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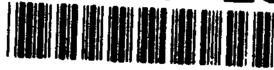

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The overall goal of this proposal is to develop sequence specific DNA binding compounds which are targeted by triplex DNA formation to the promoters of the c-myc, neu, and c-Ha-ras genes which play an important role in the malignant phenotype of breast carcinoma cells. We have made significant progress in the first year of funding. In addition, we have made several unexpected observations which have substantially enhanced the likelihood that this approach will be successful. During the first year of funding of this grant, we have established the animal models which we proposed in the original application, we have demonstrated the nebularine substituted TFO's have markedly increased binding affinity, we have shown that murine targeted acridine-oligonucleotide conjugates have very little, if any, toxicity, and we have been able to demonstrate markedly enhanced antitumor effects of neu-targeted TFO's delivered by liposomes. This progress has encouraged us to push forward with the clinical applications of this work. We are quite confident that within the next year of funding we will be prepared to embark on clinical trials of triplex forming agents in breast cancer.

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A. Introduction

The overall goal of this proposal is to develop sequence specific DNA binding compounds which are targeted by triplex DNA formation to the promoters of genes which play an important role in the malignant phenotype of breast carcinoma cells. Our proposal was based largely on our extensive experience with the transcriptional inhibitory effects of G-C specific DNA binding drugs such as mithramycin. All of the test systems and reagents necessary for the proposed experiments were available at the beginning of the funding period, which has allowed us to make substantial progress during the past year. In addition, we have made several unexpected observations which have substantially enhanced the likelihood that this approach will be successful. During the first year of funding of this grant we have established the animal models which we proposed in the original application, we have demonstrated that nebullarine substituted TFO's have markedly increased binding affinity, we have shown that murine targeted acridine-oliognucleotide conjugates have very little, if any, toxicity, and we have been able to demonstrate markedly enhanced antitumor effects of neu-targeted TFO's delivered by liposomes. This progress has encouraged us to push forward with the clinical applications of this work. We are quite confident that within the next year of funding we will be prepared to embark on clinical trials of triplex forming agents in breast cancer.

B. Body

Specific milestones accomplished during the first year of funding of this proposal are outlined below.

a. Determination of whether neu, c-myc, and c-Ha-ras targeted acridine-oligonucleotide conjugates inhibit expression of the target genes in breast cancer cells in culture (Specific Aim 1)(J. Clin. Invest. 92:2433-2439, 1993). The HER-2/neu oncogene promoter sequence contains a polypurine region from -42 to -69 upstream of the transcription start site which is a potential triplex forming sequence. Gel shift analysis shows that a 28-mer oligonucleotide which is identical to the target sequence but in antiparallel orientation will form triplex DNA with the target duplex in a concentration dependent manner. Triplex formation occurs at 100 fold excess of TFO, but does not occur with a control parallel oligonucleotide. The Kd of triplex formation by the TFO is 4.5×10^{5} M. The sequence specificity of this intermolecular triplex in the HER-2/neu promoter was confirmed by DNAse I protection footprinting. A gel mobility shift experiment in which a HeLa nuclear extract was added to the HER-2/neu promoter demonstrates that triplex formation prevents nuclear protein binding. This indicates that triplex formation prevents the binding of one or more nuclear proteins to the HER-2/neu promoter. In addition, triplex formation by this sequence results in specific inhibition of in vitro transcription activity. The triplex target site in the neu promoter is one of importance to the transcription of the HER-2/neu oncogene since it lies between the CCAAT and TATA boxes, binding sites for two factors necessary for transcription.

b. Characterization of the antiproliferative effects of the neu, c-myc, and c-Ha-ras targeted acridine-oligonucleotide conjugates, individually and in combination, in

breast carcinoma cells (Specific Aim 2). We have extensively characterized the antiproliferative effects of the neu and c-myc triplex forming oligonucleotides and acridine-oligonucleotide conjugates on breast carcinoma cells in culture. These compounds have demonstrated significant antiproliferative activity, but only at relatively high concentrations (> 10 uM). The inhibition of cellular proliferation is accompanied by downregulation of neu expression. There does not appear to be synergism at lower concentrations, although the combination of the neu and c-myc targeted conjugate appears to be quite toxic at higher combinations. Because of the relative lack of antiproliferative activity by the neu targeted TFO and acridine-oligonucleotide conjugate at lower concentrations, we have begun to investigate various means of achieving higher intracellular concentrations of phosphorothioate oligonucleotides.

