

CPEC induces erythroid differentiation of human myeloid leukemia K562 cells through CTP depletion and p38 MAP kinase

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Cyclopentenyl cytosine (CPEC) is a carbocyclic cytidine analog inhibitor of CTP synthetase and experimental drug for combination chemotherapy. CPEC treatment (50 nM) depleted intracellular CTP and induced a specific S-phase arrest and erythroid differentiation of human erythroleukemia K562 cells. The equilibrative nucleoside transporters (ENT1, 2) facilitated uptake of CPEC into K562 cells as evidenced by both NBMPR and dipyrindamole inhibition of CPEC-mediated CTP depletion and erythroid differentiation. Incubation with the pyridinylimidazole p38 MAPK inhibitors, SB203580 or SB220025, suppressed both the CPEC-induced cell cycle arrest and differentiation of K562 cells. SB203580 also prevented the cell cycle arrest and erythroid differentiation of K562 cells induced by Leflunomide (LEF), a non-nucleoside inhibitor of the *de novo* pyrimidine pathway, without affecting LEF-induced depletion of pyrimidine pools. Finally, selective knockdown of p38 MAPK by using Smart Pool™ siRNA to p38 MAPK significantly decreased the CPEC-induced differentiation of K562 cells. These results suggest that endogenous activity of p38 MAP kinases may be required for committing K562 cells to cell cycle arrest and erythroid differentiation under conditions of CTP depletion.

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Introduction

Cyclopentenyl cytosine (CPEC), a carbocyclic analogue of cytidine, is a potent and specific inhibitor of CTP synthetase (CTP-S). Inhibition of CTP-S results in depletion of cellular CTP and dCTP pools^{1,2} and CPEC has been shown to be effective *in vitro* treatment of leukemia cells obtained from children with acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML).^{3,4} CPEC has inhibitory activity against several malignant human cell lines including tumor KB cells,⁵ L1210 cells,⁶ HT-29 colon carcinoma cells,⁷ promyelocytic leukemia cell line HL60,¹ SK-N-BE(2)c and SK-N-SH neuroblastoma cell lines,^{8,9} glioblastoma cells¹⁰ *in vitro* and showed significant antitumor activity in mice bearing L1210, P388 leukemias, B16 melanoma,¹¹ human colon carcinoma HT-29 tumor¹² and in athymic mice bearing human tumor xenografts⁷ *in vivo*.

Recently, CPEC has generated considerable interest as a promising modulator for cancer chemotherapy. Nanomolar concentrations of CPEC were reported by Verschuur *et al*¹³ to increase AraC (1- β -D-arabinofuranosylcytosine)-induced apoptosis in MOLT-3, a human T-lymphoblastic leukemic cell line. Pretreatment of this leukemia cell line with 100–400 nM of CPEC increased the phosphorylation and incorporation of AraC into

DNA,¹⁴ and similar proliferation-inhibiting effects of AraC in combination with CPEC were observed in a neuroblastoma cell line SK-N-BE(2)c.¹⁵ Synergistic antitumor activity has been demonstrated *in vivo* in mice transplanted with HT-29 tumor when treated with a combination of CPEC and cisplatin (CDDP).¹² Moreover, pretreatment of SK-N-BE(2)c neuroblastoma cells with CPEC has been recently shown to lead to accumulation in the S-phase of the cell cycle,¹⁵ which correlated well with the maximum incorporation of [³H]AraC into DNA and elevated activity of deoxycytidine kinase (dCK).¹⁵

The p38 MAP kinases (p38 MAPKs) are a class of kinases activated by cellular stresses and inflammatory cytokines that have been implicated in a range of biological responses including the regulation of inflammation, cell cycle procession,¹⁶ cell differentiation¹⁷ and apoptosis.¹⁸ Four isoforms of p38 MAPKs (p38 α , β , γ and δ) have been identified which are selectively phosphorylated and activated by the MAP kinase kinases, MKK3 and MKK6 (reviewed in Johnson and Lapadat,¹⁹ Ono and Han²⁰). Activation of p38 MAPKs results in phosphorylation/activation of specific downstream substrates including the MAPK-activated protein kinase (MAPKAP K2/3), PRAK, MSK (RSK-B or RLPK), and multiple transcription factors that are believed to mediate the cellular effects of p38 MAPK signaling (reviewed in Ono and Han²⁰).

