Anti-Cancer Drugs 1994, **5**, pp. 437–442

Oligonucleotides protect cells from the cytotoxicity of several anti-cancer chemotherapeutic drugs

Mikhail V Blagosklonny and Leonard M Neckers
Clinical Pharmacology Branch, National Cancer Institute, National Institutes of Health, Bldg 10, Room 12N226, Bethesda, MD 20892, USA. Tel: (+1) 301 496 7641; Fax: (+1) 301 402 1608.

The possibility of inhibiting gene expression with antisense oligonucleotides (AS ODNs) in combination with more conventional chemotherapy is a very attractive modality in oncology. However, possible interaction between the ODN and drug must be considered. Here we show that ODNs protect cells from the cytostatic/cytotoxic action of actinomycin D (AMD), adriamycin, daunomycin or quinacrine, but not mitomycin, camptothecin, vincristine, cisplatin, etoposide (VP-16) or cycloheximide. The cytoprotective effect depends on ODN length as well as ability to interact directly with the cytotoxic drug and is only slightly sequence selective.

Key words: Antineoplastic drugs, antisense, cytoprotection, drug interaction, oligonucleotides.

Introduction

Antisense DNA oligonucleotides (AS ODNs) offer the promise of specifically modulating gene expression. Development of ODN-based therapeutics raises the possibility of potential combination therapy with conventional cytostatic drugs. However, given the non-antisense effects which ODNs can produce,[1,2] the possibility of direct interference with various drugs must be examined.

The interaction of actinomycin D (AMD) with double-stranded DNA is well studied. AMD has a strong binding affinity for 5′-GC-3′, although a weaker affinity for other sequences has also been demonstrated.[3–6] Besides binding to double-stranded DNA, AMD has been shown to bind to single-stranded DNA as well.[7] The binding occurs with particular DNA sequences containing guanosine residues, but the exact sequences required for binding of AMD to single-stranded DNA are not known.

AMD, at non-toxic concentrations, is able to potentiate the cytotoxicity produced by tumor necrosis factor (TNF) and is currently used in many *in vitro* systems studying TNF. Recently it has been reported that TNF AS ODNs, as well as unrelated ODNs, directly interact with AMD and prevent TNF induced, AMD-dependent, cytotoxicity.[8] Independently, we observed a similar phenomenon while using AS ODNs directed toward the TNF receptor in order to block TNF toxicity in AMD-pretreated U937 cells.[9] In this report we characterize the cytoprotection phenomenon and demonstrate that it not only applies to AMD but to a group of DNA intercalating drugs, including adriamycin and daunomycin.

Correspondence to MV Blagosklonny

Materials and methods

Reagents

The chemotherapeutic drugs AMD, 7-aminoactinomycin D (7-aAMD), adriamycin, daunomycin, VP-16, cycloheximide, mitomycin C, cisplatin and quinacrine were purchased from Sigma (St Louis, MO). Camptothecin was kindly provided by Dr B Sinha (NCI, NIH, Bethesda, MD). Human recombinant TNF-α was purchased from R & D Systems.

ODN description and synthesis

Nuclease-resistant phosphorothioate ODNs (PS ODNs) were prepared using an Applied Biosystems DNA synthesizer (model 380B). PS ODNs were purified by double ethanol precipitation in the presence of sodium acetate.

TNF receptor (three versions) and BCL-2 AS ODNs were synthesized to straddle the predicted translation-initiation site of human TNF receptor or *bcl-2* mRNA. A scrambled (SCR) version of BCL-2 AS was also prepared.

AS TNFR[1]: 5′-GCCCATGCCAGACAGCTATGG
AS TNFR[2]: 5′-GGAGAGGCCCATGCCAGACAG
AS TNFR[3]: 5′-ACGGTGGAGAGGCCCATGCCA
AS BCL2: 5′-AGCGTGCGCCATCCTTCCCAG
SCR BCL2: 5′-CCGTCTAGGACCTTCGAGCCC

Three additional sequences were selected based on their previously reported binding affinity for AMD.[7]

F9: 5′-TTT TTA TGA AAT ATA (does not bind AMD)
D1: 5′-AAA AAA AAA ATA ATT TTA AAT ATTT (does not bind AMD)
PL7: 5′-**CTC GAC GG** (binds AMD)

In addition, we synthesized the chimeric F9 + PL7 ODN:

5′-TTT TTA TGA AAT ATA **CTC GAC GG**

Spectroscopic experiments

UV spectra of AMD were obtained using a Hewlett Packard 8452A diode array UV-visible spectrophotometer. All experiments were performed in water at room temperature. AMD (10 μM; absorption max 440 nm) was incubated with 10 μM ODNs and absorption spectra were determined.