Liposomes represent an attractive way to increase the delivery of adequate concentrations of TFO to the nucleus to form triplex structures with a large proportion of target sequences and to inhibit expression of the target gene. We have established a collaboration with Applied Immune Sciences, Santa Clara, CA in order to develop a liposome delivery system for triplex based gene therapy for breast cancer. Our preliminary experiments have demonstrated that liposome delivery of neu targeted triplex forming oligonucleotide results in a dramatic increase in the ability of this oligonucleotide to inhibit expression of the gene and proliferation of MCF-7 cells. This suggests that liposome delivery may substantially increase the intranuclear concentration of TFO, perhaps allowing the complete inhibition of neu expression. This approach is attractive because it can very rapidly be adapted to clinical trials.

c. Determination of the effect of the murine c-myc targeted conjugate on expression of the murine and human target genes in intact animals (Specific Aim 5) (manuscript in preparation). Since the triplex forming oligonucleotides which we have characterized are sequence specific, they are also species specific, i.e. the human c-myc triplex forming oligonucleotide does not form triplex DNA with the corresponding portion of the murine c-myc promoter. This creates a significant problem for conducting toxicity studies, and indeed, we have shown that large amounts of the human c-myc TFO or its acridine conjugate have very little effect on mice. Therefore, we have now identified a triplex forming region in the murine c-myc promoter. This sequence binds a murine protein which is quite similar to the Puf protein (nm23) which binds the human c-myc triplex forming sequence. We have shown that triplex formation by this sequence prevents protein binding and in vitro transcription. Administration of the acridine conjugate of this oligonucleotide to mice in relatively large doses (1400 ug as a single dose, or 100 ug/day for 14 days) resulted in no significant morphologic or physiologic toxicity. The white blood cell number and distribution did not change during the treatment period, and the weight of the animals was not different from untreated controls. The liver and kidneys of treated animals were examined morphologically and demonstrated no adverse effect from the TFO treatment. This is consistent with the data of Gewirtz, and others, who have demonstrated little toxicity with c-myb antisense oligonucleotide administration. Likewise the clinical trial of antisense p53 phosphorothioate oligonucleotide at the University of Nebraska has demonstrated very little toxicity.

Experiments to characterize the murine c-H-ras and neu targeted conjugates are currently being planned. They will be performed during Year 2 of the funding period. The absence of significant toxicity is extremely important to the utilization of this approach as an anticancer therapeutic modality.

d. Adenovirus Delivery of Oligonucleotides to Human Breast Cancer Cells. There have been a series of studies which have demonstrated that inactivated adenovirus complexed with polylysine can serve as a very potent potentiator of DNA cellular entry. An important aspect of this proposal will be to investigate the potential role of this delivery system in the development of triplex based gene therapy approaches. We have evalulated the ability of the HER-2/neu targeted TFO to inhibit HER-2/neu transcription in human breast cancer cells by reporter gene expression and direct measurement of HER-2/neu specific mRNA. The minimal HER-2/neu promoter containing the triplex forming target sequence and sequences necessary for complete transcriptional activity (267 base pairs including 247 base pairs upstream of the major transcription start site) was cloned upstream of the luciferase gene in plasmid neu/pGL. This plasmid was transduced into MCF-7 human breast cancer cells, and HER-2/neu transcription was measured by luciferase activity. HER-2/neu transcription in MCF-7 cells is easily detectable over background by luciferase assay, although the light activity is modest (on the order of 10^3 light units) consistent with the low level of HER-2/neu expression in this cell line. Delivery of the TFO to the MCF-7 cells in a separate adenovirus-polylysine-DNA complex resulted in a significant decrease in HER-2/neu expression. Thus, triplex formation in human breast cancer cells results in significant inhibition of HER-2/expression when measured by reporter gene activity.

