Biochemical Pharmacology, Vol. 60, pp. 83–90, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00310-5

SHORT COMMUNICATION

Antisense Inhibition of P-glycoprotein Expression Using Peptide–Oligonucleotide Conjugates

Anna Astriab-Fisher,† Dimitri S. Sergueev,†‡ Michael Fisher,* Barbara Ramsay Shaw‡ and Rudolph L. Juliano*§*

*Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC; and ‡Department of Chemistry, Duke University, Durham, NC, U.S.A.

ABSTRACT. Antisense oligonucleotides are potentially a powerful tool for the therapeutic manipulation of genes associated with cancer. However, pharmacological applications of oligonucleotides have been hindered by the inability to effectively deliver these compounds to their sites of action within cells. In this study, we have prepared peptide–oligonucleotide conjugates with the intent of improving intracellular delivery. The phosphorothioate oligonucleotide component of the conjugates was complementary to a site flanking the AUG of the message for P-glycoprotein, a membrane ATPase associated with multidrug resistance in tumor cells. Two types of peptide–antisense oligonucleotide conjugates, but not mismatched control conjugates, provided substantial inhibition of cell surface expression of P-glycoprotein. Surprisingly, the peptide–oligonucleotide conjugates were more potent in the presence of serum than when used under serum-free conditions; this is in striking contrast to most other approaches for intracellular delivery of nucleic acids. Effective inhibition of P-glycoprotein expression was attained with submicromolar concentrations of antisense conjugates under serum-replete conditions. The combination of relatively modest molecular size and good efficacy in the presence of serum proteins suggests that peptide–antisense oligonucleotide conjugates may have significant promise for *in vivo* therapeutic applications. BIOCHEM PHARMACOL **60**;1:83–90, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. antisense; drug resistance; drug delivery

Antisense oligonucleotides offer the possibility of highly selective pharmacological manipulation of gene expression [1–4]. This strategy may be particularly important in cancer therapeutics, allowing the regulation of genes involved in tumor progression or resistance to therapy [5, 6]. P-glycoprotein, the product of the *MDR1* gene, is an ATP-driven transmembrane pump that can expel a wide variety of drug molecules from cells [7, 8]. Overexpression of P-glycoprotein in tumor cells confers a MDR‖ phenotype that can impede the effectiveness of cancer chemotherapy [9, 10]. Thus, a number of approaches have been devised to attempt to reverse the MDR phenotype, either through inhibition of P-glycoprotein function [11] or through reduction in its expression using molecular techniques [12, 13].

We and others have sought previously to inhibit expression of P-glycoprotein message and protein using antisense oligonucleotides [14–18], but with only moderate success. One of the major problems with antisense pharmacology is the inefficient delivery of oligonucleotides to their sites of action in the cytoplasm and nucleus [3, 19]. Most attempts to overcome this problem have involved complexing oligonucleotides with polycationic carrier entities, such as cationic liposomes [20]. One major liability with this approach is that most of the carrier or delivery entities tested thus far do not function well in the presence of serum [19, 21], although there are some exceptions to this [22]. In addition, liposomal complexes are particulate in nature, with sizes ranging up to microns. Thus, cationic liposome/oligonucleotide complexes would be unlikely to work in the *in vivo* milieu where plasma proteins are abundant, and where the complexes would be subject to rapid clearance by filtration and other mechanisms [19].

Recently, a new approach to the intracellular delivery of large molecules has emerged. This is based on the use of several types of "delivery peptides" that seem to have the ability to carry large, polar molecules including peptides, oligonucleotides, and even proteins across cell membranes. Two examples of delivery peptides are a 35-amino-acid sequence ("Tat") from the HIV Tat protein [23, 24], and a 16-amino-acid sequence ("Ant") from the *Drosophila* Antennapedia protein [25, 26]. Antennapedia-type peptides have already been used to deliver oligonucleotides, including peptide–nucleic acids, into neuronal cells [26, 27], but their general applicability is unclear. Other types of pep-

† Contributed equally.

§ Corresponding author: R. L. Juliano, Ph.D., CB#7365, 1106 Jones Building, Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599-7365. Tel. (919) 966-4383; FAX (919) 966-5640; E-mail: arjay@med.unc.edu

‖ *Abbreviations*: MDR, multidrug resistance; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; Tat, peptide sequence from the HIV Tat transcription factor; and Ant, peptide sequence from the Antennapedia transcription factor.

Received 23 August 1999; accepted 10 December 1999.

tides, containing hydrophobic motifs, have also been used for antisense delivery [28].

Inhibition of *MDR1* gene expression is a challenging task for antisense approaches since, in highly drug-resistant cells, the gene is amplified, the message is abundant and stable, and the protein has a long half-life [14]. Nonetheless, here we report significant inhibition of P-glycoprotein expression using conjugates formed from versions of the Tat and Ant delivery peptides and a phosphorothioate oligonucleotide that flanks the AUG site of the MDR1 message [14]. Importantly, these peptide–oligonucleotide conjugates are quite effective in the presence of serum, unlike the situation with many other nucleic acid delivery agents.

MATERIALS AND METHODS

Cells

NIH 3T3 cells transfected with a plasmid containing the human *MDR1* gene (pSK1 MDR) were a gift from M. M. Gottesman [29]. The MDR-3T3 cells were grown in DMEM containing 10% FBS and 60 ng/mL of colchicine in an atmosphere of 95% air, 5% CO_2.

