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Curcumin synergistically augments bcr/abl phosphorothioate antisense oligonucleotides to inhibit growth of chronic myelogenous leukemia cells[1]

Kun-zhong ZHANG[2], Jian-hua XU[2,4], Xiu-wang HUANG[2], Li-xian WU[2], Yu SU[2], Yuan-zhong CHEN[3]

[2]Institute of Clinical Pharmacology, School of Pharmacy, Fujian Medical University, Fujian 350004, China; [3]Fujian Institute of Hematology, Fujian 350004,China

Key words

combination drug therapy; curcumin; abl genes, antisense oligonucleotides; chronic myelogenous leukemia; bcr-abl fusion proteins

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[4] Correspondence to Dr Jian-hua XU.
Phn 86-591-2286-2019.
Fax 86-591-8356-9307.
E-mail xjh@mail.fjmu.edu.cn

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Abstract

Aim: To investigate the growth inhibition effect of the combination of bcr/abl phosphorothioate antisense oligonucleotides (PS-ASODN) and curcumin (cur), and the possible mechanisms of cur on the chronic myelogenous leukemia cell line K562. **Methods:** The K562 cell line was used as a $P210^{bcr/abl}$-positive cell model *in vitro* and was exposed to different concentrations of PS-ASODN (0–20 μmol/L), cur (0–20 μmol/L), or a combination of both. Growth inhibition and apoptosis of K562 cells were assessed by MTT assay and AO/EB fluorescent staining, respectively. The expression levels of $P210^{bcr/abl}$, NF-κB and heat shock protein 90 (Hsp90) were assessed by Western blot. **Results**: Exposure to cur (5–20 μmol/L) and PS-ASODN (5–20 μmol/L) resulted in a synergistic inhibitory effect on cell growth. Growth inhibition was associated with the inhibition of the proliferation and induction of apoptosis. Western blot analysis showed that the drugs synergistically downregulated the level of $P210^{bcr/abl}$ and NF-κB. Cur downregulated Hsp90, whereas no synergism was observed when cur was combined with PS-ASODN. **Conclusion**: PS-ASODN and cur exhibited a synergistic inhibitory effect on the cell growth of K562. The synergistic growth inhibition was mediated through different mechanisms that involved the inhibition of $P210^{bcr/abl}$.

Introduction

Chronic myelogenous leukemia (CML) is characterized by a reciprocal t (9; 22) chromosomal translocation, known as the Philadelphia chromosome, which fuses parts of the c-abl gene, located on chromosome 22, and is the hallmark of chronic myeloid leukemia[1–3]. Today, $P210^{bcr/abl}$, the gene product of the resultant bcr/abl hybrid gene, is one of the most intensively studied signaling proteins in cancer research[4]. $P210^{bcr/abl}$-initiated signaling such as NF-κB decreases the ability of a variety of stimuli to induce apoptosis *in vitro*[5,6]. Therefore, it is a new and attractive therapeutic strategy to target $P210^{bcr/abl}$ and $P210^{bcr/abl}$ signaling.

In recent years, antisense therapy with phosphorothioate oligodeoxynucleotides has emerged as a potentially useful strategy for inhibiting CML[7]. Bcr/abl phosphorothioate antisense oligonucleotides (PS-ASODN) might be synthesized for uses as therapeutic agents. The idea is that PS-ASODN would block disease processes by blocking the synthesis of a protein encoded by bcr/abl. The binding of the PS-ASODN to the bcr/abl mRNA would normally result in the downregulation of that protein, thus preventing malignant transformation and development of resistance to apoptosis. However, their use as therapeutic agents is limited by inefficient cellular uptake, scare nuclear internalization, and oligonucleotide self-aggregation[8].

