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Inhibition of Hypoxia Inducible Factor-2 Transcription: Isolation of Active Modulators from Marine Sponges

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Abstract

Renal or kidney cancer accounts for about 3% of all cancer cases reported each year in the US. Molecular signatures that define the cancer, such as the loss of functional VHL, are found in both sporadic and familial cases of cancer. In clear cell renal cancer, the transcription factor HIF-2 α has been shown to have a distinct role in tumorigenesis. Our laboratories developed a cell-based screen to identify modulators of HIF-2 α . Screening of the NCI's Natural Product Extract Repository resulted in the identification of 10 sponge extracts from which 12 compounds were isolated. The biological evaluation of these compounds will be discussed including evaluation of HIF-1 α vs. HIF-2 α selectively and the isolated compounds' effects on mRNA from several pathways regulated by HIF.

Approximately 58,000 individuals were diagnosed with renal cell carcinoma (RCC, kidney cancer) in 2011,¹ accounting for 3% of the cancers in the USA.² RCC is not a single disease, but can be separated into several distinct diseases based on clinical, morphological and molecular features. Many of these molecular features have been identified based on the study of families that are affected by inherited renal cancer syndromes.² Certain molecular features of patients with Von Hippel Lindau (VHL) disease are also present in individuals who develop sporadic clear cell RCC, the most prevalent RCC subtype.³ Specifically, loss of *VHL* gene function as occurs in Von Hippel Lindau patients is seen in > 90% of sporadic RCC tumors. VHL protein (pVHL) is part of an E3 ubiquitin ligase system that recognizes and marks substrates for degradation.⁴ The primary substrate of the pVHL complex is hypoxia inducible factor–alpha subunit (HIF- α). There are three known isoforms of HIF- α :

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ASSOCIATED CONTENT:

Supporting Information: Details of the collection, identification and isolation of compounds **1-12**. qPCR results for compounds **7, 8** and **9** and pictures of the sponges *Amphimedon* sp. (0CDN481), *Xestospongia* sp. (0CDN7718), *Haliclona* sp. (Q66C334), *Petrosia* sp. (0M9H2607) and *Fascaplysinopsis* sp. (0M9I5678) are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

HIF-1 α , HIF-2 α and HIF-3 α . HIF is a dimeric transcription factor [HIF- α isoform with aryl hydrocarbon receptor nuclear translocator, ARNT or HIF- β] that is used by cells to respond and adapt to low levels of oxygen or hypoxia.

Under normal levels of oxygen, specific proline residues in HIF-1 α and HIF-2 α are hydroxylated. This reaction allows VHL to bind and ubiquitylate the α -subunit leading to its degradation. Under hypoxic conditions, this hydroxylation does not take place, HIF- α subunits accumulate within the cell, dimerize with ARNT, and translocate to the nucleus where they bind to hypoxia response elements (HREs), turning on transcription of a large number of genes. HIF regulated genes function in a variety of pathways including angiogenesis (*e.g.*, vascular endothelial growth factor, VEGF), cell survival (*e.g.*, erythropoietin, EPO) and glucose metabolism (*e.g.*, glucose transporter-1, GLUT1) that together allow the cell to cope with and survive under the hypoxic conditions.⁵ It is therefore not surprising that HIF has been shown to be upregulated in many tumors due to intratumoral hypoxia or by genetic mutation. Indeed upregulation of HIF is often associated with aggressive growth, treatment resistance, metastasis, and poor prognosis.⁶ In renal cancer, HIF-2 α plays a primary role in tumorigenesis.⁷⁻⁹