Considerable recent evidence points to a requirement for p38 MAPKs in the regulation of cellular differentiation (reviewed in Ono and Han²⁰). Specifically, p38 MAPK has been shown to contribute to the differentiation of 3T3-L1 cells, MC3T3-E1, PC12 cells, SKT6 and C2C12 into adipocytes, osteoblasts, neurons, haemoglobin-positive cells and myotubes, respectively.^{21–24} K562 is a pluripotent, human erythroleukemia cell line capable of differentiating into a megakaryocytic, erythroid, or to a lesser extent, monocytic lineage. Although both the ERK MAP kinase and p38 MAPKs have been implicated in hydroxyurea-, cyclosporin A-, or butyrate-induced erythroid differentiation of K562 leukemia cells,^{17,25,26} constitutive activation of ERKs has been shown to be required for megakaryocytic differentiation of K562 cells.²⁷

The equilibrative nucleoside transporters (ENTs) are a major class of facilitative transporters that transport both natural and non-natural nucleosides.²⁸ The ENTs can be classified by differential sensitivity to nitrobenzylthioinosine (NBMPR nitrobenzylmercaptapurine ribonucleoside (6-[(4-nitrobenzyl) thiol]-9- β -D-ribofuranosyl purine)) with ENT1 inhibited by nanomolar concentrations of this drug whereas ENT2 is only slightly inhibited by micromolar concentrations.²⁸ Both ENT1 and ENT2 are inhibited by cardioprotective agents (diazepam, drafazine and dipyrindamole (DP)), although species differences of these effects have been observed.²⁸ The uptake of clinically important nucleoside analogs (eg gemcitabine, capecitabine, cytarabine (AraC)) is mediated primarily by ENT1 in human cells,²⁸ and recent studies indicate that the development of resistance to both gemcitabine and capecitabine correlates strongly with a deficiency of hENT1 expression in human breast cancer cells.²⁹

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Intriguingly, our results provide evidence that nanomolar concentrations of CPEC induce growth inhibition and erythrocyte differentiation in K562 cells through ENT1 and ENT2 mediated uptake of CPEC. Co-incubation with p38 MAPK inhibitors or p38 siRNA (short-interfering RNA) demonstrated both direct effects on nucleoside transport and effects on p38 MAPK that were necessary for CPEC-induced differentiation of K562 cells. Thus, our studies point to an important role of p38 MAPK in determining cell cycle progression and differentiation in response to cellular CTP depletion.

Materials and methods

Cell culture and reagents

Human erythroleukemia K562 cells were cultured in RPMI 1640 (GIBCO) medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were kept at 37°C in an atmosphere containing 5% CO₂. These cells were tested negative for mycoplasma contamination using the Gen-Probe[®] MTC-NI Kit for mycoplasma contamination from Pall Life Sciences. CPEC was obtained from the Pharmaceutical Resources Branch, National Cancer Institute, Bethesda, MD, USA. The stock solution of CPEC was prepared as a 20 mM PBS solution and stored at -20°C. Benzidine, propidium iodide (PI), NBMPR, DP, thymidine, cytidine and uridine were from Sigma (Sigma-Chemical Co., USA). SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridinyl) imidazole), SB220025 (5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl) imidazole) and SB202474 (4-(ethyl)-2-(4-methoxyphenyl)-5-(4-pyridyl)-1H-imidazole) were purchased from Calbiochem (San Diego, CA, USA). Anti-ERK2 (C-14), anti-p38 (C-14) and antiphospho-p44/42 MAP kinase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-p38 MAP kinase was from Cell Signaling Technology (Beverly, MA, USA).

Cell cycle analysis, DNA fragmentation analysis, erythroid differentiation, analysis of intracellular nucleotides by high-performance liquid chromatography (HPLC) and cell proliferation

Cell cycle analysis, fragmentation of cellular DNA, HPLC analysis of nucleotides, determination of erythroid differentiation and cell proliferation of K562 were performed as described previously.^{30,31}

Western blotting conditions

Cells were rinsed twice with phosphate-buffered saline and lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 150 µM Na₃VO₄, 0.25 mM phenylmethanesulfonyl fluoride (PMSF), 5 µg/ml leupeptin, 10 nM microcystin LR. An equivalent amount protein (20 µg for ERK, p-ERK, p38 and 40 µg for P-p38) was electrophoresed on 12.5% SDS-polyacrylamide gels. The blots were incubated with the primary antibody, washed and exposed to horseradish peroxidase-conjugated secondary antibody. Specific antibody binding was visualized using enhanced chemiluminescence (ECL) detection reagents.

Transfection of K562 cells with double-stranded RNA (siRNA)

RNA interference of p38 MAPK was performed using p38 MAPK Smart Pool[™] siRNA (Dharmacon). K562 cells were washed once in complete medium, resuspended to 4 × 10⁶/ml in complete medium and then 500 µl of cell mixture was transferred to a 0.4-cm electroporation cuvette. A total of 1 nmol of p38 siRNA or mock solution (universal buffer only) was added and the cuvette was electroporated at 260 V, 960 µF (Gene Pulser; Bio-Rad Laboratories). After electroporation the cuvettes were put on ice for 10 min to allow the siRNA to enter the cells. The cells were incubated at 37°C at a density of 5 × 10⁵/ml for 24–94 h and 5 × 10⁵ cells were harvested for immunoblot analysis for p38 MAPK every 24 h. Cells were immunoblotted for β-actin as a control to assess siRNA specificity.