Curcumin (cur) is a diferuloylmethane derived from the plant *Curcuma longa*. It is a potent antioxidant that possesses both anti-inflammatory and antitumor activities, and has been regarded as one of the prosperous antitumor agents[9]. In our previous works, cur has been proved to inhibit the proliferation of K562 cells correlates with the downregulation of the $P210^{bcr/abl}$ protein[10]. Therefore, we supposed that cur might synergistically inhibit the growth of CML with PS-ASODN. If there is a combination, what would the action of cur after bcr/abl mRNA be blocked by PS-ASODN?

We selected NF-κB to investigate whether the combination of cur and PS-ASODN exerted a synergistic effect on the downstream of $P210^{bcr/abl}$ signaling, thus affecting cell growth. NF-κB, a transcription factor with a critical role in promoting inflammation and which is connected with multiple aspects of oncogenesis and cancer cell survival, appears to be involved in the regulation of cell proliferation, control of apoptosis, promotion of angiogenesis, and the stimulation of invasion/metastasis[5,11]. For further analysis of the inhibitory mechanism of PS-ASODN and cur, we studied the molecular chaperone heat shock protein 90 (Hsp90), which is important in maintaining the conformation, stability, and function of $P210^{bcr/abl}$[12].

In this study, the $P210^{bcr/abl}$-positive K562 cell line was used as a cellular model of CML for drug screening (Figure 1).

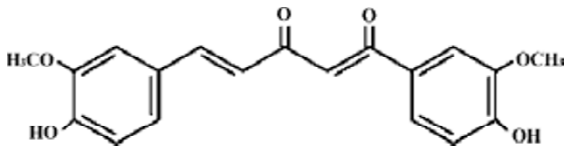

Figure 1. Structure of curcumin ($C_{21}H_{20}O_6$, M_r 368.4).

Materials and methods

Drugs and antibodies Cur was purchased from Shanghai No 3 Reagent Company (Shanghai, China; No 980825). It had a purity of 99%, and was dissolved in DMSO as stock solution. The sequence of primers are as follows[13]: bcr/abl anti-sense 5'-GCT GAA GGG CTT TTG AAC TCT GCT-3'; bcr/abl nonsense 5'-CTC TGG ATA GGA TCA TGT-3'. Both were synthesized by Bioasia Bio-technology Inc (Shanghai, China); c-Abl, NF-κB, Hsp90, and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA); Acridine orange (AO) and ethidium bromide (EB) were purchased from Amresco (Solon, OH, USA), and MTT was from Sigma (St Louis, MO, USA).

Cell culture The human leukemia cell line K562 (purchased from Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) was cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum and gentamicin (80 U/100 mL) in a humidified atmosphere with 5% CO_2 at 37 ºC.

Cell growth inhibition assay The K562 cells were plated in 0.1 mL in triplicate at a density of (10–20)×10^4 cells/mL in 96-well plates and were exposed to the tested agents: PS-ASODN, cur, and a combination of both, which were added at the selected concentrations to the culture medium with a final volume of 0.2 mL per well for 48 h. Nonsense was used as a control. At the indicated time, 20 μL of 5 mg/mL MTT solution in phosphate-buffered saline (PBS pH 7.2) was added to each well for 4 h. After removal of the medium, 170 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 490 nm was determined using a Biokinetics plate reader (Bio-Rad Laboratories, Hercules, CA, USA). Triplicate wells were assayed, and standard deviations were determined.

Assessment of apoptosis The K562 cells were plated in 0.1 mL in triplicate at a density of (10–20)×10^4 cells/mL in 96-well plates and were exposed to the tested agents: PS-ASODN, cur, or a combination of both. They were added at the selected concentrations to the culture medium, with a final volume of 0.2 mL per well for 48 h. After the indicated time, 100 cells were counted under a microscope after AO/EB fluorescent staining to determine the apoptotic rate[14]. One hundred micrograms of AO and EB were dissolved, respectively, in 1 mL PBS (pH 7.2) as stock solution. To further confirm the synergistic apoptosis, the K562 cells were treated with selected concentrations of 10 μmol/L PS-ASODN, 10 μmol/L cur, or a combination of both for 48 h. After the indicated time, the apoptotic cells were detected by nuclear morphologic changes using AO/EB fluorescent staining. Four μL of AO/EB mixture was added with 100 μL of K562 cells on the cover slip, and then the stained nuclei were viewed under an Olympus microscope (Hatagaya, Tokyo, Japan) to evaluate the extent of apoptosis (ie cell shrinkage, nuclear condensation, formation of apoptotic bodies etc).