Peptide–Oligonucleotide Conjugate Synthesis

Fluorescently labeled peptide–oligonucleotide conjugates were synthesized via disulfide bond formation [30, 31]. Specifically, phosphorothioate 20-mer anti-MDR 5′-d(CCA-TCC-CGA-CCT-CGC-GCT-CC)-3′ and mismatch 5′-d(CCA-TAC-CAA-CAT-CAC-GCT-CC)-3′ deoxyoligonucleotides were derivatized with 3′-Amino-Modifier C7 and 5′-Thiol-Modifier C6 S-S (Glen Research) during solid phase synthesis. After ammonia deprotection, the 5′-thiol function was activated with the 2-pyridylthio (PyS) moiety [30] followed by HPLC purification of the activated oligonucleotides. The resulting PyS-S-5′-oligo-3′-NH_2 compounds were coupled with a 6-fold excess of 5 (and 6)-carboxytetramethylrhodamine *N*-hydroxysuccinimidyl ester (TAMRA, SE) (Molecular Probes) and subsequently were isolated by gel filtration on Sephadex G-25. Before the final conjugation, the availability and content of the SH groups in the peptides were assessed by reaction with Ellman's reagent [32]. Disulfide bond formation between the labeled PyS-activated oligonucleotides and Tat and Ant peptides (3-fold excess) was carried out in 0.3 M KBr, 0.02 M K_2HPO_4 (pH 7.5), 5 M urea, 20% CH_3CN. After ion-exchange HPLC purification and desalting, the purity of the conjugates was assessed by HPLC (see Fig. 1) and PAGE. Cleavage of the conjugate with dithiothreitol regenerated the original peptide and oligonucleotide.

Treatment of Cells with Peptide–Oligonucleotide Conjugates

The experimental protocols were similar to those described previously [15]. Briefly, MDR 3T3 cells were grown in 162-mm flasks to 95% confluency and then seeded into 100-mm dishes at 5×10^6/dish in 10% FBS/DMEM and incubated for 24 hr. The cells were washed twice with PBS. Peptide–oligonucleotide conjugates, oligonucleotides complexed with Lipofectin (as a positive control), and oligonucleotides with no delivery agent (as negative controls) were mixed in Opti-MEM and incubated with cells at 37° overnight. Oligonucleotide concentrations ranged from 0.05 to 1.0 μM in various experiments (see legends).

Uptake and Subcellular Distribution of Peptide–Oligonucleotide Conjugates

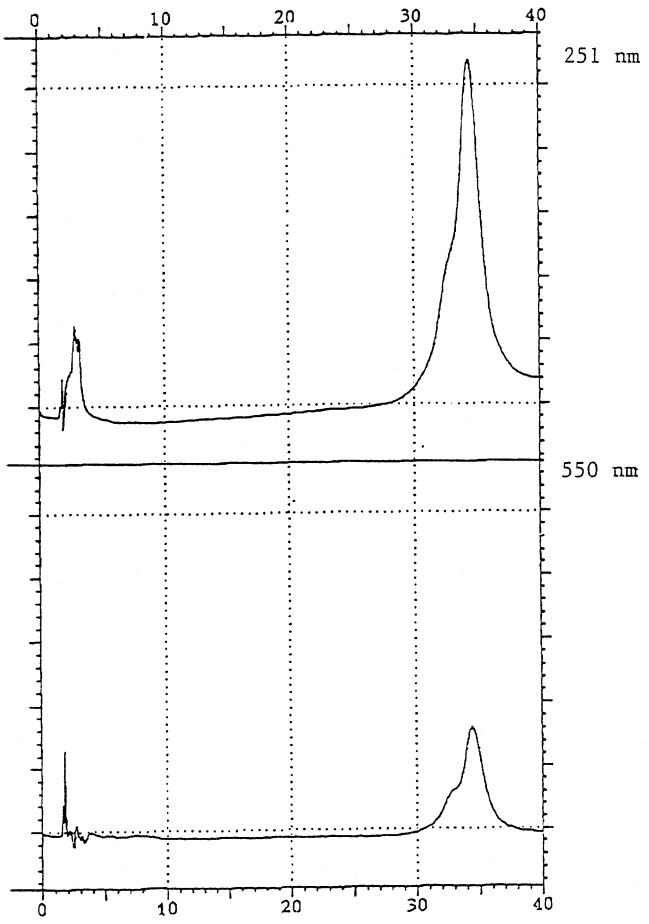
Cells treated with fluorescent oligonucleotides or peptide–oligonucleotide conjugates were harvested by trypsinization and then examined by fluorescence microscopy or by flow cytometry. For microscopy, cells resuspended in 1 mL of 10% FBS/DMEM were incubated for 6 hr on fibronectin-coated cover slips. The fluorescence patterns were analyzed on a Zeiss Axiophot equipped with a 100-W mercury lamp, an oil immersion objective (FLUAR 40X/1.30 Oil/0.17), and an H5546 filter. Images were captured with a slow scan charge-coupled device 3CCD Video Camera System (Carl Zeiss, Inc., ZVSTM3C75DE) interfaced to a PC using the MetaMorph Imaging System (Version 3.5, Universal Imaging Corp.). For flow cytometry analysis, cells were resuspended in 500 μL PBS and measured for the accumulation of TAMRA marker using a Becton Dickinson flow cytometer with Cicero software (Cytomation).

