# Antisense candidates against protein kinase C-α designed based on phylogenesis and simulant structure of mRNA[1]

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## ABSTRACT

**AIM:** To optimize the antisense drug design by the combined method of phylogenetic analysis and secondary structure prediction and to get ideal candidates. **METHODS:** The phylogenetic analysis and the secondary structure simulation were performed by computer. Oligodeoxynucleotides (ODN) were designed against the full-conserved blocks with low local reaction free energy of protein kinase C (PKC)-α mRNA. The *in vitro* effects of ODN were evaluated by human A549 lung carcinoma cells and mouse B16-BL6 melanoma cells, the expression of target mRNA was detected by *in situ* hybridization and RT-PCR. The *in vivo* effects of ODN were also evaluated by models of A549 xenografts in nude mice and B16 melanoma in mice. **RESULTS:** Three ODN had significantly lower $IC_{50}$ values than that of ISIS3521, the positive control, on A549 cells *in vitro*. Five ODN inhibited the growth of B16-BL6 cells with $IC_{50}$ <100 nmol/L, while $IC_{50}$ of ISIS3521 was >200 nmol/L. *In situ* hybridization and RT-PCR showed that the best candidate AP1261 inhibited the expression of PKC-α at mRNA level in a dose-dependent manner. AP1261 inhibited the growth of A549 and B16 tumors *in vivo* at 0.005-0.5 $mg \cdot kg^{-1} \cdot d^{-1}$. The inhibitory rate of AP1261 on A549 tumors was greater than that of ISIS3521 at the same dose. ISIS3521 did not affect the growth of B16 tumors. **CONCLUSION:** AP1261 may be of value as an antitumor agent or adjuvant and the combined method of phylogenetic analysis and secondary structure prediction is a potential helpful tool for antisense drug design.

## INTRODUCTION

Antisense strategies capture the imagination with their promise of rational drug design and exquisite specificity[1]. Up to now, more than 20 antisense oligodeoxynucleotides (ODN) are undergoing clinical trials as anti-neoplasm, anti-virus, or anti-inflammation agents. One of them, ISIS2922, was approved by FDA to treat cytomegalovirus (CMV) retinitis[2]. However, the antisense drug design is still low-efficient and, sometimes by random[3]. Activities of different antisense ODNs targeting different local sequences on the same mRNA varied dramatically[4,5]. Obviously, how to select efficient antisense drugs from numerous possible candidates against target mRNA is quite difficult.

In this study, we tried to screen efficient ODN

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comprehensively based on the phylogenesis and computational secondary structure prediction of target mRNA. On the one hand, in the process of phylogenesis, both the nucleotide composition and the codon usage could vary to different extent in mRNA[6]. Normally, high conservation in the sequence during the evolution suggests that the primary sequence may be important for its function in post-transcriptional regulation[7]. Given an example of enzyme, the mRNA sequences corresponding to the amino acid residues involved in vital function of catalysis, substrate, and inorganic phosphate binding are often highly conserved, whereas the variation often corresponding to the lesser function such as the cofactor-binding, *etc*[6]. Data suggested that it would be helpful for the efficacy of antisense drugs to select the highly conserved sequences as targets. On the other hand, previous results[8] also showed that the quantitative structure-activity relationship (QSAR) analysis based on simulant secondary structure and reaction free energy ($DG^o$) did help to improve the antisense drug design and to predict the activity of new designed ODN. Then, would the ODN designed against highly conserved blocks with low $DG^o$ of target mRNA possess satisfactory activities to some extent?

To verify the hypothesis, antisense ODN against PKC-α mRNA was designed and screened. PKC-α, one of classical PKC isoforms, overexpressed in many tumors, and the selected reduction in PKC-α expression caused growth inhibition of tumors or tumor cells[9]. An antisense inhibitor directly against the 3'-untranslated region of PKC-α mRNA, ISIS3521, was now in clinical trials as anti-neoplasm agent[10]. *In vitro* and *in vivo* bioactivities of designed ODN in this study were evaluated by human A549 lung carcinoma cell line and mouse melanoma B16/B16-BL6 cell line, ISIS3521 was set as positive control in human cell line and negative control in mouse cell line. ODN with good *in vitro* and *in vivo* activities might be served as potent anti-neoplasm agent in the future.