Protein extraction and Western blotting After treatment with drugs for 48 h, the cells were collected by centrifugation and washed 3 times in ice-cold PBS (pH 7.2). The cell pellets were resuspended in lysis buffer (Tris-HCl 50 mmol/L, pH 8.0, NaCl 150 mmol/L, dithiothreitol 1 mmol/L, EDTA 0.5 mmol/L, NP40 0.1% (*v/v*), sodium dodecyl sulfate 0.1% (*w/v*) containing protease inhibitors (aprotinin 1 mg/L, leupeptin 2 mg/L, sodium orthovanadate 100 μmol/L and phenylmethylsulfonly fluoride 10%). The protein concentration of each sample was estimated using the Coomassie brilliant blue kit (Nanjing Jiancheng Biotechnology, Jiangsu, China). After boiling for 5 min, the lysates were subjected to electrophoresis on 8% polyacrylamide gels and transferred to Hybond-C membranes (Amersham, Arlington Heights, IL, USA) in transfer buffer [Tris 25 mmol/L, glycine1 90 mmol/L, methanol 20% (*v/v*)] using a transfer apparatus at 150 mA for 2 h. The membranes were blocked with blocking-buffer [5% (*g/v*) nonfat milk] at 4 °C overnight and were incubated with mouse anti-primary antibodies: c-Abl, Hsp90, NF-κB, and β-actin for 2 h at room temperature. They were washed 4 times in TBST [Tris-buffered saline supplemented with 0.03% (*v/v*)

Tween-20], and incubated with horse anti-mouse antibody (1:500) for 1.5 h at room temperature; they were washed 4 times with Tris-HCl (pH 7.2), and the membranes were detected with substrate according to the product kits (Gene Lab, Singapore). Intensities of the different proteins were quantified by densitometric scanning. All experiments were repeated at least 3 times and yielded similar results.

Statistical analysis Data were expressed as mean±SD. Jin's formula was used to evaluate the synergistic effects between the drugs[15]. The formula is: $Q=E_{a+b}/(E_a+E_b-E_a\times E_b)$; Q is the combination index; E_{a+b} which represents the cell proliferative inhibition rate of the combined drug; E_a and E_b are signs of the cell proliferative inhibition rate of each drug. After calculation: Q=0.85-1.15, means indication of simple addiction (+); Q=1.15-2.0 → synergism (+ +); Q>2.0 → significant synergism (+ + +); Q=0.85-0.55 → antagonistic effect (–); Q<0.55 → significant antagonistic effect (– –). The differences between treatment and control groups were evaluated with Student's *t*-test. The differences were considered significant at $P<0.05$.

Results

Effects of cur and/or PS-ASODN on cell growth Treatment with either cur (5–20 μmol/L) or PS-ASODN (5–20 μmol/L) resulted in an inhibition of cell growth of 40%, 55%, 90%, 30%, 45%, and 65%, respectively (Figure 2A,B). In combination, the cell number was reduced greatly (Figure 2C), cur (5–20 μmol/L) and PS-ASODN (5–10 μmol/L) showed synergism (Q>1.15), that is, a combination of 10 μmol/L cur and 10 μmol/L PS-ASODN induced an inhibition of cell growth of 90%, compared to cur (55%) or PS-ASODN (45%), and showed synergism (Q=1.19).

