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European Journal of Pharmacology 488 (2004) 27–34
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# Suppression of cell proliferation with induction of p21 by $Cl^-$ channel blockers in human leukemic cells

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Received 11 August 2003; received in revised form 27 January 2004; accepted 6 February 2004

**Abstract**

The existence of $Cl^-$ channels in lymphocytes and neutrophils has been increasingly recognized, but the biological functions are not yet clear. We examined the effects of $Cl^-$ channel blockers on the cell proliferation and the cell cycle of human leukemic cell lines. The growth of leukemic cells was suppressed most efficiently by NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid), partially by 9-AC (9-anthracenecarboxylic acid) and tamoxifen, but not by stilbene compounds. NPPB increased the G0/G1 population and induced the expression of p21, one of the critical molecules for G1/S checkpoint. Antisense oligonucleotide for a NPPB-sensitive and stilbene-insensitive $Cl^-$ channel, ClC-2, sufficiently suppressed the ClC-2 protein synthesis, but did not affect the growth of leukemic cells. These findings suggest that NPPB-sensitive and stilbene-insensitive $Cl^-$ channels other than ClC-2 play important roles in cell cycles and cell proliferation of human leukemic cells.


*Keywords:* Leukemia; Proliferation; $Cl^-$ channel blocker; Cell cycle; ClC-2

## 1. Introduction

Many $Cl^-$ channels have been functionally identified based on electrophysiological characteristics, ion selectivity, gating behavior and pharmacological sensitivities, such as volume regulated anion channels (VRAC), $Ca^{2+}$-activated $Cl^-$ channels (CaCC) or high-conduction anion channels (Strange et al., 1996). On the other hand, several $Cl^-$ channels have been molecularly identified such as ligand gated $Cl^-$ channels (gamma-aminobutyric acid (GABA) and glycine receptors), the cystic fibrosis transmembrane conductance regulator (CFTR) and the ClC family of $Cl^-$ channels (ClC-1, 2, 3, 4, 5, 6, 7, Ka, Kb) (Xin et al., 2001; Jiang and Ye, 2003; Lambert et al., 2001; Jentsch et al., 2002). Several types of $Cl^-$ channel blockers are available to inhibit specific $Cl^-$ currents; disulfonic stilbene group such as DIDS (4, 4′-diisothiocyanatostilbene-2,2′-disulfonic acid) and SITS (4-acetamido-4′-isothiocyanato-stilbene-2, 2′-disulfonic acid), arylaminoalkyl benzoates group such as NPPB, 9-AC and DPC (diphenyl-amine-2-carboxylate), and antiestrogen group such as tamoxifen ([*Z*]-1-[*p*-dimethylaminoethoxy-phenyl]-1, 2-diphenyl-1-butene) (Cabantchik and Greger, 1992; Diaz et al., 2001).

It has been recognized that some of the $Cl^-$ channel blockers affect the proliferation of a variety of cell types such as endothelial cells, vascular smooth muscle cells, glioma cells, intestinal enterocytes, hepatocytes and peripheral T lymphocytes (Voets et al., 1995; Xiao et al., 2002; Rouzaire-Dubois et al., 2000; O' Loughlin et al., 2001; Wondergem et al., 2001; Schumacher et al., 1995). However, it is not known whether the growth of leukemic cells can be affected by $Cl^-$ channel blockers.

Several $Cl^-$ channels have been electrophysiologically demonstrated in lymphocytes (a large-conductance "maxi" channel, a medium conductance channel, a small conductance "mini" channel and volume regulated anion channels VRAC) (Pahapill and Schlichter, 1992; Schumacher et al., 1995; Lepple-Wienhues et al., 1998) and in neutrophils (a glycine-gated channel, a tumor necrosis factor α-gated channel and voltage-dependent and $Ca^{2+}$ activated chan-

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doi:10.1016/j.ejphar.2004.02.008

nels) (Froh et al., 2002; Wheeler et al., 2000; Schumann and Raffin, 1994). Further, we recently found that swelling and/or pH regulated $Cl^-$ channels (ClC-2, 3, 4 and 5) were expressed in human T, B and myeloid leukemic cells (Jiang et al., 2002). Proliferating leukemic cells have higher rates of mitosis and migration, both of which require the changes in cell volume and shape compared with normal cells. It is, therefore, likely that these $Cl^-$ channels play more important roles in the proliferation of leukemic cells than that of normal cells. The aim of this study is to clarify the types of $Cl^-$ channel blockers effective to regulate leukemic cell proliferation and the underlying mechanism.

## 2. Materials and methods

### 2.1. Culture of human leukemic cells and drug application

The human leukemic cell line of Daudi was purchased from Human Science Research Resource Bank (Osaka, Japan), and Jurkat and HL-60 were from RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 50 units/ml of penicillin and 50 μg/ml of streptomycin. In a humidified incubator with a $CO_2$ concentration of 5% at 37 °C, the cells were seeded onto culture plates at a density of $1 \times 10^5$/ml. In some experiments, HL-60 cells were incubated in modified Eagle's medium or the medium in which 116 mM sodium chloride was replaced with 116 mM sodium isethionate and 35 mM sucrose was added to maintain osmolarity. Five $Cl^-$ channel blockers (Sigma, St. Louis, MO, USA): NPPB, 9-AC, tamoxifen, DIDS and SITS were dissolved in culture medium and applied to the naturally growing cells (asynchronous cells). Leukemic cells stained with 0.4% trypan blue were counted and the cell viability (total cell number-stained cell number)/total cell number × 100% was calculated. The number of living cells was also assessed by 2-(2-methoxy-4nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt reduction for mitochondrial activity using Cell Counting Kit-8 (WST-8, Dojindo, Tokyo, Japan). The O. D. (optical density) value represents the relative number of living cells.