Results

Time-dependent depletion of intracellular CTP by CPEC; induction of K562 cell differentiation in a CTP-dependent manner

Previously we reported that leflunomide (LEF) induced cell cycle arrest and the erythroid differentiation of K562 cells through inhibition of *de novo* pyrimidine nucleotide synthesis.³¹ We investigated the ability of CPEC to specifically deplete CTP and to induce erythroid differentiation of K562 cells. Incubation of K562 cells with 50 nM CPEC reduced the intracellular CTP level to 39% of control level after 6 h, which further declined to 7 and 4% of the control values after 12 and 24 h, respectively. By contrast, the amounts of intracellular ATP, GTP and UTP remained relatively unaffected or slightly increased after addition of CPEC (Figure 1a). CPEC treatment also resulted in a time and dose-dependent increase in the differentiation of these cells with the percentage of benzidine-positive cells reaching a maximum of approximately 80–90% after 72–96 h (Figure 1b).

To determine whether depletion of CTP or dCTP pools was responsible for the effects of CPEC on differentiation, cells were incubated with cytidine or deoxycytidine to increase salvage synthesis of CTP or dCTP, respectively. As shown in Figure 1b, the CPEC-induced erythroid differentiation was completely prevented by co-incubation with cytidine (100 µM). Similar to the effect of CPEC on intracellular CTP, CPEC also depleted dCTP pools, however, co-incubation with deoxycytidine (10–100 µM) failed to rescue the depleted dCTP pool (Min Huang, unpublished observations) and was unable to prevent CPEC-induced erythroid differentiation of K562 cells (see Supplementary Figure 1). Although CPEC treatment (200 nM) induced apoptosis in HL60 cells, no obvious apoptosis was observed in K562 cells after incubation with CPEC at concentrations up to 25 µM for 30 h (see Supplementary Figure 2).

Prevention of CPEC-induced erythroid differentiation of K562 cells by inhibition of equilibrative nucleoside transport

CPEC is a hydrophilic analogue of cytidine and is unlikely to passively diffuse across cell membranes. Thus, the nucleoside transporters necessary for the uptake of CPEC were investigated.

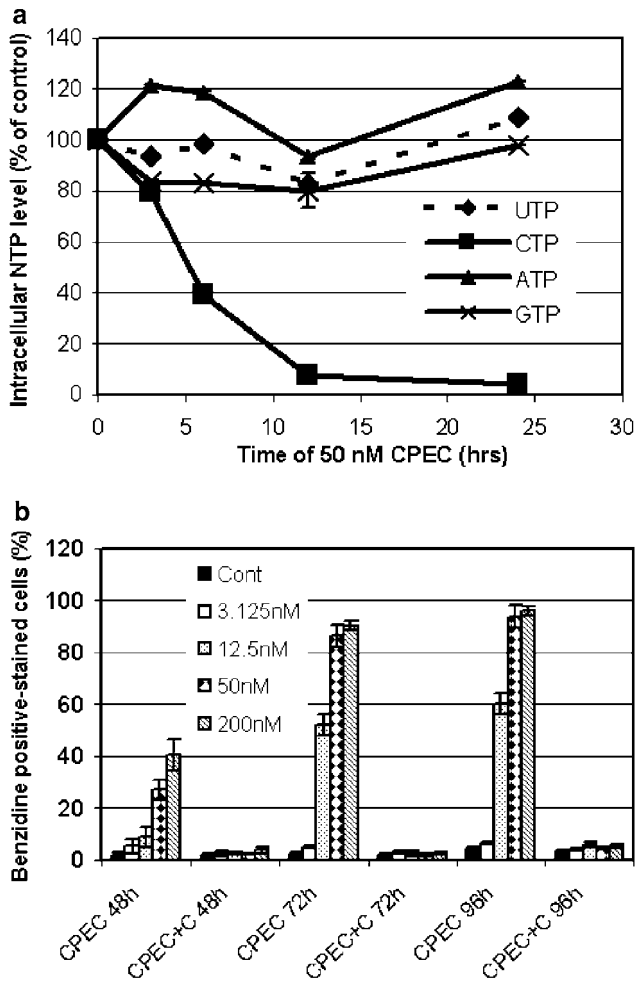


Figure 1 Effect of CPEC on intracellular nucleotide levels and differentiation of K562 erythroleukemic cells. K562 cells were exposed to 50 nM CPEC at concentrations and times indicated. (a) Intracellular ATP, CTP, UTP and GTP of 1.0×10^7 cells were extracted and measured as described under Materials and methods. The data shown are plotted as the percentage of nucleotide remaining after exposure to CPEC, and represent the mean of duplicate samples. The amount of intracellular ATP, GTP, UTP and CTP in 1.0×10^7 control K562 cells are 38.2, 11.0, 12.2 and 3.7 nmol, respectively. (b) K562 cells were exposed to CPEC at times indicated in the presence or absence of 100 μ M cytidine. The percentage of hemoglobin containing cells that stained positive for benzidine was obtained by counting at least 1000 cells per sample under microscopy using 100 \times magnification (data shown represent one of three independent experiments).