We wished to demonstrate an inhibition of HER-2/neu mRNA transcription, an indicator that the TFO is binding to the native HER-2/neu gene in human breast cancer cells. In parallel experiments, MCF-7 cells transduced with the luciferase plasmid and the TFO in an adenovirus-polylysine-DNA complex were harvested, RNA was extracted, and HER-2/neu and actin mRNA was measured by reverse transcription and PCR (RT-PCR) with HER-2/neu and actin specific primers. The control cells had readily detectable levels of HER-2/neu mRNA by RT-PCR. In cells treated with the TFO, HER-2/neu mRNA was undetectable while the actin mRNA level was identical. This experiment suggests that triplex formation in human breast cancer cells results in inhibition of target gene expression, and that delivery of a TFO to the cell nucleus is possible by means of an adenovirus-polylysine-DNA complex.

MCF-7 human breast cancer cells were also treated with various adenovirus-polylysine-DNA complexes to evaluate cell toxicity and TFO effect on cell viability. After a twenty-four hour treatment, cells were harvested. Controls included a no treatment culture, a culture treated only with adenovirus particles, and two complexes containing a control non-triplex forming oligonucleotide. All of the control cell cultures demonstrated nearly identical numbers of viable cells at twenty-four hours (approximately 80% of the number of cells seeded per plate), but the cultures treated with the TFO demonstrated significantly fewer viable cells at a level of 55-60% of the number of cells seeded per plate prior to treatment. These experiments demonstrate that the adenovirus and the adenovirus-polylysine-DNA complexes by themselves are not cytotoxic to cultured human breast cancer cells. The triplex

forming oligonucleotide shows a clear and early effect on cell viability that suggests a potential therapeutic application in human breast cancer.

e. Development of linking bases which "skip over" exception bases in triplex forming target sequences. (Nucleic Acid Res., in press). A region of the Ha-*ras* promoter (-8 to -28) which contains two of the three Sp1 binding sites essential for transcriptional activity forms sequence specific oligonucleotide-directed pur*pur:pyr triple helix. The relative binding of oligonucleotides containing different substitutions, including an abasic propanediol linker, over three potentially destabilizing C:G interruptions in the otherwise poly G:poly C target was examined. DNase I footprint titrations reveal that substitution of the positively charged abasic propanediol linker results in approximately ten fold greater binding than cytosine substitution which in turn provides greater sequence specific binding than substitution of a guanine in the third strand oligonucleotide over the C:G interruptions. As an indication of the effect of linker substitution and targeting consensus Sp1 sites on triplex specificity, the relative ability of the Ha-*ras* promoter targeted oligonucleotides to interact with non-target Sp1 sequences within the Ha-*ras* promoter as well as in the DHFR promoter and HIV-1 LTR was also investigated. At concentrations which afford complete DNase I protection of the target sequence, HR21ap does not bind to the non-target sequences while HR21Xap interacts weakly only at a distal site in the DHFR promoter. These results suggest that the propanediol linker is able to skip over interruptions in a target sequence thereby stabilizing triplex but, slightly compromises sequence specificity and the ability to sterically occlude protein binding. We have recently characterized nebularine substituted TFOs which have a 1,000 fold increased binding affinity.

f. Acridine conjugation increases intracellular and intravascular stability of triplex forming oligonucleotides in vitro and in vivo. (manuscript submitted). When MCF-7 breast carcinoma cells are treated with radiolabelled c-myc targeted oligonucleotide, 57-68% of intracellular oligonucleotide is found in the nuclei in cells treated with oligonucleotide for periods as short as five minutes, or as long as 48 hours. Importantly, there is very little degradation of the oligonucleotide over this period. Interestingly, much of the oligonucleotide which is taken up (75-85%) is retained by these cells after the cells are placed into fresh medium, which does not contain oligonucleotide. Acridine conjugation improves the ability of triplex forming oligonucleotides to enter the cell. When the NALM/6 preB cell line is incubated ^{32}P radiolabelled conjugate or oligonucleotide for 24 hours it is clear that the addition of an acridine conjugate significantly increases the ability of the oligonucleotide to enter the cell. In fact, the concentration of intracellular conjugate is almost double that of the oligonucleotide. Later time points (24 and 48 hours) demonstrated that both the oligonucleotide and conjugate reach equilibrium and are present at similar intracellular concentrations. We have also determined the effect of acridine conjugation on intracellular stability of the c-myc targeted oligonucleotide. When the conjugate and oligonucleotide are added to NALM/6 cells and the intracellular compound isolated at 24 and 48 hours, both are relatively intact. However, the relative retention of the acridine-conjugate is significantly higher than that of the unconjugated oligonucleotide.

