Chapter 4

Modulating Hypoxia-Inducible Transcription by Disrupting the HIF-1-DNA Interface

The text of this chapter was taken in part from a manuscript coauthored with Claire S. Jacobs, Michelle E. Farkas, and Peter B. Dervan (Caltech).

(Nickols, N. G., Jacobs, C. S., Farkas, M. E. & Dervan, P. B. (2007) *submitted)*

Abstract

Transcription mediated by hypoxia inducible factor (HIF-1) contributes to tumor angiogenesis and metastasis but is also involved in the activation of cell-death pathways and normal physiological processes. Given the complexity of HIF-1 signaling it could be advantageous to target a subset of HIF-1 effectors rather than the entire pathway. We compared the genome-wide effects of three molecules that each interfere with the HIF-1-DNA interaction: a polyamide targeted to the hypoxia response element (HRE), siRNA targeted to HIF-1α, and echinomycin, a DNA binding natural product with a similar but less specific sequence preference to the polyamide. The polyamide affects a subset of hypoxia-induced genes that are consistent with the binding site preferences of the polyamide. For comparison, siRNA targeted to HIF-1α and echinomycin each affect the expression of nearly every gene induced by hypoxia. Remarkably, the total number of genes affected by either polyamide or HIF-1α siRNA over a range of thresholds is comparable. The data show how polyamides can be used to affect a subset of a pathway regulated by a transcription factor. In addition, this study offers a unique comparison of three complementary approaches toward exogenous control of endogenous gene expression.

4.1 Introduction

Exogenous chemical and biological methods to control directly the expression of selected endogenous genes could have broad implications for human medicine. Toward this goal, a number of technological approaches are currently being investigated. Polydactyl zinc finger proteins are a programmable class of DNA binding proteins capable of sequence-specific binding (*1, 2*). These designed proteins have been used to inhibit expression of target genes (*3*), and conjugates of zinc finger proteins and transcriptional activator domains have been used to activate the expression of target genes (*4*). The RNA-interference pathway can be used to regulate gene expression at the post-transcriptional level (*5*). siRNA and shRNA molecules enlist cellular machinery to degrade selected mRNA targets (*6, 7*). RNAi technology has been highly effective in achieving potent and specific knock-down of target mRNAs and is now widely used to probe functions of target genes (*8*). However, bioavailability and delivery of zinc finger proteins and siRNA, or vectors that code for them, to targets in humans could be an obstacle to their therapeutic application and continues to receive considerable attention (*8*). Cell-permeable small molecules that modulate protein-protein or protein-DNA interactions offer another approach to the control of endogenous gene regulation. Screening small molecule and natural product libraries for a desired effect can identify candidate molecules that have a high likelihood of possessing drug-like bioavailability. Drawbacks include the need to screen anew for each target protein-protein or protein-DNA interaction. Polyamides containing *N*-methylimidazole (Im) and *N*-methylpyrrole (Py) are a class of programmable DNA-binding small molecules that have been used to disrupt protein-DNA interactions in a sequence specific manner in cell culture (*9*).

Controlling the transcriptional activity of the hypoxia inducible factor (HIF-1), a heterodimer of HIF-1α and HIF-1β (ARNT), is a goal with clinical relevance (*10-13*). HIF-1 is the principal mediator of the adaptive cellular response to hypoxia (*14*). Under normoxic conditions, the α subunit of HIF-1 is specifically hydroxylated by an iron-dependent proline hydroxylase, ubiquitinated by the von Hippel-Lindau (VHL) tumor suppressor protein-ubiquitin ligase protein complex, and degraded by the proteosome (*15*). Iron chelators, such as deferoxamine (DFO), can be used to mimic hypoxia in cell culture (*15*). Through interaction with co-activators p300/CBP, HIF-1 directly activates the expression of at least 100 genes that are involved with cellular and tissue adaptation to hypoxia (*12*). This set includes pro-angiogenic factors such as vascular endothelial growth factor (VEGF), glycolytic enzymes, extra-cellular matrix remodeling enzymes, and genes involved in both pro- survival and death pathways (*10*). HIF-1 activation by the hypoxic microenvironment of solid tumors, or additionally by deactivating mutations in VHL, contributes to an aggressive phenotype of increased cell proliferation, invasion, metastasis, and drug resistance (*10*). Given the complexity of HIF-1 signaling in cellular survival and death pathways, and its critical role in physiological processes in normal tissues, it could be advantageous to target a subset of HIF-1 effectors rather than the entire pathway (*12*).

In important proof of principle experiments, introduction of siRNA against HIF-2α to $VHL^{-/-}$ renal carcinoma cells was sufficient to abrogate tumor formation by these cells in mice (*16*). Screening for small molecules capable of disrupting the HIF-1-p300 interaction identified chetomin, a natural product that binds p300 that was shown to inhibit expression of HIF-1 regulated genes and exhibit anti-tumor activity in a mouse

model (*17*). In an effort to inhibit directly the HIF-1 binding to DNA, a hairpin polyamide was designed to bind the sequence 5'-ATACGT-3' found in the HRE of VEGF. This polyamide bound its target site and prevented HIF-1-DNA binding in a sequence specific manner, and inhibited hypoxia-induced expression of VEGF and several other HIF-1 regulated genes in cultured cells without the use of transfection agents (*18, 19*). Melillo and colleagues screened a library of small molecules for inhibition of HIF-1 mediated transcription in a cell-based assay and identified the natural product echinomycin, a DNA-binding bisintercalator (*20*). Echinomycin binds the four base pair sequence 5'-NCGN-3' found in the consensus HRE 5'-NACGTG-3' and inhibited expression of VEGF in cultured cells (*21*).

To establish a benchmark of comparison for the global effects of polyamides, we compare the genome-wide effects on U251 cells induced with DFO of a polyamide targeted to the HRE sequence 5'-WTWCGW-3', echinomycin, and siRNA targeted against HIF-1α. siRNA mediated destruction of HIF-1α mRNA establishes a maximum level of inhibition that can be achieved for HIF-1 target genes through disruption of the HIF-1-HRE interaction. Nearly all transcripts induced by DFO are inhibited by both HIF-1α siRNA and echinomycin. Polyamide **1**, however, inhibits only a subset of these genes, and shows a preference for genes containing HREs of the sequence 5'-(T/A)ACGTG-3', consistent with the predicted binding preferences of this molecule. Remarkably, the total number of genes affected by either polyamide **1** or HIF-1α siRNA over a range of thresholds is comparable. We show that HIF-1 occupancy at the HREs of two genes affected by polyamide **1** is reduced in the presence of the polyamide, while HIF-1 occupancy at the HREs of two unaffected genes is unchanged. We also show that

a polyamide that binds a site immediately 5' to the HRE of the VEGF promoter inhibits induced expression of VEGF but not of FLT1, a HIF-1 target gene lacking this flanking site.

