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Spirohexenolide A Targets Human Macrophage Migration Inhibitory Factor (hMIF)

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Abstract

Spirohexenolides A and B comprise a unique family of spirotetronate natural products. We report on the identification of their binding to and modulation of human macrophage migration inhibitor factor (hMIF). Using an immunoaffinity–fluorescent labeling method, the properties of this interaction are detailed and evidence is provided that hMIF plays a key role in the cytostatic activity of the spirohexenolides.

We recently described the discovery of two bioactive polyketides, spirohexenolide A (**1a**) and B (**1b**) from the application of mutagenesis and clonal selection techniques to strains of *Streptomyces platensis*.¹ The structures of **1a** and **1b** (Scheme 1) are composed of four fused rings including a polyunsaturated spirotetronate and pyran core. The NCI–60 screening of **1a** (raw data deposited under NSC Number 746076) provided an average GI₅₀, TGI and LC₅₀ values of 2.16 μ M, 8.81 μ M and 33.2 μ M, respectively, over all cell lines. Enhanced activity was observed in leukemia cell lines and select colon cancer cell lines. Subsequent, COMPARE analyses suggested a unique mode of action (MOA).

We further investigated this activity by evaluating the uptake and localization of spirohexenolide A (**1a**) in live tumor cells. Using its native fluorescence, we observed that **1a** was readily taken up in HCT–116 tumor cells, with distinct lysosomal localization within 12 h.¹ This activity, combined with a unique activity profile from cancer cell line screening, prompted further investigations into the MOA of **1a**. To this end, we chose the use of a tagged-analogue with dual fluorescent and affinity reporter, or immunoaffinity–fluorescent (IAF) tag.² Based on the availability of centralized synthetic methods³ and milligram quantities of an associated anti-tag antibody,² we used the IAF dye, 7–dimethylamino–4–coumarinacetamide (Scheme 1), conjugated to **1a** as the probe for this MOA study.

RESULTS AND DISCUSSION

Given that spirohexenolide B (**1b**) displayed activity comparable to **1a**,¹ the C–8 hydroxyl group provided a logical position for tag attachment. We first prepared acetate **1c** (Scheme

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Author Contributions

MJK, JCH, BDJ and JLL conducted the natural product culturing and purification of the samples of spirohexenolide A; BDJ and JLL synthesized the analogs and probes; WLY and JLL conducted the biological studies including confocal microscopy, immunoprecipitation studies, target identification, and target validation efforts; JLL and MDB designed the experiments; WLY, JLL and MDB drafted the manuscript, and all authors participated in the editing process.

Supporting Information Available. Protein ID data, p-ratio data from the Akt phosphorylation assay, as well as ¹H and ¹³C for all new compounds associated with synthesis of probes **2** and **3** are available free of charge via the Internet at <http://pubs.asc.org>.

1) to evaluate the reactivity at this position. Compound **1a** proved unreactive to mild acetylation conditions, requiring treatment with acetyl chloride in a mixture of pyridine and dichloromethane to generate **1c**. Acylation of **1a** with 3-azidopropanoyl chloride (**4a**) affording azide **5a**, followed by installation of the IAF tag by Huisgen cycloaddition with alkyne **6a**,⁴ provided our first probe **2** in 14% yield from **1a** (Scheme 1). Samples of probe **2** were screened for activity using the MTT assay in HCT-116 cells as applied for **1a** and **1b**,¹ returning an activity (IC_{50} value of $21.2 \pm 1.5 \mu M$) comparable to that of **1a** (IC_{50} value of $36.0 \pm 5.1 \mu M$). HCT-116 cells were also chosen as they were one of the more responsive cell lines during the NCI-60 cell line screen with GI_{50} , TGI, and LC_{50} values of 0.79 nM, 5.01 μM and 100.0 μM , respectively. We also examined a second linker strategy. Slow addition of **1a** to a solution of succinyl chloride (**4b**) effected formation of a carboxylic acid intermediate **5b**, which was then coupled to an amine-terminal IAF dye **6b** to afford probe **3** in a 60% overall yield from **1a** (Scheme 1). Bioactivity assays of **3** returned an IC_{50} value of $6.0 \pm 0.4 \mu M$ in HCT-116 cells, showing activity greater than both probe **2** and parent compound **1a**.

With this activity data in hand, we next performed cellular imaging to expand the activity comparisons between probes **2–3** and the natural product **1a**. Fortunately, the native fluorescence of **1a** ($\lambda_{ex} = 435 \text{ nm}$, $\lambda_{em} = 466 \text{ nm}$)¹ was orthogonal to that of the IAF tag ($\lambda_{ex} = 360 \text{ nm}$, $\lambda_{em} = 470 \text{ nm}$), thereby allowing the use of two-color confocal microscopy.^{2,3} Here the blue and green channels are tuned to selectively resolve the IAF tag (blue) and natural product (green), respectively (Fig. 1a).⁵ The uptake and subcellular localization of probe **2** (Fig. 1b) and probe **3** (Fig. 1c) in HCT-116 tumor cells matches that of the natural product **1a** (Fig. 1a). Identical images were obtained in the blue and green channels for both probes (Figs. 1b–1c), indicating that the IAF-tags were metabolically stable. While both probes performed similarly, we focused our studies on probe **3** due to its more efficient preparation and increased bioactivity. With this data at hand, we were convinced that probe **3** provided a sufficient representation of the activity of **1a**.