We have also determined the stability of a triplex forming acridine-

oligonucleotide conjugate in whole animals. When the conjugate is administered intravenously to mice it has a serum half-life of approximately ten minutes. However, we noted that radioactive conjugate was present in the animal at relatively high levels at 24 and 48 hours after administration, in contrast to radiolabelled oligonucleotide which is largely excreted in urine during the first hour after IV administration. Further investigation revealed that the "whole animal half-life' is between 24 and 36 hours in contrast to the serum half-life of 5 to 10 minutes. Both the intestine and liver demonstrate extremely high levels of conjugate uptake. In fact, 25% of the administered dose can be found in the intestine at 24 hours following administration, compared to none of the oligonucleotide. Likewise, 8% of the total administered dose is found in the liver of treated animals, with significant but lower levels found in the bone marrow and kidney. Gel electrophoretic analysis of the conjugate at 24 and 48 hours reveals that the majority of radiolabelled conjugate remains intact at these time points. These data suggest that the addition of acridine to the 5' and of the oligonucleotide results in substantial stabilization of this molecule and it is now feasible to begin to consider the administration of these agents in therapeutic settings.

g. Triplex DNA formation is dependent on divalent cation concentration. (J. Biol. Chem., submitted; Nucleic Acids Res., submitted). In order to characterize intracellular conditions which may affect the therapeutic usefulness of triplex forming oligonucleotides, we have characterized the effects of monovalent metal cations on triplex formation by the DHFR promoter. Quantitative DNAse I protection analysis demonstrated that potassium has a potent, concentration dependent inhibitory effect on triplex formation by this sequence. However, potassium does not alter the rate of triplex disassociation. Sodium also has an inhibitory effect on triplex formation, but only at concentrations which are three times as high as those required for maximal inhibition by potassium. Since potassium represents the major intracellular cation, this inhibition of triplex formation by K^+ at concentrations well below the physiologic range, may represent an impediment to the utilization of triplex forming molecules for transcriptional inhibitors. Therefore, we have also characterized the relationship between divalent and monovalent cations on triplex formation. We have demonstrated a positive effect of Mn^{2+} or Co^{2+} in promoting triplex formation, event as the K+ concentration approaches physiologic levels. We have constructed a model to account for the effects of these monovalent and divalent cations on the reactivity of the components of triplex DNA formation.

h. Effective treatment of murine lymphoma. We have utilized the murine c-myc TFO to perform preliminary animal studies with murine tumors. Since the murine TFO inhibits murine c-myc expression, we studied the effect of pretreatment of the YC8 murine lymphoma cell line prior to inoculation of mice with 5 X 10^6 cells. These cells were exposed to 10 and 100 uM concentrations of acridine-oligonucleotide conjugate or 3'-amine blocked oligonucleotides. Following a two hour exposure to TFO, the cells were inoculated into the flank of BalbC mice. The mice were observed for tumor growth. After 10 days the mice were sacrificed and tumor size and weight determined. The tumors treated with 10 uM acridine conjugate demonstrated 35% inhibition of growth, while those treated with 100 uM acridine conjugate were inhibited by 63%. The amine-blocked oligonucleotide demonstrated

an even greater inhibition of cell growth. These experiments are currently being repeated, but strongly suggest that these oligonucleotides will be capable of inhibiting cell growth.