Pharmacological Effects of Peptide–Oligonucleotide Treatment

Analysis of the pharmacological effects of peptide–oligonucleotide conjugates was based on a previously described assay [14] involving antisense inhibition of P-glycoprotein expression in mouse 3T3 cells stably transfected with the human *MDR1* gene. The cell surface expression of P-glycoprotein was determined by immunostaining and quantitation by flow cytometry, as described [14, 15]. Briefly, the MRK16 anti-P-glycoprotein antibody (Kamiya Biochemicals), which is directed against an external epitope, was employed as the primary antibody. An R-phycoerythrin-conjugated goat anti-mouse IgG (Sigma) was used as the second antibody. The level of R-phycoerythrin fluorescence in viable cells (as determined by light scatter) was quantitated using the Cicero software application (Cytomation) on a Becton-Dickinson flow cytometer.

RESULTS AND DISCUSSION

Peptide–oligonucleotide conjugates joined by S-S linkages were prepared as described above and were analyzed by ion-exchange HPLC. The HPLC profile of the Ant-20 conjugate seen in Fig. 1 is typical for these compounds. The chart below Fig. 1 indicates the amino acid and base sequence for each of the conjugates used in this study, as



Name	Sequence
Ant-20	NH2RQIKIWFQNRRMKWKKGGC(COOH)S-S-5'CCA-TCC-CGA-CCT-CGC-GCT-CC-3-'NH2-TAMRA
Ant mismatch	NH2RQIKIWFQNRRMKWKKGGCCOOHSS5'CCA-TAC-CAA-CAT-CAC-GCT-CC-3'-NH2-TAMRA
Tat-20	NH2RKKRRQRRRPPQC(COOH)-S-S-5'CCA-TCCCGA-CCT-CGC-GCT-CC-3'NH-TAMRA
Tat 20mismatch	NH2RKKRRQRRRPPQC(COOH)-S-S-5'CCA-TAC-CAA-CAT-CAC-GCT-CC-3-'NH-TAMRA
20	5'CAA-TCC-CGA-CCT-CGC-GCT-CC-3'-NH-TAMRA

FIG. 1. Analysis of peptide–oligonucleotide conjugates. The figure illustrates an HPLC profile for the Ant-20 conjugate with a TAMRA fluorophore. A Polysil CA 4 × 150 mm column was used with a linear gradient of 0.18 to 1.35 M KBr over 60 min in 0.1 M KH_2PO_4 (pH 6.5), 5 M urea, 25% CH_3CN. The ordinates are absorbance at 251 nm (top panel, for oligonucleotide absorbance) and 550 nm (bottom panel, for fluorophore absorbance), and the abcissa is time in minutes. The chart under the HPLC profile provides the peptide and nucleic acid sequences of the antisense and mismatch peptide–oligonucleotide conjugates used in this study.

well as for the parent anti-MDR phosphorothioate oligonucleotide [14]. The Tat conjugate uses an active fragment [33] of the larger Tat sequence [23]. All of the conjugates readily dissolved in buffer, at least at the micromolar levels used in these studies. More complete studies of the physicochemical properties of the peptide–oligonucleotide conjugates are currently underway.

As illustrated in the flow cytometry profiles in Fig. 2, the conjugates accumulated in cells to a much greater degree than "free" oligonucleotide (oligo-20), but to a lesser degree than oligonucleotide complexed with a cationic lipid carrier. The accompanying fluorescence micrographs illustrate that both the cationic lipid carrier (Fig. 2A) and the peptide–oligonucleotide conjugate (Fig. 2B) resulted in accumulation of fluorescent oligonucleotide in the cell nucleus. In addition, punctate cytoplasmic fluorescence indicative of endosomal vehicle was also observed with both delivery agents, but to a greater extent with the