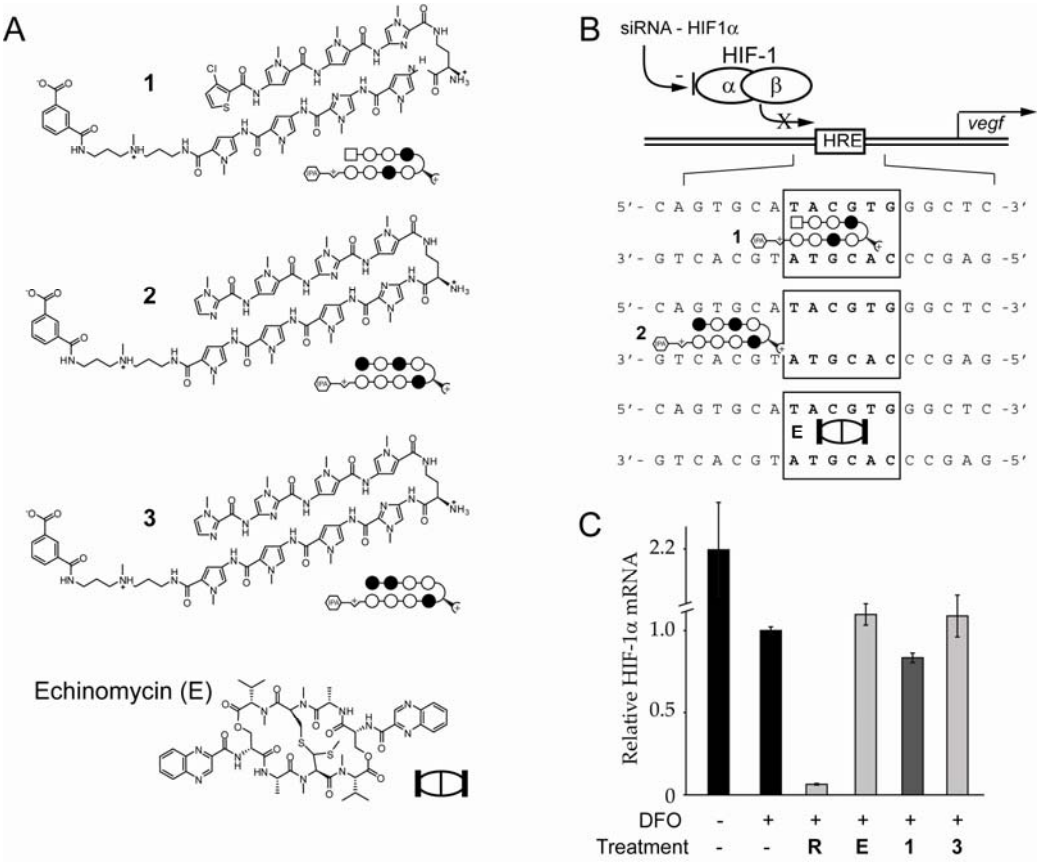

Figure 4.1 (A) Structures of polyamides **1**-**3** and echinomycin. (B) Three approaches to inhibiting HIF-1 induced gene expression: sequence specific small molecule binding to the HRE by a polyamide or echinomycin, and reduction in HIF-1α mRNA using siRNA. (C) Measurement of HIF-1α, mRNA by quantitative real-time PCR: siRNA against HIF-1α, **R**; echinomycin (100 nM), **E**; and polyamides **1**, and **3** (1 μM). Treatment with siRNA decreases HIF-1α mRNA by more than 95%.

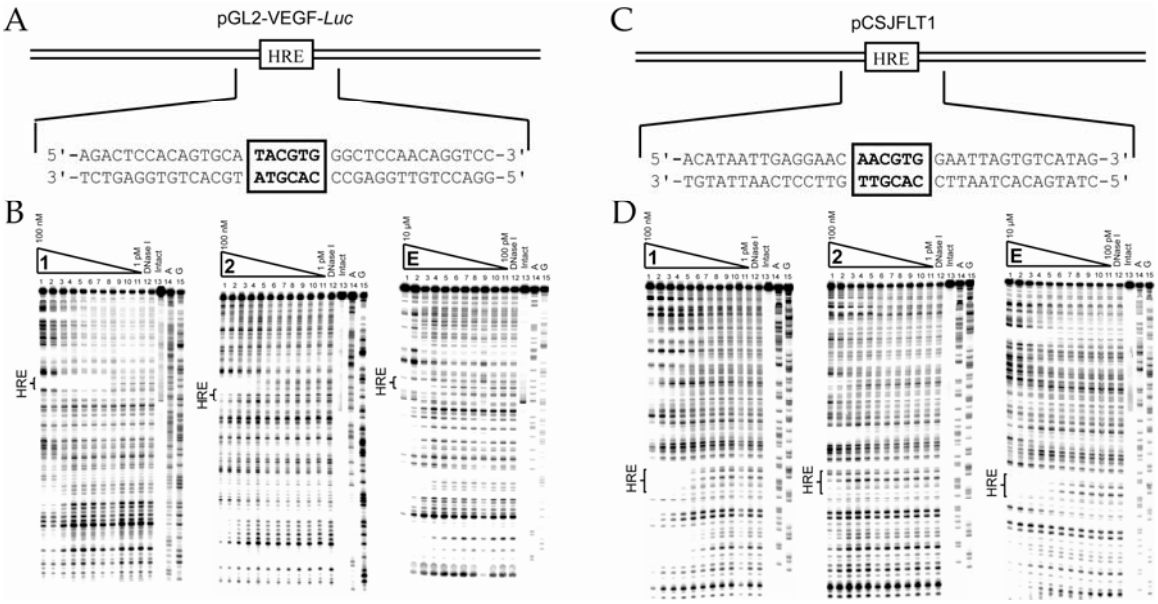

Figure 4.2 (A) Illustration of pGL2-VEGF-*Luc* and partial sequence containing the VEGF HRE and putative binding sites for polyamides **1**, **2** and echinomycin. (B) Quantitative DNase I footprint titration experiments for polyamides **1, 2** and echinomycin, **E**, on the 5'-end-labeled PCR product of plasmid pGL2-VEGF-*Luc*. For polyamides **1** and **2**: lanes 1–11, 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 300 pM, 100 pM, 30 pM, 10 pM, 3 pM and 1 pM polyamide, respectively; lane 12, DNAse I standard; lane 13, intact DNA; lane 14, A reaction; lane 15, G reaction. For echinomycin, **E**: lanes 1-11, 10 μM, 3 μM, 1 μM, 300 nM, 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 300 pM, 100 pM echinomycin, respectively; lane 12, DNAse I standard; lane 13, intact DNA; lane 14, A reaction; lane 15, G reaction. Polyamide **1** and echinomycin have K_a = 2.6 (±0.4) x 10^{10} M^{-1} and K_a = 8.4 (±2.1) x 10^6 M^{-1}, respectively, at the VEGF HRE. Polyamide **2** has a K_a = 3.2 (±0.6) x 10^9 M^{-1} for the site 5'-AGTGCA-3' immediately 5' to the VEGF HRE. (C) Illustration of pCSJFLT1 and partial sequence containing the FLT1 HRE and putative binding sites for polyamides **1** and echinomycin. (D) Quantitative DNase I footprint titration experiments for polyamides **1**, **2**, and echinomycin, **E**, on the 5' end-labeled PCR product of plasmid pCSJFLT1. For polyamides **1** and **2**: lanes 1–11, 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 300 pM, 100 pM, 30 pM, 10 pM, 3 pM and 1 pM polyamide, respectively; lane 12, DNAse I standard; lane 13, intact DNA; lane 14, A reaction; lane 15, G reaction. For echinomycin, **E**: lanes 1-11, 10 μM, 3 μM, 1 μM, 300 nM, 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 300 pM, 100 pM echinomycin, respectively; lane 12, DNAse I standard; lane 13, intact DNA; lane 14, A reaction; lane 15, G reaction. Polyamide **1** and echinomycin have K_a = 2.7 (±0.2) x 10^9 M^{-1} and K_a = 2.9 (±0.7) x 10^7 M^{-1}, respectively, at the FLT1 HRE. Polyamide **2** has a K_a = 2.2 (±0.8) x 10^8 at this site.