Addition of NBMPR, a potent ENT1 inhibitor or DP, an inhibitor of both ENT1 and ENT2, completely blocked CPEC-induced depletion of intracellular CTP pools (Figure 2a) and the CPEC-induced erythroid differentiation of K562 cells (Figure 2b). Since K562 cells express predominantly ENT1,³² to determine if CPEC was also a substrate for ENT2, C6 rat glioma (C6) cells that express ENT2 but not ENT1³³ were investigated. The CPEC-induced cytotoxicity of C6 cells was significantly suppressed by 10 μ M DP, but not 1 μ M NBMPR (see Supplementary Figure 3), suggesting that either ENT1 or ENT2 are required for uptake of CPEC in K562 cells whereas ENT2 is predominately required in C6 cells.

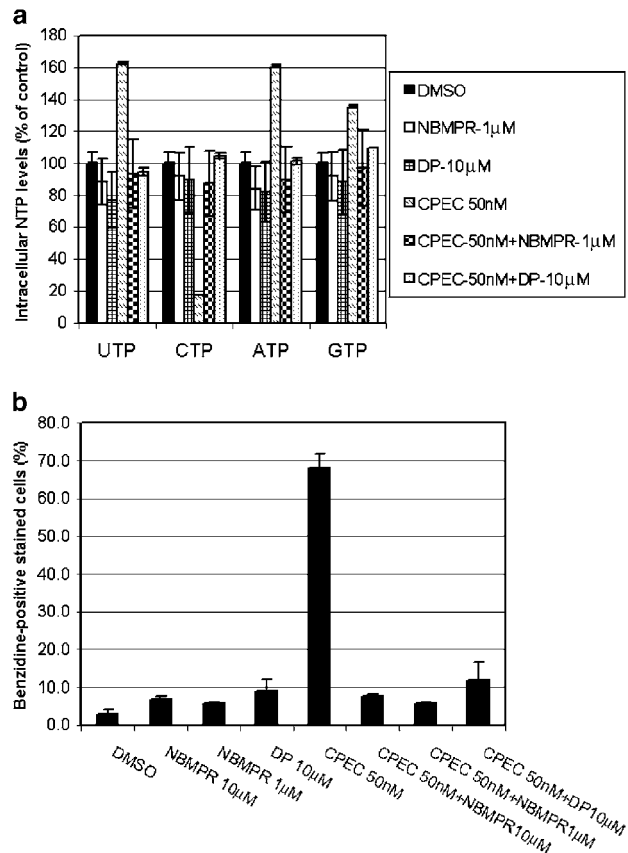


Figure 2 Effect of NBMPR and DP on CPEC-induced depletion of CTP and erythroid differentiation of K562 erythroleukemic cells. K562 cells were exposed to vehicle (DMSO) or 50 nM CPEC for 24 h in the presence or absence of 1 μ M NBMPR, or 10 μ M DP. (a) Intracellular ATP, CTP, UTP and GTP of 1.0×10^7 cells were extracted and measured in the legend to Figure 1a. The data shown are plotted as the percentage of nucleotide remaining after exposure to CPEC, and represent the mean of duplicate samples. (b) Percentage of hemoglobin containing cells was determined as described in the legend to Figure 1b.

CPEC induces a CTP-depletion-dependent S-phase arrest that precedes the differentiation of K562 cells

Since we observed CPEC induced K562 cell differentiation, we examined the affect of CPEC on cell cycle progression. Flow cytometric analysis demonstrated that in untreated control cell cultures, G1-, S- and G2-M-phase cells represented approximately 28, 59 and 12%, respectively, of the total cell population. After treatment with CPEC (50 nM, 6 h), the cell cycle distribution remained comparable to the control cells. Increasing the time of exposure to CPEC increased the percentage of cells in S-phase (12 h, 68%; 24 h, 87%; 48 h, 92%), which correlated with the selective reduction of the intracellular CTP. After 24 h, the S-phase-arrested K562 cells were unable to proceed into the G2-M phase (see Supplementary Figure 4). Co-incubation of CPEC-treated cells with 100 μ M cytidine or 30 μ M uridine completely blocked the CPEC-induced cell cycle arrest after 48 h (Figure 3), whereas cytidine alone did not affect the cell cycle distribution (data not shown). By comparison, incubation with physiological concentrations of either cytidine (0.5 μ M) or uridine (5 μ M) only slightly affected the cell cycle arrest induced by 50 nM CPEC (Figure 3).