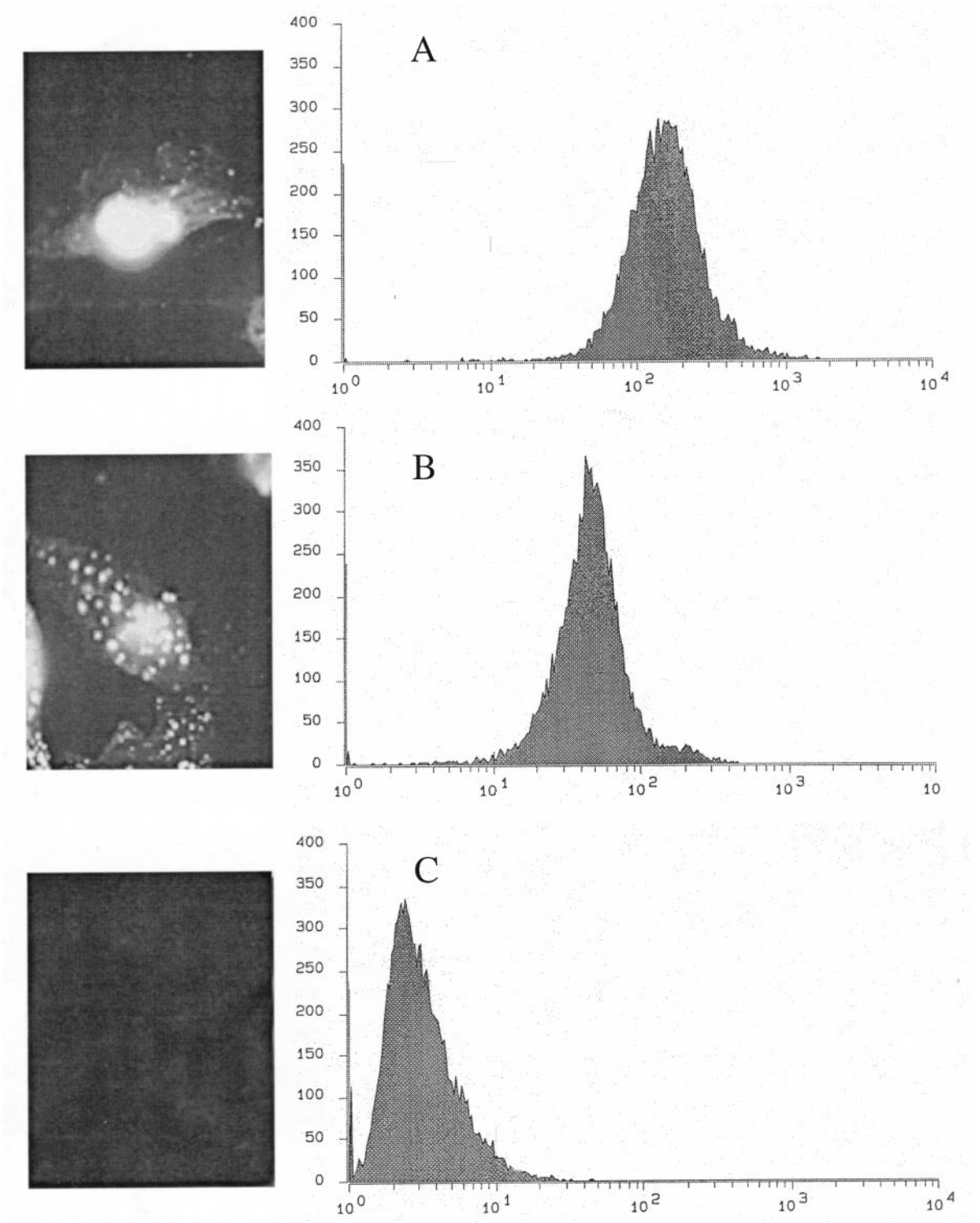


FIG. 2. Analysis of the cellular uptake of oligonucleotide by MDR-3T3 cells. The left panels are fluorescence microscopy images of the subcellular distribution of TAMRA-fluorophore-labeled oligonucleotides. The right panels are flow cytometry profiles reflecting total cell uptake of TAMRA-fluorophore-labeled oligonucleotides. (A) Oligonucleotide 20 complexed with Lipofectin (20 μg/mL) (positive control); (B) peptide–oligonucleotide conjugate Tat-20; (C) oligonucleotide 20 with no delivery agent (negative control). The concentration of TAMRA-labeled oligonucleotide 20 in panels A–C was 0.5 μM. Similar patterns of uptake and subcellular distribution were seen with the Ant-20 conjugate. In all flow cytometry profiles, the abcissa represents relative fluorescence, while the ordinate is the number of cells at each level of fluorescence.

peptide conjugate. Cells treated with "free" oligonucleotide (Fig. 2C) had virtually no intracellular fluorescence.

The pharmacological effects of the peptide–oligonucleotide conjugates in inhibiting expression of P-glycoprotein are illustrated in Fig. 3 for the case of the Ant-20 sample and its controls. The expression of cell surface P-glycoprotein was quantitated by flow cytometry, using an antibody that is directed to a P-glycoprotein epitope displayed on the external surface of cells. In the experiments illustrated in Fig. 3, the cells were exposed to the peptide–oligonucleotide conjugates in serum-free culture medium. The P-glycoprotein expression profile observed in cells that were not exposed to oligonucleotides (Fig. 3E) and the profile observed in cells treated with a control peptide–oligonucleotide conjugate having a 4-base mismatch (Fig. 3F) are essentially the same. However, in cells exposed to increasing concentrations of the Ant-20 antisense peptide-oligonucleotide conjugate (Fig. 3, A–C), there is a progressive left-shift of the flow cytometry profile, indicating reduced P-glycoprotein expression. Thus, treatment with 1 μM Ant-20 shifts 34% of the population into the window that encompasses the unstained control (Fig. 3D), where 1 μM of the mismatch conjugate does not result in any significant shift. Very similar results were obtained with the Tat-20 peptide–oligonucleotide conjugate and its controls (not shown). Treatment with 1 μM antisense oligonucleotide complexed with Lipofectin, a cationic lipid carrier, shifted 19% of the cell population (Fig. 3G); the unstained control for this experiment is Fig. 3H. This degree of reduced expression of P-glycoprotein is comparable with our previous results using this same antisense sequence and cationic lipids [14].