Fluorescence spectra of 7-aAMD were obtained using a PTI Deltascan fluorometer. The excitation wavelength was set at 540 nm and fluorescence emission was scanned from 600 to 700 nm. 7-aAMD (2 μM, in water) was analyzed first; the cuvette was then removed, ODNs were added to a 4 μM final concentration, and the sample was mixed and re-analyzed.

Scoring of growth and viability

U937 and MCF7 cell lines (ATCC) were maintained in RPMI 1640 or Dulbecco's minimal essential medium, respectively, supplemented with 10 mM HEPES and 10% FBS. For assay, cells were plated in 96-well plates (Costar) in 200 μl of medium containing test materials (15 000 and 5000 cells per well for U937 and MCF7, respectively). The plates were then incubated for 36 h (if not otherwise indicated) at 37°C. Triplicate wells were assayed for each condition and standard deviations were determined. The following tests were employed.

MTT assay. Twenty microliters of 5 mg/ml MTT solution in PBS was added to each well and the plates were incubated for an additional 4 h. After removal of the medium, 150 μl of dimethylsulfoxide was added to dissolve the formazan crystals and absorbance was measured at 540 nm.

[^{3}H]Thymidine incorporation. [^{3}H]Thymidine (1 μCi; 6.7 Ci/mmol) was added to each well and the plates were incubated for an additional 4 h. Cells were harvested onto glass fiber filters and incorporated radioactivity was determined.

Determination of protection index. The ratio of IC_{50} of drug alone to IC_{50} of drug in the presence of ODN, designated the protection index, was used to quantify the protective capacity of various ODNs against different drugs.

Results

A low dose of AMD (8 nM), while only cytostatic alone, caused the death of U937 cells after 24 h incubation together with TNF (Figure 1B), while co-incubation with AS TNFR[1] ODN prevented cell death (Figure 1C). This effect of the ODN was dependent on its continuous presence in the culture medium and was not sequence specific since AS BCL2 and SCR BCL2 ODNs, as well as other AS TNFR ODNs, were also effective (data not shown).

In order to show that cytoprotection is not due to interference with TNF action we excluded TNF from the testing system in the following experiments. U937 cells are very sensitive to the cytostatic (Figure 2A) and cytotoxic (Figure 2B) action of AMD alone. ODN completely reversed the effects of AMD at low to moderate AMD concentrations. Although higher amounts of AMD overcame ODN protection (Figure 2), 300- to 1000-fold more AMD was necessary to reach the IC_{50} for [^{3}H]thymidine incorporation (Figure 2A, Tables 1 and 2). The shorter the incubation time with AMD, the more protective was a single ODN concentration. Thus, 1000-fold more AMD was needed to overcome 5 μM ODN in a 20 h experiment (Figure 2A), but only 300-fold more AMD could overcome the same amount of ODN if the experiment was carried out for 40–48 h (Tables 1 and 2).

ODNs exerted the most effective cytoprotection when they were used in excess compared with the cytostatic drug. Thus, 5 μM ODN was necessary to completely reverse the cytostatic effect of 10 nM AMD (Figure 3). The minimal ODN concentration at which protection against 10 nM AMD could be detected was 80 nM. Each of five 21-mer ODNs tested (three antisense to the TNF receptor, one to the *bcl-2* oncogene and one scrambled ODN) were cytoprotective, suggesting a sequence-independent phenomenon. However, cytoprotection depended on both the ability of the ODN to bind AMD and on the length of the ODN (Table 1). Thus, the non-AMD binding ODNs D1 (25-mer) and F9 (15-mer) were not cytoprotective, but the protective