### 2.2. Measurement of $^{36}Cl^-$ efflux from HL-60 cells

HL-60 cells suspended at $4 \times 10^7$ cells/ml in RPMI-1640 medium were incubated with [$^{36}Cl^-$] NaCl (3.0 μCi/ml) at 37 °C for 4 h in a shaking water bath to equilibrate $^{36}Cl^-$ between the intracellular and extracellular compartments. The cells were washed twice with pre-warmed unlabeled buffer to remove the tracers, suspended into the medium with or without 100 μM NPPB, and incubated at 37 °C with shaking. One milliliter of the cell suspension was collected at 0, 10, 20, 40 and 60 min and centrifuged for 30 s at 12,000 rpm. The supernatant was carefully removed and the radioactivity remained in the cells was counted by liquid scintillation counter (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA).

### 2.3. Fluorescence activated cell sorting (FACS)

Leukemic cells were harvested, washed with phosphate-buffered saline (PBS) and fixed in cool 70% ethanol for 1 h at 4 °C. Cellular DNA was stained with 20 μg/ml propidium iodide (Molecular Probes, Eugene, OR, USA) containing 500 U/ml RNase (Roche Diagnostics, Mannheim, Germany) for at least 30 min, and measured using Becton Dickinson FACScan (Becton Dickinson, San Jose, CA, USA). The populations of cells in the G0/G1, S and G2/M phases were estimated using the Modifit cell cycle analysis program (Verity Software House, Topsham, ME, USA).

### 2.4. RNA isolation and Northern blot analysis for p21

Total RNA was isolated from human leukemic cells using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Gibco BRL, Rockville, MD, USA), followed by extraction and precipitation with isopropyl alcohol. The amounts of RNA were quantitated using UV spectroscopy (absorption at 260 nm). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a commercially available RT-PCR system (Promega, Madison, WI, USA) in 50 μl of 10 mM Tris–HCl buffer containing 1 μM each of sense and antisense primers, 0.1 U/μl avian myeloblastosis virus (AMV) reverse transcriptase, 0.1 U/μl Thermus flavus (Tfl) DNA polymerase, 0.2 mM dNTP, 1.2 mM $MgSO_4$ and 0.5 μg RNA, which was pretreated with DNase I (Gibco BRL) to remove trace amounts of genomic DNA. After producing cDNA by RT at 48 °C for 30 min, 40 cycles of PCR were carried out in a Gene Amp PCR System 9600 (Perkin-Elmer Life and Analytical Sciences) under the following conditions: denaturing at 94 °C for 15 s, annealing at 58 °C for 30 s, and extension at 70 °C for 1 min. The primers were 5′-CCTCTTCGGCCCAGTGGAC-3′ (sense) and 5′-CCGTTTTCGACCCTGAGAG-3′ (antisense), which were designed on the cDNA sequence of p21 (Harper et al., 1993). The predicted length of the PCR product of p21 was 367 bp. p21 cDNA fragment made by the above method was labeled with digoxigenin-11-dUTP by PCR (DIG DNA labeling mixture; Boehringer Mannheim, Mannheim, Germany) and used as a probe for Northern blotting (Jiang et al., 2002). Denatured total RNAs (20 μg/lane) were separated by electrophoresis on a 1% agarose-formaldehyde gel. The gel was washed in 20 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and the RNA was transferred onto positively charged nylon membranes (Boehringer Mannheim) by capillary blotting with 20 × SSC for 15–17 h. The RNA was fixed by UV cross-linking at 254 nm (0.16 J/cm$^2$). The membrane was pre-hybridized at 42 °C for 1–2

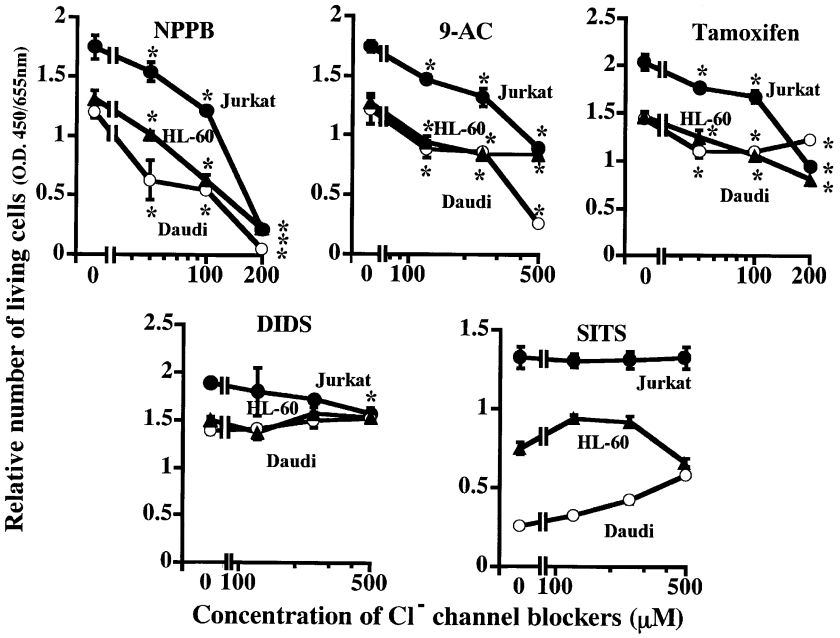


Fig. 1. Dose–response curves of chloride channel blockers on the proliferation of human leukemic cells. NPPB, 9-AC, tamoxifen, DIDS or SITS was applied to the culture medium of human T cell (● Jurkat), B cell (○ Daudi) and myeloid (▲ HL-60) leukemic cell lines. Cell proliferation was assessed by a spectrometric method using the WST-8 assay system. The ratio of O.D. 450/655 nm represents mitochondrial activity reflecting the number of living cells. The values are means ± S.E.M. ($n=6$). * significantly lower versus each control without chloride channel blockers.