We wished to evaluate whether the peptide–oligonucleotide conjugates would also function in the presence of serum proteins. Thus, experiments with the Ant-20 and Tat-20 conjugates and their controls were done, exposing the cells to the conjugates in the presence of 10% FBS or in serum-free medium. As seen in Fig. 4, both conjugates caused a dramatic left-shift of the flow cytometry profiles when used in the presence of serum. Indeed, using either the Tat-conjugate or the Ant-conjugate, 0.1 μM peptide–oligonucleotide conjugate was sufficient to shift over 50% of the population into the window overlapping the unstained controls. The effects seen in the presence of serum (Fig. 4, D and E) were substantially greater than in its absence (Fig. 4, A and B). The mismatched controls had essentially no effect (not shown). The greater effectiveness of the peptide–oligonucleotide conjugates in serum was a very surprising result, and stands in contrast to any other antisense delivery approach of which we are aware. For example, antisense oligonucleotides complexed with Lipofectin are virtually without effect in the presence of 10% serum ([34], and data not shown).

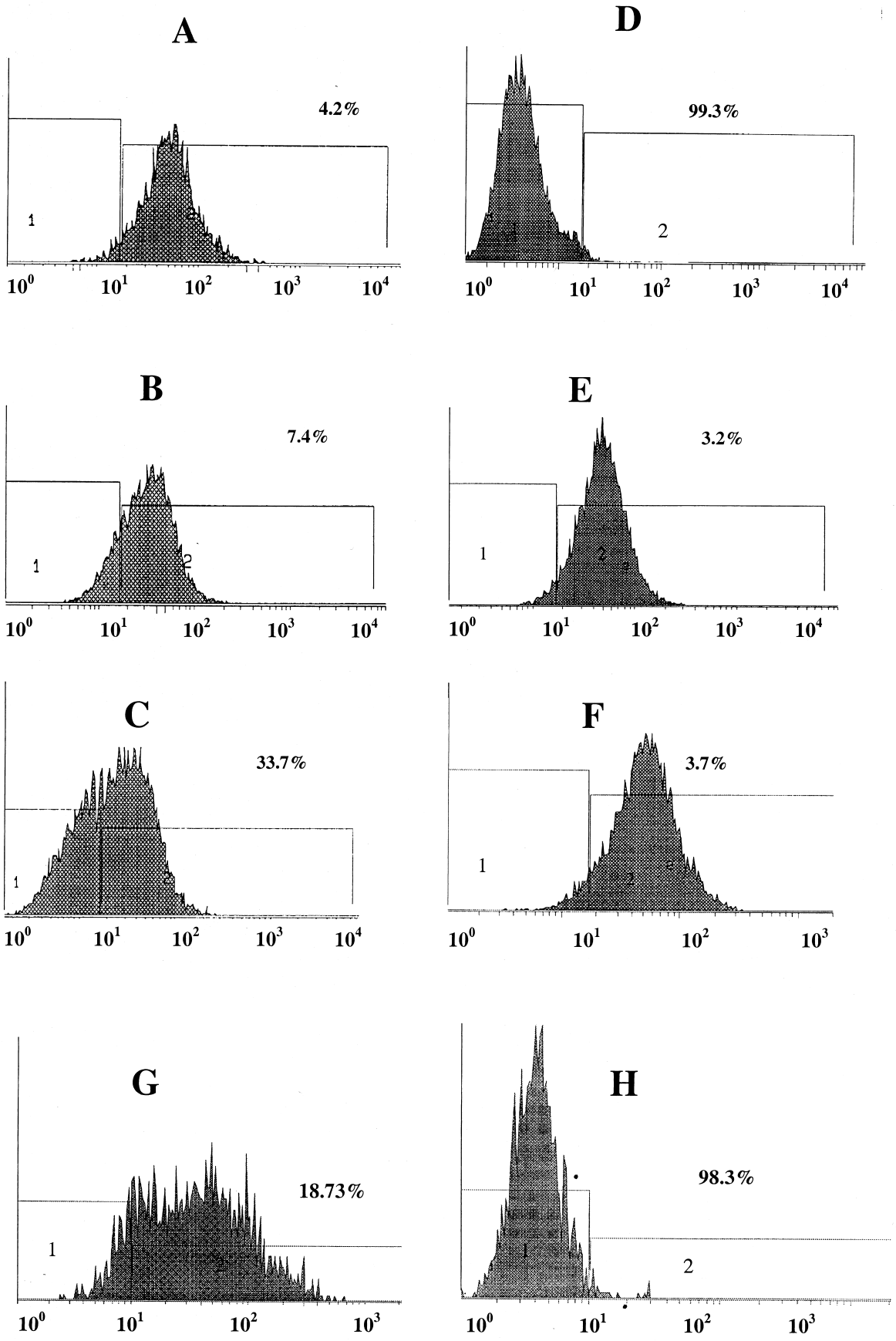
The shape of the flow cytometry profile in Fig. 4 indicates that only a fraction of the total population was affected by treatment with conjugates, but in that fraction there was a dramatic reduction in P-glycoprotein expression. However, as seen in Fig. 2, most of the cells took up the conjugate. One possible explanation is that a threshold level must be reached before the conjugate is effective; alternatively, there may be intrinsic heterogeneity in the responsiveness of the cell population.