4.2 Results

Binding of Polyamide 1, 2 and Echinomycin at the VEGF and FLT1 HREs

Polyamide sequence specificity is programmed by side-by-side pairings of the heterocyclic amino acids in the minor groove of DNA: Im/Py distinguishes G•C from C•G; Py/Py binds both A•T and T•A; 3-chlorothiophene/N-methylpyrrole (Ct/Py) prefers T•A at the N-terminus position (*22-24*). Polyamide **1** and echinomycin are expected to bind at the VEGF HRE sequence 5'-TACGTG-3'. Polyamide **2** is expected to bind the sequence 5'-AGTGCA-3' immediately 5' to the VEGF promoter HRE. The DNA-binding affinities of **2** and echinomycin for the VEGF HRE were measured by quantitative DNase I footprint titrations using a 5' ^{32}P-labeled PCR amplification product of the plasmid pGL2-VEGF-*Luc*, which contains the VEGF HRE (Figure 4.2A and B). Polyamide **1** was previously found to have a K_a = 2.6 (±0.4) x 10^{10} M^{-1} at this site (*19*). For ease of comparison, a footprinting gel of **1** is included in Figure 4.2B. Polyamide **2** has a K_a = 3.2 (±0.6) x 10^9 M^{-1} for the site 5'-AGTGCA-3' immediately 5' to the VEGF HRE. To our knowledge, the DNA binding properties of echinomycin have not been quantitatively examined previously with DNase I footprint titration experiments. We find that echinomycin binds the VEGF HRE with a K_a = 8.4 (±2.1) x 10^6 M^{-1}.

The DNA-binding affinities of **1**, **2** and echinomycin for the HRE of FLT1 were also measured by quantitative DNase I footprint titrations using a 5' ^{32}P-labeled PCR amplification product of the plasmid pCSJFLT1 (Figure 4.2C and D). Although formally targeted to the sequence 5'-WTWCGW-3', polyamide **1** would still be expected to bind 5'-CAACGT-3', albeit with a moderate decrease in affinity (*24*). The sequence preference of a Ct/Py pair for T•A is approximately 4-fold over A•T, but 50-fold over a

G•C (*24*). Polyamide **1** has a K_a = 2.7 (±0.2) x 10^9 M^{-1} at the FLT1 HRE. Polyamide **2** has a K_a = 2.2 (±0.8) x 10^8 M^{-1} at this site. Echinomycin binds the FLT1 HRE with a K_a = 2.9 (±0.7) x 10^7 M^{-1}.

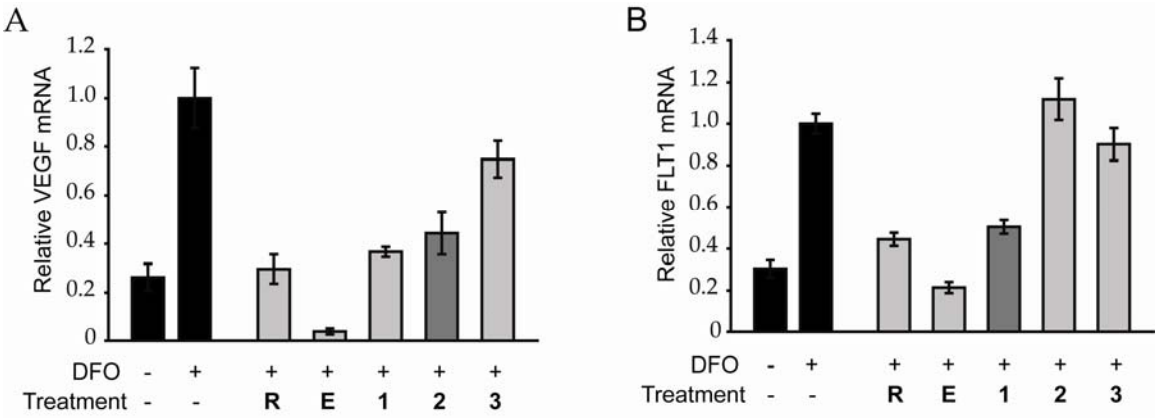

Figure 4.3 (A) Induction of VEGF mRNA by the hypoxia mimetic deferoxamine (DFO) measured by quantitative real-time PCR: siRNA against HIF-1α, **R**; echinomycin (100 nM), **E**; and polyamides **1**, **2**, and **3** (1 μM). Treatment with siRNA, **1**, or **2** decrease VEGF mRNA levels to near non-induced levels. Echinomycin potently inhibits VEGF mRNA to below non-induced levels. Polyamide **3** has a more modest effect. (B) Induction of FLT1 mRNA by the hypoxia mimetic deferoxamine (DFO) measured by quantitative real-time PCR: siRNA against HIF-1α, **R**; echinomycin (100 nM), **E**; and polyamides **1**, **2**, and **3** (1 μM). The siRNA, echinomycin, and **1** decrease FLT1 mRNA levels. Polyamides **2** and **3** have minimal or no effects.

Suppression of induced VEGF and FLT1 expression

HIF-1 induces VEGF expression by binding to the 5'-TACGTG-3' HRE located approximately 950 base pairs upstream of the transcription start site (*25, 26*). The effect on induced VEGF expression by siRNA silencing of HIF-1α mRNA establishes a theoretical maximum level of inhibition gained from disruption of HIF-1/DNA binding. HIF-1α mRNA was reduced by approximately 95% in the presence of the HIF-1α siRNA, but was minimally affected by polyamides **1** and **2** or echinomycin under DFO induced

conditions (Figure 4.1C). Polyamides **1** and **2** (1 μM) and HIF-1α siRNA had a similar effect on induced VEGF expression; both treatments inhibited most of the increase in VEGF mRNA following DFO treatment, but not to levels below that observed for non-induced controls (Figure 4.3A). As previously reported, 100 nM echinomycin potently inhibits VEGF expression to levels below the non-induced control (*20*). Polyamide **3**, which targets the sequence 5'-WGGWCW-3', not found at or near the VEGF HRE, had a more modest effect on VEGF mRNA levels.