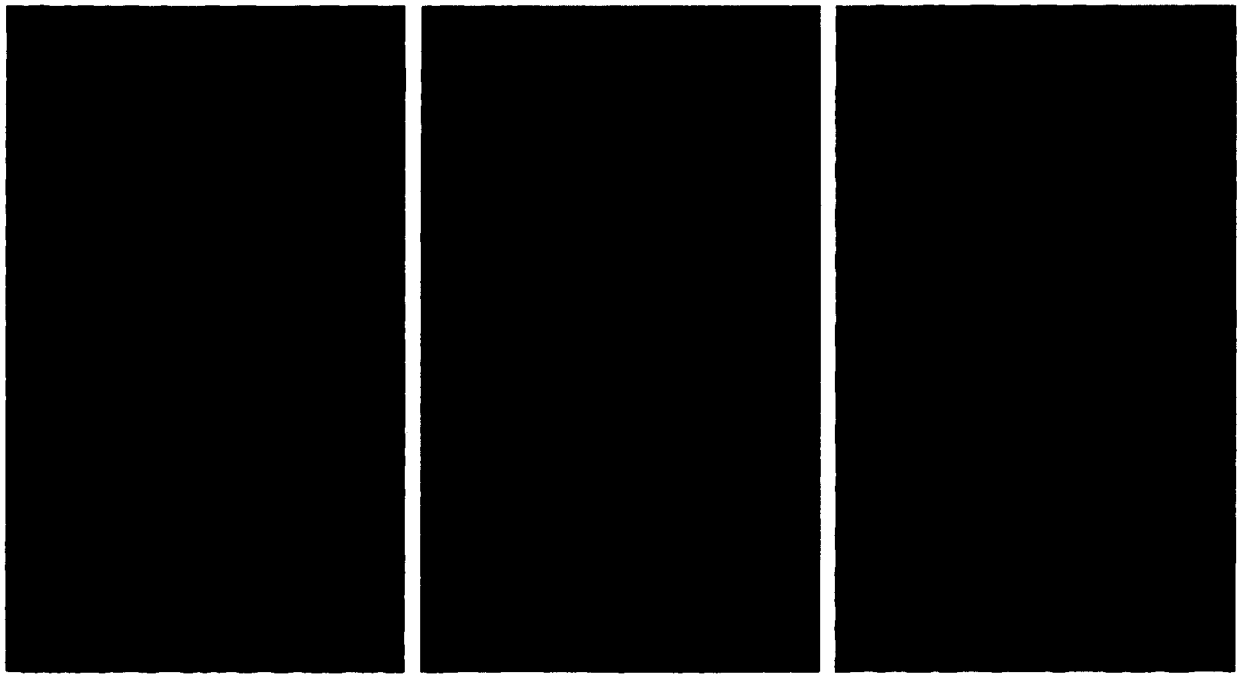
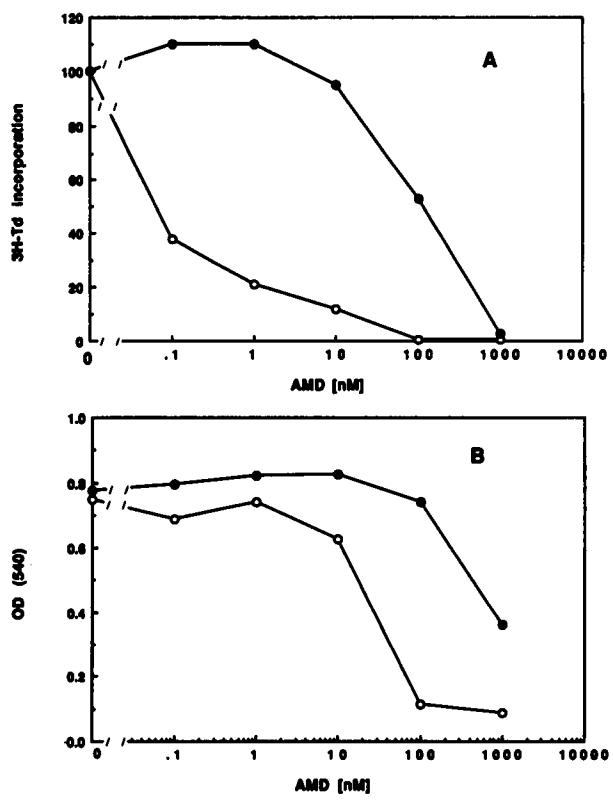


Figure 1. ODNs block TNF/AMD induced cell death. Micrographs of untreated U937 cells (A) or U937 cells incubated for 24 h with 5 ng/ml TNF and 8 nM AMD (B), or with 5 ng/ml TNF, 8 nM AMD and 2 μM ODN (AS TNFR[1]) (C).



capacity of the 8-mer ODN PL7 was also very low, even though this ODN is reported to bind AMD.[7] However, the 23-mer chimeric ODN produced by combining F9 and PL7 sequences bound AMD and was strongly cytoprotective (Table 1).