## MATERIALS AND METHODS

**Phylogenetic comparison analysis of mRNA** Phylogenetic comparison analyses were performed by the computer program ClustalW (ver 1.74) Multiple Sequence Alignments. The whole sequences of human, mouse, and rat PKC-α mRNA (GenBank entry code: X52479, M25811, and X07286) were obtained from GenBank. The sequences were input into the Clustal W window, and then the function "multiple sequence alignments" was executed.

**Simulation of secondary structures of human PKC-a mRNA** The computer program RNAstructure (version 3.5, 1999) was kindly permitted to get, update, and use by Turner (Department of Chemistry, University of Rochester, New York 14627, USA) after our registration. The secondary structures of the mRNA were calculated based on the principle of minimizing free energy. Then the values of $DG^o$ were calculated as described previously[11].

**Design and synthesis of the antisense ODN** Based on the results of the phylogenetic comparison and the calculation of $DG^o$ [8], seven 20-mer ODN directly against the fully conserved "motif" among species with low $DG^o$ were designed, and were synthesized by Sangon Bioengineering Company, Shanghai. All oligonucleotides tested in the study were phosphorothioate modified ODN (Tab 1). ISIS3521[12] was used as positive control, two scrambled sequences [scrambled AP1261 (SCR1261) and scrambled ISIS3521 (SCR3521), which were randomized sequences with the same base composition of AP1261 and ISIS3521, respectively] and a totally randomized sequence (Random) were also synthesized and tested as control.

**Cell culture** Human A549 lung carcinoma and mouse B16-BL6 melanoma cells were grown in RPMI-1640 (Gibco, BRL) containing 10 % fetal bovine serum (FBS, HyClone), and in 37 °C, 5 % $CO_2$. The cells were routinely passaged when 85 %-90 % confluent.

**Treatment of tumor cells by ODN *in vitro*** A549 or B16-BL6 cells were seeded in 96-well plates (NUNC, Denmark) until 50 %-60 % confluent. At this time, the cells were washed twice with serum-free RPMI-1640, and the ODNs of required concentration were then transfected into cells by DOTMA/DOPE solution (Lipofectin, Gibco BRL) according to the instruction of the manufacturer directions. The cells were incubated at 37 °C for 6 h, washed twice with RPMI-1640 containing 10 % FBS to remove the DOTMA / DOPE, and allowed to recover for an additional 66 h. The thiazolyl blue (MTT, SERVA) solutions (0.5 g/L) were then added and incubated for another 4 h, $Me_2SO$ was used to dissolve the precipitation. The absorbance at 570 nm (reference wavelength was set at 450 nm) was determined by the Wellscan MK-2 microplate reader (Labsystems Dragon).

**Evaluation of *in vitro* inhibitory effect of antisense ODN on tumor cell proliferation** A series

**Tab 1. ODN sequences tested in this study and inhibitory effects ($IC_{50}$) on proliferation of human lung carcinoma A549 cell line and mouse melanoma B16-BL6 cell line *in vitro*. *n*=4 parallel experiments. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* ISIS3521.**

| ODN | Sequence (5' – 3') | $\Delta G^{o}_{37\,^{\circ}C}$/kJ· mol$^{-1}$ | $IC_{50}$ on A549/ nmol· L$^{-1}$ | $IC_{50}$ on B16-BL6/ nmol· L$^{-1}$ |
|---|---|---|---|---|
| AP0155 | GAAGGTGGGCTGCTTGAAGA | -110.1 | 58±12$^{b}$ | 73±50$^{b}$ |
| AP0166 | CAGTGGCTGCAGAAGGTGGG | -110.1 | 82±17 | 73±17$^{c}$ |
| AP0186 | ACCCCCAGATGAAGTCGGTG | -131.5 | 48±11$^{b}$ | 44±38$^{c}$ |
| AP0413 | GCAGGTGTCACATTTCATCC | -123.9 | 158±12 | 109±42 |
| AP0478 | TCAGTGTGATCCATTCCGCA | -109.7 | 68±17 | 83±40$^{b}$ |
| AP1261 | TCCATGACGAAGTACAGCCG | -126.0 | 47±8$^{b}$ | 35±6$^{c}$ |
| AP1999 | ACAAACTGGGGGTTGACATA | -134.0 | 128±51 | 99±38 |
| ISIS3521 | GTTCTCGCTGGTGAGTTTCA | -28.0 | 82±25 | 201±53 |
| SCR1261 | CGAGCACGCAGTATCACTAG | - | 175±52 | 154±11 |
| SCR3521 | GGTTTTACCATCGGTTCTGG | - | 164±34 | 262±29 |
| Random | CGAGCACGGAGTATCGGTAG | - | 181±54 | 169±33 |