Because of the limited amount of peptide–oligonucleotide conjugates currently available, we have not tested the degree to which these conjugates can affect drug resistance. Based on previous experience [14] with anti-MDR antisense, we believe that the degree of inhibition of P-glycoprotein expression seen here would result in a 2- to 3-fold increase in drug uptake in the total cell population. Thus, although current results are encouraging, they would result in only a modest reversal of MDR. Use of more potent chemically modified oligonucleotides [15] rather than the phosphorothioates used here might further enhance the efficacy of the peptide–oligonucleotide conjugates.

The discovery of relatively short peptide sequences that promote the intracellular delivery of large molecules promises to open up interesting avenues for using macromolecules as therapeutic agents. The Tat sequence has been shown to permit the delivery of proteins into cells [23, 35], and the Ant sequence has been used to deliver peptides [36] and oligonucleotide derivatives [26, 27]. The mechanism of transmembrane delivery via the Tat and Ant peptides is still somewhat mysterious; however, it seems likely that neither specific cell surface receptors nor active endocytosis is involved [25, 33].

Here we have extended the use of the Tat and Ant delivery peptides to the challenging problem of modulating the expression of the P-glycoprotein drug efflux pump. Previous work from our laboratory [14, 15] and from others [16, 17] has demonstrated the possibility of inhibition of P-glycoprotein expression using antisense oligonucleotides. However, current results seem to be a substantial improvement over previous efforts. Perhaps most importantly, the peptide–oligonucleotide conjugates work extremely well in the presence of serum, unlike most other oligonucleotide delivery agents [19]. This, coupled with the relatively small size of these agents (MW = 9,000–10,000), opens up interesting possibilities for using conjugates of delivery peptides and antisense oligonucleotides in the *in vivo* setting.

Another indication of the value of the peptide–oligonucleotide conjugates lies in the fact that we have attained significant inhibition of P-glycoprotein expression with submicromolar amounts of a relatively ineffective phosphorothioate oligonucleotide. This is quite striking; in previous studies, much higher levels of this same phosphorothioate antisense compound were required when used with a cationic lipid delivery system [14], and effects at submicromolar levels were attained only with novel, chemically modified oligonucleotides such as methoxyethoxy derivatives [15]. In summary, conjugates of polybasic delivery peptides such as Tat and Ant and antisense oligonucleotides may offer a promising means for inhibition of the expression of cancer-associated genes both in cell culture and in the *in vivo* milieu.


A
4.2%
1
2
10^0 10^1 10^2 10^3 10^4
D
99.3%
1
2
10^0 10^1 10^2 10^3 10^4
B
7.4%
1
2
10^0 10^1 10^2 10^3 10^4
E
3.2%
1
2
10^0 10^1 10^2 10^3 10^4
C
33.7%
1
2
10^0 10^1 10^2 10^3 10^4
F
3.7%
1
2
10^0 10^1 10^2 10^3
G
18.73%
1
2
10^0 10^1 10^2 10^3
H
98.3%
1
2
10^0 10^1 10^2 10^3