Effects of cur and/or PS-ASODN on cell apoptosis The extent of apoptosis was more significant when the K562 cells were treated with the combination of cur and PS-ASODN than each treated alone. Exposure of cells for 48 h to cur (5–20 μmol/L) or PS-ASODN (5–20 μmol/L) induced apoptosis of 25%–55% and 18%–45% (Figure 3A, 3B). However, when exposed to the combination of cur and PS-ASODN, it significantly increased the percentage of the hallmark of apoptosis, that is, when 20 μmol/L cur (55%) and 20 μmol/L PS-ASODN (45%) were treated in combination, a marked increase (80%) in apoptosis was induced (Q=1.06), corresponding to a synergistic interaction (Figure 3C). The drug-treated K562 cells were examined for morphologic evidence of apoptosis using AO/EB fluorescent staining under an Olympus microscope (×400). Typical apoptotic features of chromatin condensation and nuclear fragmentation were observed in the treated cells when they were exposed to both drugs in combination (Figure 4).

Cur and PS-ASODN synergistically downregulated $P210^{bcr/abl}$, NF-κB, and Hsp90 expression To determine whether the potentiality of apoptosis in the K562 cells treated with PS-ASODN combined with cur would be associated with the downregulation of $P210^{bcr/abl}$ and selected $P210^{bcr/abl}$ signaling- NF-κB, Western blot was performed to evaluate their expression. As a result, treatment with a combination of PS-ASODN and cur resulted in a marked reduction of $P210^{bcr/abl}$ compared to each drug treated alone (Figures 5, 8). When the cells were exposed for 48 h to 10 μmol/L PS-ASODN in combination with increasing concentrations of cur (5–20 μmol/L), the $P210^{bcr/abl}$ level decreased by 75%, 80%, and 90%,

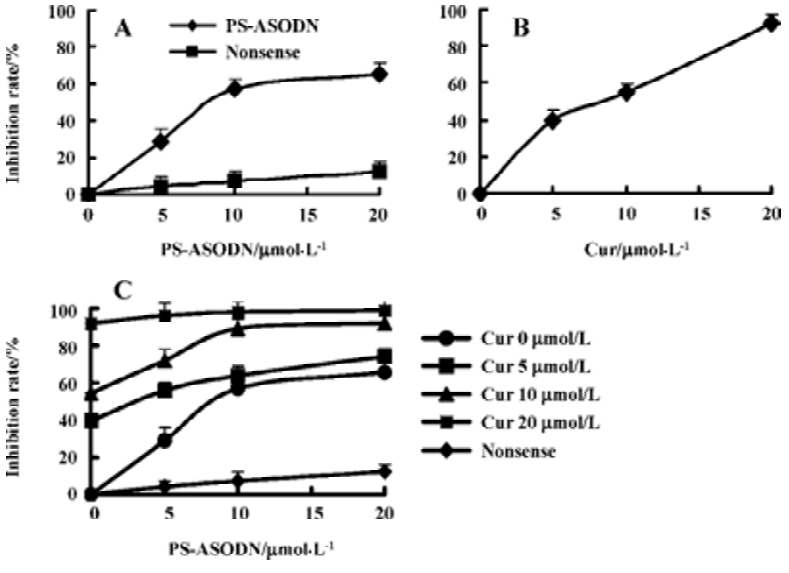

Figure 2. K562 cells were exposed to cur, PS-ASODN, or a combination of PS-ASODN with increasing concentration of cur (5–20 μmol/L) for 48 h. MTT assay was performed. Results were calculated as the percent of values obtained and are represented as mean±SD. (A) Inhibition of cur on K562 cells; (B) Inhibition of PS-ASODN on K562 cells; (C) Inhibition of the combination of PS-ASODN with increasing concentration of cur (5–20 μmol/L) on K562 cells.