of concentrations (32-1000 nmol/L) were set and 2-4 duplicated wells per concentration were performed to evaluate the antisense activities *in vitro* of every ODN. The positive control ISIS3521 was set as working standard on every plate, and each ODN was tested for 4 times in order to obtain reliable results. The concentration *vs* $A_{570\,nm}$ data of each ODN were plotted, and fifty-percent inhibitory concentrations ($IC_{50}$) and slopes of ODN were calculated using the method of logit analysis with MicroCal Origin software. Mean $IC_{50}$ value of ODN was used as the major criterion of ODN inhibitory potency.

**Measurement of PKC-a mRNA level by *in situ* hybridization** Cells were treated with ODN and DOTMA/DOPE for 4 h and were then washed once in RPMI-1640 plus 10 % FBS and allowed to recover for an additional 20 h. Then 200 μL harvested cell suspension ($1\times10^{8}$ cell/L) was smeared by automatic centrifuge smearer at 50 ×*g* for 5 min. The slides were immersed in ice-cold 4 % paraformaldehyde for 15-30 min and subsequently washed by phosphate-buffered saline (PBS) and distilled water twice for 5 min, respectively. The fixed cells were then digested by the proteinase K (1 mg/L) at 37 °C for 20-30 min. *In situ* hybridization with a digoxigenin (DIG)-labeled ODN probe (sequence: 5'-AACTCCCCTTTCCCAACACCATGA-3') was performed as described previously[13]. Probes hybridized to mRNA were visualized and detected by the DIG nucleic acid detection kit (Roche). Briefly, the DIG was captured by an Alkaline Phosphatase (AP)-labeled anti-DIG antibody, then the nitro-blue-tetrazolium (NBT)-5-bromo-4-chloro-3-indolyl-phosphate (BCIP) system was utilized to fulfil the color reaction. The stereological analysis was carried out with the software of pathological image analysis system (Beijing University of Aeronautics and Astronautics).

**Reverse transcriptase-polymerase chain reaction (RT-PCR)** Total RNA (5 μg) was primed with oligo $(dT)_{15}$ (Gibco BRL) and then reverse transcribed[14]. The prepared cDNA then underwent differential PCR (30 cycles: 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C with a 5-min extension) with human specific primers for PKC-α. Human specific primers for β-actin were added as internal standard. The primers were PKC-α, forward 5'-GGGACGAGGAAGGAAACATGG-AAC-3' and reverse 5'-AACTCCCCTTTCCCAACACC-ATGA-3'[15], which amplified a 172-bp region of the human PKC-α cDNA species. The β-actin primers were forward 5'-AGGGTACATGGTGGTGCCACCAG-AC-3' and reverse 5'-CCAAGGCCAACCGCGAGAAGATGAC-3'[16], which amplified a 584-bp region of the human β-actin cDNA species. The reaction products were subjected to electrophoresis in 1.5 % agarose gel (Pharmacia) containing ethidium bromide and photographed under ultraviolet light.

**Oligonucleotide effects on the growth of B16 xenografts in mice and A549 xenografts in nude mice** Male C57BL/6 mice (Grade II, Certificate No B98004) were obtained from the Experimental Animal Center of Academy of Military Medical Sciences. The mean body weight was 20±2 g. Mouse B16 melanoma cells were implanted sc ($1\times10^{7}$ cell per animal) and then

serially passaged by a minimum of three consecutive transplantations prior to the start of treatment. The tumors were taken out and ground by proportion of 300 g/L saline through a mesh with the sieve number of 50. Then 200 μL suspension of tumor cells was implanted sc into the right flank of every animal. ODN (formulated in saline) were administered ip daily at indicated doses when the tumor volumes reached a mean value of 100 $mm^3$. Twenty-four hours after the last treatment, the animals were sacrificed and the tumors were taken out and weighed. The inhibitory rate of the growth of the tumors was obtained by the following formula: $(a–b)/a \times 100$ %, where $a$=mean tumor weight of the saline-treated animals, $b$=mean tumor weight of the test-treated animals.