We then turned to time-course imaging to further detail the movement of probe **3** over a 24 h treatment. Within 1 h of treatment, probe **3** was rapidly uptaken in HCT-116 cells, as blue fluorescence within the cell gradually increased compared to surrounding media (Fig. 1d). During this time, fluorescence did not localize and was observed throughout the cell membrane and cytoplasm. After 6 h, the probes localized within the lysosomes¹ and remained localized (Figs. 1b–1c) until the start of cell death, typically after 12 h. Dead cells displayed a combination of compacted chromatin and blebbing (Fig. 1e). These studies allowed us to characterize the functional modifications within cells treated with probe. With this data in hand, we turned our attention to screen for biomolecular targets by shifting to apply the affinity properties of the IAF tag in probe **3**.

Next, we screened for biomolecular targets using the affinity properties of probe **3**. We examined two procedures. The first applied lysates prepared from live HCT-116 cells that were treated with probe **3**, while the second evaluated whole HCT-116 cell lysates that were treated with probe **3** after lysate preparation. Samples of these cell lysates (lane L1, Fig. 2a) were then subjected to immunoprecipitation using agarose beads with a tethered anti-IAF antibody.² While washing and elution with an IAF competitor, 7-dimethylamino-4-coumarinacetic acid (lane L2, Fig. 2a), returned only traces of the anti-IAF antibody, elution with acidic glycine (lane L3, Fig. 2a) returned a 12 kDa protein from both lysate preparations.⁶ The negative control experiment (treating cells with the IAF tag alone) was also conducted and failed to return the 12 kDa protein. We also noted that the 12 kDa band was not fluorescent, suggesting that the protein was not isolated by covalent probe-modification, as seen in prior studies.⁵

Samples of this 12 kDa band were excised from SDS-PAGE gels and submitted for trypsin digestion and LC-MS/MS protein ID analysis, positively identifying it as human macrophage migration inhibitory factor (hMIF, Fig. 2b) with 54% peptide coverage (see the Supporting Information). The identified protein was verified by Western blot analysis using an hMIF monoclonal antibody⁷ against the immunoprecipitated fraction of both HCT-116 lysates (lanes L4-L5, Fig. 2c) and *E. coli* lysate expressing the recombinant hMIF⁸ (lanes L6-L7, Fig. 2c). We further confirmed hMIF as the target of spirohexenolide A (**1a**) by using isothermal titration calorimetry (ITC) to determine the affinity of recombinant hMIF to **1a** (Figs. 3a-3c). Over multiple analyses, ITC confirmed the binding of hMIF to **1a** with a K_d of $35.9 \pm 15.9 \mu\text{M}$.

Given that cellular uptake of hMIF has been well documented,⁹ we tested the uptake of hMIF in the presence of **1a**. Alexa Fluor 488 labeled-hMIF (Alexa-hMIF), was prepared containing ~0.3 molecules of dye per protein using established protocols¹⁰ and presented to HCT-116 cells. Alexa-hMIF was readily taken up by HCT-116 cells, with clear localization within the lysosomes after 2 h incubation at 37 °C (Fig. 1f). However, treatment with both Alexa-hMIF and spirohexenolide A (**1a**) inhibited uptake of Alexa-hMIF (Fig. 1g). Using filter sets that isolated the fluorescence from **1a** and Alexa 488 tag, we observed fluorescence from **1a** and the absence of Alexa emission in cells treated with **1a** prior to exposure to Alexa-hMIF, suggesting that **1a** targets a cellular uptake domain on the hMIF protein.

We also examined other assays used to evaluate binding to hMIF. To date, a tautomerase activity (Figs. 3d) has been used to screen compounds that inhibit the catalytic function of hMIF.¹¹ However, the tautomerase activity has been shown not be relevant to the cytokine function of hMIF. Application of this assay indicated spirohexenolide A (**1a**) and probe **3** not appear to block tautomerase activity (Fig. 3e), suggesting that the binding site for **1a** lies within a distinct binding site. This fact combined with the observation that **1a** attenuated the cellular uptake of hMIF suggest that spirohexenolide binds to a site critical to cell entry.¹²⁻¹³

We then examined the effects of **1a** on the downstream signaling. Recently, hMIF has been shown to regulate tumor cell proliferation through the PI3K/Akt pathway.¹⁴ Using this model, we observed that spirohexenolide A (**1a**) reduced hMIF-induced Akt phosphorylation. As shown in Fig. 2d, the addition of hMIF to the media surrounding NIH/3T3 fibroblasts results in the upregulation of Akt phosphorylation, as expected.¹⁴ The addition of spirohexenolide A (**1a**) reduced Akt phosphorylation towards levels that were observed in native cells (see the p-ratios provided in Fig. 2d). This observation not only provides further evidence validating the targeting of **1a** to hMIF, but also suggests that the inhibition of hMIF uptake by **1a** leads to a reduced tumor cell growth.¹⁵