Induction of FLT1 (VEFG receptor type 1) is mediated by HIF-1 binding to the 5'-AACGTG-3' HRE in the FLT1 promoter (*27*). Polyamide **1** and HIF-1α siRNA both inhibited expression of FLT1 following DFO induction (Figure 4.3B). Echinomycin reduced expression of FLT1 to below that of the non-induced control. Polyamides **2** and **3** both had a minimal effect. Given the relative binding affinities of polyamide **1** and echinomycin, it may be surprising that 1 μM of polyamide **1** is necessary to inhibit VEGF and FLT1 expression to an extent similar to HIF-1α siRNA, while 100 nM echinomycin reduces expression of these genes to below basal levels. The structure of double-helical DNA is not greatly perturbed by minor groove binding hairpin polyamides (*22*); echinomycin binding to DNA causes local unwinding and lengthening of the DNA helix, which might account for its greater potency in these experiments (*28, 29*). Polyamide-intercalator conjugates have been shown to unwind DNA in a sequence specific fashion and to improve the ability of a polyamide to inhibit the binding of several DNA binding proteins *in vitro* (*30, 31*). Attempts to use polyamide-intercalator conjugates to target the VEGF HRE, however, have not been successful due to poor nuclear uptake.

The ability to target DNA sequences flanking critical protein-DNA binding sites while maintaining productive inhibition of protein-DNA binding expands the repertoire of such interactions amenable to inhibition by polyamides. In a similar approach, Kageyama et al. showed that polyamides targeted to sequences flanking the VEGF HRE could inhibit VEGF expression (*32*). Polyamides targeted to flanking sites have previously been successful in inhibiting protein-DNA binding in the cases of TATA binding protein and LEF-1 (*33*). It should be noted, however, that minor-groove binding polyamides and some major-groove binding proteins can co-occupy DNA sequences in some cases (*34*).

Microarray analysis of gene expression

One potential limitation to the use of hairpin-polyamides for regulation of target genes is the binding site size and specificity for match versus mismatch sites, which may result in a prohibitively large number of affected genes. To examine this, the global effects of polyamide treatment on hypoxia-induced gene expression were measured using Affymetrix Human Genome U133 Plus 2.0 Arrays which contain oligonucleotide sequences representing more than 50,000 transcripts. To establish a benchmark for comparison, the effects of siRNA against HIF-1α and echinomycin were also measured. Experiments were conducted in triplicate, and gene expression levels were normalized to DFO-treated controls. Cells not treated with DFO were also normalized to DFO-treated controls. Microarray data was deposited in the NCBI Gene Expression Omnibus repository.

Polyamide **1** (1 μM) affected the expression of 2,284 transcripts by more than 2-fold ($p \leq 0.01$) (Table 4.1). At the same threshold, siRNA against HIF-1α affected 3,190

transcripts and echinomycin (100 nM) affected the expression of 10,906. In all cases, a majority of affected genes were down-regulated and a minority up-regulated. For comparison, DFO treatment by itself affected expression of 2,142 transcripts (4.6% of interrogated transcripts), with a majority up-regulated. A clustering analysis was performed to identify similarities in the expression profiles between the different treatments (Figure 4.4A). The expression profile of cells treated with siRNA against HIF-1α is similar to that of cells not treated with DFO under the conditions of the analysis; the expression profiles of echinomycin-treated and polyamide-treated cells are less similar to each other and to the other treatments. Analysis of transcripts affected by both **1** and HIF-1α siRNA shows that 395 and 150 transcripts are commonly down- and up- regulated, respectively, at least 2-fold ($p \leq 0.01$) (Figure 4.5). A similar analysis of transcripts affected by both **1** and echinomycin show that 731 and 112 transcripts are commonly down- and up- regulated, respectively.

Table 4.1 Number of transcripts affected ($p \leq 0.01$).

Treatment	**-**	**R**	**E**	**1**
DFO	**-**	**+**	**+**	**+**
up-regulated (fold change ≥ 2.0)	662	1,380	3,480	709
down-regulated (fold change ≤ -2.0)	1,480	1,810	7,426	1,575
up-regulatd (fold change ≥ 4.0)	62	122	413	57
down-regulated (fold change ≤ -4.0)	296	356	4,133	336

We find that DFO induced the expression of a set of 297 transcripts by at least 4-fold ($p \leq 0.01$) (Figure 4.4B) (Appendix A, Table A1). Of this set, 69 were inhibited by polyamide **1** by at least 2-fold ($p \leq 0.01$) (Appendix A, Table A2). For comparison, HIF-1α siRNA inhibited 244, and echinomycin 263 of the 297 DFO-induced transcripts (Figure 4.4B). It is not known what proportion of these affected transcripts are direct

HIF-1 targets. To examine more closely the effects of polyamide **1**, HIF-1α siRNA, and echinomycin on transcripts induced directly by HIF-1, we examined a limited set of 31 transcripts consisting of previously identified direct HIF-1 targets that were induced at least 1.5-fold ($p \leq 0.01$) by DFO in this experiment (Figure 4.4C) (*27, 35-44*) Nearly all 31 transcripts in this set were down-regulated by HIF-1α siRNA. In most cases, the expression was reduced to levels observed in cells untreated with DFO. Echinomycin treatment resulted in down-regulation of all 31 transcripts. For some genes, including VEGF, expression was reduced to levels far below those of the siRNA-treated cells and non-induced controls. Polyamide **1** inhibited the expression of 14 of these but displayed minimal effect on the others.

The HRE sequences for these genes, where known, are displayed in Table 4.2. Quantitative real-time RT–PCR was used to confirm the effects of polyamide and siRNA treatments on these genes. Chromatin immunoprecipitation was used to measure HIF-1 occupancy at the HREs of VEGF and carbonic anhydrase IX (CA9), which were both affected by polyamide **1**, and those of BNIP3, and PGK1, which were unaffected (Figure 4.6). HIF-1α occupancy at the VEGF HRE was decreased by HIF-1α siRNA, echinomycin, and polyamide **1**, but less so by treatment with **3**. HIF-1 occupancy at the carbonic anhydrase IX locus was also decreased in the presence of **1**, but not at that of BNIP3 or PGK1, consistent with the observed gene expression changes. It is interesting to note that all of the genes affected by polyamide **1** displayed in Table 2 have HREs that fall within the sequence 5'-(T/A)ACGTG-3', consistent with the expected DNA binding preferences for this molecule.