ODN binding in solution to 7-aAMD has been demonstrated as well[7] and the cytotoxicity of this compound was also blocked by ODNs (Table 2). ODNs also protected cells against adriamycin, daunomycin and quinacrine (Table 2). The difference in degree of protection may be related to the differences in IC_{50} of these drugs. For example, the high IC_{50} of quinacrine (2 μM) required that more ODN be used to achieve cytoprotection. At an ODN concentration of 5 μM, only a 2-fold protective effect was seen, while in the presence of 50 μM ODN, the protection index for quinacrine was increased to nearly 10 (Table 2 and Figure 4). Lack of ODN protection against other drugs cannot be explained by differences in IC_{50}, however (Table 2). For ex-

Figure 2. ODNs block the antiproliferative and cytotoxic activity of AMD. U937 cells were incubated with increasing concentrations of AMD in the absence (○) or presence (●) of 5 μM ODN (SCR BCL2). After 20 h, [^{3}H]thymidine incorporation (A) or MTT reduction (B) tests were performed.

Table 1. The cytoprotective activity of ODNs against AMD depends both on length and drug binding capability

ODN	Base length	Drug binding[a]	Protection index[b]
AS BCL2	21	yes	290
SCR BCL2	21	yes	280
AS TNFR[1]	21	yes	280
AS TNFR[2]	21	not tested	240
AS TNFR[3]	21	not tested	260
F9	15	no[c]	4
D1	25	no	1
PL7	8	yes	6
F9 + PL7	23	yes	250

[a] Drug binding measured by alteration in UV absorbance of AMD or fluorescence of 7-aAMD as described in text.
[b] U937 cells were incubated with AMD for 48 h and [^{3}H]-thymidine incorporation was measured during the last 4 h. Ratio of IC_{50} for AMD in the presence of 5 μM ODNs to IC_{50} of AMD alone was calculated and displayed as the protection index.
[c] Binding data according to Wadkins and Jovin[7].

Table 2. Cytoprotection of SCR BCL2 ODN against several cytostatic drugs

Drug	ODN IC_{50} (nM)		Protection index
	−	+	
Actinomycin D	0.2	60	300
7-aAMD	4.0	500	125
Adriamycin	10	300	30
Daunomycin	0.6	70	117
Quinacrine	2000	4000	2
Vincristine	0.05	0.07	1.4
Mitomycin C	18	18	1.0
Cycloheximide	60	80	1.3
Cisplatin	400	600	1.5
VP-16	120	120	1.0
Camptothecin	3	3	1.0

U937 cells were incubated with test drug in the absence (−) or presence (+) of 5 μM SCR BCL2 ODN for 40 h and [^{3}H]thymidine incorporation was measured during the last 4 h. Drug dose–response curves were generated and data converted to IC_{50} values.

ample, even 50 μM ODN did not alter the IC_{50} for VP-16, although the IC_{50} for VP-16 is 120 nM (Table 2 and Figure 4). We also observed the phenomenon of ODN cytoprotection in other cell types. Thus, quinacrine exerts a cytostatic/cytotoxic action on the breast cancer cell line MCF7 with an IC_{50} of 2 μM, whereas a 2-fold higher dose of quinacrine is required to obtain the same response in the presence of 5 μM ODN (data not shown).

Stull *et al.*[8] demonstrated that those antisense phosphodiester and phosphorothioate ODNs which protected cells from TNF plus AMD-induced cytotoxicity also shifted the absorbance maximum of AMD from 440 to 460 nm, similar to results reported by Wadkins and Jovin[7] with defined ODN sequences. We found that the AS TNFR[1] ODN, as well as the AS and SCR BLC2 ODNs, produced a similar shift in AMD absorbance maximum, from 440 to 458 nm. ODN D1, reported by Wadkins and Jovin not to bind AMD,[7] did not shift its absorbance maximum (data not shown). Additionally, Wadkins and Jovin[7] reported that ODNs which bound to 7-aAMD caused a marked fluorescence enhancement and hypsochromic shift from 670 nm for the free dye to 630 nm for the complex. Again, we observed a similar phenomenon. The cytoprotective ODNs tested (AS TFR,[1] AS and SCR BCL2) all produced a significant hypsochromic shift in, and a marked

