Induction of HL-60 cell differentiation by the p38 mitogen-activated protein kinase inhibitor SB203580 is mediated through the extracellular signal-regulated kinase signaling pathway

Jin-Xia Zhang[a], Wie-Jian Zhuang[a], Ka-Hung Poon[a], Michael Yang[a] and Wang-Fun Fong[a]

The pyridinyl imidazole p38 kinase inhibitor, SB203580, was initially used to block inflammatory cytokine synthesis. Here we report that SB203580 by itself could induce human promyeloid leukemic HL-60 cells to differentiate mainly along the granulocytic lineage, as evidenced by cellular morphological changes, and the concurrent expression of cell surface markers CD11b and CD14. This differentiation induction was time and dose dependent. After 12 h exposure to 10 μM SB203580, 12.5% of the cells became CD11b$^+$ as compared to only 2.6% in untreated control cells. By 96 h, CD11b$^+$ cells increased to 72.3%, and among them, 26% were CD14$^+$. Morphologically, the cells were smaller in size with lower nuclear/cytoplasmic ratio. The nucleus was indented and nucleoli markedly reduced. However, 10 μM SB203580 had little effect on HL-60 cell growth and survival during the first 72 h, but by 96 h the percentage of cells in G_1 phase was markedly increased. These effects of SB203580 were not attributable to its inhibition of p38 kinase activity. Instead, the essential kinases in the extracellular signal-regulated kinase (ERK) pathway such as phospho-Raf-1, phospho-MEK1/2, phospho-ERK1/2 and phospho-p90RSK were all elevated dramatically shortly after cells were exposed to SB203580 and lasted for 24 h before declining. Pre-incubation of cells with 20 μM of PD98059 1 h before addition of SB203580 could completely block the expression of differentiation markers. Our results suggest that SB203580-induced differentiation in HL-60 cells was mediated by activation of MEK/ERK signaling. In conclusion, our data have shown that SB203580 possessed biological activities other than inhibition of p38 and these activities could make it a potential candidate as an inducing agent for cell differentiation in the therapeutic treatment of leukemia. *Anti-Cancer Drugs* 14:31–38 © 2003 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2003, **14**:31–38

Keywords: differentiation, HL-60, mitogen-activated protein kinase, p38 kinase, p38 kinase inhibitor, SB203580

[a]Bioactive Products Research Group, Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong SAR, China.

Sponsorship: Supported by City University of Hong Kong Strategic research grant 7001110.

Correspondence to W.F. Fong, Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong SAR, China.
Tel: +852 2788 9181; fax: +852 2788 7406;
e-mail: bhwffong@cityu.edu.hk

Received 10 September 2002 **Accepted** 6 October 2002

Introduction

The mitogen-activated protein kinases (MAPKs) are proline-directed serine–threonine kinases, which serve as mediators for cellular responses to a variety of extracellular stimuli. The superfamily of MAPKs consists of at least three major groups, i.e. the extracellular signal-regulated kinases (ERK1 and ERK2), the c-jun N-terminal kinases (JNKs), and p38 MAP kinase. ERKs are characteristically activated by various growth factors in association with cell proliferation and protection against apoptosis. Members of the JNK and p38 subfamilies are strongly activated in response to stress stimuli such as ultraviolet radiation, heat shock and hyperosmolarity [1–3], and thus they have been given the name 'stress-activated protein kinases' (SAPKs). The p38 MAPKs are also strongly activated by pro-inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α. They have been shown to play important roles in inflammatory-related diseases, thus making them a possible therapeutic target for inflammatory diseases. However, a number of cellular functions unrelated to stress responses, including cell differentiation, proliferation and survival, have also been shown to be related to P38 MAPK activities [4].

The pyridinyl imidazole p38 kinase inhibitors, such as SB203580, SB202190 and other similar p38 kinase inhibitors, were originally prepared as inflammatory cytokine synthesis inhibitors [5,6] exerting potent anti-inflammatory function both *in vitro* and *in vivo* [7]. Subsequently, they were found to be specific inhibitors of p38 MAP kinase [8]. Structural analysis shows that SB203580 is bound inside the ATP pocket and, thereby, specifically inhibits ATP's enzymatic activity [9].

0959-4973

Interestingly, besides inhibiting p38 activity, reports of other new functions of SB203580 began to emerge recently. It activates Raf-1 in smooth muscle cells [10], and potentiates ERK pathways in the 1α,25-dihydroxyvitamin D_3-induced monocytic differentiation in HL-60 cells [11,12] and epidermal growth factor-stimulated PC12 cell differentiation [13]. However, all these studies indicate that SB203580 by itself exerts no effects on ERK activity or cell differentiation. A controversial report by Yuki showed that SB203580 alone could induce HL-60 cell differentiation [14]. The present study was aimed at resolving this controversy by studying the role of SB203580 with regard to cell differentiation and ERK activation. We found here that SB203580 and SB202190 could, not only induce cell differentiation, but also moderately inhibit cell growth in HL-60 cells. In addition, these effects were not due to the inhibition of p38 MAPK kinase activity, but probably involve ERK kinase activation.