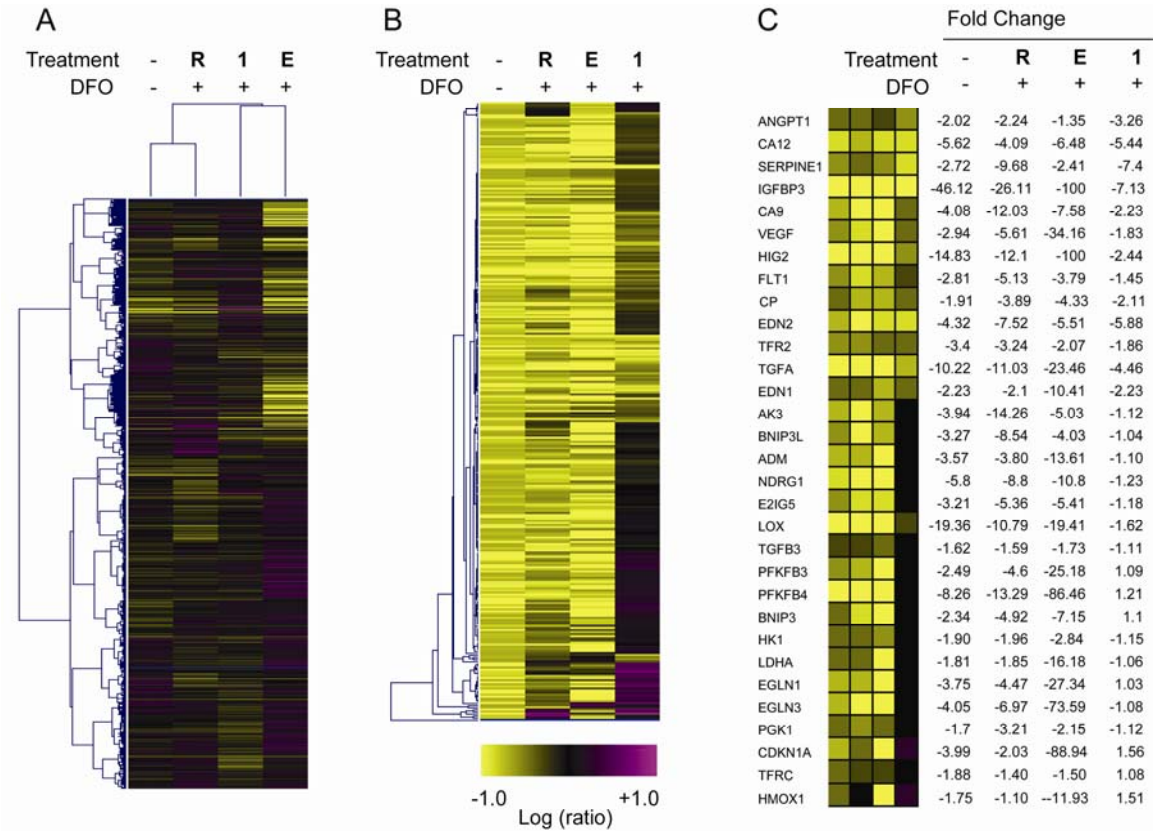

Figure 4.4 (A) Divisive clustering analysis over all interrogated transcripts for DFO induced cells: siRNA against HIF-1α, **R**; echinomycin (100 nM), **E**; and polyamides **1** (1 μM). (B) Agglomerative clustering analysis over all 297 transcripts induced by DFO at least 4-fold ($p \leq 0.01$). Of this transcript set, HIF-1α siRNA inhibited 244, echinomycin inhibited 263, and polyamide **1** inhibited 69 by at least 2-fold ($p \leq 0.01$). (C) Effects of the indicated treatments on a panel of genes that have been previously characterized as direct targets of HIF-1 that were also induced by DFO at least 1.5-fold ($p \leq 0.01$) in this experiment. Treatments reported are an error-weighted average from three experiments.

5.3 Discussion

In this experiment, polyamide **1** (1 μM) affected the expression of 2,284 transcripts by more than 2-fold ($p \leq 0.01$), which represents less than 5% of the transcripts assayed. A search of the publicly available human genome for the sequence 5'-WTWCGW-3' finds 1,876,480 potential match sites for polyamide **1.** This corresponds to an average of one binding site every 1,600 base pairs. However, the

proportion of these sites accessible to polyamide binding in the context of heterochromatin *in vivo* is currently unknown. Additionally, data from *in vitro* transcription experiments suggest that a polyamide non-covalently bound within the coding region of a gene would not interfere with RNA polymerase activity at that locus (*33*). It would thus not be surprising if a significant fraction of polyamide-DNA binding events occurring in a cell do not have a direct affect on gene expression. In parallel to this, global analysis of transcription factor binding to chromatin *in vivo* has shown occupancy at promoters and enhancers without an associated change in gene expression at that locus (*45*).

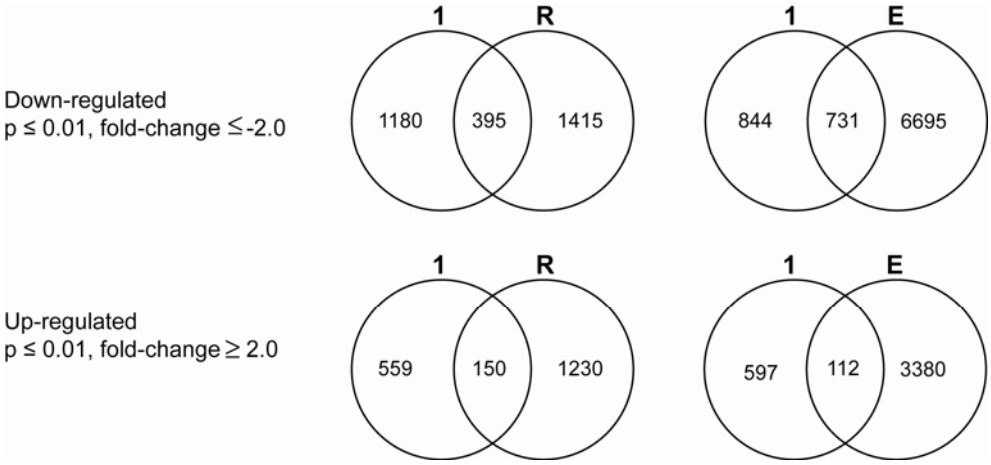

Figure 4.5 Ven diagrams representing transcripts commonly down- and up-regulated (|fold-change| ≥ 2.0, p ≤ 0.01) by **1** and HIF-1α siRNA, and by **1** and echinomycin. Numbers inside the intersections represent transcripts affected by both treatments.

Table 4.2 HIF-1 induced genes affected by HIF-1α siRNA (**R**) and 1 (**1** μM) expressed as fold-change relative to DFO-induced controls.

	HRE(s) (5′ to 3′)	R	1
TFRC	agcg **TACGTG** cctc	-2.0	1.1
PFKFB3	gcgg **GACGTG** acgc gacg **CACGTG** ggca	-5.5	1.0
LDHA	ggcg **GACGTG** cggg ctca **CACGTG** ggtt	-1.7	1.3
BNIP3	gccg **CACGTG** ccac	-9.4	1.3
EGLN3	gggc **TACGTG** cgct	-5.3	1.0
EGLN1	ggtg **TACGTG** caga	-3.4	1.1
PGK1	gtga **GACGTG** cggc tgcc **GACGTG** cgct	-5.4	-1.2
CA9	gctg **TACGTG** catt	-89.0	-2.1
VEGF	tgca **TACGTG** ggct	-3.4	-2.0
FLT1	gaac **AACGTG** gaat	-2.2	-2.0
EDN1	aggc **AACGTG** cagc	-3.5	-2.5