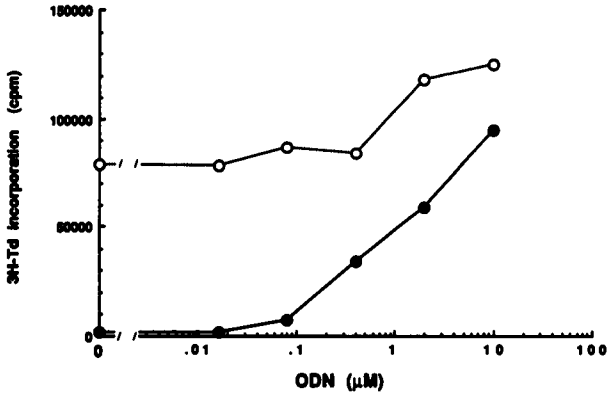


Figure 3. The cytoprotective effect of ODNs is dose dependent. U937 cells were incubated in the presence (●) or absence (○) of 10 nM AMD together with increasing concentrations of ODN (SCR BCL2). [^{3}H]Thymidine incorporation was performed after 24 h of incubation.

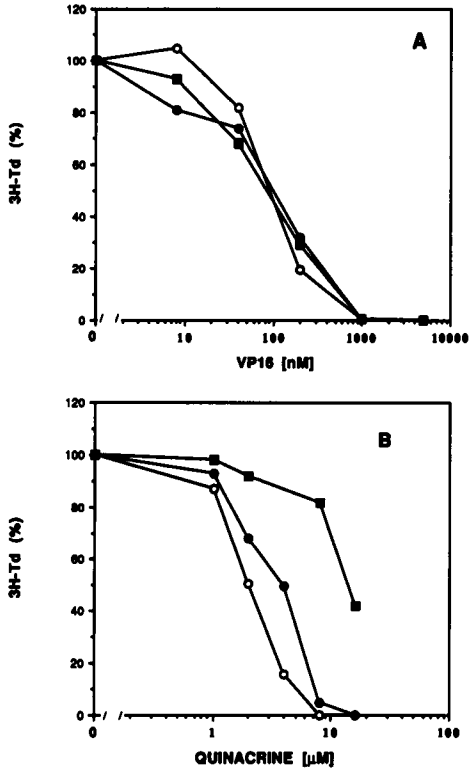


Figure 4. The cytoprotective activity of ODNs against quinacrine, but not VP-16, is dose dependent. U937 cells were incubated with increasing concentrations of quinacrine in the absence (○) or presence of 5 μM (●) or 50 μM (■) ODN (F9+PL7 chimeric ODN). After 36 h, [^{3}H]thymidine incorporation (A) or MTT reduction (B) tests were performed.

enhancement of, 7-aAMD fluorescence, while the non-cytoprotective ODN D1 did not significantly alter the fluorescence characteristics of the dye (Figure 5).

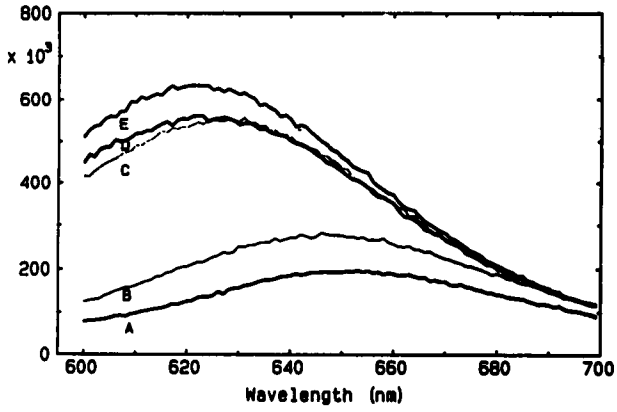


Figure 5. Cytoprotective ODNs alter the fluorescence characteristics of 7-aAMD. Three cytoprotective ODNs (C = AS TNFR[1], D = AS BCL2, E = SCR BCL2) induce a hypsochromic shift in, and markedly enhance, 7-aAMD fluorescence in solution. A non-cytoprotective ODN (B = ODN D1) does not alter the spectral properties of the dye. A = 7-aAMD alone. Excitation wavelength was 540 nm.