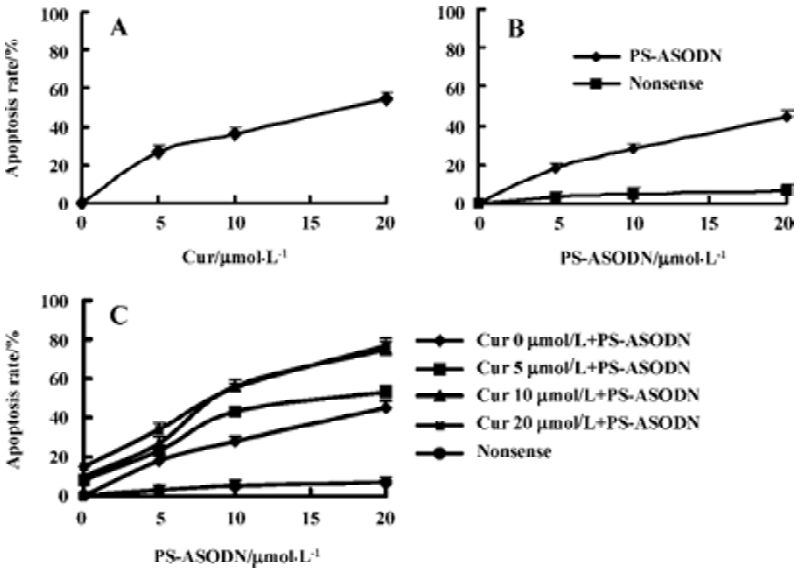

Figure 3. K562 cells were exposed to cur, PS-ASODN, or a combination of PS-ASODN with increasing concentrations of cur (5–20 μmol/L) for 48 h. AO/EB method was performed. Results were calculated as the percent of values and are represented as mean±SD. (A) Apoptosis induced by cur on K562 cells; (B) Apoptosis induced by PS-ASODN on K562 cells; (C) Apoptosis induced by the combination of PS-ASODN with increasing concentrations of cur (5–20 μmol/L) on K562 cells.

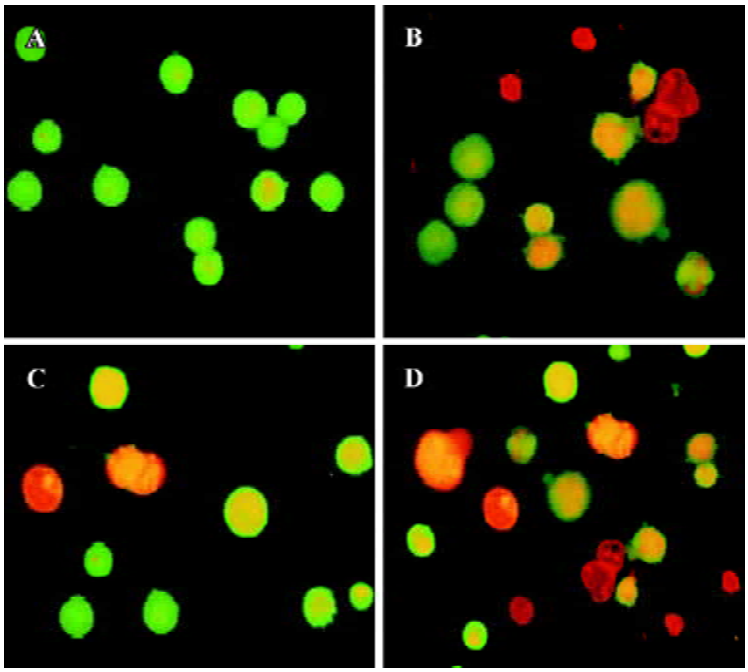

Figure 4. Nuclei staining by AO/EB after the K562 cells were treated with cur and/or PS-ASODN (×400). The K562 cells were treated with selected concentrations of 10 μmol/L PS-ASODN, 10 μmol/L cur, or a combination of both for 48 h. After the indicated time, the apoptotic cells were detected by nuclear morphologic changes using AO/EB fluorescent staining. Four μL of AO/EB mixture was added with 100 μL cells on a cover slip, and then the stained nuclei were viewed under an Olympus microscope to evaluate the extent of apoptosis. (A) Control; (B) Cur 10 μmol/L; (C) PS-ASODN, 10 μmol/L; (D) PS-ASODN, 10 μmol/L+cur, 10 μmol/L.

respectively, compared to the treatment of PS-ASODN (50%) or cur (20%, 40%, and 60%) alone.