h in a hybridization solution containing 50% formamide, 5 × SSC, 50 mM sodium phosphate (pH 7.4), 2% blocking reagent (Boehringer Mannheim), 50 μg/ml salmon testes DNA, and 0.5% sodium dodecyl sulfate (SDS). Denatured digoxigenin-labeled probe and dextran sulfate were added to the hybridization solution at final concentrations of 12.5 ng/ml and 5%, respectively, and incubation was continued for 16 h. The blots were rinsed briefly and washed twice at 65 °C for 20 min in 1 × SSC with 5% SDS. The hybridized probes were detected using alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Boehringer Mannheim) and a chemiluminescent substrate, 0.26 mM disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1.13,7]-decan}-4-yl) phenyl phosphate (CSPD) (Tropix, Bedford, MA, USA), according to the manufacturer's instructions. The membrane sealed in the hybridization bag was exposed to Kodak-OMAT AR film. To normalize the amount of total RNA, the membrane was reprobed with a β-actin cDNA probe after stripping twice with 1 × SSC and 5% SDS at 95 °C for 10 min each. The density of the band was quantitated by Fluorochem IS-8000 (Alpha Innotech, CA, USA). Phorbolmyristate acetate (PMA) (100 nM) was applied and the p21 mRNA expression was examined 24 h later as a positive control.

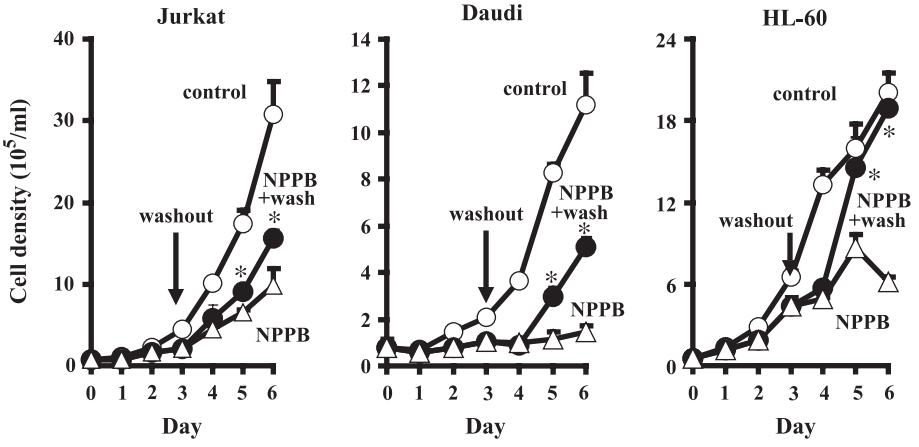


Fig. 2. Washout effects on the proliferation of NPPB-treated human leukemic cells. Control (○) and 100 μM NPPB-treated (△) cells were cultured for 6 days. In washout experiments in NPPB-treated cells, the medium was changed to NPPB-free one on day 3 (●). The values are means ± S.E.M.($n=4$). *Significantly different vs. NPPB treated cells.

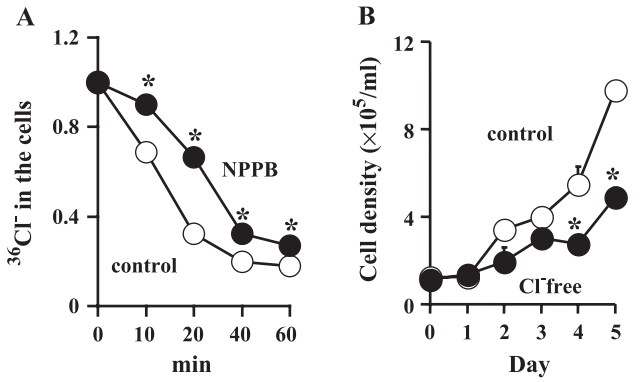


Fig. 3. Effects of NPPB on chloride efflux and those of chloride on leukemic cell proliferation in HL-60 cells. (A) HL-60 cells were preloaded with $^{36}Cl^-$ (3.0 μCi/ml) and put into tracer free medium with (●) or without (○) NPPB (100 μM). Radioactivity remained in the cells was counted with liquid scintillation counter. *Significantly higher than that in NPPB free medium. (B) HL-60 cells were incubated in modified Eagle's medium with (○) or without (●) chloride. *Significantly lower than those in chloride containing medium.

### 2.5. Transfection of antisense oligonucleotides for ClC-2

The antisense and sense oligonucleotides corresponding to the codon region (922 to 943) of human ClC-2 mRNA were synthesized and purified by high performance liquid chromatography. The antisense sequence was 5′-GG-AAGTCGAGCCGGAATCGGGT-3′, and the sense sequence was 5′-ACCCGATTCCGGCTCGACTTCC-3′. The oligonucleotides were labeled with Cy3 at the 5′ end to examine the uptake into the cells. HL-60 leukemic cells were plated at a density of $10^5$/ml onto 24-well tissue culture plates. The sense or antisense oligonucleotide (1.5 μM each) was added to the cell suspension. To examine the effects of NPPB on the growth of HL-60, to which ClC-2 antisense oligonucleotide was transfected, the cells cultured in the presence of the oligonucleotides for 2 days were adjusted at a cell density of $10^5$/ml, and treated with 100 μM of NPPB. The Cy3 fluorescence, which was trapped in the