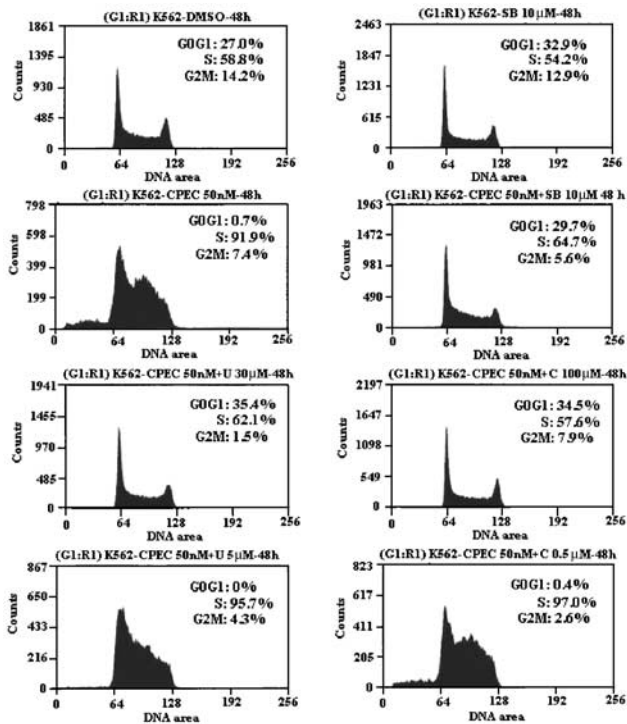


Figure 3 Effects of CPEC on cell cycle arrest of K562 cells and reversal by exogenous cytidine and SB203580. K562 cells were then stained with PI and were analyzed for DNA content by flow cytometry. A total of 20000 cells were analyzed from each sample, and the percentage of cells in G1, S and G2/M phases of cell cycle was determined. K562 cells were treated in the absence (control) or presence of 50 nM CPEC or 10 μM SB203580, or with 50 nM CPEC plus 10 μM SB203580 (SB), 0.5 or 100 μM cytidine (C), and 5 or 30 μM uridine (U), respectively, for 24 h.

Inhibition of p38 MAPK prevents both CPEC- and LEF-induced cell cycle arrest and erythroid differentiation of K562 cells

Since recent studies have implicated p38 MAPK in drug-induced differentiation of K562 cells,¹⁷ we investigated the effects of CPEC on both p38 MAPK (p38) and Erk MAPK (Erk) activity by Western immunoblotting for the phosphorylated, active forms of these enzymes. No obvious alteration in the phosphorylation of Erk and p38 MAPK was found after treatment of K562 cells with 50 nM CPEC for various time intervals (1.5, 3, 6, 12, 24, 48 and 72 h) (data not shown). However, addition of the p38 MAPK inhibitors SB203580 or SB220025 dose-dependently inhibited the CPEC-induced cell cycle differentiation (Figure 4a), whereas SB203580 (10 μM) alone did not affect cell cycle progression (Figure 3).

We recently observed that SB203580 inhibited nucleoside transport in K562 cells.^{34,35} As expected, the CPEC-mediated loss of intracellular CTP pool was largely prevented by SB203580 co-incubation (Figure 4b). However, to distinguish the effects of p38 inhibition from that on nucleoside transport, we examined SB220025, a p38 inhibitor that does not inhibit nucleoside transport in K562 cells.³⁵ SB220025 dose-dependently blocked CPEC-induced erythroid differentiation (Figure 4a) without significantly altering the CPEC-induced reduction of intracellular CTP pools (Figure 4b). These results indicated that SB220025 prevented CPEC-induced erythroid differentiation independently of nucleoside transport inhibition.