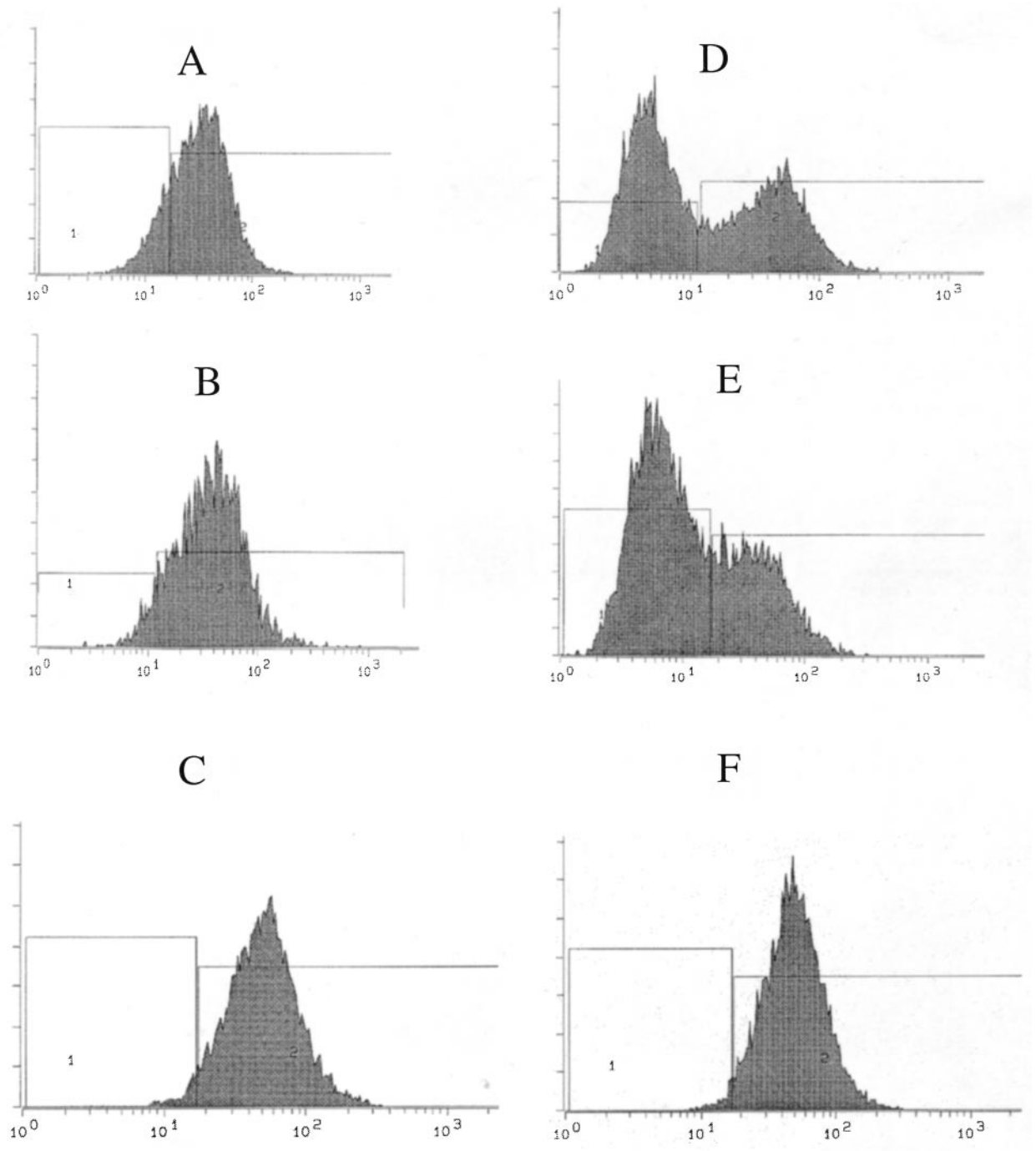


FIG. 4. Serum dependence of antisense effects. Inhibition of P-glycoprotein expression in the absence (A–C) or presence (D–F) of serum (10%) using peptide–oligonucleotide conjugates: Tat-20 (A and D), Ant-20 (B and E), untreated cells (C and F). The concentration of peptide–oligonucloetide conjugates in all cases was 0.1 μM. P-glycoprotein expression was measured by flow cytometry. The abcissa represents relative fluorescence, while the ordinate is the number of cells at each level of fluorescence. Results are representative of several independent experiments.

←

FIG. 3. Concentration-dependent inhibition of P-glycoprotein expression using the Ant-20 conjugate. P-glycoprotein expression was measured by immunostaining and flow cytometry after overnight incubation of MDR-3T3 cells in serum-free Opti-MEM with various concentrations of Ant-20 conjugate or with controls. (A) 0.05 μM Ant-20; (B) 0.1 μM Ant-20; (C) 1 μM Ant-20; (D) unstained control (no anti-P-glycoprotein antibody); (E) untreated, stained positive control cells; (F) 1 μM Ant-20 mismatch control; (G) treated with a 1 μM concentration of the 20-mer antisense oligonucleotide complexed with Lipofectin; (H) unstained control for G. The abcissa represents relative fluorescence, while the ordinate is the number of cells at each level of fluorescence. The numbers on the scans represent the percent of the total population that falls into the window (window 1) that encompasses an unstained control. Results are representative of several independent experiments.