To better understand the importance of HIF-2 α to RCC, our laboratories generated a cell-based screen to identify inhibitors of HIF-2 α using the RCC 786-0 cell line that expresses functional HIF-2 α and a truncated, non-functional HIF-1 α .¹⁰ The intent of these experiments was to identify compounds with selective HIF-2 α modulating activity. Whether known or new natural products, they would be instrumental in increasing our understanding of HIF-2 α function. Over 146,000 plant, marine invertebrate and microbial extracts available from the NCI Open Repository were screened for activity in the assay. From among the active extracts, 10 extracts from marine sponges were identified as active. Chemical exploration of these sponge extracts led to isolation of 12 known metabolites: aaptamine (**1**) from *Aaptos aaptos*¹¹ Schmidt, 1864 (Suberitidae), mimosamycin (**2**) from *Amphimedon* sp.^{12,13} (Niphatidae), xestoquinone (**3**) from *Xestospongia* sp.¹⁴ (Petrosiidae), *O*-demethylrenierone (**4**) and *N*-formyl-1,2-dihydrorenierone (**5**) from *Haliclona velinea*^{15,16} De Laubenfels, 1954 (Chalinidae), puupehenone (**6**) from *Hyrtios reticulatus*¹⁷⁻¹⁹ Thiele, 1899 (Thorectidae), variolin B (**7**) from *Kirkpatrickia variolosa*²⁰ Kirkpatrick, 1907 (Hymedesmiidae), haliclonadiamine (**8**) from *Haliclona* sp.^{21, 22} (Chalinidae), neopetroformyne A (**9**) from *Petrosia* sp.²³ (Petrosiidae), 12 α -acetoxy-24-methyl-24-oxoscalar-16-en-22,25-dial (**10**) and (-)-22-acetoxy-16 β -hydroxy-24-methyl-24-oxoscalarano-25,12 β -lactone (**11**) from *Lendenfeldia chondrodes*²⁴ Lendenfeld, 1889 (Spongiidae) and heteronemin (**12**) from *Fascoplysinopsis* sp.²⁵⁻²⁷ Berquist, 1980 (Thorectidae). The compounds were identified by comparison of their spectroscopic data with literature values.

The purified metabolites were tested in the HIF-2 screening assay for their ability to inhibit the luciferase reporter expression in an engineered cell line. The cell line was stably transfected with five tandem copies of the minimal VEGF promoter. The most important piece of this promoter is the hypoxia responsive element (HRE) that is common to all HIF regulated genes and is not specific for HIF-2 α . These results provided an EC₅₀ (the concentration at which the luciferase signal decreased to 50% of control) for all the compounds. An arbitrary cut-off for an “active” designation was set at 100 μ M. The results for compounds **1-12** are detailed in Table 1. Aaptamine (**1**) and heteronemin (**12**) have been isolated within our group on numerous occasions and are regarded as nuisance compounds based on activity in a variety of screens and as such, were not evaluated further. Compounds **5-10** were considered active. All others (**2-4**, **11**) were considered inactive. Compound **10**, although active in the HIF-2 α screen, was not available for additional testing and therefore was not evaluated further.

The primary HTS assay used the 786-0 cell line because it produces only HIF-2 α protein. HIF-1 α mRNA is present but translation results in a truncated, non-functional protein. The reporter plasmid in the engineered cell line used in the primary screen was generated using five copies of the minimal VEGF promoter containing the core HRE sequence RCGTG common to all HIF responsive genes. A logical extension of the primary screen, as an initial biological evaluation of these compounds, was to examine the ability of compounds **5-9** to inhibit expression of VEGF protein under the control of the complete, endogenous promoter in unmodified 786-0 cells. Our intent was not to discover VEGF inhibitors, but rather to document the effect of treatment on the response of VEGF under the control of its complete promoter sequence. Comparing this response with that of the concentration of total protein

was also a simple measure of specific versus general inhibition of protein synthesis. These results are summarized in Figure 1.

We observed a 50% decrease in VEGF secretion upon treatment with **7** and **8**. With **8**, this decrease in VEGF was coupled with a 20% decrease in total protein, suggesting that at least some of the observed decrease was related to cellular toxicity. In contrast, inhibition of VEGF expression by compounds **6** or **9** was mirrored by a similar decrease in total protein, indicating the result was entirely due to toxicity. The results observed for compound **5** showed a net gain of both VEGF and total protein over untreated cells that was unexpected, novel and not easily explained by the data in hand. Future experiments will address these results in detail.

We next examined cells treated with **5-9** for their effects on HIF-induced transcription of mRNAs under the control of HIF-1 α , HIF-2 α , or both. The goal of this study was to identify HIF-2 α selectivity. This study would also provide additional information about activity of the compounds on several HIF-regulated pathways, as well as some indication of selectivity for HIF-1 α vs. HIF-2 α . For these experiments we utilized three different cell lines: 786-0 (functional HIF-2 α only), RCC4 (both HIF-1 α and HIF-2 α), and SK-RC-28 (HIF-1 α only; expression of HIF-2 α had been suppressed by stable transfection with a HIF-2 α shRNA. (data not shown)). The three cell lines were first compared for their sensitivity to compounds **5-9** using a XTT-based cytotoxicity screen. The IC₅₀ values for each compound against the three cell lines are summarized in Table 2. Interestingly only compound **7** displayed selective cytotoxicity for RCC4 (IC₅₀ 8.8 μ M) but was non-toxic to either 786-0 or SK-RC-28 cell lines (IC₅₀ > 100 μ M for both cell lines) while the other compounds had generally comparable toxicities against each of the three cell lines.