Female BALB/c nude mice (18±2 g) were provided by the Experimental Animal Center of Academy of Military Medical Sciences (Grade SPF, Certificate No B98007) and used when 12 weeks old. Cultured A549 cells were implanted sc and passaged as described above. The ODN (formulated in saline) was begun to be administered sc 24 h after the implantation of the tumors. Tumors were weighed and the inhibitory rate of the growth of the tumors was calculated as described above too.

## RESULTS

**Phylogenetic comparison analysis and the design of antisense ODN** The Clustal W multiple sequence alignment showed that there were 13 conserved sequences (length >20-mer) among human, mouse, and rat PKC-α mRNA, we decided to make the choice of targets for ODN from these sequences. Local secondary structures of the 13 sequences above were also considered when performing antisense drug design. Finally, seven ODN against conserved blocks with low $DG^o$ were designed (Tab 1) and synthesized based on the results of previous QSAR equation[8].

***In vitro* effects of ODN on tumor cells** The *in vitro* antitumor activities of ODN designed were tested against human A549 lung carcinoma cell lines and mouse B16-BL6 melanoma cell line. On A549 cell line, $IC_{50}$ value of ISIS3521 was 82±25 nmol/L, which was similar to that reported in previous document, 50-100 nmol/L[9]. Five out of the 7 designed ODN had $IC_{50}$ values less than 100 nmol/L on proliferation of A549 cell line, and $IC_{50}$ values of AP0155, AP0186, and AP1261 were significantly lower than that of ISIS3521 ($P$<0.05). On the other hand, against the B16-BL6 cell line, activity of ISIS3521 was poor (with $IC_{50}$ value more than 200 nmol/L), while AP0155, AP0166, AP0186, AP0478, AP1261, and AP1999 still had low $IC_{50}$ values (Tab 1).

**Reduction in PKC-a expression at mRNA level** ISIS3521 had previously been demonstrated to reduce the expression of PKC-α mRNA in A549 cells grown in tissue culture[12]. Effect on expression of PKC-α mRNA in A549 cells of AP1261, which was with the lowest inhibitory $IC_{50}$ against A549 cells in culture was tested and compared with ISIS3521 too. The *in situ* hybridization showed that treatment of A549 cells with AP1261 resulted in a reduction in PKC-α mRNA content, at the concentration of 100 nmol/L, the expression of PKC-α mRNA was 32 %±6 % of control. It was significantly lower than that of ISIS3521-treated cells (40 %±4 % of control, $P$<0.05). While the expression of PKC-α mRNA in A549 cells treated with SCR1261 and SCR3521 were 102 %±11 % and 113 %±10 % of control, respectively (Fig 1).

Similarly, the result of RT-PCR also showed that AP1261 could inhibit the expression of PKC-α mRNA in A549 cells in a dose-dependent manner (Fig 2).

**Effects of ODN on tumor cell growth *in vivo*** On the basis of *in vitro* results, *in vivo* antitumor activities of the ODN were tested on the mouse melanoma B16 tumor cell line in mice and the effect was evaluated at different doses. The treatment was initiated 6 d after tumor implantation (when the mean tumor volume was reached about 100 $mm^3$) and continued daily for additional 12 d. At this time, average weight of B16 tumors in control animals (saline-treated) was 3.9±1.0 g. Doses of the AP1261 at 0.5, 0.05, 0.005 $mg \cdot kg^{-1} \cdot d^{-1}$ inhibited the growth of the tumors at the rate of about 75.4 %, 57.8 %, and 55.4 %, respectively. The scrambled ODNs and ISIS3521 had no significant inhibitory effect on the B16 tumors at a high dose of 0.5 $mg \cdot kg^{-1} \cdot d^{-1}$ (Fig 3). Dose of AP0155 and AP0186 at the medium level, 0.05 $mg \cdot kg^{-1} \cdot d^{-1}$, also inhibited the growth of the tumors at the rate of 48.1 % and 49.4 % respectively (data not shown).