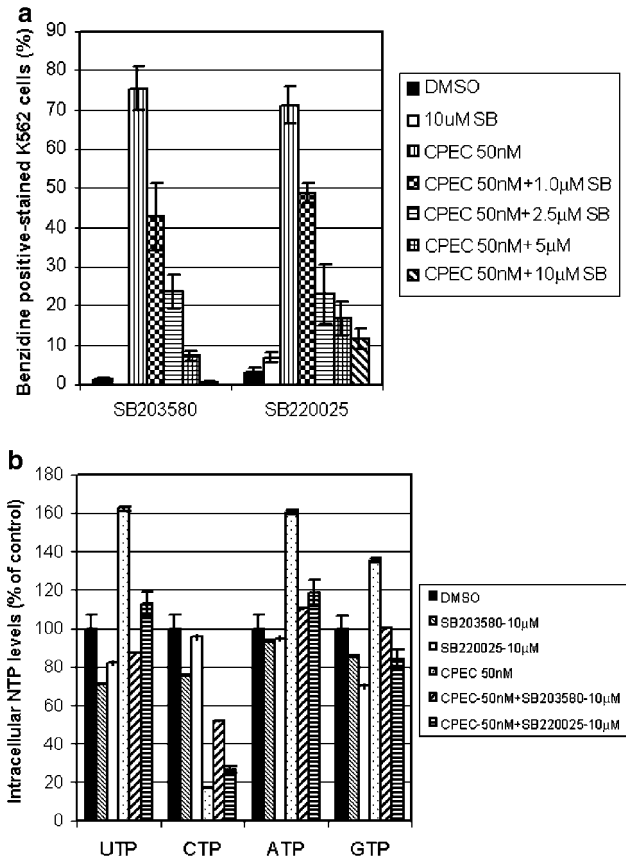


Figure 4 Inhibition of CPEC-induced differentiation of K562 cells by p38 MAPK inhibitors. (a) K562 cells were pre-incubated with or without 1–10 μM SB203580 or SB220025 for 15 min and then treated with 50 nM CPEC for 48 and 72 h. Percentage of benzidine positive-stained cells was determined as described above. (b) K562 cells were treated with vehicle control, or 50 nM CPEC in the presence or absence of 10 μM SB203580 or SB220025, or 10 μM SB203580, or 10 μM SB220025 for 24 h and the intracellular nucleotides were measured and analyzed as described above.

Moreover, SB203580, strongly reduced the LEF-induced S-phase arrest and erythroid differentiation of these cells (Figure 5a, b) without affecting the CTP and UTP pool depletion induced by LEF (Figure 5c). Similarly, neither NBMPR nor DP alone affected the LEF-mediated loss of intracellular pyrimidine nucleotides (Figure 5c). Thus, these results demonstrate that the differentiation-inducing effects of LEF were dependent on activation of p38 and independent of nucleoside transport.

Inhibition of p38 MAPK by p38 siRNA reduces CPEC-induced erythroid differentiation of K562 cells

To confirm the role of p38 in regulating CPEC-induced erythroid differentiation of K562 cells, functional gene silencing of p38 was achieved using the Smart Pool™ siRNA as described in Materials and methods. A significant reduction of p38 protein levels was observed as early as 24 h and sustained until 96 h following transfection (Figure 6a). At 24 h after electroporation, the transfected cells were exposed to CPEC (50 and 200 nM) or vehicle control for an additional 48 or 72 h. Consistent with the pyridinylimidazole p38 inhibitor data, the CPEC-induced erythroid differentiation of K562 cells was significantly reduced