Subsequent experiments measured the effect of treatment with compounds **5-9** on the transcription of HIF-inducible genes. Results were obtained by measuring mRNA levels of five HIF-induced genes using quantitative polymerase chain reaction (qPCR) in the three cell lines. The genes were chosen on the basis of published evidence of induction by a specific HIF- α isoform and the availability of tested, reliable primers and antibodies. The five mRNA transcripts that were measured encoded carbonic anhydrase 9 (CA-IX; mediated by HIF-1 α), transforming growth factor alpha, glucose transporter 1 and cyclin D1 (TGF α , GLUT1 and CCND1, respectively; mediated by HIF-2 α), and vascular endothelial growth factor A (VEGF-A; mediated by both HIF-1 α and HIF-2 α). In addition, the mRNA transcripts for HIF-1 α and HIF-2 α were also quantified in all three cell lines. The relative abundance of each mRNA was normalized to the mRNA of actin. The purpose of these experiments was two-fold: First and foremost, they were designed to see which (if any) compounds were truly HIF-2 α selective by looking at the overall patterns of inhibition of the mRNA transcripts in each of the cells lines. Specifically, a HIF-2 α selective compound would inhibit mRNA in 786-0 cells (HIF-2 α only) and RCC4 (both HIF-1 α and HIF-2 α , present). Inhibition of mRNA in RCC4 would indicate that HIF-1 α could not compensate for inhibition of HIF-2 α . Finally, one would expect relatively less inhibition of SK-RC-28 with a selective compound as only HIF-1 α is present. Secondly, the results were anticipated to indicate how the transcription of each gene was affected by treatment with each compound. The results for **5** and **6** are summarized in Figure 2.

Compounds **5** and **6** were much less active against the engineered SK-RC-28 cell line (HIF-1 α only) since the mRNA levels were not significantly reduced, but mRNA levels in both 786-0 and RCC4 cells were substantially decreased for all target genes except CA-IX (not detected in RCC4; highly induced in 786-O cells with **5**). These results, coupled with the modest effects observed in the SK-RC-28 cells where HIF-2 α is suppressed, suggest that these compounds may be selective inhibitors of HIF-2 α . Moreover, HIF-1 α did not appear

to compensate for most of the HIF-2 α inhibition observed, since mRNA levels were also suppressed in RCC4 where both HIF- α isoforms were present. Interestingly, these mRNA results were not consistent with the VEGF protein levels measured in earlier experiments with compound **5**. Compounds **4** and **7** were inactive (most mRNA levels were not significantly changed compared to actin), while compounds **8** and **9** appeared to inhibit transcription since all mRNA levels, including those for the non-HIF regulated “housekeeping genes,” were significantly decreased. (Results for **7-9** are available in Supporting Information.) Based on its potency and its HIF-2 α selectivity, compound **5**, *N*-formyl-1,2-dihydrorenierone, was selected for further biological evaluation; results from these studies will be the subject of a future report.

Experimental Section

Collection and Extraction of Sponge Materials

All of the sponge material was collected under contract to the National Cancer Institute. Materials were frozen immediately upon collection and stored frozen until extraction. Extraction of the frozen sponge materials was done as described by McCloud.²⁸ Voucher specimens are stored at the Smithsonian Institution. Additional details are provided in the Supporting Information.

Isolation of Compounds 1–12

Compounds **1-12** were isolated from a variety of different sponge extracts and the detailed description of the isolation procedures for each compound is provided in the Supporting Information. Compounds **2-3**, and **7-12** were purified by HPLC (> 95% purity) while the purity of compounds **1**, and **4-6** was determined to be > 95% by LCMS. All structures were determined by comparison of acquired NMR spectra (generally ¹H, ¹³C, HSQC and HMBC experiments) and mass spectrometry data with literature values.