The inhibitory effect of AP1261 on human A549 cell line in nude mice was also evaluated and compared with ISIS3521. ODN treatment was initiated 24 h after tumor implantation and continued to be administered sc daily for an additional 14 d until the study was terminated and the animals were sacrificed. At this time, the mean tumor weight in control (saline-treated) animals was 2.0±0.3 g. ISIS3521 was set as positive control in

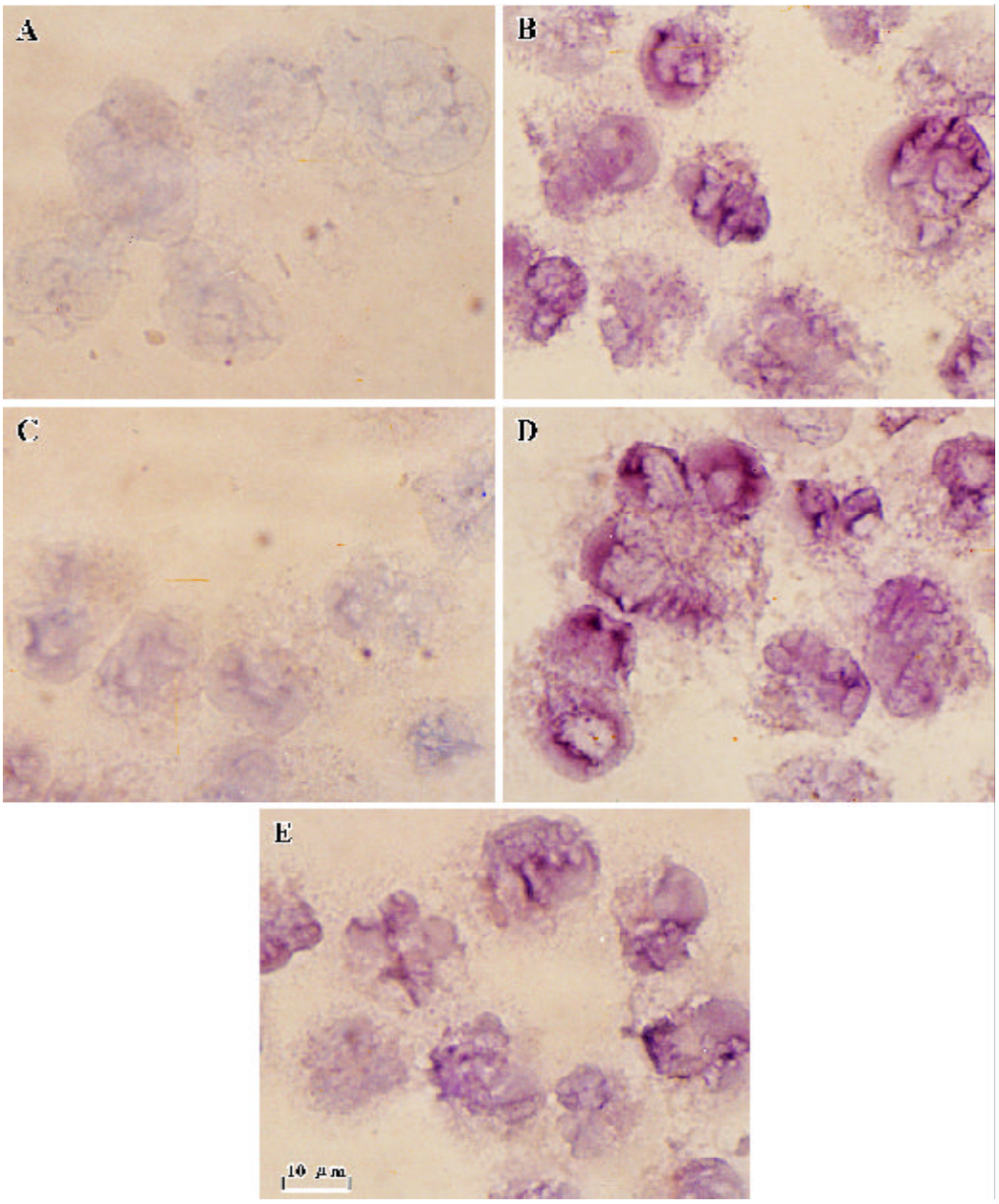


**Fig 1. Expression of PKC-a mRNA in A549 cells detected by *in situ* hybridization. (A) AP1261, (B) SCR1261, (C) ISIS3521, (D) SCR3521. Concentrations of all ODN were 100 nmol/L. (E) lipofectin control (NBT-BCIP coloration system, ×1000).**