The identification of hMIF as a target of spirohexenolide (**1a**) is in accordance with the established cellular uptake and lysosomal localization of hMIF in non-immune cells, as shown in Fig. 1.¹⁶ The fact that **1a** blocks the uptake and subcellular localization of Alexa-hMIF suggests that spirohexenolide A (**1a**) targets a domain on hMIF that plays a key role in its cellular transport.¹⁷ While there are distinct relationships between hMIF immune regulation¹⁸ and non-immune functions,¹⁹ the discovery of binding by a fluorescent natural product **1a**, not only provides a next step in the development of probes and inhibitors for hMIF,²⁰⁻¹² but also offers a new tool to examine hMIF regulation of tumorigenesis within a diversity of cellular and *in vivo* models.^{16,18,22} Interestingly, both hMIF and spirohexenolide A (**1a**) localize within the lysosomes of HCT-116 cells.¹⁶ This observation suggests that **1a** not only interferes the endocytosis of hMIF but may also play a role in its intracellular signaling by hMIF.¹⁶ This combined with fact that **1a** did not regulate the

catalytic activity of hMIF, as evident by other small molecules,^{20–21} suggests that **1a** may act through a unique binding site.

Remarkably, hMIF was one of the first cytokines to be described,²³ yet our understanding of this ubiquitously expressed protein remains incomplete. Ongoing studies evaluating hMIF as a drug target²⁴ provide an impetus to further evaluate the activity of **1a** and its interaction with hMIF. The unique structure of **1a** combined with its activity not only support further biological examination but also justify further synthetic chemical studies to identify the core structural motif responsible for this activity and to deliver optimized synthetic analogs for potential clinical evaluation.^{25–26} While **1a** did not display potent cytostatic or cytotoxic activities in tumor cells, the discovery of its binding to and targeting of hMIF offers strong potential for the advance of small molecule cytokine regulators.

EXPERIMENTAL SECTION

General Experimental Procedures

Unless otherwise noted, all reagents and chemical compounds were purchased from Alfa Aesar, GFS Chemicals, Strem Chemicals, Sigma–Aldrich or TCI and used without further purification. Spirohexenolide (**1**) was obtained from cultures of *S. platensis* using reported procedures.¹ High purity anhydrous dichloromethane and *N,N*-dimethylformamide were obtained by passing through a solvent column composed of dry activated A1 alumina. *N,N*-diisopropylethylamine was distilled from ninhydrin, dried (Na₂SO₄), and then redistilled from sodium. All reactions were performed under a positive pressure of dry Ar in oven-dried glass scintillation vials and stirred with a Teflon coated stir bar. Flash chromatography was performed on Silica Gel 60, 230–400 mesh (EMD Chemicals). TLC analyses were conducted on 250 μ m Silica Gel 60 F254 glass plates (EMD Chemicals). Visualization was achieved with UV light and stained with ceric ammonium molybdate. Yields and characterization data correspond to isolated, homogeneous materials. Unless otherwise noted all solvent mixtures are given in v:v ratios. NMR spectra were recorded on a Varian Mercury Plus 400 MHz, Jeol ECA 500 MHz, Bruker DMX 500 MHz or Varian VX 500 MHz (equipped with XSENS cold probe) spectrometer. FID files were processed using MestRenova version 6.0.2 (Mestrelab Research) and were referenced residual solvent peaks according to S. Budavari, M.J. O’Neil, A. Smith, P.E. Heckelman, The Merck Index, an Encyclopedia of Chemicals, Drugs, and Biologicals, Eleventh Edition, Merck Co., Inc. Rahway, NJ, 1989. Mass spectral data was collected by Dr. Yongxuan Su (UC San Diego). Electrospray (ESI) and atmospheric pressure chemical ionization (APCI) analysis was performed using a Finnigan LCQ Deca mass spectrometer, and fast atom bombardment (FAB) analysis was carried out using a Thermo Finnigan MAT 900 XL mass spectrometer. Spectral data and procedures are provided for all new compounds and copies of select spectra have been provided. For biological experiments common buffers were used.

Synthesis of Probe 2

3–Azidopropionic acid (360 mg, 3.10 mmol) was dissolved in thionyl chloride (2 mL), and the solution was heated to reflux for 12 h. The excess thionyl chloride was removed by distillation under reduced pressure to afford 3–azidopropionyl chloride, which was used without further purification. A solution of 3–azidopropionyl chloride (**4a**) (250 μ L of a 0.74 M solution in CH₂Cl₂, 0.185 mmol) was added to a mixture of spirohexenolide A (**1a**) (25 mg, 0.061 mmol), pyridine (74 μ L, 0.92 mmol) and 4–dimethylaminopyridine (1 mg, 0.006 mmol) in CH₂Cl₂ (2 mL). The reaction was monitored by TLC analysis, and additional acid chloride was added as needed until the starting material was no longer observed. Upon completion, the reaction was quenched by the addition of satd. NaHCO₃ (10 mL) and the aqueous layer was extracted twice with CH₂Cl₂ (2 $\times$ 10 mL). The combined organic layers