The synergistic inhibition was observed in the inhibition of NF-κB expression (Figures 6, 8), that is, the individual effect of 10 μmol/L PS-ASODN or (5–20 μmol/L) cur caused a 48%, 31%, 42%, and 48% decrease of NF-κB expression, respectively. However, when both of the drugs were combined, the NF-κB level markedly decreased by 66%, 77%, and 86%. This was consistent with the downregulated proportion of $P210^{bcr/abl}$.

Individual treatment of PS-ASODN had little effect on Hsp90 (P>0.05 *vs* control), whereas (5–20 μmol/L) cur caused a 26%, 72%, and 81% decrease of Hsp90. The Hsp90 treated with cur, or the combination of PS-ASODN and cur, had no significant difference (P>0.05). This suggested that the downregulation of Hsp90 was through the effect of cur, but not PS-ASODN (Figures 7, 8).

Discussion

ASODN is the high degree of specificity, without

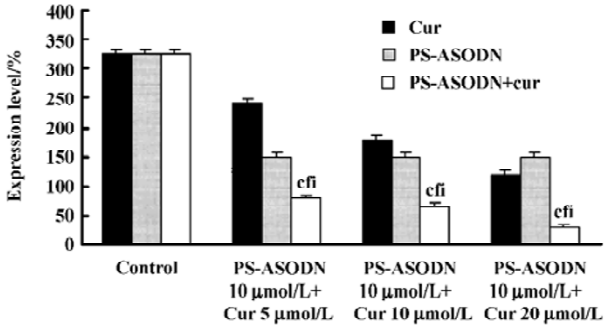

Figure 5. After quantification, the P210$^{bcr/abl}$ level was expressed as arbitrary units based on its ratio to β-actin. n=3. Mean±SD. ^{c}P<0.01 *vs* cur, ^{f}P<0.01 *vs* PS-ASODN, ^{i}P<0.01 *vs* control.

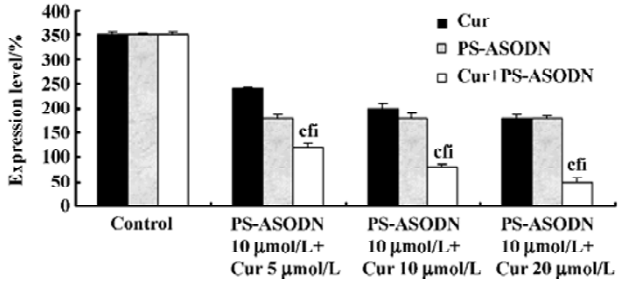

Figure 6. After quantification, the NF-κB level was expressed as arbitrary units based on its ratio to β-actin. n=3. Mean±SD. ^{c}P<0.01 *vs* cur, ^{f}P<0.01 *vs* PS-ASODN, ^{i}P<0.01 *vs* control.

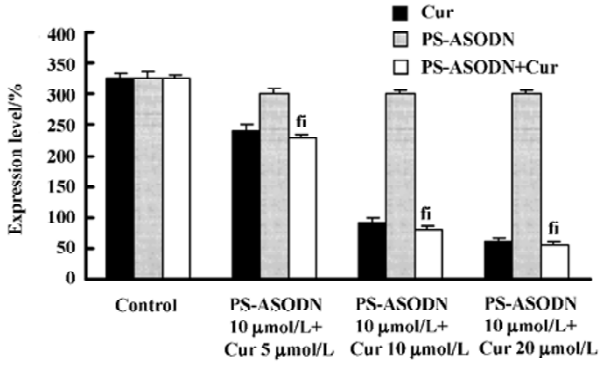

Figure 7. After quantification, the Hsp90 level was expressed as arbitrary units based on its ratio to β-actin. n=3. Mean±SD. ^{c}P<0.01 *vs* cur, ^{f}P<0.01 *vs* PS-ASODN, ^{i}P<0.01 *vs* control.