HIF-2 Assay

A renal clear cell carcinoma cell line 786-0 was engineered with five copies of the HIF-2 α hypoxia response element (HRE) linked to a luciferase output signal as previously described.¹⁰ Briefly, 5000 cells/well were plated in 384 well white plates and left overnight to attach. Then cells were treated with compound for 24 h. After incubation in the presence of test compounds, luciferin was added to cells and the luciferase signal was measured. In the presence of an inhibitor, HIF-2 α will not bind to the HRE or inhibit transcription, leading to a decrease in the presence of luciferase in the cells. At the end of the incubation period when luciferin was added, the luminescent signal of the cells was decreased. Since a false-positive signal could also be caused by cytotoxicity, the cytotoxicity of the compound or extract tested was also measured concurrently. Actinomycin D was used as a positive control. Compounds were considered hits if displaying 44% inhibition of the luciferase signal and 22% cytotoxicity. Hits were confirmed via duplicate testing followed by triplicate dose response testing.

Secondary Evaluations of HIF-2 Activity

For all cell-based studies, cells were serum deprived for 24 h prior to treatment, then treated in serum free media for 48 h at the following doses: 79, 20, 1, 21, 20 μ M for compounds **5-9**, respectively.

VEGF-A Protein Concentration Determination

Media was collected and measured according to the manufacturers protocol (Meso Scale Discovery; Cat. No. K151BMC-3). Unknown VEGF-A concentrations were calculated

using a non-linear regression using GraphPad Prism software, based on a standard curve. Each experiment was done in duplicate.

shRNA Knockdown of HIF-2 α in SK-RC-28

The SK-RC-28 cells were generously provided by Lloyd Old (Ludwig Institute for Cancer Research). Cells were stably transfected with α -HIF-2 α shRNA plasmids (GI361930 & GI361931) or a control shRNA plasmid (TR30007). Stable transfections were maintained in 375 ng/mL of puromycin. Knockdown of HIF-2 α alpha was verified by immuno-blotting with a HIF-2 α antibody (AF2886, R&D).

XTT Cytotoxicity Screen

The *in vitro* XTT cytotoxicity bioassay is a 2-day bioassay. Cells were grown in RPMI-1640 without L-glutamine (Gibco), supplemented with 10% fetal bovine serum (FBS, Hyclone), 5.0 mL of a 200 mM glutamine stock (Gibco), and penicillin-streptomycin (at 100 IU/mL and 100 μ g/mL, respectively; Gibco). Once the cells were confluent, they were harvested by trypsinization, spun down and resuspended in phenol red-free RPMI-1640 with L-glutamine (Gibco), supplemented with 10% FBS (Hyclone). The dilutions for each data point were run in triplicate for each compound.

To yield optical density readings in the range of 1–2.0, cells were plated in 384-well microtiter flat-bottom plates at a seeding density of 5,000 cells per well and incubated for up to 4 h in a 37 °C, 5% CO₂ incubator. After the 4 h incubation, the cells were then introduced to the test samples. 5 μ L of test compounds in phenol-red free RPMI-1640 assay medium was added via a Biomek FX (Beckman Coulter) automated liquid handling workstation.

The test plates were then returned to the incubator. After the 2-day incubation, the cells were exposed to 10 μ L/well of phenol-red RPMI-1640 with 0.1 % tetrazolium salt 2,3-bis[2-methoxy-4-nitro-5- sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute) and 0.63% phenazine methosulfate (PMS, Sigma) for a 4 h incubation in a 37 °C incubator, where metabolically viable cells reduced the tetrazolium salt to a colored formazan product. Once the incubation was completed, the plates were read at 450 nm, (0.1 s/well) using a Wallac Victor² 1420 plate reader (Perkin Elmer). A positive control of 20% DMSO was used.

Quantitative Real-time PCR of mRNA

Total RNA was isolated using QIAGEN RNeasy Mini Kit (Cat. No. 74104) and concentrations were determined using a Nano Drop UV/Vis Spectrophotometer. 20 ng of RNA were reverse transcribed according to the manufactures protocol (Invitrogen; Cat. No. 11904-018). PCR reactions were carried out using SYBR Green PCR Master Mix and 1 ng of cDNA (Cat. No. 4334973). Relative Quantities of transcripts were calculated using the $2^{-\Delta\Delta CT}$ method, normalized to actin levels and using the mean from duplicate experiments: $RQ = 2^{-(\Delta\Delta CT)}$; $\Delta\Delta CT = \Delta CT(\text{compound}) - \Delta CT(\text{control})$; $\Delta CT(\text{compound}) = CT(\text{target gene, with compound}) - CT(\text{actin, with compound})$; $\Delta CT(\text{control}) = CT(\text{target gene, control}) - CT(\text{actin, control})$