were washed with brine (10 mL), dried over Na₂SO₄, and then filtered through a short plug of silica gel with 5:4:1 EtOAc/CH₂Cl₂/Et₃N. The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography (1:1 hexanes/EtOAc) to provide the azide **5a** (5 mg, 16%). Azide **5a**: ¹H NMR (CDCl₃, 400 MHz) δ 7.46 (d, *J* = 10.0 Hz, 1H), 7.01 (d, *J* = 10.0 Hz, 1H), 5.72 (d, *J* = 15.6 Hz, 1H), 5.65 (d, *J* = 8.8 Hz, 1H), 5.55 (ddd, *J* = 15.6, 10.4, 5.2 Hz, 1H), 5.51–5.45 (m, 1H), 5.28 (s, 1H), 5.08 (s, 1H), 4.79 (d, *J* = 12.7 Hz, 1H), 4.61 (d, *J* = 12.7 Hz, 1H), 3.58 (t, *J* = 6.3 Hz, 2H), 2.70–2.62 (m, 1H), 2.60 (t, *J* = 6.3 Hz, 2H), 2.44–2.30 (m, 2H), 2.24–2.13 (m, 1H), 1.77 (s, 3H), 1.76 (s, 3H), 1.71 (d, *J* = 13.7 Hz, 1H), 1.33 (d, *J* = 7.2 Hz, 3H), 1.19 (s, 3H). Azide **5a** was used immediately after preparation and hence ¹³C NMR data was not collected. A mixture of the azide **5a** (5 mg, 0.010 mmol) and alkyne-terminal IAF tag **6a** (2.4 mg, 0.011 mmol) was dissolved in a mixture of CH₂Cl₂ (80 μL) and H₂O (80 μL). Solutions of sodium ascorbate (10 μL of an 87 g/L solution in H₂O, 0.87 mg, 0.0043 mmol) and CuSO₄ (10 μL of a 23 g/L solution in H₂O, 0.23 mg, 0.0014 mmol) were added to the biphasic mixture. The mixture was stirred at room temperature for 1 h, and the layers were separated. The aqueous phase was extracted with EtOAc (2 × 10 mL), and the combined organic layers were dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (1:1 hexanes/EtOAc to 5:1 EtOAc/MeOH) to provide the probe **2** (6 mg, 86%). Probe **2**: ¹H NMR (CD₃OD, 400 MHz) δ 7.79 (s, 1H), 7.50 (d, 1H, *J* = 9.0 Hz), 7.31 (d, 1H, *J* = 10.0 Hz), 7.25 (d, 1H, *J* = 10.0 Hz), 6.74 (dd, 1H, *J* = 9.0, 2.6 Hz), 6.56 (d, 1H, *J* = 2.6 Hz), 6.01 (s, 1H), 5.72–5.60 (m, 3H), 5.54–5.45 (m, 1H), 5.31 (s, 1H), 4.66–4.56 (m, 4H), 4.46 (d, 1H, *J* = 15.3 Hz), 4.42 (d, 1H, *J* = 15.3 Hz), 3.70 (s, 2H), 3.08 (s, 6H), 2.97 (td, 2H, *J* = 6.2, 2.3 Hz), 2.57–2.07 (m, 4H), 1.77 (s, 3H), 1.75 (s, 3H), 1.70 (d, 1H, *J* = 14.0 Hz), 1.34 (d, 3H, *J* = 7.2 Hz), 1.16 (s, 3H); ¹³C NMR (CD₃OD, 125 MHz) δ 198.2, 171.4, 171.2, 168.7, 164.6, 157.5, 155.0, 152.9, 144.8, 142.3, 137.8, 137.3, 135.5, 134.6, 129.5, 129.2, 127.1, 124.9, 122.2, 120.5, 110.7, 110.6, 110.0, 101.1, 99.0, 90.5, 72.9, 65.8, 49.7, 47.1, 45.5, 40.5, 40.1, 39.7, 36.0, 35.6, 34.8, 34.4, 27.7, 22.2, 20.3, 14.7; ESI-MS *m/z* 790.17 [M+H]⁺, 812.27 [M+Na]⁺; HR-ESI-MS *m/z* calcd. for C₄₄H₄₇N₅O₉ [M+H]⁺: 790.3447, found 790.3461.

Succinate half-ester **5b**

A solution of spirohexenolide A (**1a**) (11.2 mg, 0.027 mmol) in CH₂Cl₂ (2 mL) was added drop wise over 30 min to a second flask containing succinyl chloride **4b** (15.1 μL, 0.14 mmol) and *N,N*-diisopropylethylamine (47.8 μL, 0.27 mmol) in CH₂Cl₂ (5 mL) at –20 °C. The reaction mixture was stirred at –20 °C for 1 h, diluted with satd. NaHCO₃ (10 mL). The resulting mixture was extracted with CH₂Cl₂ (3 × 25 mL). The combined organic layers were washed with brine (10 mL), dried (Na₂SO₄), and the solvent removed by rotary evaporation. Purification by flash chromatography (3:1 hexanes/EtOAc to EtOAc) provided 10.8 mg (77%) of acid **5b** as a yellow wax. Succinate half-ester **5b**: ¹H NMR (500 MHz, CDCl₃) δ 7.47 (d, *J* = 9.9 Hz, 1H), 7.00 (d, *J* = 9.7 Hz, 1H), 5.72 (d, *J* = 15.6 Hz, 1H), 5.65 (d, *J* = 8.6 Hz, 1H), 5.55 (ddd, *J* = 5.3, 10.5, 15.5 Hz, 1H), 5.45 (m, 1H), 5.29 (s, 1H), 5.09 (s, 1H), 4.78 (d, *J* = 12.7 Hz, 1H), 4.60 (d, *J* = 11.4 Hz, 1H), 2.75–2.58 (m, 4H), 2.46–2.32 (m, 3H), 2.20 (d, *J* = 11.0 Hz, 1H), 2.16 (d, *J* = 12.1 Hz, 1H), 1.78 (d, *J* = 0.9 Hz, 3H), 1.77 (s, 3H), 1.35 (d, *J* = 9.9 Hz, 1H), 1.20 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 195.7, 171.4, 169.0, 165.1, 141.6, 141.3, 136.0, 135.1, 134.1, 133.4, 127.8, 127.7, 120.7, 119.4, 100.9, 89.1, 71.1, 64.5, 44.1, 38.8, 33.4, 33.2, 28.8, 27.0, 21.8, 19.5, 13.9; HR-ESI-MS *m/z* calcd. for C₂₉H₃₃O₈ [M+H]⁺: 509.2097, found 509.2109.