Materials and methods

Chemicals and antibodies

P38 inhibitors SB203580, SB202190 and MEK inhibitor PD98059 were purchased from Calbiochem (La Jolla, CA). All-*trans*-retinoic acid (ATRA) and Wright–Giemsa staining solution were purchased from Sigma (St Louis, MO). Anti-CD11b and anti-CD14 antibodies were purchased from PharMingen (San Diego, CA); phospho-Erk1/2 pathway sampler kit was from Cell Signaling Technology (Bedford, MA); mouse anti-phospho-p38 and mouse anti-p38 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

The human promyelocytic leukemia cell line HL-60 was obtained from the ATCC (Manassas, VA) and cultured in RPMI 1640 medium (Gibco/BRL, Grand Island, NY) supplemented with 15% fetal bovine serum. Cells were subcultured 2–3 times weekly to maintain a log phase growth.

Testing of cell proliferation and cell viability

Cells were seeded at 1.5×10^5/ml in 24-well plates and were allowed to grow for 4 days in normal growth medium or medium containing various amounts of SB203580 as indicated in the text. Their numbers were counted each day using a hemocytometer. Cell viability was determined by Trypan blue dye exclusion method. All the experiments were repeated at least 3 times.

Cell differentiation assay

HL-60 cells were suspended at 3.5×10^5 cells/ml in fresh medium, and various amounts of SB203580 were added as indicated in the text and incubated at 37°C with 5% CO_2 for various durations. After incubation, the cells were harvested and aliquots were used for cell cycle and differentiation analysis.

Cell differentiation was determined by cell morphological changes and cell surface markers (CD11b and CD14) expression. For detection of cell surface markers, 1×10^6 cells were harvested at indicated time, washed twice with ice-cold phosphate buffer solution (PBS) before 20 μl of phycoerythrin (PE)-conjugated anti-CD11b and FITC-conjugated anti-CD14 antibodies were added and incubated for 45 min at room temperature. After incubation, the cells were washed twice again with cold PBS and re-suspended in 0.5 ml of PBS. Two-parameter analysis was performed by FACSCalibur flow cytometry with the aid of CellQuest software (Becton Dickinson, Mountain View, CA). All the differentiation-induction experiments were independently performed at least 3 times.

The morphology of differentiated cells was examined microscopically using the Wright–Giemsa stain according to the manufacturer's instruction.

Analysis of cell cycle distribution

Aliquots of 1×10^6 cells from the same differentiation-induction experiments were washed twice with ice-cold PBS and fixed in 70% ethanol at −20°C overnight. The cells were washed with PBS and incubated with 100 μg/ml RNase at 37°C for 30 min. Propidium iodide (PI) solution was added at a final concentration of 40 μg/ml. Analysis was performed immediately after PI staining by a FACSCalibur flow cytometer (Becton Dickinson).

Cell extracts and immunoblotting

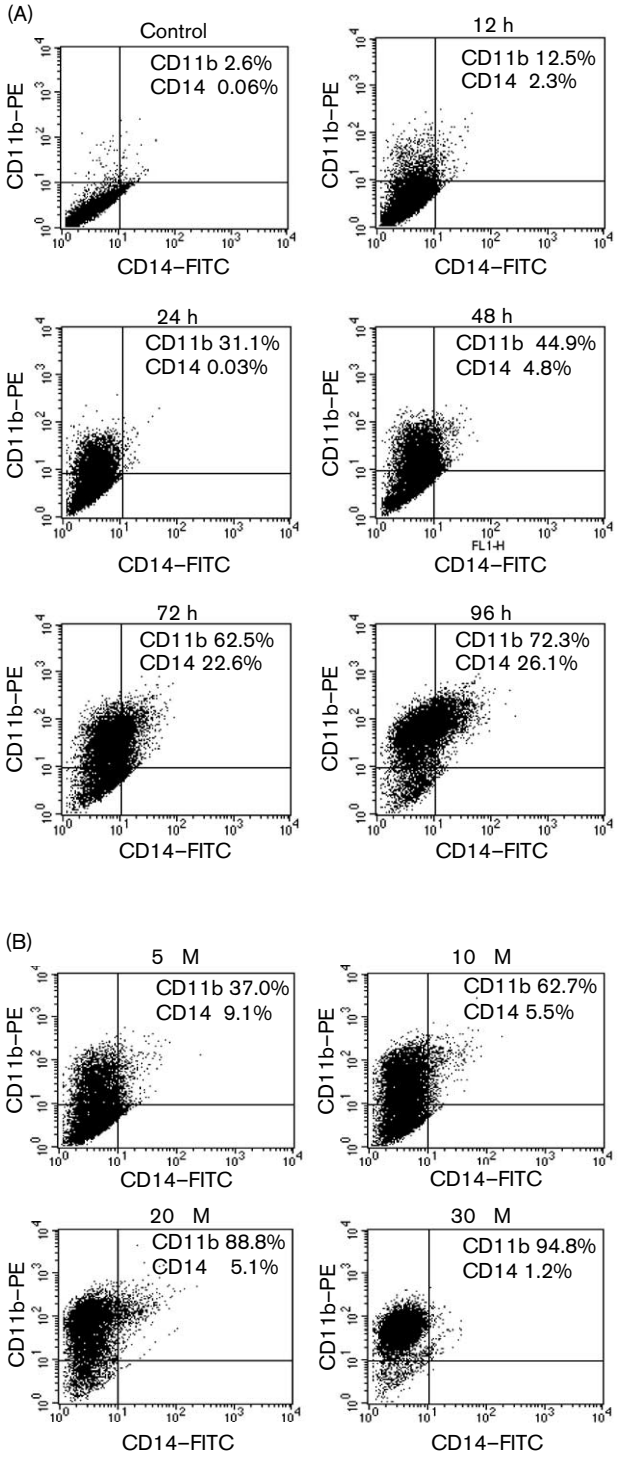
Cells were washed with ice-cold PBS and were lysed in a lysis buffer (1% NP-40, 50 mM Tris pH 7.4, 100 mM NaCl, 0.5 mM $MgCl_2$, 2 mM EDTA, 10 mM EGTA, 5% glycerol, 2 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin) for 10 min on ice. The whole-cell lysates were then centrifuged at 14 000 r.p.m. for 15 min at 4°C. Protein concentration was determined by the Bradford assay. Extracted samples containing 40 μg of protein were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 5% non-fat milk in PBS and then incubated with primary antibodies at 4°C overnight followed by horseradish peroxidase-conjugated secondary antibody at room temperature for 30 min.The protein bands were visualized using a chemiluminescence assay system (Amersham, Piscataway, NJ).