Discussion

We have shown that PS ODNs protect cells against the cytostatic/cytotoxic action of several cytotoxic drugs. A similar finding with respect to AMD has been recently reported.[8] These authors demonstrated that both phosphorothioate and phosphodiester ODNs were also cytoprotective in the TNF assay, and that the cytoprotection was not due to the polyanionic nature of the ODNs. Although initially these ODNs were designed to protect against TNF cytotoxicity (AS toward TNF receptors in our experiments and AS toward TNF itself in ref. 7), these studies show that the protective effect of ODNs has no relation to TNF-induced cytotoxicity, but rather to a direct interaction with AMD. Moreover, the cytoprotective capacity of ODNs is not restricted to AMD, but can be expanded to include a broad group of chemotherapeutic agents: actinomycin D, adriamycin, daunomycin and quinacrine. ODNs were poorly protective against mitomycin, camptothecin, vincristine, cisplatin, VP-16 or cycloheximide.

Although each of the tested drugs has several mechanisms of action, one can correlate sensitivity to ODN interference with a drug's intercalating ability. Thus, ODN-sensitive drugs possess intercalating activity among their properties, while ODN-insensitive drugs are not intercalators. Although sequence-specific binding of AMD by intercalation occurs with $[d(ATCGAT)]_2$,[5] the ability of AMD to bind single-stranded ODNs is not a result of inter-

calation.[7] The mechanism of this binding appears to involve stacked complexes between the drug and the single-stranded DNA.[7] Whether a similar mechanism underlies the antagonistic effects of ODNs toward daunomycin, adriamycin and quinacrine remains to be determined.

Cytoprotection requires the continuous presence of ODN in the cell culture, since removal of ODNs rapidly restores cell sensitivity to the cytostatic agent. A requirement for direct ODN–drug interaction is suggested by our data and those of Stull *et al.*[8] We have demonstrated that cytoprotective ODNs, originally designed for diverse antisense experiments, directly interact with and alter the physicochemical characteristics of both AMD and 7-aAMD. Conversely, we have demonstrated that an ODN which does not interact with either AMD or 7-aAMD in solution (ODN D1) is not cytoprotective.

Although ODN binding to drug may be necessary, it is not sufficient for effective cytoprotection, since an 8-mer ODN which binds to AMD in solution (PL7) is not cytoprotective. However, when the 8-mer was linked to a 15-mer non-AMD binding (and non-cytoprotective) ODN (F9), the resultant chimeric molecule was fully cytoprotective. Thus, ODN chain length as well as drug binding capacity would appear to be an important determinant of cytoprotection. One can speculate that longer ODNs may have more favorable binding kinetics in the physiologic system used to test cytotoxicity.

Although this phenomenon appears to be independent of ODN sequence, we have observed that two non-AMD binding ODNs primarily composed of adenosine and thymidine residues (14/15 and 25/25) were not cytoprotective. Since guanosines have been implicated in the ability of ODNs to bind to AMD,[7] a certain number of these residues may be critical for cytoprotection. However, since most antisense ODNs, or their controls, contain at least several guanosine residues, for all practical purposes this phenomenon can be considered sequence-independent. Determination of an unknown ODN's effect on the UV absorbance of AMD or on the fluorescence characteristics of 7-aAMD should serve as a rapid screening technique to estimate the cytoprotective ability of the ODN in question.

In summary, direct interaction between ODNs and intercalating anti-cancer drugs must be taken into account in all *in vitro* experiments, and in planned *in vivo* applications of AS technology. Finally, it may be possible to utilize the cytoprotective property of ODNs for the *in vivo* reversal of AMD-, adriamycin- or daunomycin-related toxicities.

References

1. Stein CA, Cheng YC. *Science* 1993; **261**: 1004–12.
2. Neckers L, Whitesell L. *Am J Physiol* 1993; **9**: L1–12.
3. Wells RD, Larson JE. *J Mol Biol* 1970; **49**: 319–42.
4. Sobel HM, Jain SC, Sakore TD, *et al. Nature* 1971; **231**: 200–5.
5. Zhou N, James TL, Shafer RH. *Biochemistry* 1989; **28**: 5231–9.
6. Kamitori S, Takusagawa F. *J Mol Biol* 1992; **225**: 445–56.
7. Wadkins RM, Jovin TM. *Biochemistry* 1991; **30**: 9469–78.
8. Stull RA, Zon G, Szoka FC. *Antisense Res Dev* 1993; **3**: 295–300.
9. Biagosklonny MV, Neckers LM. *Eur Cytokine Netw.* 1993; **4**: 279–83.

(Received 30 March 1994; accepted 7 April 1994)