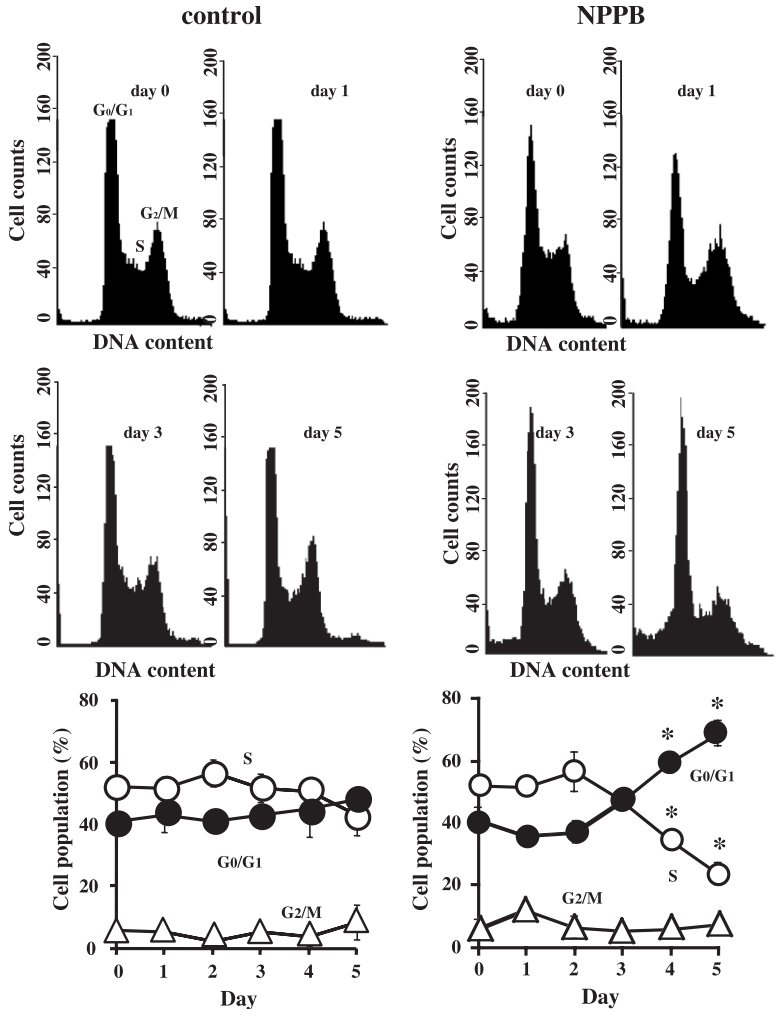


Fig. 4. FACS analysis of cell cycle of HL-60 cells treated with NPPB. Representative profile of DNA histogram (upper panel) and the time course (lower panel) of the changes in the G0/G1, S and G2/M phases in HL-60 cells with (right) or without (left) 100 μM NPPB. HL-60 cells were seeded at a density of $5 \times 10^4$ cells/ml and harvested on day 0, 1, 3, 5. The population of cells in each phase was determined using Modifit cell cycle analysis program. *Significantly different than those on day 0.

cells, was detected using a Zeiss laser confocal microscope (Carl Zeiss, Jena, Germany).

### 2.6. Western blot for ClC-2

Cells were homogenized in 1 mM $NaHCO_3$ with Dounce homogenizer and the protein concentration was measured by the Lowry method. An aliquot (50 μg protein) of the homogenate was treated with 2% SDS, 4 M urea, and 5% mercaptoethanol at 100 °C for 3 min, and electrophoresed on a 10% polyacryamide gel containing 0.375 M Tris–HCl (pH 8.8) and 0.1% SDS. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad laboratories, Hercules, CA, USA) and blotted with rabbit anti-human ClC-2 antibody (Alomone Labs, Jerusalem, Israel) followed by goat anti-rabbit $F(ab')_2$-horse radish peroxidase conjugate (ICN Pharmaceuticals, Aurora, OH, USA). The bands of ClC-2 were visualized by the chemiluminescence method (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, England) and the density of the band was quantitated by Fluorochem IS-8000 (Alpha Innotech).

### 2.7. Statistical analysis

All values are presented as means ± standard error (S.E.M). Analysis of variance (ANOVA) was used to test for statistical significance ($P < 0.05$).

## 3. Results

### 3.1. Effects of $Cl^-$ channel blockers on the proliferation of human leukemic cells

Fig. 1 shows the dose responses of $Cl^-$ channel blockers (NPPB, 9-AC, tamoxifen, DIDS and SITS) on the proliferation of human leukemic cells with T cell (Jurkat), B cell (Daudi) and myeloid cell (HL-60) origins, measured by the WST assay 4 days after the drug administration. NPPB significantly and dose-dependently suppressed the proliferation of all leukemic cells with $IC_{50}$ of 110, 50 and 72 μM for Jurkat, Daudi and HL-60, respectively. 9-AC and tamoxifen also significantly suppressed the growth of all cell lines but not to the degree of NPPB. On the other hand, DIDS and SITS up to 500 μM did not show such an inhibitory effect on the proliferation of human leukemic cells. Trypan blue staining showed that the cell viability was 85 ~ 90% throughout the first 4 days in the presence of $Cl^-$ channel blockers, suggesting that the reduced proliferation was not due to cell death. Since NPPB was the most potent inhibitor for the growth of these leukemic cells, we used NPPB in the following experiments.

Fig. 2 shows the time course of leukemic cell proliferation. The growth rate began to slow down from day 2 by 100 μM NPPB treatment, but washout of the drug from the culture medium on day 3 significantly restored the rate of proliferation thereafter, indicating that NPPB suppressed the growth rate, but did not kill the cells.

### 3.2. Specific inhibitory effects of NPPB on leukemic cell proliferation via $Cl^-$ channel blockade

To examine whether NPPB actually inhibits $Cl^-$ flux in HL-60 cells, we performed $Cl^-$ efflux study using $^{36}Cl^-$. HL-60 cells which were preloaded with $^{36}Cl^-$ were put into tracer free medium with or without NPPB (100 μM). The $Cl^-$ efflux was significant slower in medium with NPPB than that without NPPB (Fig. 3A), suggesting that NPPB inhibits $Cl^-$ efflux via $Cl^-$ channel blockade in HL-60 cells.

Then, we examined whether $Cl^-$ ion was necessary for leukemic cell growth. When HL-60 cells were incubated in $Cl^-$ free medium, the cell growth was significantly inhibited (Fig. 3B), suggesting that $Cl^-$ plays an important role in leukemic cell proliferation.