Results

SB203580- and SB202190-induced cell surface markers and morphological changes during differentiation

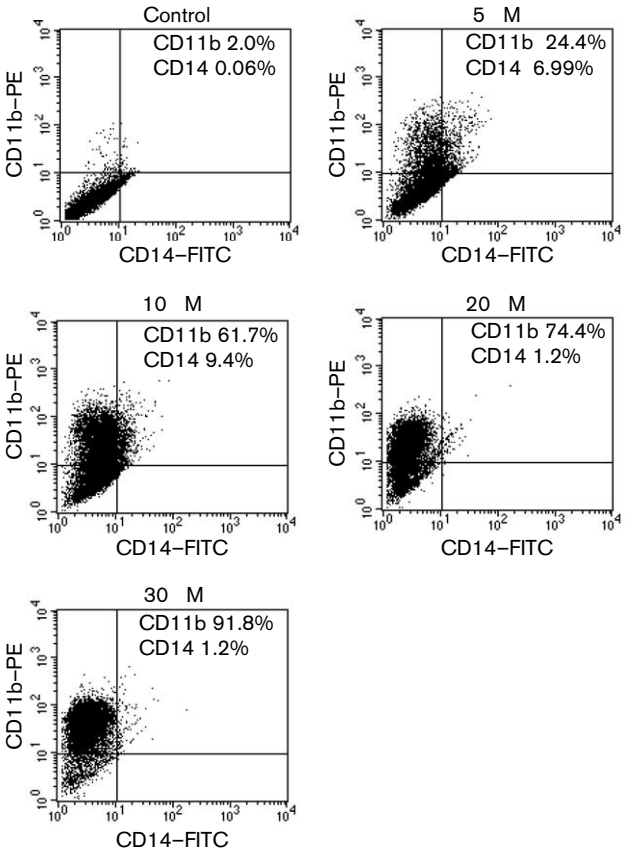
When induced to differentiate, HL-60 cells may express a myeloid cell surface marker, CD11b, and/or a monocytic

Fig. 1



Dose- and time-dependent expression of cell surface differentiation markers in SB203580-treated HL-60 cells. HL-60 cells were harvested at 12, 24, 48, 72 and 96 h. Untreated HL-60 cells were used as the control. Surface differentiation markers, i.e. CD11b (vertical scale) and CD14 (horizontal scale), were determined by flow cytometry as described in Materials and methods. The total percentages of positive cells (CD11b—two upper quadrants; CD14—two right-side quadrants) are indicated within each panel. Experiment were repeated 3 times with similar results. (A) Time dependent. (B) Dose dependent.