References

1. Crooke ST, An overview of progress in antisense therapeutics. *Antisense Nucleic Acid Drug Dev* **8:** 115–122, 1998.
2. Agrawal S, Antisense oligonucleotides: Towards clinical trials. *Trends Biotechnol* **14:** 376–378, 1996.
3. Wagner RW, Gene inhibition using antisense oligodeoxynucleotides. *Nature* **372:** 333–335, 1994.
4. Stein CA and Cheng Y-C, Antisense oligonucleotides as therapeutic agents—is the bullet really magical? *Science* **261:** 1004–1012, 1993.
5. Flanagan WM, Antisense comes of age. *Cancer Metastasis Rev* **17:** 169–176, 1998.
6. Ho PT and Parkinson DR, Antisense oligonucleotides as therapeutics for malignant diseases. *Semin Oncol* **24:** 187–202, 1997.
7. Licht T, Pastan I, Gottesman M and Herrmann F, P-glycoprotein-mediated multidrug resistance in normal and neoplastic hematopoietic cells. *Ann Hematol* **69:** 159–171, 1994.
8. Bradley G and Ling V, P-glycoprotein, multidrug resistance and tumor progression. *Cancer Metastasis Rev* **13:** 223–233, 1994.
9. Chabner BA and Wilson W, Reversal of multidrug resistance. *J Clin Oncol* **9:** 4–6, 1991.
10. Dalton WS, Is P-glycoprotein a potential target for reversing clinical drug resistance? *Curr Opin Oncol* **6:** 595–600, 1994.
11. Dalton WS, Crowley JJ, Salmon SS, Grogan TM, Laufman LR, Weiss GR and Bonnet JD, A phase III randomized study of oral verapamil as a chemosensitizer to reverse drug resistance in patients with refractory myeloma: A Southwest Oncology Group Study. *Cancer* **75:** 815–820, 1995.
12. Scanlon KJ, Ishida H and Kashani-Sabet M, Ribozyme-mediated reversal of the multidrug resistant phenotype. *Proc Natl Acad Sci USA* **91:** 11123–11127, 1994.
13. Kobayashi H, Dorai T, Holland JF and Ohnuma T, Reversal of drug sensitivity in multidrug-resistant tumor cells by an MDR1 (PGY1) ribozyme. *Cancer Res* **54:** 1271–1275, 1994.
14. Alahari SK, Dean NM, Fisher MH, DeLong R, Manoharan M, Tivel KL and Juliano RL, Inhibition of expression of the multidrug resistance-associated P-glycoprotein by phosphorothioate and 5′ cholesterol-conjugated phosphorothioate antisense oligonucleotides. *Mol Pharmacol* **50:** 808–819, 1996.
15. Alahari SK, DeLong R, Fisher MH, Dean NM, Viliet P and Juliano RL, Novel chemically modified oligonucleotides provide potent inhibition of P-glycoprotein expression. *J Pharmacol Exp Ther* **286:** 419–428, 1998.
16. Corrias MV and Tonini GP, An oligomer complementary to the 5′ end region of MDR1 gene decreases resistance to doxorubicin of human adenocarcinoma-resistant cells. *Anticancer Res* **12:** 1431–1438, 1992.
17. Efferth T and Volm M, Modulation of P-glycoprotein-mediated multidrug resistance by monoclonal antibodies, immunotoxins or antisense oligodeoxynucleotides in kidney carcinoma and normal kidney cells. *Oncology* **50:** 303–308, 1993.
18. Cucco C and Calabretta B, *In vitro* and *in vivo* reversal of multidrug resistance in a human leukemia-resistant cell line by *mdr1* antisense oligodeoxynucleotides. *Cancer Res* **56:** 4332–4337, 1996.
19. Juliano RL, Alahari S, Yoo H, Kole R and Cho M, Antisense pharmacodynamics: Critical issues in the transport and delivery of antisense oligonucleotides. *Pharm Res* **16:** 494–502, 1999.
20. Bennett CF, Chiang MY, Chan H, Shoemaker JE and Mirabelli CK, Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol Pharmacol* **41:** 1023–1033, 1992.
21. DeLong RK, Yoo H, Alahari SK, Fisher M, Short SM, Kang SH, Kole R, Janout V, Regan SL and Juliano RL, Novel cationic amphiphiles as delivery agents for antisense oligonucleotides. *Nucleic Acids Res* **27:** 3334–3341, 1999.
22. Lewis JG, Lin K-Y, Kothavale A, Flanagan WM, Matteucci MD, DePrince RB, Mook RA Jr, Hendren RW and Wagner RW, A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. *Proc Natl Acad Sci USA* **93:** 3176–3181, 1996.
23. Falwell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B and Barsoum J, Tat mediated delivery of heterologous proteins into cells. *Proc Natl Acad Sci USA* **91:** 664–668, 1994.
24. Kim DT, Mitchell DJ, Brockstedt DG, Fong L, Nolan GP, Fathman CG, Engleman EG and Rothbard JB, Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV *tat* peptide. *J Immunol* **159:** 1666–1668, 1997.
25. Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G and Prochiantz A, Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem* **271:** 18188–18193, 1996.
26. Aldrian-Herrada G, Desarmenien MG, Orcel H, Boissin-Agasse L, Mery J, Brugidou J and Rabie A, A peptide nucleic acid (PNA) is more rapidly internalized in cultured neurons when coupled to a retro-inverso delivery peptide. The antisense activity depresses the target mRNA and protein in magnocellular oxytocin neurons. *Nucleic Acids Res* **26:** 4910–4916, 1998.
27. Pooga M, Soomets U, Hallbrink M, Valkna A, Saar K, Rezaei K, Kahl U, Hao JX, Xu XJ, Wiesenfeld-Hallin Z, Hokfelt T, Bartfai T and Langel U, Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission *in vivo*. *Nat Biotechnol* **16:** 857–861, 1998.
28. Chaloin L, Vidal P, Lory P, Mery J, Lautredou N, Divita G and Heitz F, Design of carrier peptide-oligonucleotide conjugates with rapid membrane translocation and nuclear localization properties. *Biochem Biophys Res Commun* **243:** 601–608, 1998.
29. Kane SE, Reinhard DH, Fordis CM, Pastan I and Gottesman MM, A new vector using the human multidrug resistance gene as a selectable marker enables overexpression of foreign genes in eukaryotic cells. *Gene* **84:** 439–446, 1989.
30. Zuckerman R, Corey D and Schultz P, Efficient methods for attachment of thiol specific probes to the 3′-ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Res* **15:** 5305–5321, 1987.
31. Vives E and Lebleu B, Selective coupling of a highly basic peptide to an oligonucleotide. *Tetrahedron Lett* **38:** 1183–1186, 1997.
32. Riddle PW, Blakeley RL and Zerner B, Reassessment of Ellman's reagent, *Methods Enzymol* **91:** 49–60, 1983.
33. Vives E, Brodin P and Lebleu B, A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* **272:** 16010–16017, 1997.
34. Yoo H, Sazani P and Juliano RL, PAMAM dendrimers as delivery agents for antisense oligonucleotides. *Pharm Res* **16:** 1799–1804, 1999.
35. Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevshy SA and Dowdy SF, Transduction of full-length TAT fusion proteins into mammalian cells: TAT-$p27^{Kip1}$ induces cell migration. *Nat Med* **4:** 1449–1452, 1998.
36. Troy CM, Stefinis L, Prochiantz A, Greene LA and Shelanski ML, The contrasting roles of ICE family proteases and interleukin-1β in apoptosis induced by trophic factor withdrawal and by copper/zinc superoxide dismutase down-regulation. *Proc Natl Acad Sci USA* 93: 5635–5640, 1996.