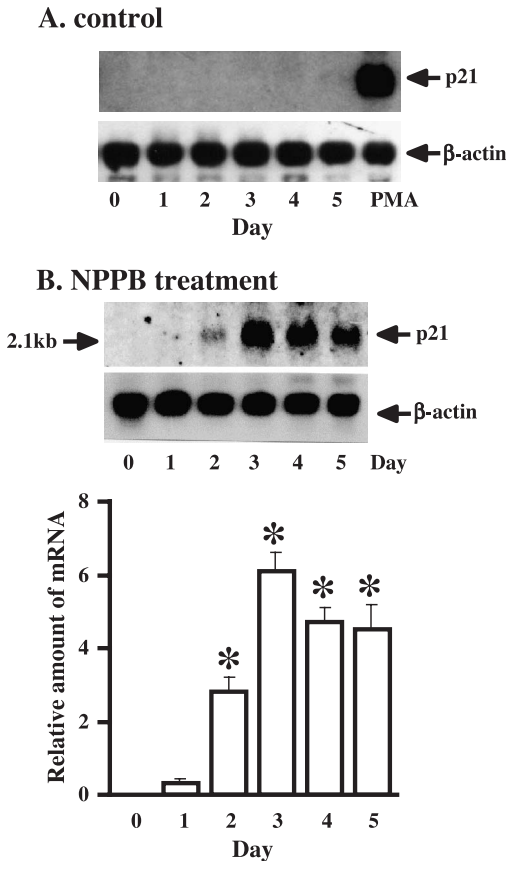


Fig. 5. Induction of p21 by NPPB. The expression of p21 in HL-60 cells was examined by Northern blotting with (right) or without (left) NPPB (100 μM). In control experiment, aliquots of cells were treated with PMA (100 nM) and the expression of p21 mRNA was examined 24 h later as a positive control. Lower panel shows the quantitative analysis by densitometer shown as means ± S.E.M. ($n = 3$). The ratio of the density of the band to that of background is shown as relative amounts of mRNA. *Significantly higher than that on day 0.

### 3.3. Effects of NPPB on cell cycle and p21 expression

Inhibition of leukemic cell proliferation by NPPB was further analyzed by FACS (Fig. 4). NPPB treatment of HL-60 leukemic cells significantly increased the population of cells in G0/G1 phase (first peak) and decreased those in S (plateau) phase on days 4 and 5, indicating that G0/G1 arrest occurred. On the other hand, no significant changes were observed in the FACS pattern with NPPB non-treated cells. Jurkat and Daudi leukemic cells showed similar changes (data not shown).

The expression of p21, one of the critical molecules for G1/S checkpoint, was examined by Northern blotting (Fig. 5). NPPB treatment significantly increased p21 mRNA level after day 2 with the peak on day 3, while p21 mRNA was not expressed without NPPB despite the strong expression by phorbolmyristate acetate (PMA) as a positive control. The time course of p21 mRNA expression by NPPB treatment was similar to the profile of cell growth inhibition.

### 3.4. Effects of ClC-2 antisense oligonucleotide on the proliferation of HL-60

Because the blocker studies strongly suggested that a NPPB-sensitive and stilbene-insensitive $Cl^-$ channels such as ClC-2 was the most probable candidate, we examined the effects ClC-2 antisense oligonucleotide on the proliferation of HL-60. Cy3-labeled antisense and sense oligonucleotides for ClC-2 were transfected into HL-60 leukemic cells. Two days later, the Cy3 fluorescence was observed in most cells, showing that the transfection efficiency was good (date not shown). The ClC-2 protein expression in the cells harvested on day 3 was examined by Western blotting. Antisense oligonucleotide for ClC-2 significantly diminished the ClC-2 protein expression to the level of 10% of that in sense oligonucleotide-treated cells or in control non-transfected cells (Fig. 6A). On the other hand, the cell proliferation was not affected by the transfection of ClC-2 antisense oligonucleotide (Fig. 6B). When NPPB was administered to the antisense oligonucleotide-transfected cells, the proliferation rate was significantly decreased (Fig. 6C), suggesting that NPPB sensitive $Cl^-$ channels other than ClC-2 are involved in human leukemic cell proliferation.

## 4. Discussion

We demonstrated that leukemic cell proliferation was suppressed most efficiently by NPPB, moderately by 9-AC and tamoxifen, and almost resistant to stilbene compounds such as DIDS and SITS, and the G0/G1 cell cycle arrest concurred with the expression of p21 in leukemic cells by NPPB treatment.

In order to clarify that NPPB suppresses leukemic cell proliferation via $Cl^-$ channel blockade, we examined the effects of NPPB on $Cl^-$ efflux and those of $Cl^-$ ion on cell growth (Fig. 3). Since NPPB significantly blocked $Cl^-$ efflux from the cells and the leukemic cell growth was inhibited in $Cl^-$ free medium, NPPB appeared to reduce cell proliferation via blockade of $Cl^-$ flux. Similar effects of NPPB on the proliferation have been reported in liver cell line (AML12) (Wondergem et al., 2001).

