) of HL-60 cells to low concentrations of genistein resulted in a suppression of cell progression through S or through both S and $G_2$ phases. Longer exposure (24 hrs) to low concentrations of genistein leads to an S-phase arrest (Traganos et

al., 1992). In our study, HL-60 cells were exposed for only one hour to genistein and therefore S and $G_2$ phase arrest could not account for the decrease in PTP-1B expression shown in our result, suggesting that another mechanism must be responsible for the decreased phosphorylation.

Hyperphosphorylation and migration shifts of total cellular protein in MCF7 cells were noted when adding protein phosphatase inhibitors, okadaic acid and calyculin-A, and examining on SDS-PAGE (Favre et al., 1997). Similar migration shifts in PTP-1B were seen by in HL-60 cells after one hour of exposure to okadaic acid. Okadaic acid has been shown to increase pp76 serine phosphorylation by increasing pp76 kinase activity 7-fold (Cengel et al., 1998). A similar 4-fold increase in PTP-1B tyrosine phosphorylation was seen by us in HL-60 cells after only one hour exposure to okadaic acid.