Fig. 2

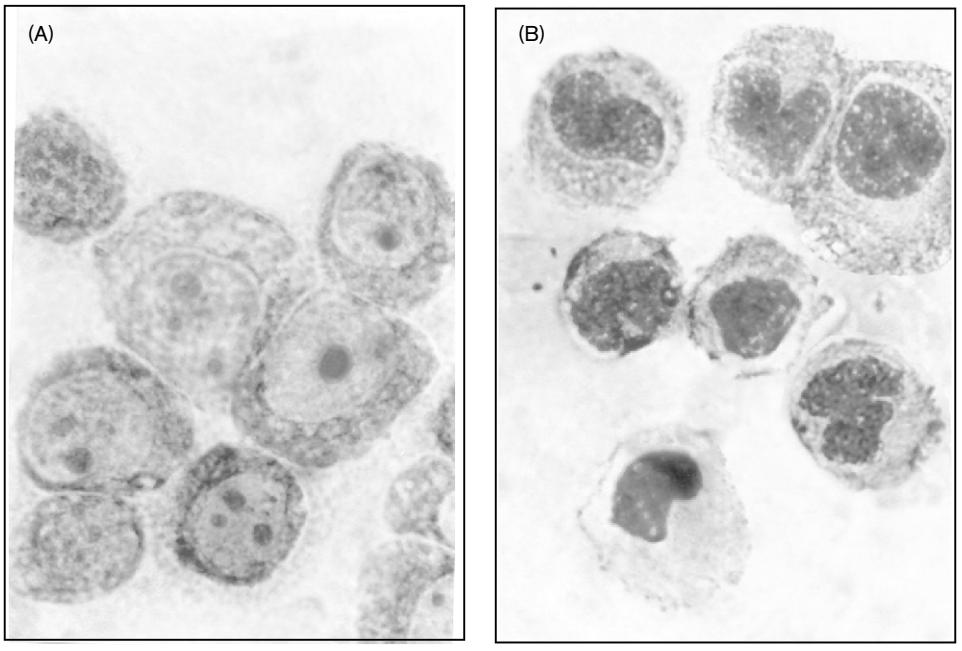


Dose-dependent expression of cell surface differentiation markers in SB202190-treated HL-60 cells.

cell marker, CD14. After SB203580 treatment, we found that HL-60 cells expressed only the myeloid cell surface marker, CD11b, but the monocytic cell marker, CD14 was not visible. The expression of CD11b was dose and time dependent (Fig. 1A and B), and could be observed as early as 12 h, with 12.5% cells labeled positively compared to 2.6% in control cells. By 96 h, the percentage of $CD11b^+$ cells increased up to 72.3% (Fig. 1A). At the same time, CD14 marker began to express in 26% of the cells implying that SB203580 induced HL-60 cells to differentiate mainly, but not exclusively, towards the granulocyte lineage. Similar results were found when the cells treated with another pyridinyl imidazole p38 inhibitor, SB202190 (Fig. 2).

As positive controls, we studied effects of ATRA (1 μM), a well-known granulocyte differentiation inducer and 1α,25-dihydroxyvitamin D_3, a monocytic differentiation inducer. As expected, ATRA induced HL-60 cells to express only CD11b marker and 1α,25-dihydroxyvitamin D_3 to induce CD11b and CD14 markers (data not shown).

Fig. 3



Morphological changes in SB203580-treated HL-60 cells. (A) Untreated HL-60 cells, (B) HL-60 cells were treated with 10 μM SB203580 for 72 h before being stained with Wright–Giemsa.

Besides induction of cell surface marker expression, SB203580 could induce changes in cell morphology. In the absence of SB203580, HL-60 cells were predominantly promyelocytes with large round nuclei, each of which contained two to four nucleoli with dispersed nuclear chromatin. The nuclear:cytoplasmic ratio was relatively high (Fig. 3A). After treatment with 10 μM SB203580, the cells became smaller in size and the nuclear:cytoplasmic ratio lower. The nucleoli were markedly reduced or completely disappeared, and the nucleus was indented and convoluted. These changes became dominant after 72 h of SB203580 treatment (Fig. 3B).