the study. At the dose of 0.5 mg· kg$^{-1}$· d$^{-1}$, ISIS3521 inhibited the growth of A549 xenograft tumors by 69.2%. AP1261 at 0.5 mg· kg$^{-1}$· d$^{-1}$ inhibited the growth of the tumors about 89.2 %. This effect was stronger than that of ISIS3521 at the same dose ($P$<0.01). Other two lower doses of AP1261 (0.05 and 0.005 mg· kg$^{-1}$· d$^{-1}$) inhibited the growth of the tumors at 64.1% and 48.8%, respectively. The randomized and scrambled control ODN at the high dose of 0.5 mg· kg$^{-1}$· d$^{-1}$ was also tested. At this dose, the control ODN did not affect the growth of the A549 tumors compared with the saline-treated

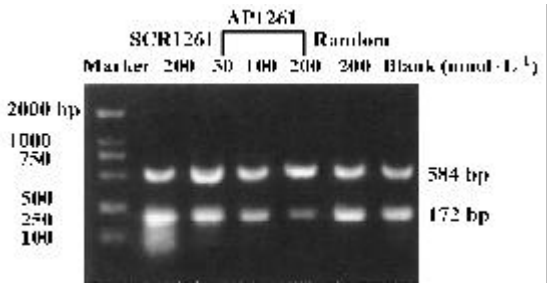


**Fig 2. Inhibition of PKC-a mRNA expression in A549 cells detected by RT-PCR. The amplification product of b -actin was set as internal standard (584 bp), the target amplification product of PKC-a mRNA was 172 bp.**

animals (Fig 3).

## DISCUSSION

In this paper, we reported novel potent antitumor ODN, AP1261, targeted against PKC-α mRNA. The *in vitro* activity of AP1261 on A549 cells ($IC_{50}$=47 nmol/L±8 nmol/L) was statistically lower than that of ISIS3521. The *in vivo* inhibitory effect of AP1261 on A549 tumors in nude mice was somewhat better than that of ISIS3521 too. The results implicated that AP1261 could be served as a potential antitumor agent with less non-specific adverse effects because of the lower dose of administration.

PKC-α mRNA is a 2245-base-mRNA. From the view of probability, every site of the sequence could be designed as target of antisense drug by random, then the number of possible candidates for 20-mer ODN would reach 2225. Whereas, if the full conserved blocks of the mRNA were selected out for antisense attack, the number of possible candidates will drop dramatically. Although the PKC-α mRNA is a well-conserved sequence, there are no more than 13 full-homologous blocks longer than 20 bases among human, mouse, and rat species. The number of possible candidates against these blocks is only 220. Further, the number of possible targets for antisense design will continue to shrink if the secondary structure and $DG^{o}$ of the conserved blocks were considered. The final result that we obtained potent ODN from 7 ODN candidates suggests that the high efficiency may be contributed to the first time of using the new approach of the combination of phylogenetic analysis and the principle of minimizing free energy for antisense drug design.

The phylogenetic comparison is the method of finding structural features that are conserved during evolution by comparing sequences with identical function in different organisms. This method is also involved in the prediction of the secondary and the three-dimensional structure of RNA[11]. Setting the conserved sequences of mRNA as the antisense target is inclined to be helpful for seeking ODN with outstanding activity from numerous candidates. However, further comprehensive investigations have to be carried out before the phylogenetic analysis could be accepted as one validated principle for antisense drug design. For instance, different mRNA with different function and different extent of conservation should be involved in further studies. ODN against the variational and partly conserved sequences should be considered and compared with those against the full homologous blocks in order to observe the importance of the phylogenetic conservation for antisense drug design. Furthermore, much more controls will be involved to demonstrate the exact "sequence-specific antisense effect" of the ODN with good activities.