altering the expression of genes with closely-related sequences. Theoretically, it is one of the ideal therapeutic agents of CML[9,16]. However, they are very poorly soluble, exhibit low affinity for their target complementary RNA sequences, and target RNase-H poorly. The solubility of PS-linked ASODN may be improved in that it can direct

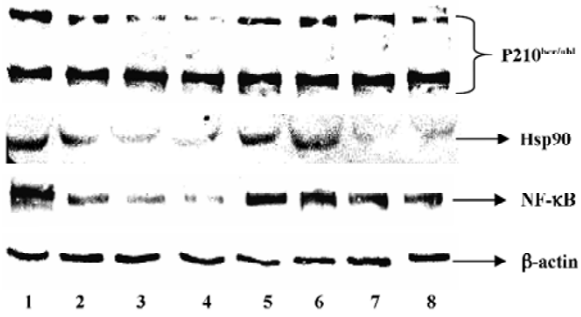

Figure 8. After the K562 cells were treated with PS-ASODN, cur, and a combination of both, Western blot was used to assess expression of P210$^{bcr/abl}$, Hsp90, NF-κB, and β-actin. Each lane contained 50 μg of protein. Blots were stripped and reprobed to ensure equivalent loading and transfer. Two additional studies yielded equivalent results. (1) Control; (2) PS-ASODN, 10 μmol/L+cur, 5 μmol/L; (3) PS-ASODN, 10 μmol/L+cur, 10 μmol/L; (4) PS-ASODN, 10 μmol/L+cur, 20 μmol/L; (5) PS-ASODN, 10 μmol/L; (6) Cur, 5 μmol/L; (7) Cur, 10 μmol/L; (8) Cur, 20 μmol/L.

RNase-H-mediated destruction of the target RNA, but this may result in non-antisense inhibition of growth[13]. What's more, PS-ASODN are very poorly transported into human cells; liposomes may enhance cellular ASODN uptake, but it seems, can not meet its initial high expectations. In this study, treatment with PS-ASODN (5–20 μmol/L) alone resulted in an inhibition of cell growth (30%–60%).

Recently, cur has been commonly studied as a prosperous drug in its anticancer effect. It can inhibit the proliferation and induce the apoptosis of many kinds of cancer cells *in vitro*, and cur is safe, with human clinical trials indicating no dose-limiting toxicity when administered at doses up to 10 g/d[9,17–19]. In this study, cur synergistically augmented the growth inhibitory effects of PS-ASODN in human leukemic cells *in vitro*. This observed synergistic effect may be clinically important, and the combinatorial strategies in cancer therapy can provide dramatic improvements in safety and efficacy over monotherapy regiments.

Our current results show that the combination of low concentrations of cur (5–20 μmol/L) and PS-ASODN (5–20 μmol/L) were sufficient to inhibit cell growth by inhibiting proliferation and inducing apoptosis, downregulating P210$^{bcr/abl}$, NF-κB, and Hsp90. The consistent downregulation of NF-κB with P210$^{bcr/abl}$ showed that the effect of the combination of PS-ASODN and cur also exerted a synergistic inhibition on the downstream signals of P210$^{bcr/abl}$, thus affecting the inhibition of cell growth. The possible rationales for combining PS-ASODN and cur were that both drugs inhibit P210$^{bcr/abl}$ by different mechanisms: PS-ASODN might downregulate the P210$^{bcr/abl}$ protein by blocking bcr/abl

mRNA, while cur might accelerate the degradation of Hsp90[12] which is important in maintaining the conformation, stability, and function of $P210^{bcr/abl}$, thus downregulating $P210^{bcr/abl}$ after bcr/abl mRNA was blocked by PS-ASODN.

In summary, PS-ASODN and cur exhibited a synergistic inhibitory effect on the cell growth of K562. The synergistic growth inhibition was mediated through different mechanisms that involved the inhibition of $P210^{bcr/abl}$. The synergistic effect is clinically important and may provide the combinatorial strategies in cancer therapy.

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