FACS analysis revealed that NPPB arrested HL-60 cell proliferation in the G0/G1 phase, suggesting that the NPPB-

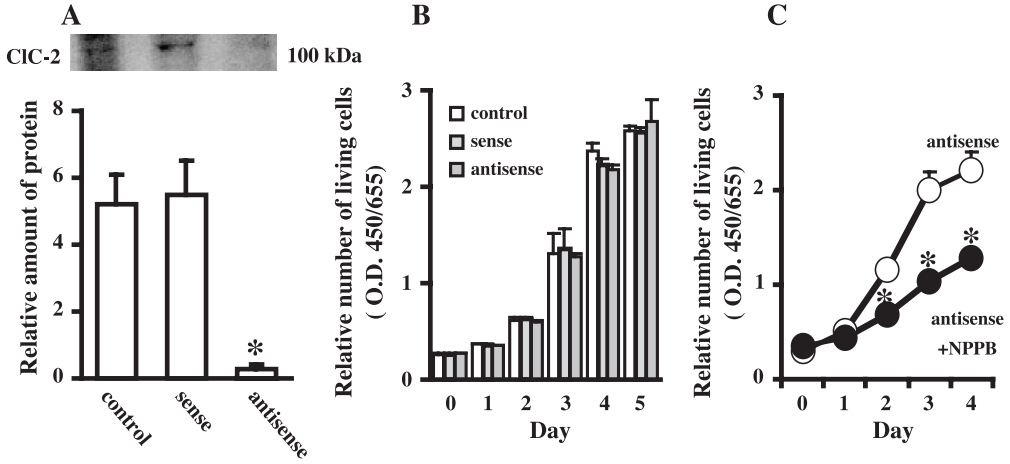


Fig. 6. Transfection of Cy3-labeled ClC-2 antisense and sense oligonucleotides to HL-60 human leukemic cells. (A) Expression of ClC-2 protein in HL-60 cells transfected by ClC-2 sense or antisense oligonucleotide on day 3 and that in control non-transfected cells shown by Western blotting. Lower panel shows the quantitative analysis by densitometer shown as means ± S.E.M. ($n = 3$). The ratio of the density of the band to that of background is shown as relative amounts of protein. *Significantly lower than that in control or sense oligonucleotide transfected cells. (B) Proliferation of HL-60 cells transfected by ClC-2 sense (hatched bar) or antisense oligonucleotide (gray bar) and that of control non-transfected cells (white bar) measured by WST assay ($n = 6$). (C) Effects of NPPB on the proliferation of HL-60 cells transfected with ClC-2 antisense oligonucleotide. The cells were treated with the antisense oligonucleotide for 2 days, adjusted to be $1 \times 10^5$ cells/ml and subjected to culture with (○) or without (●) 100 μM NPPB in the presence of the antisense. *Significantly lower than those without NPPB.

sensitive $Cl^-$ channel was involved in G1/S checkpoint. This is in agreement with the a previous report showing that tamoxifen or NPPB treatment arrested the cell cycle progression of human cervical cancer cells at the G0/G1 phase (Shen et al., 2000). Several molecules are involved in G1/S checkpoint progression. Retinoblastoma proteins are reportedly important in cell cycle regulation and its phosphorylation by cyclin D–cyclin-dependent kinase (CDK)-4 or cyclin D–CDK6 complex is required for G1 to S transition (Sherr, 1996). CDK4 and CDK6 activities are negatively modulated by Kip/Cip family molecules known as cyclin kinase inhibitors (CKIs) (Grana and Reddy, 1995). The first characterized member of this family, p21, is a 21-kDa protein, also called Cip1/Waf1 (Taniguchi et al., 1999) and the expression of p21 inhibits the activities of CDK4 and CDK6, resulting in G0/G1 arrest. We observed the expression of p21 in HL-60 by NPPB treatment from day 2 when the retardation of cell growth began, suggesting that this molecule is involved in the present observation.

The mechanism by which p21 is induced by $Cl^-$ channel blockade is unclear. The p21 is induced by p53, a molecule upstream of p21, whose level increases in response to DNA damaging agents (gamma or ultraviolet irradiation) (Bartek and Lukas, 2001). The p21 is also induced in a p53-independent manner by platelet-derived growth factors, fibroblast growth factors, 12-*o*-tetradecanoyl phorbol 13-acetate (TPA) or okadaic acid, which may involve PKC pathways (Michieli et al., 1994). Since the HL-60 cell line has null-p53 phenotype (Schwaller et al., 1995), it is likely that induction of p21 by $Cl^-$ channel blockade is via a p53-independent pathway. During cell cycle progression, cells undergo a significant increase in size especially at the G1/S phase, which perturbs cell volume homeostasis and is counterbalanced by regulatory volume decreased. $Cl^-$ channel is a major player of regulatory volume decreased together with the voltage-dependent $K^+$ channel. $Cl^-$ permeability reportedly varies with the cell cycle phase, being low in G0 and S phases and increasing in G1/S (Bubien et al., 1990). The blockade of the $Cl^-$ channel may result in regulatory volume decreased (Chan et al., 1994), changes in cytoskeletal organization or ionic environment (Ullrich and Sontheimer, 1997), and induction of cytosolic acidification by the blockade of $HCO3^-$ entry pathway (Mastrocola et al., 1998). Such changes in the intracellular environment may affect the expression or function of cell cycle regulating proteins such as p21.

There are several NPPB-sensitive $Cl^-$ channels including ClC-2, ClC-3, volume regulated anion channel (VRAC), $Ca^{2+}$-activated $Cl^-$ channel (CaCC) and maxi-$Cl^-$ channel (Furukawa et al., 1998; Von Weikersthal et al., 1999; Sabirov et al., 2000; Atherton et al., 2003; Pahapill and Schlichter, 1992). However, the contribution of ClC-3, CaCC and maxi-chloride channel in leukemic cell proliferation seems less because these channels are sensitive to stilbene compounds with $IC_{50}$ of 80, 50 and 81 μM for DIDS, respectively (Schmid et al., 1998; Pappone and Lee, 1995; Gosling et al., 1995). There are conflicting reports about the responsiveness of VRAC to stilbenes, sensitive (Shen et al., 1996) or insensitive (Wang et al., 1997), probably because this channel may not be a single entity, but may instead represent several different channels that are expressed to a variable extent in different tissues. Another candidate of $Cl^-$ channel involved in leukemic proliferation is ClC-2, which is sensitive to NPPB, 9-AC, tamoxifen, and resistant to stilbene compound (Jentsch et al., 2002). Since ClC-2 is only one structurally identified NPPB-sensitive and stilbene-insensitive $Cl^-$ channel, we examined the effects of ClC-2 antisense oligonucleotide on HL-60 cell proliferation. However, ClC-2 antisense oligonucleotide did not inhibit the leukemic cell growth even though the ClC-2 protein synthesis was successfully suppressed. VRAC or other NPPB sensitive and stilbene insensitive $Cl^-$ channels such as forskolin activated apical $Cl^-$ channel in bovine corneal endothelial cells (Sun and Bonanno, 2002), volume sensitive taurine permeable $Cl^-$ channel in rat supraoptic glial cells (Bres et al., 2000) or as yet unknown type may be involved.