Interestingly, polyamide **1** (1 μM) affected the expression of fewer genes than siRNA targeted to HIF-1α under the conditions of the experiment. A direct comparison in genomic specificity between polyamide and siRNA, however, cannot be drawn from these limited data because a large proportion of the genes affected by the siRNA are likely a result of silencing the target gene, HIF-1α, and not due to off-target effects involving post-transcriptional silencing of mRNA using the RNA interference pathway (*46*). If we eliminate from the total number of transcripts affected by the HIF-1α siRNA (2-fold, $p \leq 0.01$) all transcripts affected by treatment with DFO alone (1.5-fold, $p \leq 0.01$) we are left with 1,523 affected transcripts. A similar treatment of the data for polyamide **1** results in 1,626 affected transcripts. It should also be noted that in the case of most

HIF-1 regulated genes affected by both polyamide and siRNA, inhibition by the polyamide was somewhat more modest than that of siRNA, suggesting incomplete abrogation of HIF-1 DNA binding by the polyamide. This might be improved upon with molecules of higher binding affinity. Approximately 23% of the 297 transcripts induced by DFO were inhibited by polyamide **1**. For genes where the functional HRE has been identified, the effects observed upon treatment with polyamide **1** and echinomycin are, thus far, consistent with the expected binding preferences of these molecules.

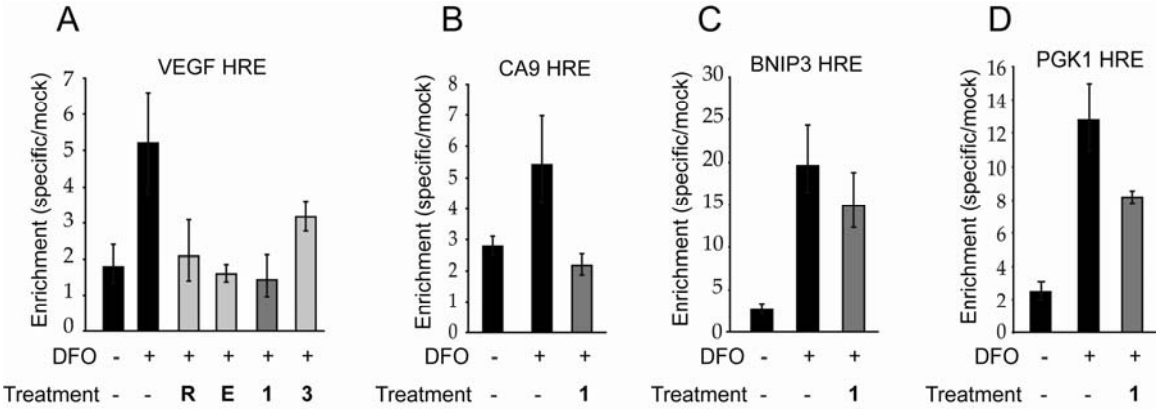

Figure 4.6 (A) Chromatin immunoprecipitation of HIF-1α at the VEGF HRE following DFO treatment: siRNA against HIF-1α, **R**; echinomycin (100 nM), **E**; and polyamides **1** and **3** (1 μM). Occupancy is decreased in the presence of **R**, **E**, and **1**, but only modestly affected by **3**. Chromatin immunoprecipitation of HIF-1α at the CA9 HRE (B), BNIP3 HRE (C), and PGK1 HRE (D) in the presence of 1 (1 μM). HIF-1α occupancy at CA9 is reduced by polyamide **1**. Occupancy at the BNIP3 and PGK1 HREs are less affected.

HIF-1 is frequently overactive in tumors and a number of direct targets in the HIF-1 pathway have become points of clinical intervention (*47*). Bevacizumab, an anti-VEGF antibody, and sorafenib and sunitinib, tyrosine kinase inhibitors with activity against the VEGF receptors, have shown some promise in clinical trials as cancer therapeutics (*48-50*). Echinomycin had been previously brought to the clinic as a cancer

therapeutic in phase I and II trials (*51*), based on observations that echinomycin exhibits potent anti-proliferative effects on several tumor derived cell lines (*51, 52*). However, survival benefit was not established (*51*). In light of the recent work by Melillo and colleagues, a re-examination of the clinical use of echinomycin in tumor types expected to be highly sensitive to HIF-1 activity may be justified (*20*).

Table 4.3 Equilibrium association constants reported are mean values from three DNase I footprint titration experiments; standard deviations are shown in parentheses.

pGL2-VEGF-*Luc*	Ligand	5'-ATACGT-3'	5'-AGTGCA-3'
	1	2.6 (±0.4) x 10^{10}	--------
	2	--------	3.2 (±0.6) x 10^{9}
	E	8.4 (±2.1) x 10^{6}	--------
pCSJFLT1	Ligand	5'-CAACGT-3'	5'-TGAGGA-3'
	1	2.7 (±0.2) x 10^{9}	--------
	2	--------	2.2 (±0.8) x 10^{8}
	E	2.9 (±0.7) x 10^{7}	--------

The induction of pro-angiogenic, proliferative, metastatic, and glycolytic genes by HIF-1 are established as contributing to the cancer phenotype (*10*). However, genes that promote cell death, such as BNIP3 and NIX (BNIP3L), are also induced by hypoxia through HIF-1 (*53*). In this sense, HIF-1 plays dual roles in the survival and death pathways of tumor cells (*11*). A functional separation of these targets of HIF-1 at the level of HIF-1 DNA binding might have clinical relevance (*11*). Given sufficient knowledge of the particular regulatory sequences involved, one could, in principle, design a polyamide or cocktail of polyamides to affect a selected subset of target genes in the HIF-1 pathway, making use of the programmability of polyamide recognition for targeting particular HREs and flanking sequences. The utility of polyamides as

regulators of hypoxia induced gene expression awaits continued study in small animal models of HIF-1 activity.

4.4 Methods

Synthesis of Polyamides. Polyamides were synthesized by solid-phase methods on Kaiser oxime resin (Nova Biochem) (*54*). Polyamides were cleaved from resin with 3,3'-diamino-*N*-methyl-dipropylamine and purified by reverse-phase HPLC. Isophthalic acid was activated with PyBOP (Nova Biochem) and conjugated to the polyamides as previously described (*19*). Purities and identities of the polyamides were assessed by HPLC, UV-visible spectroscopy, and MALDI-ToF MS.

Determination of DNA-binding affinities and sequence specificities

Quantitative DNase I footprint titration experiments were used to measure the binding affinities of polyamides **1**, **2**, and echinomycin on 5' ^{32}P labeled fragments of pGL2-VEGF-*Luc* or pCSJFLT1 that contain the promoter sequences containing the HREs of the VEGF and FLT1 genes, respectively. Quantitative DNase I footprint titration experiments were conducted as reported previously (*55*).

Measurement of hypoxia induced gene expression

U251 cells were plated in 24-well plates at a density of 20–30 x 10^3 cells per well (40-60 x 10^3 cells/ml) in RPMI (ATCC) supplemented with 5% FBS (Irvine Scientific). After 24 hours, polyamides were added to the adhered cells in solutions of cell media at the appropriate concentration and allowed to incubate with the cells for 48 hours. Hypoxic induction of gene expression was then chemically induced by adding deferoxamine (DFO) to 300 μM for an additional 16 hours. When appropriate, echinomycin was added

two hours prior to DFO stimulation. Isolation of RNA and subsequent cDNA synthesis were as previously described (*18*). When appropriate, siRNA against HIF-1α (HIF-1α validated stealth duplex, Invitrogen) was transfected 48 hours prior to RNA isolation. Transfection of the siRNA was achieved using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocols. Quantitative real-time RT–PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 instrument. Target gene mRNA was measured relative to ß-glucuronidase as an endogenous control. Primer sequences are available upon request.