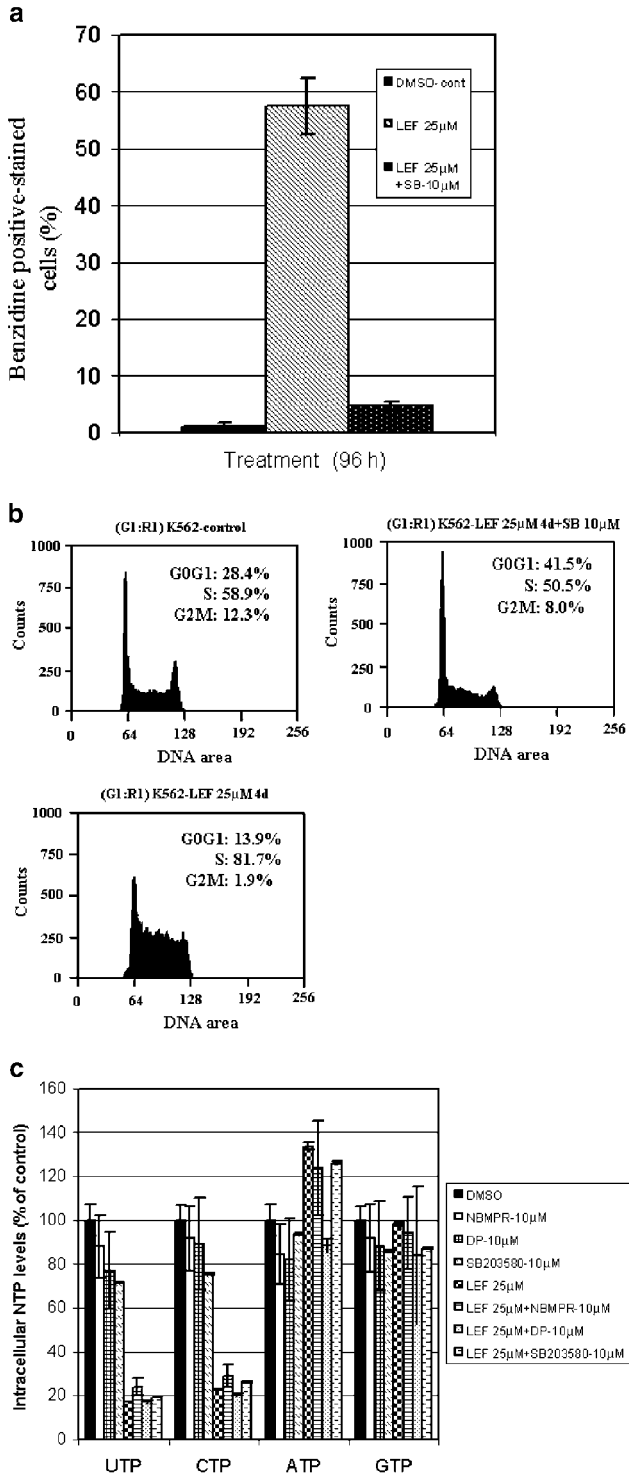


Figure 5 SB203580 suppresses LEF-induced cell cycle arrest and differentiation of K562 cells without affecting LEF-induced depletion of intracellular pyrimidine pools. K562 cells were preincubated with or without 10 µM SB203580 (SB) for 15 min and then treated with 25 µM LEF for 96 h. (a) Percentage of benzidine positive-stained cells was determined as described in Figure 1b legend. (b) Cell cycle analysis was determined as described in the legend to Figure 3. (c) K562 cells were exposed to vehicle control or 25 µM LEF for 24 h in the presence or absence of 10 µM NBMPR, 10 µM DP and 10 µM SB203580. Intracellular ATP, CTP, UTP and GTP of 1.0×10^7 cells were extracted and measured as described above. The data shown are plotted as the percentage of vehicle control nucleotide after exposure to LEF, and represent the mean of duplicate samples.

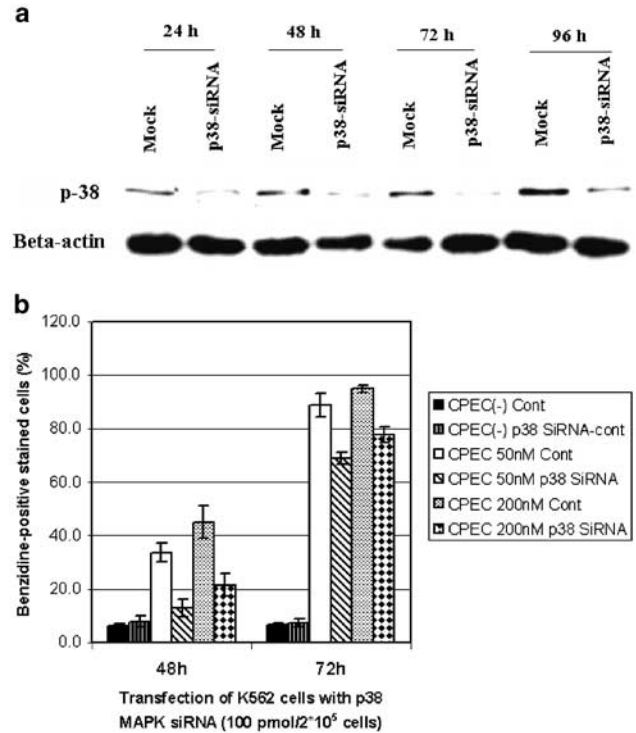


Figure 6 Effects of p38 siRNA on p38 expression and CPEC-induced erythroid differentiation of K562 cells. (a) K562 cells were treated with 100 pmol of p38 siRNA/ 2×10^5 cells or mock that consisted of complete medium with universal buffer by electroporation. After 24 h of transfection, 5×10^5 cells were harvested for immunoblot analysis for p38 MAPK every 24 h. Immunoblot for beta-actin was used as control to assess specificity. (b) After exposure of K562 cells to p38 siRNA or mock control for 24 h, siRNA or mock treated K562 cells were exposed to 50 or 200 nM CPEC for additional 48–72 h, and then the percentage of hemoglobin containing cells was determined as described above.

after 48 h with Smart Pool™ treated cells as compared to mock controls (Figure 6b). However, a decreased efficacy of p38 siRNA was observed after 72 h of CPEC exposure (Figure 6b), indicating that p38 remaining from incomplete gene silencing may be sufficient to allow erythroid differentiation.

Discussion

Previously we found that LEF induced a cell cycle arrest and erythroid differentiation of K562 cells in a pyrimidine-dependent manner.³¹ Using CPEC, a highly specific inhibitor of CTP-S, the results of the current study demonstrates the following: (1) Nanomolar concentrations of CPEC deplete intracellular CTP pools,³⁶ induce S-phase arrest¹⁵ and erythrocyte differentiation of K562 cells; (2) ENT1 or ENT2 are required for CPEC uptake in K562 and C6 rat glioma cells; (3) P38 MAP kinase is necessary for CPEC-mediated S-phase accumulation and erythrocyte differentiation of K562 cells.

CPEC cytotoxicity has been shown to correlate with the formation of CPEC-TP and the rapid depletion of intracellular CTP and dCTP pools.¹ We observed a similar depletion of CTP and dCTP pools after CPEC treatment, however, whereas cytidine addition resulted in restoration of the CTP pools, addition of deoxycytidine did not restore the dCTP pools (Min Huang, unpublished observations). The reason that deoxy-

cytidine did not rescue the CPEC-induced depletion of dCTP is unknown. Thus, although cytidine addition restored the CTP pools and prevented CPEC-induced cell cycle arrest and erythroid differentiation, depletion of dCTP cannot be excluded as the rate-limiting step in CPEC-induced differentiation of these cells.