Synthesis of Probe **3**

Succinate half-ester **5b** (3.2 mg, 0.0063 mmol) and amine-terminal IAF tag **6b** (3.3 mg, 0.0094 mmol) were dissolved in DMF (0.8 mL). *N,N*-diisopropylethylamine (8.8 μg, 0.051 mmol) and HATU (9.6 mg, 0.025 mmol) were added sequentially at rt. After stirring the

mixture at rt for 15 h, the solvent removed by rotary evaporation and the crude mixture was subjected to purification by flash chromatography (1:1 hexanes/EtOAc to 5:1 EtOAc/MeOH) afford 4.1 mg (78%) of probe **3** as a yellow wax. Probe **3**: ^1H NMR (500 MHz, CDCl_3) δ 8.77 (d, J = 4.2 Hz, 1H), 8.52 (d, J = 8.3 Hz, 1H), 7.58 (dd, J = 4.4, 8.3 Hz, 1H), 7.31 (d, J = 10.0 Hz, 1H), 7.23 (d, J = 10.0 Hz, 1H), 5.84 (d, J = 8.4 Hz, 1H), 5.67 (d, J = 3.3 Hz, 1H), 5.30 (s, 1H), 5.10 (bs, 1H), 4.99 (s, 1H), 4.89 (d, J = 12.6 Hz, 1H), 4.70 (m, 1H), 4.59 (d, J = 12.5 Hz, 1H), 4.21–4.08 (m, 2H), 3.81–3.52 (m, 2H), 3.32 (s, 2H), 3.08 (s, 6H), 2.59–2.53 (m, 1H), 2.45–2.33 (m, 1H), 2.32–2.25 (m, 2H), 2.04 (m, 4H), 1.74 (s, 3H), 1.73 (s, 3H), 1.65 (d, J = 14.3 Hz, 1H), 1.33 (d, J = 7.4 Hz, 3H), 1.12 (s, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 198.0, 171.2, 171.0, 171.0, 168.5, 165.2, 157.2, 154.8, 152.7, 146.0, 144.7, 142.1, 137.6, 137.1, 135.3, 134.4, 129.4, 129.0, 127.0, 122.0, 120.3, 110.6, 110.4, 109.8, 100.9, 98.9, 90.3, 72.6, 65.6, 49.8, 46.9, 45.3, 40.3, 39.9, 39.5, 35.8, 35.4, 34.7, 34.1, 27.5, 22.0, 20.1, 14.5; HR-ESI-MS m/z calcd. for $\text{C}_{48}\text{H}_{58}\text{O}_{10}$ $[\text{M}+\text{H}]^+$: 836.4044, found 836.4069.

Cell culture

HCT-116 cells (ATCC CCL-247) were propagated in McCoy's SA media (GIBCO-BRL, Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin (GIBCO-BRL, Invitrogen). NIH/3T3 fibroblast cells were cultured in DMEM containing 10% FCS. All cells were incubated at 37 °C in a 5% CO_2 atmosphere. For routine passage, cells were split 1:3 to 1:6 when they reached confluence, generally every 1–3 days. HCT-116 cells were chosen for this study due to our prior experience with their use in the IAF procedure.^{2,5,6}

Activity assays

A conventional MTT assay protocol was used.²⁷ Data presented was collected in triplicate by screening with multiple 5–6 concentrations from 0 to 0.035 mg/mL. All assays were repeated until the deviation was within 5% error.

Uptake and subcellular localization studies

Cell uptake and localization studies were conducted by treating cells at a density of 10^6 cells/cm² in a 35 mm glass-bottom dish (MatTek Corporation). Each compound was added as a 10 $\times$ stock in media such that the net DMSO content remained under 0.5%. Studies began by conducting time-lapse microscopy on a Nikon TE2000. This was conducted by incubating the cells at 37 °C under a 5% CO_2 atmosphere with a given concentration of probe and imaging over the course of 24 h. In each experiment, greater than 90% of the fluorescence (from compound **1a** or probes **2** or **3**) absorbed within the cells within the first hour and therefore washing was not required. Images were collected at 10 – 15 min intervals with a 500 μs exposure to ensure minimal phototoxicity to the cells or photobleaching of the probes. Select time points from these studies were then subjected to confocal microscopy. Confocal fluorescent images were collected on a Leica DMI6000 inverted confocal microscope with a Yokogawa spinning disk confocal head, ORCA-ER High Resolution B&W Cooled CCD camera (6.45 μm /pixel at 1 $\times$), Plan-Apochromat[®] 40 $\times$ /1.25 na and 63 $\times$ /1.40 na objective (Zeiss). After imaging the cells were fixed by treatment with 500 μL of 2% formaldehyde with 0.2% glutaraldehyde in Dulbecco's Phosphate-Buffered Saline (D-PBS) (2.7 mM KCl, 1.6 mM KH_2PO_4 , 137.9 mM NaCl, 8.1 mM Na_2HPO_4) pH 7.2 for 30 min followed by washing three times with D-PBS pH 7.2. The fixed cells were then costained by first fixing with SYTO[®] 60 red (Invitrogen) for the nucleus, LysoTracker[®] Red DND-99 (Invitrogen) for lysosomes, BODIPY[®] TR glibenclamide (Invitrogen) for the endoplasmic reticulum, or MitoTracker[®] Red 580 (Invitrogen) for mitochondria. This staining was conducted by slow addition of each stain, monitoring the uptake on a Nikon