Chromatin Immunoprecipitation

U251 cells were plated in 15 cm diameter plates and left to attach overnight. Media, time course, DFO, polyamide, echinomycin, and siRNA treatments were the same as described above. After the 16 hour DFO treatment, cells were treated with 1% formaldehyde for 10 minutes. Chromatin was isolated and sheared. HIF-1α antibodies (Novus Biologicals) were used to immunoprecipitate HIF-1 bound DNA fragments. After crosslink reversal, PCRs using primers targeted to the regions of interest were used to assess enrichment of bound fragments as compared to mock-precipitated (no antibody) controls. PCRs were monitored either using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 instrument, or directly visualized using gel electrophoresis. See Appendix C for a more detailed protocol.

Analysis of Gene Expression with Oligonucleotide Microarrays

U251 cells were plated in 12-well plates at a density of 40-60 x 10^3 cells per well. Media, time course, DFO, polyamide, echinomycin, and siRNA treatments were the same as described above. RNA was isolated as previously described. From this point, sample

preparation for the microarray experiments were carried out at the Millard and Muriel Jacobs Gene Expression Facility at Caltech. Labeled mRNA was hybridized to affymetrix Human 133 arrays according to established protocols. Gene expression was analyzed using Resolver (Rosetta Biosoftware, Seattle).

References

1. Blancafort, P., Segal, D. J., and Barbas, C. F. (2004) Designing transcription factor architectures for drug discovery, *Mol. Pharmacol.* 66, 1361-1371.

2. Beerli, R. R., and Barbas, C. F. (2002) Engineering polydactyl zinc-finger transcription factors, *Nat. Biotechnol.* 20, 135-141.

3. Beerli, R. R., Segal, D. J., Dreier, B., and Barbas, C. F. (1998) Toward controlling gene expression at will: Specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks, *Proc. Natl. Acad. Sci. U. S. A.* 95, 14628-14633.

4. Liu, P. Q., Rebar, E. J., Zhang, L., Liu, Q., Jamieson, A. C., Liang, Y. X., Qi, H., Li, P. X., Chen, B. L., et al. (2001) Regulation of an endogenous locus using a panel of designed zinc finger proteins targeted to accessible chromatin regions - Activation of vascular endothelial growth factor A, *J. Biol. Chem.* 276, 11323-11334.

5. Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans, *Nature* 391, 806-811.

6. Meister, G., and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA, *Nature* 431, 343-349.

7. Mello, C. C., and Conte, D. (2004) Revealing the world of RNA interference, *Nature* 431, 338-342.

8. Hannon, G. J., and Rossi, J. J. (2004) Unlocking the potential of the human genome with RNA interference, *Nature* 431, 371-378.

9. Dervan, P. B., and Edelson, B. S. (2003) Recognition of the DNA minor groove by pyrrole-imidazole polyamides, *Curr. Opin. Struct. Biol.* 13, 284-299.

10. Semenza, G. L. (2003) Targeting HIF-1 for cancer therapy, *Nat. Rev. Cancer* 3, 721-732.

11. Zhou, J., Schmid, T., Schnitzer, S., and Brune, B. (2006) Tumor hypoxia and cancer progression, *Cancer Lett.* 237, 10-21.

12. Pouyssegur, J., Dayan, F., and Mazure, N. M. (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression, *Nature* 441, 437-443.

13. Melillo, G. (2006) Inhibiting hypoxia-inducible factor 1 for cancer therapy, *Molecular Cancer Research* 4, 601-605.

14. Semenza, G. L. (2000) HIF-1 and human disease: one highly involved factor, *Genes Dev.* 14, 1983-1991.

15. Ivan, M., Kondo, K., Yang, H. F., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., et al. (2001) HIF alpha targeted for VHL-mediated destruction by proline hydroxylation: Implications for O-2 sensing, *Science* 292, 464-468.

16. Zimmer, M., Doucette, D., Siddiqui, N., and Iliopoulos, O. (2004) Inhibition of hypoxia-inducible factor is sufficient for growth suppression of VHL-/- tumors, *Molecular Cancer Research* 2, 89-95.

17. Kung, A. L., Zabludoff, S. D., France, D. S., Freedman, S. J., Tanner, E. A., Vieira, A., Cornell-Kennon, S., Lee, J., Wang, B. Q., et al. (2004) Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway, *Cancer Cell* 6, 33-43.

18. Olenyuk, B. Z., Zhang, G. J., Klco, J. M., Nickols, N. G., Kaelin, W. G., and Dervan, P. B. (2004) Inhibition of vascular endothelial growth factor with a sequence-specific hypoxia response element antagonist, *Proc. Natl. Acad. Sci. U. S. A.* 101, 16768-16773.

19. Nickols, N. G., Jacobs, C. S., Farkas, M. E., and Dervan, P. B. (2007) Improved nuclear localization of DNA-binding polyamides, *Nucleic Acids Res.* 35, 363-370.

20. Kong, D. H., Park, E. J., Stephen, A. G., Calvani, M., Cardellina, J. H., Monks, A., Fisher, R. J., Shoemaker, R. H., and Melillo, G. (2005) Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity, *Cancer Res.* 65, 9047-9055.

21. Van Dyke, M. M., and Dervan, P. B. (1984) Echinomycin Binding-Sites on DNA, *Science* 225, 1122-1127.

22. Kielkopf, C. L., Baird, E. E., Dervan, P. D., and Rees, D. C. (1998) Structural basis for G•C recognition in the DNA minor groove, *Nat. Struct. Biol.* 5, 104-109.

23. White, S., Szewczyk, J. W., Turner, J. M., Baird, E. E., and Dervan, P. B. (1998) Recognition of the four Watson-Crick base pairs in the DNA minor groove by synthetic ligands, *Nature* 391, 468-471.

24. Foister, S., Marques, M. A., Doss, R. M., and Dervan, P. B. (2003) Shape selective recognition of T•A base pairs by hairpin polyamides containing N-terminal 3-methoxy (and 3-chloro) thiophene residues, *Bioorg. Med. Chem.* 11, 4333-4340.

25. Liu, Y. X., Cox, S. R., Morita, T., and Kourembanas, S. (1995) Hypoxia Regulates Vascular Endothelial Growth-Factor Gene-Expression in Endothelial-Cells - Identification of a 5'-Enhancer, *Circ.Res.* 77, 638-643.

26. Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., and Semenza, G. L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1, *Mol. Cell. Biol.* 16, 4604-4613.

27. Gerber, H. P., Condorelli, F., Park, J., and Ferrara, N. (1997) Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes - Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia, *J. Biol. Chem.* 272, 23659-23667.