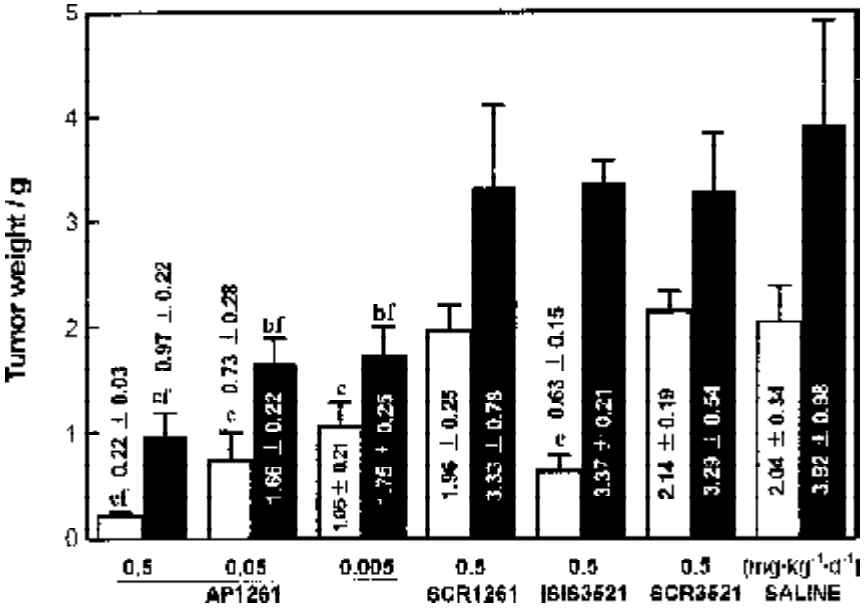


**Fig 3. Inhibitory effects of ODN on tumors *in vivo*. The animals were sacrificed and tumors were taken out and weighed 24 h after the last treatment. □: Human A549 carcinoma/nude mice; ■: Mouse B16 melanoma/mice. *n*=4 or 5. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* saline-treated control. [f]*P*<0.01 *vs* ISIS3521.**

Since AP1261 was targeting the conserved sequences among species, it was effective on both human and mouse cell lines *in vitro* and *in vivo*. On the other hand, the conservation of target site of ISIS3521, which was initially designed specially targeting human PKC-α mRNA, was poor. So ISIS3521 only behaved the activity on the human cell line A549, but did not influence the growth of mouse cell lines B16/B16-BL6. The results demonstrated that the "sequence-specific antisense effect" did exist to some extent. However, in this study, *in vitro* $IC_{50}$ values on cells in culture of all ODN including the scrambled and random controls were not higher than 300 nmol/L. It was implicating the "non-specific" cytotoxicity of ODN. Since the ODN is a kind of exogenous compound, presentation of side effects at a high dose level is seemingly inevitable. Maybe other chemical modifications of the ODN[17,18] could avoid the nonspecific side effects to some extent, but what the most important is still to look for the optimum ODN, which has already totally worked at an enough low dose under the emergence of side effects.

Besides, results also suggested that maybe the activities of antisense drug designed on the basis of phylogenetic analysis could be evaluated by the model of other species. For example, in this paper, the antitumor ODN designed against the conservative blocks between human and mouse mRNA could be tested by xenograft models of both human and mouse species. Our preliminary result showed that, for the same ODN, its inhibitory effect on human A549 tumors was significantly correlated with the effect on mouse B16 carcinoma ($r$=0.88, $n$=9, $P$<0.01). Thus, researcher may enjoy using the simple, cheap model of implanted mouse tumor in mice instead of the difficult and expensive model of human tumor grafted in nude mice for preliminary screening of ODN before the formal and comprehensive evaluation.

Although AP1261 had a satisfactory effect, not all designed ODN presented high efficacies. Once again the fact that activities of different antisense ODN targeting different local sequences on the same mRNA varied remarkably was demonstrated. There are still some unknown mysteries. Some of them may be related to the interaction of mRNA with proteins or other endogenous components, which result in the fact that only part of the three-dimensional structure of mRNA was exposed and approachable for antisense match.

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