TE2000 microscope. The cells were washed twice with D–PBS pH 7.2 and mounted with Vectashield® Mounting Media (Vector Laboratories). The resulting samples were then imaged confocally using multiple color channels. Images from these studies are presented in Figs. 1a–1c.

Cell lysate preparation

Cells were grown at a density of 10^6 cells/cm² on a 75 cm² culture flask in fresh media. The media was removed and the cells were washed three times with D–PBS pH 7.2 buffer cooled to 4 °C. For the live cell labeling, 15 µg of probe **3** was added into 15 mL fresh media. This solution was added to HCT–116 cells at a density of 10^6 cells/cm² growing on 75 cm² culture flask at 37 °C in a 5% CO₂ atmosphere. After 4 h incubation, the labeling media was discarded and cells were washed three times with D–PBS pH 7.2 buffer cooled to 4 °C. The resulting cells were either flash frozen in their flasks and stored at –80 °C or used immediately. To prepare cell lysates, the cells were scraped from each flask and suspended in 1–2 mL of lysis buffer (D–PBS with 5 mM EDTA, 1% NP–40) with 50 µL of mammalian protease inhibitor cocktail P8340 (Sigma Aldrich). The cell suspension was then passed through 27.5 Gauge needle at 5–10 times with syringe, centrifuged, and filtered through Corning 0.45 µm membrane. All the lysate preparation procedures were performed on ice. The protein concentration of each cell lysate was determined by Bradford's assay. When necessary, lysates were concentrated using a Millipore 3 kDa cutoff spin filter.

Immunoprecipitation of hMIF

Samples of HCT–116 cell lysate (~2 mg/mL in net protein) or *E. coli* protein lysate (1–2 mg in net protein) were precipitated with XRI–TF35 mAb–conjugated Affi–Gel 10 resin (50 µL). A 1 µg sample of probe **3** was added into the precipitation mixture. The immunoprecipitation mixture was incubated with agitation by Labquake rotator (ThermoScientific) for 4 h at 4 °C. The resin was then washed three times with ice–cold wash buffer (D–PBS pH 7.2, 5 mM EDTA, 1% NP–40 and 0.1 % SDS) and one time with D–PBS. The bound protein was eluted twice from the resin with 150 µL D–PBS containing 1 mM IAF–tag or 0.1 M glycine–HCl (pH 2.5) for 10 min at 4 °C. After neutralization with 1M Tris buffer (pH 9.5, only for glycine elute), the eluate protein was concentrated with Millipore 3 kDa centrifugal membrane. The eluted proteins were analyzed by NuPAGE 4–12% Bis–Tris gels (Invitrogen) and stained with Coomassie blue R–250 (EMD Chemicals) or Silver Quest staining kit (Invitrogen). Images of gels from these studies are provided in Fig. 2a.

Mass spectral protein identification

Samples of the Coomassie blue stained bands were excised from the polyacrylamide gel and submitted to LC–MS/MS Protein–ID analysis conducted by the Biomolecular and Proteomics Mass Spectrometry Facility at UC San Diego. General procedure was using in–gel trypsin digest and then the trypsin–digested peptides were analyzed by liquid chromatography LC–MS/MS with electrospray ionization. All nanospray ionization experiments were performed by using a QSTAR–Elite hybrid mass spectrometer (AB/MDS Sciex) interfaced to a nanoscale reversed–phase high–pressure liquid chromatograph (Tempo) using a 10 cm–180 ID glass capillary packed with 5–µm C₁₈ Zorbax™ beads (Agilent). The buffer compositions were as follows. Buffer A was composed of 98% H₂O, 2% CH₃CN, 0.2% formic acid, and 0.005% trifluoroacetic acid (TFA); buffer B was composed of 100% CH₃CN, 0.2% formic acid, and 0.005% TFA. Peptides were eluted from the C₁₈ column into the mass spectrometer using a linear gradient of 5–60% Buffer B over 60 min at 400 µL/min. LC–MS/MS data were acquired in a data–dependent fashion by selecting the 4 most intense peaks with charge state of 2 to 4 that exceeds 20 counts, with

exclusion of former target ions set to “360 seconds” and the mass tolerance for exclusion set to 100 ppm. Time-of-flight MS were acquired at m/z 400 to 1600 Da for 1 s with 12 time bins to sum. MS/MS data were acquired from m/z 50 to 2,000 Da by using enhance all and 24 time bins to sum, dynamic background subtract, automatic collision energy, and automatic MS/MS accumulation with the fragment intensity multiplier set to 6 and maximum accumulation set to 2 s before returning to the survey scan. Peptide identifications were made using paragon algorithm executed in Protein Pilot 2.0 software (Life Technologies). Further data is provided in Fig. 2b and in the Supporting Information.