Phipps et al. (1996) examined the effects of $Cl^-$ channel blockers on T-cell proliferation and found that mitogen (phytohemagglutinin) induced and interleukin 2-dependent T cell proliferation was inhibited by NPPB and DIDS with about the same potency ($IC_{50}$ value: 100 μM). The discrepant responsiveness of lymphocyte to DIDS may be explained by the different cell types and/or experimental conditions. They used normal human lymphocytes and examined the effects of $Cl^-$ channel blockers on the proliferative response to mitogen and interleukin-2. On the other hand, we used human leukemic cell lines and examined the effects of $Cl^-$ channel blockers on the spontaneous growth. It is of interest that $Cl^-$ channels involved in lymphocyte proliferation may be different in normal and malignant cells.

In summary, $Cl^-$ channel blockers inhibit the proliferation of human leukemic cells with a profile different from that in normal lymphatic cell proliferation. Blockade of NPPB-sensitive $Cl^-$ channels may perturb the intracellular ionic environment, resulting in G0/G1 arrest via the p21 pathway.

## Acknowledgements

This work was supported by Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Japan Private School Promotion Foundation and Salt Science Research Foundation. We thank Kansai Medical University Laboratory Center for providing experimental instruments.

## References

Atherton, H., Mesher, J., Poll, C.T., Danahay, H., 2003. Preliminary pharmacological characterisation of an interleukin-13-enhanced calcium-activated chloride conductance in the human airway epithelium. Naunyn-Schmiedeberg's Arch. Pharmacol. 367, 214–217.

Bartek, J., Lukas, J., 2001. Mammalian G1- and S-phase checkpoints in response to DNA damage. Curr. Opin. Cell Biol. 13, 738–747.

Bres, V., Hurbin, A., Duvoid, A., Orcel, H., Moos, F.C., Rabie, A., Hussy, N., 2000. Pharmacological characterization of volume-sensitive, taurine permeable anion channels in rat supraoptic glial cells. Br. J. Pharmacol. 130, 1976–1982.

Bubien, J.K., Kirk, K.L., Rado, T.A., Frizzell, R.A., 1990. Cell cycle dependence of chloride permeability in normal and cystic fibrosis lymphocytes. Science 248, 1416–1419.

Cabantchik, Z.I., Greger, R., 1992. Chemical probes for anion transporters of mammalian cell membranes. Am. J. Physiol. 262, C803–C827.

Chan, H.C., Fu, W.O., Chung, Y.W., Huang, S.J., Chan, P.S.F., Wong, P.Y.D., 1994. Swelling-induced anion and cation conductances in human epididymal cells. J. Physiol. 478, 449–460.

Diaz, M., Bahamonde, M.I., Lock, H., Munoz, F.J., Hardy, S.P., Posas, F., Valverde, M.A., 2001. Okadaic acid-sensitive activation of Maxi $Cl^-$ channels by triphenylethylene antioestrogens in C1300 mouse neuroblastoma cells. J. Physiol. 536, 79–88.

Froh, M., Thurman, R.G., Wheeler, M.D., 2002. Molecular evidence for a glycine-gated chloride channel in macrophages and leukocytes. Am. J. Physiol. 283, G856–G863.

Furukawa, T., Ogura, T., Katayama, Y., Hiraoka, M., 1998. Characteristics of rabbit ClC-2 current expressed in Xenopus oocytes and its contribution to volume regulation. Am. J. Physiol. 274, C500–C512.

Gosling, M., Smith, J.W., Poyner, D.R., 1995. Characterization of a volume-sensitive chloride current in rat osteoblast-like (ROS 17/2.8) cells. J. Physiol. 485, 671–682.

Grana, X., Reddy, E.P., 1995. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growh suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11, 211–219.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., Elledge, S.J., 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75, 805–816.

Jentsch, T.J., Stein, V., Weinreich, F., Zdebik, A.A., 2002. Molecular structure and physiological function of chloride channels. Physiol. Rev. 82, 503–568.

Jiang, Z.L., Ye, J.H., 2003. Protein kinase $C_\xi$ is involved in ethanol potentiation of glycine-gated $Cl^-$ current in rat neurons of ventral tegmental area. Neuropharmacology 44, 493–502.

Jiang, B.H., Hattori, N., Liu, B., Kitagawa, K., Inagaki, C., 2002. Expression of swelling- and/or pH-regulated chloride channels (ClC-2, 3, 4 and 5) in human leukemic and normal immune cells. Life Sci. 70, 1383–1394.

Lambert, G., Becker, B., Schreiber, R., Boucherot, A., Reth, M., Kunzelmann, K., 2001. Control of cystic fibrosis transmembrane conductance regulator expression by BAP31. J. Biol. Chem. 276, 20340–20345.

Lepple-Wienhues, A., Szabo, I., Laun, T., Kaba, N.K., Gulbins, E., Lang, F., 1998. The tyrosine kinase p56lck mediates activation of swelling-induced chloride channels in lymphocytes. J. Cell Biol. 141, 281–286.

Mastrocola, T., Porcelli, A.M., Rugolo, M., 1998. Role of CFTR and anion exchanger in bicarbonate fluxes in C127 cell lines. FEBS Lett. 440, 268–272.