28. Waring, M. J., and Wakelin, L. P. G. (1974) Echinomycin - Bifunctional Intercalating Antibiotic, *Nature* 252, 653-657.

29. Low, C. M. L., Drew, H. R., and Waring, M. J. (1984) Sequence-Specific Binding of Echinomycin to DNA - Evidence for Conformational-Changes Affecting Flanking Sequences, *Nucleic Acids Res.* 12, 4865-4879.

30. Fechter, E. J., and Dervan, P. B. (2003) Allosteric inhibition of protein-DNA complexes by polyamide-intercalator conjugates, *J. Am. Chem. Soc.* 125, 8476-8485.

31. Fechter, E. J., Olenyuk, B., and Dervan, P. B. (2004) Design of a sequence-specific DNA bisintercalator, *Angew. Chem.-Int. Edit.* 43, 3591-3594.

32. Kageyama, Y., Sugiyama, H., Ayame, H., Iwai, A., Fujii, Y., Huang, L. E., Kizaka-Kondoh, S., Hiraoka, M., and Kihara, K. (2006) Suppression of VEGF transcription in renal cell carcinoma cells by pyrrole-imidazole hairpin polyamides targeting the hypoxia responsive element, *Acta Oncol.* 45, 317-324.

33. Dickinson, L. A., Gulizia, R. J., Trauger, J. W., Baird, E. E., Mosier, D. E., Gottesfeld, J. M., and Dervan, P. B. (1998) Inhibition of RNA polymerase II transcription in human cells by synthetic DNA-binding ligands, *Proc. Natl. Acad. Sci. U. S. A.* 95, 12890-12895.

34. Oakley, M. G., Mrksich, M., and Dervan, P. B. (1992) Evidence That a Minor Groove-Binding Peptide and a Major Groove-Binding Protein Can Simultaneously Occupy a Common Site on DNA, *Biochemistry* 31, 10969-10975.

35. Tacchini, L., Bianchi, L., Bernelli-Zazzera, A., and Cairo, G. (1999) Transferrin receptor induction by hypoxia - HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation, *J. Biol. Chem.* 274, 24142-24146.

36. Lok, C. N., and Ponka, P. (1999) Identification of a functional hypoxia-response element in the transferrin-receptor gene, *Exp. Hematol.* 27, 91-91.

37. Fukasawa, M., Tsuchiya, T., Takayama, E., Shinomiya, N., Uyeda, K., Sakakibara, R., and Seki, S. (2004) Identification and characterization of the hypoxia-responsive element of the human placental 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene, *J. Biochem.* 136, 273-277.

38. Obach, M., Navarro-Sabate, A., Caro, J., Kong, X. G., Duran, J., Gomez, M., Perales, J. C., Ventura, F., Rosa, J. L., et al. (2004) 6-phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia, *J. Biol. Chem.* 279, 53562-53570.

39. Semenza, G. L., Jiang, B. H., Leung, S. W., Passantino, R., Concordet, J. P., Maire, P., and Giallongo, A. (1996) Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1, *J. Biol. Chem.* 271, 32529-32537.

40. Semenza, G. L., Roth, P. H., Fang, H. M., and Wang, G. L. (1994) Transcriptional Regulation of Genes Encoding Glycolytic-Enzymes by Hypoxia-Inducible Factor-1, *J. Biol. Chem.* 269, 23757-23763.

41. Pescador, N., Cuevas, Y., Naranjo, S., Alcaide, M., Villar, D., Landazuri, M., and Del Peso, L. (2005) Identification of a functional hypoxia-responsive element that regulates the expression of the egl nine homologue 3 (egln3/phd3) gene, *Biochem. J.* 390, 189-197.

42. Willam, C., Nicholls, L. G., Ratcliffe, P. J., Pugh, C. W., and Maxwell, P. H. (2004), The prolyl hydroxylase enzymes that act as oxygen sensors regulating destruction of hypoxia-inducible factor alpha. In *Advances in Enzyme Regulation, Vol 44*; Vol. 44, pp 75-92.

43. Grabmaier, K., de Weijert, M. C. A., Verhaegh, G. W., Schalken, J. A., and Oosterwijk, E. (2004) Strict regulation of CAIX(G250/MN) by HIF-1 alpha in clear cell renal cell carcinoma, *Oncogene* 23, 5624-5631.

44. Hu, J., Discher, D. J., Bishopric, N. H., and Webster, K. A. (1998) Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand, *Biochem. Biophys. Res. Commun.* 245, 894-899.

45. Zhang, X. M., Odom, D. T., Koo, S. H., Conkright, M. D., Canettieri, G., Best, J., Chen, H. M., Jenner, R., Herbolsheimer, E., et al. (2005) Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues, *Proc. Natl. Acad. Sci. U. S. A.* 102, 4459-4464.

46. Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. S. (2003) Expression profiling reveals off-target gene regulation by RNAi, *Nat. Biotechnol.* 21, 635-637.

47. Cohen, H. T., and McGovern, F. J. (2005) Renal-cell carcinoma, *N. Engl. J. Med.* 353, 2477-2490.

48. Escudier, B., Eisen, T., Stadler, W. M., Szczylik, C., Oudard, S., Siebels, M., Negrier, S., Chevreau, C., Solska, E., et al. (2007) Sorafenib in advanced clear-cell renal-cell carcinoma, *N. Engl. J. Med.* 356, 125-134.

49. Motzer, R. J., Hutson, T. E., Tomczak, P., Michaelson, M. D., Bukowski, R. M., Rixe, O., Oudard, S., Negrier, S., Szczylik, C., et al. (2007) Sunitinib versus interferon alfa in metastatic renal-cell carcinoma, *N. Engl. J. Med.* 356, 115-124.

50. Yang, J. C., Haworth, L., Sherry, R. M., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Steinberg, S. M., Chen, H. X., and Rosenberg, S. A. (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer, *N. Engl. J. Med.* 349, 427-434.

51. Foster, B. J., Clagettcarr, K., Shoemaker, D. D., Suffness, M., Plowman, J., Trissel, L. A., Grieshaber, C. K., and Leylandjones, B. (1985) Echinomycin - the 1st Bifunctional Intercalating Agent in Clinical-Trials, *Invest. New Drugs* 3, 403-410.

52. May, L. G., Madine, M. A., and Waring, M. J. (2004) Echinomycin inhibits chromosomal DNA replication and embryonic development in vertebrates, *Nucleic Acids Res.* 32, 65-72.

53. Kothari, S., Cizeau, J., McMillan-Ward, E., Israels, S. J., Bailes, M., Ens, K., Kirshenbaum, L. A., and Gibson, S. B. (2003) BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF, *Oncogene* 22, 4734-4744.

54. Belitsky, J. M., Nguyen, D. H., Wurtz, N. R., and Dervan, P. B. (2002) Solid-phase synthesis of DNA binding polyamides on oxime resin, *Bioorg. Med. Chem.* 10, 2767-2774.

55. Trauger, J. W., and Dervan, P. B. (2001) Footprinting methods for analysis of pyrrole-imidazole polyamide/DNA complexes, *Methods Enzymol.* 340, 450-466.