i. Triplex formation by the human c-Ki-ras promoter prevents regulatory protein binding. (Biochemistry, In Press). The human Ki-*ras* promoter contains a 22 base pair homopurine:homopyrimidine (pur:pyr) motif within a region that is nuclease hypersensitive in both native chromatin and supercoiled plasmids. Gel mobility shift analysis and competition experiments show that this pur:pyr motif binds a nuclear protein(s) which is an important regulatory factor. Gel mobility shift analysis and DNase I footprinting demonstrate that oligonucleotides can form triplex DNA with this sequence [purine*purine:pyrimidine (pur*pur:pyr) or mixed purine/pyrimidine*purine:pyrimidine (pur/pyr*pur:pyr) intermolecular triple helices through guanine (G) recognition of guanine:cytosine (G:C) base pairs and either adenine (A) or thymine (T) recognition of adenine:thymine (A:T) base pairs in the target sequence]. Triple helices containing either T*A:T or A*A:T triplets are formed exclusively with oligonucleotides antiparallel to the homopurine target strand. The affinity of the oligonucleotide which forms T*A:T triplets is approximately equal to or, slightly greater than, the affinity of an oligonucleotide which forms A*A:T triplets. Oligonucleotide-directed triplex formation inhibits sequence specific nuclear protein binding to the K-*ras* promoter. These observations suggest that triplex formation by the oligonucleotides described here may provide a means to specifically inhibit transcription of the K-*ras* oncogene.

j. Triplex formation by the human c-Ha-*ras* promoter prevents regulatory protein binding. (J. Biol. Chem., In Press). Recent work in our laboratory has also documented triplex DNA formation by two of the Spl binding sites in the promoter of the c-Ha-ras gene. This 21 bp triplex fomring oligonucleotide is G-rich and homologous to the sequence from -8 to -28, but is oriented in the antiparallel direction. As we have shown for Ki-ras, c-myc, and neu, triplex formation occurs at relatively low concentrations of oligonucleotide. DNAse I footprint analysis, has been used to confirm that this triplex formation is sequence specific. The TFO binds exclusively to the proximal target Sp1 sites over a similar non-target distal sequence which, like the target, contains a consensus Sp1 site. In order to document that triplex formation inhibits protein binding to this sequence, we have performed competitive gel mobility shift experiments with this fragment. It is clear from these experiments that Sp1 binds to this region of the Ha-ras promoter. Protein binding assays demonstrate that triplex formation by the ras targeted TFO completely blocks Sp1 binding to two sites in the target sequence and also appears to partially inhibit Sp1 binding to the distal site in the Ha-*ras* promoter. Moreover, oligonucleotide-directed triplex formation inhibits Ha-*ras* promoter dependent transcription *in vitro*. This indicates that triplex formation by this sequence may allow the transcriptional inhibition of this promoter. The results presented here suggest that triplex formation by the Ha-*ras* promoter targeted oligonucleotide may provide a means to specifically inhibit transcription of this oncogene *in vivo*.

C. Conclusions

DNA binding drugs can inhibit the binding of regulatory proteins to the promoters of

eukaryotic genes, preventing formation of the transcription initiation complex. However, currently available DNA binding drugs are inadequately sequence specific to allow modulation of the expression of single genes in a therapeutically useful way. Triplex DNA provides a sequence specific mechanism which may allow the targeting of DNA binding compounds to specific sequences. We have shown that triplex forming oligonucleotides can bind specifically to their target sequences, prevent regulatory protein binding, and inhibit transcriptional activity. We have developed a set of "gene specific" triplex forming molecules which prevent regulatory protein binding and inhibit expression of the c-myc, c-Ha--ras, and neu genes in vitro. During the first year of funding of this grant we have established the animal models which we proposed in the original application, we have demonstrated that nebullarine substituted TFO's have markedly increased binding affinity, we have shown that murine targeted acridine-oliognucleotide conjugates have very little, if any, toxicity, and we have been able to demonstrate markedly enhanced antitumor effects of neu-targeted TFO's delivered by liposomes. We anticipate that we will continue to make substantial progress towards achieving the remaining aims of the original proposal.

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