Michieli, P., Chedid, M., Lin, D., Pierce, J.H., Mercer, W.E., Givol, D., 1994. Induction of WAF1/CIP1 by p53-independent pathway. Cancer Res. 54, 3391–3395.

O'Loughlin, E.V., Pang, G.P., Noltorp, R., Koina, C., Batey, R., Clancy, R., 2001. Interleukin 2 modulates ion secretion and cell proliferation in cultured human small intestinal enterocytes. Gut 49, 636–643.

Pahapill, P.A., Schlichter, L.C., 1992. $Cl^-$ channels in intact human T lymphocytes. J. Membr. Biol. 125, 171–183.

Pappone, P.A., Lee, S.C., 1995. α-Adrenergic stimulation activates a calcium-sensitive chloride current in brown fat cells. J. Gen. Physiol. 106, 231–258.

Phipps, D.J., Branch, D.R., Schlichter, L.C., 1996. Chloride-channel block inhibits T lymphocyte activation and signalling. Cell. Signal. 8, 141–149.

Rouzaire-Dubois, B., Milandri, J.B., Bostel, S., Dubois, J.M., 2000. Control of cell proliferation by cell volume alterations in rat C6 glioma cells. Pflugers Arch. 440, 881–888.

Sabirov, R.Z., Prenen, J., Tomita, T., Droogmans, G., Nilius, B., 2000. Reduction of ionic strength activates single volume-regulated anion channels (VRAC) in endothelial cells. Pflugers Arch. 439, 315–320.

Schmid, A., Blum, R., Krause, E., 1998. Characterization of cell volume-sensitive chloride currents in freshly prepared and cultured pancreatic acinar cells from early postnatal rats. J. Physiol. 513, 453–465.

Schumacher, P.A., Sakellaropoulos, G., Phipps, D.J., Schlichter, L.C., 1995. Small-conductance chloride channels in human peripheral T lymphocytes. J. Membr. Biol. 145, 217–232.

Schumann, M.A., Raffin, T.A., 1994. Activation of a voltage-dependent chloride current in human neutrophils by phorbol 12-myristate 13-acetate and formyl–methionyl–leucyl–phenylalanine. J. Biol. Chem. 269, 2389–2398.

Schwaller, J., Koeffler, H.P., Niklaus, G., Loetscher, P., Nagel, S., Fey, M.F., Tobler, A., 1995. Posttranscriptional stabilization underlies p53-independent induction of $p21^{WAF1/CIP1/SDI1}$ in differentiating human leukemic cells. J. Clin. Invest. 95, 973–979.

Shen, M.R., Wu, S.N., Chou, C.Y., 1996. Volume-sensitive chloride channels in the primary culture cells of human cervical carcinoma. Biochim. Biophys. Acta 1315, 138–144.

Shen, M.R., Droogmans, G., Eggermont, J., Voets, T., Ellory, J.C., Nilius, B., 2000. Differential expression of volume-regulated anion channels during cell cycle progression of human cervical cancer cells. J. Physiol. 529, 385–394.

Sherr, C.J., 1996. Cancer cell cycles. Science 274, 1672–1677.

Strange, K., Emma, F., Jackson, P.S., 1996. Cellular and molecular physiology of volume-sensitive anion channels. Am. J. Physiol. 270, C711–C730.

Sun, X.C., Bonanno, J.A., 2002. Expression, localization, and functional evaluation of CFTR in bovine corneal endothelial cells. Am. J. Physiol. 282, C673–C683.

Taniguchi, T., Endo, H., Chikatsu, N., Uchimaru, K., Asano, S., Fujita, T., Nakahata, T., Motokura, T., 1999. Expression of $p21^{Cip1/Waf1/Sdi1}$ and $p27^{Kip1}$ cyclin-dependent kinase inhibitors during human hematopoiesis. Blood 93, 4167–4178.

Ullrich, N., Sontheimer, H., 1997. Cell cycle-dependent expression of a glioma-specific chloride current: proposed link to cytoskeletal changes. Am. J. Physiol. 273, C1290–C1297.

Voets, T., Szucs, G., Droogmans, G., Nilius, B., 1995. Blockers of volume-activated $Cl^-$ currents inhibit endothella cell proliferation. Pflugers Arch. 431, 132–134.

Von Weikersthal, S.F., Barrand, M.A., Hladky, S.B., 1999. Functional and molecular characterization of a volume sensitive chloride current in rat brain endothelial cells. J. Physiol. 516, 75–84.

Wang, Z., Mitsuiye, T., Rees, S.A., Noma, A., 1997. Regulatory volume decrease of cardiac myocytes induced by β-adrenergic activation of the $Cl^-$ channel in guinea pig. J. Gen. Physiol. 110, 73–82.

Wheeler, M.D., Rose, M.L., Yamashima, S., Enomoto, N., Seabra, V., Madren, J., Thurman, R.G., 2000. Dietary glycine blunts lung inflammatory cell influx following acute endotoxin. Am. J. Physiol. 279, L390–L398.

Wondergem, R., Gong, W., Monen, S.H., Dooley, S.N., Gonce, J.L., Conner, T.D., Houser, M., Ecay, T.W., Ferslew, K.E., 2001. Blocking swelling-activated chloride current inhibits mouse liver cell proliferation. J. Physiol. 532, 661–672.

Xiao, G.N., Guan, Y.Y., He, H., 2002. Effects of $Cl^-$ channel blockers on endothelin-1-induced proliferation of rat vascular smooth muscle cells. Life Sci. 70, 2233–2241.

Xin, Y.R., Yu, L., Chen, Z., Zheng, L.H., Fu, Q., Jiang, J.M., Zhang, P.Z., Gong, R.M., Zhao, S.Y., 2001. Cloning, expression patterns, and chromosome localization of three human and two mouse homologues of $GABA_A$ receptor-associated protein. Genomics 74, 408–413.