Our studies demonstrate an essential role for the ENTs in facilitating the uptake of CPEC into K562 cells. K562 express only ENTs with approximately 90% ENT1 and 10% ENT2.³² The ENT1 and ENT2 transport inhibitors, NBMPR or DP almost completely blocked CPEC-induced depletion of CTP and differentiation of K562 cells indicating that uptake of CPEC in these cells is highly dependent on these transporters. The finding that DP significantly inhibited CPEC-induced cytotoxicity of C6 rat glioma cells, a cell type that expresses predominantly ENT2,³³ provides further evidence that CPEC is also a substrate for ENT2. Taken together, these results suggest that the ENTs are necessary for the uptake of CPEC into K562 or C6 cells although these results cannot be generalized to include other cell types based only on these observations.

Recent studies suggest that p38 is essential for the regulation of cell differentiation in response to cytokine, stress signals or antimetabolites such as hydroxyurea.^{17,20,26} The direct role of p38 in mediating γ -globin gene induction in erythroid progenitors has been demonstrated by expression of MAPK kinase kinase 3 (MKK3) and MKK6, the immediate upstream activators of p38.³⁷ The contribution of p38 to pyrimidine starvation-induced differentiation is supported by evidence that pyridinylimidazole p38 inhibitors prevented K562 cell differentiation. However, since some of the pyridinylimidazole p38 inhibitors also prevent nucleoside transport,^{34,35} it was essential to distinguish the effects of these compounds on p38, from those on CPEC uptake. Consistent with the inability of SB220025 to inhibit nucleoside transport, SB220025 did not affect CPEC uptake but blocked CPEC-induced differentiation. Moreover, SB203580 blocked K562 cell differentiation induced by LEF, a non-nucleoside inhibitor that does not require ENT1 or ENT2 for uptake.

Although we were unable to consistently detect p38 activation after exposure of K562 cells to CPEC, the application of the Smart Pool™ siRNA reduced p38 expression and erythroid differentiation suggesting that the endogenous activity of p38 was necessary for CPEC-induced erythroid differentiation of K562 cells. The fact that this did not completely suppress CPEC-induced erythroid differentiation may have resulted from incomplete gene silencing. Alternatively, in addition to p38, other signaling pathways may be involved in modulating CPEC-induced erythrocyte differentiation. Several pathways including JAK-STAT signaling pathway and MKK4-JNK have been implicated in drug and the heat shock factor 2- β -mediated erythrocyte differentiation and the production of hemoglobin in erythroid progenitors.^{38–40} Thus, SB220025 or SB203580, at a concentration of 10 μ M, may also concurrently inhibit other events required for erythrocyte differentiation and the production of hemoglobin. In summary, our results demonstrate that two independent events contribute to the reversal of CPEC-induced differentiation, one, inhibition of p38 and two, inhibition of CPEC uptake by ENT1 or ENT2-dependent transport.

K562 cells expressing the p210 Bcr-Abl and p145 Abl proteins are particularly resistant to induction of apoptosis by various antileukemia drugs. The MAPK/Erk kinases and the phosphatidylinositol (PI-3) kinases^{41,42} may contribute to the inherent resistance of K562 cells to apoptosis. Consistent with this, we observed that K562 cells showed sustained activation of Erk and

did not show CPEC-induced apoptosis (data not shown). Instead, K562 cells were highly sensitive to CPEC-induced differentiation and after 4 days of CPEC treatment (50 nM), more than 90% of the K562 cells had undergone differentiation. By contrast, HL-60 cells did not express sustained Erk activity and rapidly underwent apoptosis (> 90%, 50 nM CPEC), suggesting that enhanced Erk or other anti-apoptotic signals may be necessary to prevent CPEC-induced apoptosis in K562 cells.

Studies have shown that decreased uridine/cytidine kinase activity, and/or altered CTP synthetase activity were involved in the acquired resistance of human T-cell leukemia cell (Molt-4^R)² or murine leukemia L1210 to CPEC.³⁶ In the present study we found that low concentrations of CPEC (nM) significantly reduced the intracellular CTP pools and induced erythroid differentiation of K562 cells, indicating that these cells were highly sensitive to CPEC. Alternatively, reduced catabolism of CPEC-TP as a result of reduced 5'-nucleotidase or reduced deamination of CPEC may result in the accumulation of CPEC-TP in K562 cells. Thus, multiple factors including enhanced influx of CPEC by the ENT1 and ENT2 transporters and/or decreased catabolism may contribute to the high sensitivity of K562 cells to CPEC.

Acknowledgements

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Supplementary Information

Supplementary Information accompanies the paper on Leukemia website (<http://www.nature.com/leu>).

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