PCR Primers Used

ACTIN-F: TGGGCATGGGTCAGAAGGAT; ACTIN-R: CGTCCCAGTTGGTGACGATG; CA9-F: CAGCAACTGCTCATAGGCAC; CA9-R: CTTTGCCAGAGTTGACGAGG; CCND1-F: ACGAAGGTCTGCGCGTGTT; CCND1-R: CCGCTGGCCATGAACTACCT; GAPDH-F: GGAGTCCACTGGCGTCTTCAC; GAPDH-R: GAGGCATTGCTGATGATCTTGAGG; GLUT1-F:

GATTGGCTCCTTCTCTGTGG; GLUT1-R: TCAAAGGACTTGCCCAGTTT; HIF1A-F: GAAGTGGCAACTGATGAGCA; HIF1A-R: GCGCGAACGACAAGAAA; HIF2A-F: ACGACCTGAAGATTGAAGTGATTG; HIF2A-R: GGTGCCAGTGTCTCCAAGTC; TGFA-F: GTGGCCCTGGCTGTCCTTAT; TGFA-R: CGGTTCTTCCCTTCAGGAGG; PPIA-F: ACTTCTTGGTATCCAGGCC; PPIA-R: ATTAGCCATGGTCAACCCC; VEGFA-F: GCCTTGCCTTGCTGCTCTACC; VEGFA-R: TGATGATTCTGCCCTCCTCCTTCTG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. VEGF protein and total protein (TP) in the presence of compounds **5-9**, compared to untreated 786-O cells.

Figure 2. qPCR results for compounds **5** and **6** in three different cell lines: 786-0 (HIF-2α only), RCC4 (both HIF-1α and HIF-2α) and engineered SK-RC-28 (HIF-1α only). The effect of treatment on the transcription of mRNA of five HIF-inducible genes was quantified relative to actin. Of the five mRNA transcripts measured, carbonic anhydrase 9 (CA-IX) is mediated by HIF-1α, transforming growth factor alpha (TGFα), glucose transporter 1 (GLUT1) and cyclin D1 (CCND1) are mediated by HIF-2α, and vascular endothelial growth factor A (VEGF-A) is mediated by both HIF-1α and HIF-2α. Bars represent the mean of two replicate experiments. *Indicates that mRNA was detected, but below quantifiable levels. **X** Indicates that mRNA was not detected. Results that exceeded 2-fold induction are indicated by values above the respective bars.

Table 1Activity of Compounds **1-12** in HIF-2 Induced Gene Expression Screen.

Compound	EC ₅₀ μ M
aaptamine (1)	>100
mimosamycin (2)	>100
xestoquinone (3)	>100
<i>O</i> -demethylrenierone (4)	>100
<i>N</i> -formyl-1,2-dihydrorenierone (5)	2.7 $\pm$ 0.8
puupehenone (6)	3.3 $\pm$ 0.4
variolin B (7)	0.7 $\pm$ 0.07
haliclonadamine (8)	14.8 $\pm$ 1.9
neopetroformyne A (9)	8.7 $\pm$ 1.1
12 α -acetoxy-24-methyl-24-oxoscalar-16-en-22,25-dial (10)	4.2 $\pm$ 0.3
22-acetoxy-16 β -hydroxy-24-methyl-24-oxoscalarano-25,12 β -lactone (11)	>100
heteronemin (12)	1.2 ^a

^a Considered a nuisance compound and only tested in a single replicate.

Table 2

Cellular toxicity observed for **5-9** with 786-0, RCC4 and engineered SK-RC-28 cell lines.

Comp. No.	Assay High Concentration [μ M]	786-0 IC ₅₀	RCC4 IC ₅₀	SK-RC-28 IC ₅₀
5	100	33.8 $\pm$ 3.1	26.3 $\pm$ 1.0	38.7 $\pm$ 1.7
6	100	17.7 $\pm$ 0.7	18.0 $\pm$ 0.7	15.5 $\pm$ 0.9
7	100	>100	8.8 $\pm$ 1.9	>100
8	100	15.1 $\pm$ 0.7	15.5 $\pm$ 1.2	16.1 $\pm$ 1.8
9	100	14.4 $\pm$ 0.7	27.5 $\pm$ 0.5	35.1 $\pm$ 0.4