Effects of SB203580 on HL-60 cell proliferation and cell cycle

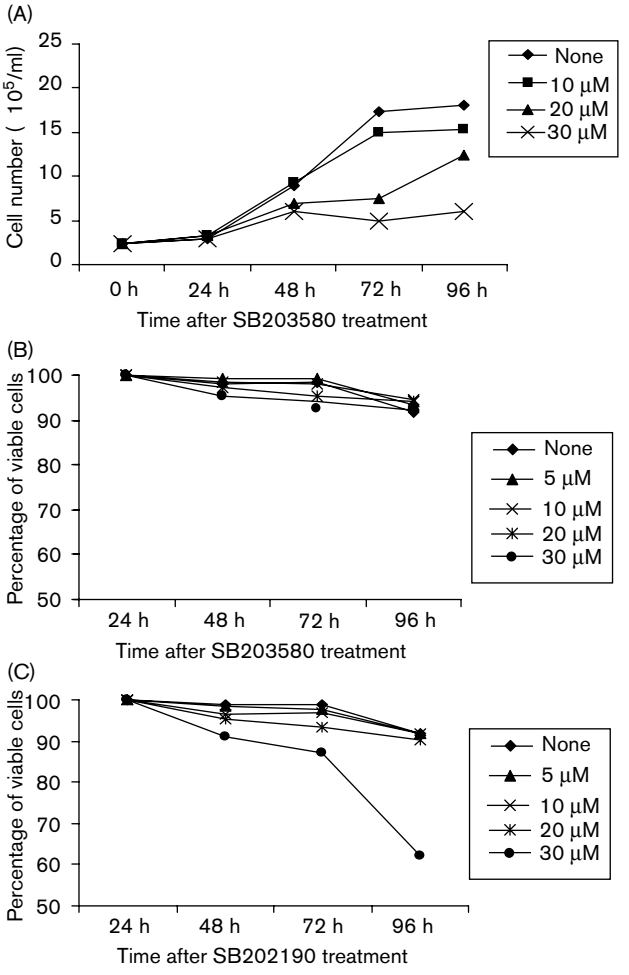
Addition of 10 μM SB203580 to HL-60 cells had little effect on cell growth and survival during the first 72 h, as evidenced by the unaltered proliferation rate and Trypan blue permeability. However, there was very little increase in cell number after 4 days of treatment with 30 μM SB203580. As a matter of fact, cell growth was inhibited after 72 h and this inhibition was dose dependent (Fig. 4A). When cell viability was tested concurrently by the Trypan blue exclusion method (Fig. 4B), SB203580 did not alter the HL-60 cell viability even at a concentration as high as 30 μM. This suggests that the lack of an increase in cell number is due to an inhibition of cell growth, but not to cell death or apoptosis. However, the other p38 inhibitor, SB202190, appears to be more toxic to HL-60 cells as the cell viability decreased appreciably when treated with 30 μM SB202190 (Fig. 4C).

To investigate further the inhibitory mechanism of SB203580 on HL-60 cell growth, Table 1 summarizes the cell cycle distribution of HL-60 after SB203580 treatment. During the first 72 h of treatment, the cell cycle distribution, as expected, did not change. However, after 96 h exposure to 10 μM SB203580, the proportion of cells in the G_1 phase was markedly increased.

Induction of cell differentiation by SB203580 was mediated by the ERK pathway

The next question was whether the induction of cell differentiation and inhibition of cell growth by SB203580 was mediated by p38 MAPK inhibition. We firstly determined the protein levels of phospho-p38; the activated form of p38 protein, in SB203580-treated HL-60 cells. As shown in Figure 5, the phospho-p38 level was not decreased, but slightly increased shortly after the drug treatment from 30 min to 6 h. From thence onward, this level fell slightly after 48–72 h. Nonetheless, total p38 protein level remained unchanged through the entire time course (Fig. 5) suggesting neither p38 protein expression nor p38 activity is drastically affected by SB203580 treatment in our experimental system.

Fig. 4



The effects of SB203580 and SB202190 on HL-60 cell proliferation and cell viability. Cells (1.5×10^5/ml) were seeded in 24-well plates and incubated for 4 days with or without the indicated drugs, and the cell numbers were counted every day (A). Cells treated with SB203580; cell viability was monitored by Trypan blue exclusion (B, cells treated with SB203580; C, cells treated with B202190). Data presented as the means of three independent experiments.

Table 1 **Effects of 10 μM SB203580 on HL-60 cell cycle distribution**

Time (h)	Treatment	G_1	S	G_2/M
24	control	42.7±0.8	20±0.2	35±0.8
	SB203580	42±1.4	18±1.9	29±2.6
48	control	49.7±0.2	18.9±1.1	31.5±3.1
	SB203580	56.2±5.2	15.1±4.7	21.6±4.4
72	control	57.7±3.8	15.5±1.8	26.6±2.4
	SB203580	60.9±4.4	21.4±3.7	17.6±3.3
96	control	53.3±3.9	27.1±2.2	19.3±1.8
	SB203580	80.2±5.1	5.9±7.2	14.1±3.7

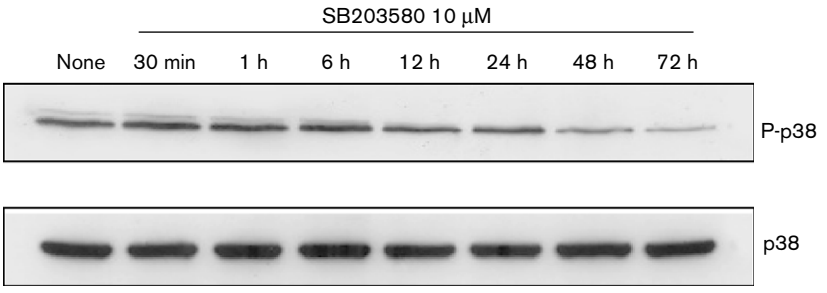
In the light of recent observations that SB203580 activates ERK signaling and the involvement of ERK activity in HL-60 cell differentiation, we next studied the effect of SB203580 on the MEK/ERK pathway in our system by examination of the levels of phospho-MEK and phospho-ERK in SB203580-treated HL-60 cells. At 30 min after 10 μM SB203580 was added to the cells, phospho-ERK1/2 proteins were elevated dramatically and this increase lasted for 24 h before the kinase activity declined. Since ERK2 is the predominant form of ERK in HL-60 cells, what we observed was mainly phophos-ERK2 (Fig. 6A). The immediate up-stream kinase of ERK1/2, i.e. MEK1/2, also showed a similar pattern of activation. The activation of the downstream target of ERKs, p90RSK, during the same time course was less significant as compared with the two upstream kinases. We then examined the activity of Raf-1 after SB203580 treatment. As shown in Figure 6(B), Raf-1 activation was observed at 30 min and lasted until 96 h. Active Raf-1 is known to be able to phosphorylate MEK at two adjacent serine residues. To sum up, our data here show that SB203580 activated the whole pathway of MEK/ERK signaling, possibly initiated through the activation of Raf-1.