Western blotting studies

Samples of immunoprecipitation elutate and 10 μ g of whole *E. coli* lysate were separated by NuPAGE 4–12% Bis–Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare). The blotting membrane was blocked with 5% non-fat milk and hybridized with mouse–anti–hMIF monoclonal antibody (M01) clone 2A10–4D3 (Abnova). The membrane was incubated with alkaline phosphatase–conjugated goat–anti–mouse secondary antibody #69266 (Novagen), washed, and stained with BCIP/NBT color development substrate S3771 (Promega). Blots from representative experiments are provided in Fig. 2c.

Recombinant hMIF preparation

The expression and purification of hMIF were followed the procedure provided by Prof. Richard Bucala.²⁸ Recombinant hMIF was expressed in *E. coli* strain BL–21 (DE3) using the pET–IIIb plasmid as the expression vector. Briefly, a 1 L culture was grown in LB medium to an OD₆₀₀ of 0.5 and induced by addition of isopropyl– β –D–thiogalactopyranoside to a final concentration of 0.4 mM. After 4 h induction, the cells were harvested, resuspended in 40 mL of 20 mM Tris containing 20 mM NaCl pH 7.4, and lysed using a French press. Cell debris was removed by centrifugation at 30,000 $\times$ g and filtration through the Corning 0.45 μ m membrane. The soluble lysate was applied to HiTrapTM SP FF and HiTrap Q FF columns (GE Healthcare). Recombinant hMIF was not retained by either column and appeared in the flow-through fraction. The purified hMIF was then concentrated with Millipore 3 kDa centrifugal filter and dialyzed against 50 mM potassium phosphate buffer pH 7.4. This procedure afforded ~25 mg of >90% pure MIF (SDS–PAGE gel analysis) per liter of growth.

Isothermal Titration Calorimetry (ITC) studies

The affinity of spirohexenolide A (**1a**) to recombinant hMIF was determined using a MicroCal VP–ITC MicroCalorimeter. After careful optimization, we found that titration of 1.25 mM purified recombinant hMIF into a chamber set at 30 °C containing 1.5 mL of 0.5 mM **1a** in 50 mM potassium phosphate buffer pH 7.4 containing 2% DMSO, as shown in Fig. 3a, provided the optimum response. Calibration with controls confirmed that this response was not due to protein oligomerization but rather was induced by the presence of compound **1a**. Data analysis was performed with Origin 5.0 software (Microcal). The ligand **1a** was quickly saturated after 6–7 injections of hMIF. This calorimetry data lead to the estimation of the dissociation constant of 35.9 ± 15.9 μ M while comparing to the control experiment of titrating to the buffer alone (Fig. 3c).

Dopachrome tautomerase assays

The dopachrome tautomerase assays were conducted using an established protocol.²⁹ Briefly, a sample of 0.24 mM 3,4–dihydroxyphenylalanine (DOPA) was mixed with 0.48 mM sodium periodate in 25 mM potassium phosphate buffer (pH 7.2) and incubated at rt for 5 min. This procedure converted DOPA to dopachrome, which was then tautomerized

spontaneously (slower) or by adding hMIF (faster) (Fig. 3d). A 100 μ L aliquot of the dopachrome mixture was added to wells of a 96-wells microtiter plate containing an 100 μ L of either: (a) buffer (control), (b) 10 μ M hMIF and 100 μ M **1a**, (c) 10 μ M hMIF and 100 μ M **3**, (d) 100 μ M **1a** (control) or (e) 10 μ M hMIF (negative control). All the reaction mixtures on one plate were immediately monitored their reaction kinetics by reading the absorbance at 485 nm on a HTS 7000 Bio Assay Reader (Perkin Elmer). Exemplary plots of this assay are provided in Fig. 3d.

Alexa 488–MIF preparation

The procedure of labeling recombinant hMIF with Alexa Fluor[®] 488 dye labeling kit (Invitrogen) was followed the manufacture's protocol. A sample of 0.1 mg recombinant hMIF in 0.1 M NaHCO₃ pH 8.3 was added to a vial of Alexa Fluor 488 dye. After incubation the solution for 1 h at rt, unreacted dye and salt were removed by spin dialysis on a 3 kDa centrifugal filter (Millipore). The labeled Alexa–hMIF was stored in 50 mM potassium phosphate buffer pH 7.4 at 4 °C. The extent of labeling was determined spectrophotometrically by evaluating the absorbance at 280 nm and 494 nm and calculating the percentage of dye per hMIF molecule. This protein was used to develop the images presented in Figs. 1g–1h.