To further determine whether the activation of ERK signaling was responsible for HL-60 cell differentiation induced by p38 inhibitors, we examined the effect of PD98059 on the expression of cell surface differentiation markers. PD98059 is considered to be a specific inhibitor to MEK1 and, thus, it selectively inhibits ERK activation [15]. When HL-60 cells were pre-treated with PD98059 (20 μM) for 1 h before addition of SB203580 for further incubation of 72 h, pretreatment of PD98059 completely blocked the expression of differentiation markers CD11b and CD14 (Fig. 7). This suggests that ERK activity is responsible for SB203580-induced differentiation in HL-60 cells. Since differentiation began only 12 h after the drug exposure, and became more prominent after 48 h, these results indicate that the ERK pathway was activated by SB203580 well before the appearance of the differentiation phenotype.

Discussion

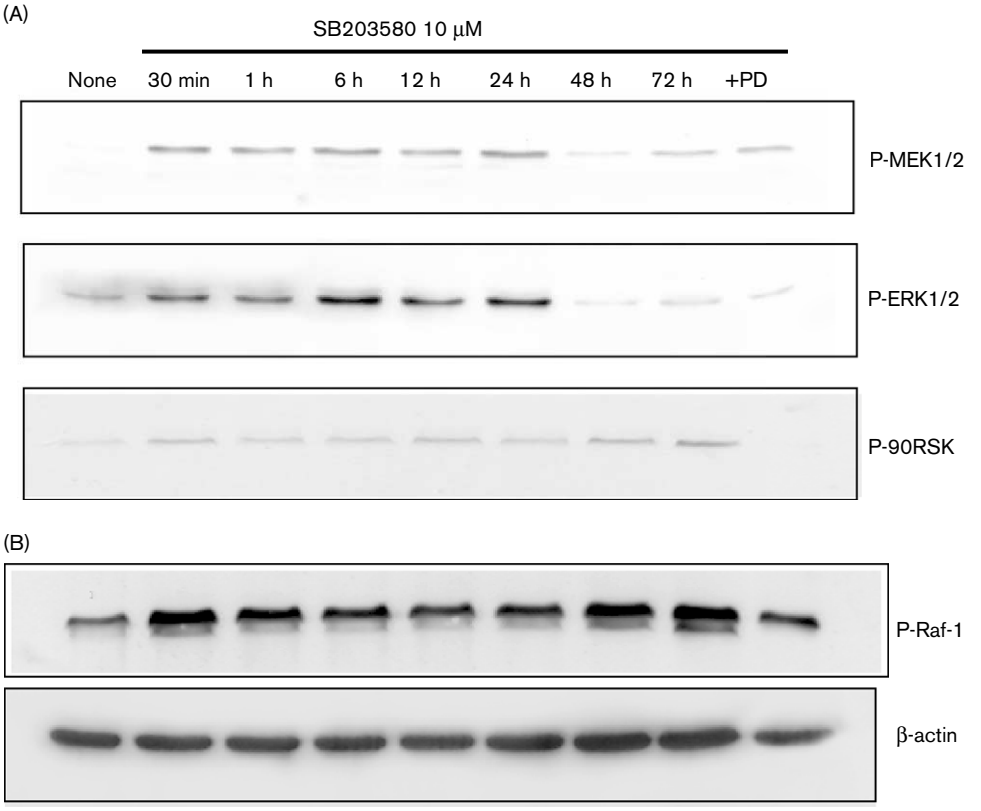
The pyridinyl imidazole p38 kinase inhibitors SB203580, SB202190 and other similar compounds were originally prepared as inhibitors against inflammatory cytokine synthesis [5,6]. Subsequent studies had revealed that these compounds selectively inhibited p38 MAPK activity in a variety of cells including HL-60 cells [16–19] but not on other MAP kinases, such as ERKs and JNKs kinases [17]. Interestingly, our present study shows that SB203580 or SB202190 alone at concentrations higher than the reported IC_{50} concentration for p38 inhibition [16,17,20] effectively induced HL-60 cell differentiation as manifested by cellular morphological changes and expression of cell surface differentiation markers (CD11b and CD14). After SB203580 or SB202190 treatment, general myeloid cell surface marker CD11b was detected as early as 24 h, but the specific monocytic surface marker CD14 was observed only in

Fig. 5



Effects of SB203580 on p38 activity. HL-60 cells exposed to 10 μM SB203580 were harvested at the indicated time and lysed. Samples of 40 μg of protein were separated on 12% SDS–PAGE gels. The protein levels of phospho-p38 and total p-38 were analyzed by immunoblotting.

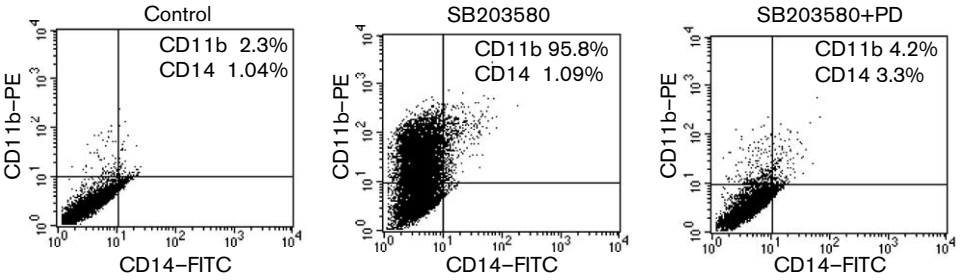
Fig. 6



Time-dependent activation of MEK/ERK pathway in HL-60 cells after exposure to 10 μM SB203580. HL-60 cells were harvested at the indicated time and lysed. Samples of 40 μg of protein were separated on 10–12% SDS–PAGE gels. The protein levels of phospho-MEK, phospho-ERK1/2, phospho-p90RSK and phospho-Raf-1 were analyzed by immunoblotting. Untreated HL-60 cells were used as the control. + PD, 20 μM PD98059 was added to the cells for 1 h before addition of SB203580.

small proportion (22.6%) of $CD11b^+$ cells after 72 h and prolonged incubation showed no further increase. Together with the morphological changes, we suggest that SB203580 induced HL-60 cells to differentiate mainly towards the granulocytic lineage. Moreover, this induction to express surface differentiation markers by SB203580 or SB202190 was both time and dose dependent. Examination of p38 kinase activity in SB203580-treated cells by Western blot shows that phospho-p38 protein did not decrease immediately as expected after the cells were exposed to SB203580, but instead increased slightly. Only a moderate decrease in

Fig. 7



Effects of MEK inhibitor PD98059 on SB203580-induced differentiation marker expression. PD98059 was added to one of the two sets of HL-60 cells 1 h before the addition of SB203580. The other set of cells was treated with SB203580 only. Untreated HL-60 cells were used as the control. The cells were incubated for 72 h, harvested, stained with anti-CD11b and anti-CD14 antibodies, and analyzed by two-parameter FACSCalibur flow cytometry as described in Materials and methods.

the phospho-p38 level was observed after 48 h of SB203580 treatment. These findings are consistent with those reported by other laboratories, demonstrating that p38 kinase activity was actually increased in HL-60 cells after exposure to SB203580 or SB202190 [11,14]. One possible explanation, as suggested by Wang *et al.* [11], is that the effect of SB203580 abrogates the negative feedback loop from a target of p38 MAPK to an upstream regulator of this pathway, leading further to a compensatory up-regulation of the p38 MAPK pathway. They showed that the immediate upstream regulators of p38 kinase, e.g. MKK3 and MKK6, were activated by SB203580 treatment [11]. Since the expression of cell surface differentiation markers was observed well before the subsequent decrease in the phospho-p38 protein level at 48 h, we conclude that the differentiation induction in HL-60 cells cannot be attributable to inhibition of p38 kinase.

ERK activities have been implicated to play important roles in differentiation in HL-60 cells [21,22,24,25]. Our data show that SB203580-induced cell differentiation in HL-60 was mediated by activation of the ERK pathway, as indicated by increased levels of different activated forms of kinases such as phospho-Raf-1, phospho-MEK, phospho-ERK2 and phospho-p90RSK in the ERK cascade. To support this observation further, we also found that SB203580-induced cell differentiation was abolished by PD98059, a specific MEK1 inhibitor. Pre-incubation of cells with 20 μM of PD98059 1 h before addition of SB203580 completely blocked the expression of differentiation markers, suggesting that ERK activities are intimately involved in the SB203580-induced differentiation. This is in contraction with several previous reports that SB203580 could neither activate ERK nor induce differentiation, although it could potentiate the function of other stimulators, such as 1α,25-dihydroxyvitamin D_3 and ATRA [11–13]. We do not know the reason for such a discrepancy amongst different laboratories. It is possible that different sublines of HL-60 cells used by different laboratories could evolve different responses to SB203580, particularly with regard to ERK activation.

In summary, p38 kinase inhibitor SB203580 could activate the ERK pathway, and induce differentiation and growth inhibition in HL-60 cells, but not affect cell viability, suggesting that SB203580 might posses certain biological activities that are not attributable solely to its better-known action of being an inhibitor of p38. Possession of these activities could make it a potential and valuable candidate as a differentiation-inducing agent for therapeutic treatment of leukemia.

References

1 Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, *et al.* The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 1994; **369**:156–160.
2 Minden A, Lin A, Smeal T, Derijard BN, Cobb M, Davis R, *et al.* c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. *Mol Cell Biol* 1994; **14**:6683–6688.
3 Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, *et al.* Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem* 1995; **270**:7420–7426.
4 Ono K, Han JH. The p38 signal transduction pathway activation and function. *Cell Signal* 2000; **12**:1–13.
5 Lee JC, Griswold DE, Votta B, Hanna N. Inhibition of monocyte IL-1 production by the anti-inflammatory compound SK&F 86002. *Int J Immunopharmacol* 1988; **10**:835–843.
6 Gallagher TF, Fier-Thompson SM, Garigipati RS, Sorenson ME, Smietana JM, Lee D, *et al.* 2,4,5-Triarylimidazole inhibitors of IL-1 biosynthesis. *Bioorg Med Chem Lett* 1995; **5**:1171–1176.
7 Badger AM, Bradbeer JN, Votta B, Lee JC, Adams JL, Griswold DE. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. *J Pharmacol Exp Ther* 1996; **279**:1453–1461.
8 Gallagher TF, Seibel GL, Kassis S, Laydon JT, Lee JC, Lee D, *et al.* Regulation of stress-induced cytokine production by pyridinylimidazoles: inhibition of CSBP kinase. *Bioorg Med Chem* 1997; **5**:49–64.
9 Prichett W, Hand A, Sheilds J, Dunnington D. Mechanism of action of bicyclic imidazoles defines a translational regulatory pathway for tumor necrosis factor alpha. *J Inflamm* 1995; **45**:97–105.

10 Kalmes A, Deou J, Clowes AW, Daum G. Raf-1 is activated by the p38 mitogen-activated protein kinase inhibitor, SB 203580. *FEBS Lett* 1999; **444**:71–74.
11 Wang X, Rao J, Studzinski FP. Inhibition of p38 MAP kinase activity up-regulates multiple MAP kinase pathways and potentiates 1,25-dihydroxyviatmin D_3-induced differentiation of human leukemia HL60 cells. *Exp Cell Res* 2000; **258**:425–437.
12 Wang X, Studzinski GP. Inhibition of p38MAP kinase potentiates the JNK/SAPK pathway and AP-1 activity in monocytic but not in macrophage or granulocytic differentiation of HL60 cells. *J Cell Biochem* 2001; **82**: 68–77.
13 New L, Li YQ, Ge BX, Zhong HY, Mansbridge J, Liu K, *et al.* SB203580 promote EGF-stimulated early morphological differentiation in PC12 cell through activation ERK pathway. *J Cell Biochem* 2001; **83**:585–596.
14 Yuki I, Shingo S, Yoshio H. Pyridinyl imidazole inhibitor SB203580 activates p44/p42 mitogen-activated protein kinase and induces the differentiation of human myeloid leukemia cells. *Leuk Res* 2001; **25**:813–820.
15 Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. *J Biol Chem* 1995; **270**:27489–27494.
16 Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, *et al.* A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* 1994; **372**:739–746.
17 Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, *et al.* SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* . 1995; **364**:229–233.
18 Schultz H, Engel K, Gaestel M. PMA-induced activation of the p42/p44 and p38 MAP kinase cascades in HL-60 cells is PKC dependent but not essential for differentiation to the macrophage like phenotype. *J Cell Physiol* 1997; **274**:310–318.
19 Meher P. p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. *J Biol Chem* 1999; **274**:17491–17498.
20 Frantz B, Klatt T, Pang M, Parsons J, Rolando A, Williams H, *et al.* The activation state of p38 mitogen-activated protein kinase determines the efficiency of ATP competition for pyridinylimidazole inhibitor binding. *Biochemistry* 1998; **37**:13846–13853.
21 Yen A, Roberson MS, Varvayanis S, Lee AT. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrested. *Cancer Res* 1998; **58**:3163–3172.
22 Yen A, Roberson MS, Varvayanis S. Retinoic acid selectively activates the ERK2 but not JNK/SAPK or p38 MAP kinase when inducing myeloid differentiation. *In vitro Cell Dev Biol Anim* 1999; **35**:527–532.
23 Hall-Jackson CA, Goedert M, Hedge P, Cohen P. Effect of SB203580 on the activity of c-Raf *in vitro* and *in vivo*. *Oncogene* 1999; **18**:2047–2054.
24 Marcinkowska E, Wiedlocha A, Radzikowski C. 1,25-dihydroxyvitamin D_3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells. *Biochem Biophys Res Commun* 1997; **241**:419–426.
25 Kharbanda S, Saleem A, Emoto Y, Stone R, Rapp U, Kufe D. Activation of Raf-1 and mitogen-activated protein kinases during monocytic differentiation of human myeloid leukemia cells. *J Biol Chem* 1994; **269**:872–878.