Akt phosphorylation assay

Akt phosphorylation assays were performed as described previously.³⁰ NIH/3T3 fibroblast cells were cultured to a growth of 10⁶ cells/well in 12-well cell culture plate using DMEM containing 10% FCS medium for 24 h. Then cells were starved for 24 h with DMEM medium without FCS. After starvation, a solution of hMIF (1 μ g) plus DMSO (control) or hMIF plus **1a** (0.1 μ g) were added in 100 μ L of the same media to an ascribed well. The cells were incubated 30 min, washed with D–PBS, and lysed in 100 μ L ice cold lysis buffer containing D–PBS (pH 7.2), 5mM EDTA, 1% Nonidet P–40, 0.1% SDS, 1% mammalian protease inhibitor cocktail P8340 (Sigma–Aldrich), and 1% phosphatase inhibitor cocktail P5726 (Sigma–Aldrich). Protein extracts were passed through Amicon Ultrafree 0.22 μ m centrifugal filter unit (Millipore) to remove cell debris. Filtered cell lysates were separated via 12% SDS–PAGE and transferred to PVDF membranes (GE Healthcare). The membrane was probed with anti–phospho–Akt (Ser₄₇₃) (4058), Akt (4691) and anti–GAPDH (2118) antibodies (Cell Signaling Technology), followed by alkaline phosphatase–conjugated anti–rabbit IgG S3731 (Promega) and BCIP/NBT color development substrate S3771 (Promega). A blot from three independent studies is presented in Fig. 2d. Densitometric analysis was performed using ImageJ 1.44p (National Institutes of Health, USA). The ratio of phosphorylated (p–ratio) Akt was calculated with dividing the intensity of p–Akt by Akt after normalization of the GAPDH intensity. To compare p–ratio between different experiments, the p–ratio of endogenous Akt phosphorylation (without any treatment) was set as 1, and other p–ratio from different treatments were adjusted as follows. The original p–ratio of three independent experiments are presented in Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fluorescence microscopy defines the cellular properties of the spirohexenolides. Confocal fluorescent images depicting live HCT-116 cells incubated in media at 37 °C containing: a) 10 μ M spirohexenolide A (**1a**) for 6 h; b) 10 μ M probe **2** for 6 h; c) 10 μ M probe **3** for 6 h; d) 10 μ M probe **3** for 1 h; or e) 10 μ M probe **3** for 24 h. Cells were treated for the ascribed period, washed twice with media, and imaged live. f) Comparative uptake of Alexa-hMIF and **1a**. HCT-116 cells treated with 1.5 μ M Alexa-hMIF. g) HCT-116 cells treated with premixed 30 μ M spirohexenolide A (**1a**) and 1.5 μ M Alexa-hMIF. Cells were treated at 37 °C for 2 h, then washed and fixed with formaldehyde-glutaraldehyde. Two-color fluorescence was collected in the spirohexenolide channel (excitation at 375 nm with emission filtered at 448 ± 20 nm) and Alexa channel (excitation at 488 nm followed by emission filtered at 524 ± 40 nm). Bars indicate 10 μ m.

Figure 2.

Target identification and validation studies. a) Anti-IAF immunoprecipitation studies identify a ~12 kDa band. Lane L1, L2 and L3 depict HCT-116 cell lysate, an IAF dye elution (bands correspond to fragments of the anti-IAF mAb), and glycine elution, respectively. The ~12 kDa band (arrowhead) was identified in the glycine elution. b) Trypsin-digest LC-MS/MS analysis identified three peptides that overlapped with the hMIF protein (shown in red). c) Western blot analysis using a mouse anti-MIF mAb confirmed the presence of hMIF in the immunoprecipitated fraction of HCT-116 cell lysate (lanes L4–L5). Comparable IP results were obtained when isolating hMIF from lysate of *E. coli* expressing recombinant hMIF (lane L7), as compared with non-hMIF-expressing BL21 *E. coli* (lane L6). d) The binding of spirohexenolide A (**1a**) to hMIF regulates the PI3K/Akt pathway. Using an established model,¹⁴ **1a** was shown to counteract hMIF-induced phosphorylation of Akt in NIH/3T3 cells. The adjusted p-ratio indicates the relative ratio of phosphorylation (lanes L8–L12). This data was collected from multiple repetitions and are standardized against normal cells in lane L8). Raw data is provided in Table S1.

Figure 3.

Binding and activity analyses. a) Evaluation of binding by means of ITC analysis. A sample of 1.25 mM purified recombinant hMIF was loaded on the syringe to titrate 1.5 mL of 0.5 mM **1a** in 50 mM potassium phosphate buffer pH 7.4 containing 2% DMSO. The reaction cell temperature was set at 30 °C. b) Data analysis was performed with Origin 5.0 software using sigmoid function fitness. The slope was 17.27 ± 7.64 . c) A control experiment of 1.25 mM purified recombinant hMIF titrating 1.5 mL of 50 mM potassium phosphate buffer pH 7.4 containing 2% DMSO was overlaid. Dopachrome tautomerization assay. d) A scheme denoting the generation and hMIF tautomerization of dopachrome. e) A reaction mixture of 0.24 mM DOPA and 0.48 mM NaIO_4 in 25 mM potassium phosphate buffer (pH 7.2) was added into the premixed 10 μM hMIF with or without **1a** on the 96-wells microtiter plate. All the reaction mixtures on one plate were immediately monitored their reaction kinetics by

reading the absorbance at 485 nm. Spirohexenolide A (**1a**) has modest absorbance at 485 nm. The activity of 10 μ M hMIF (red dashed line) is not reduced by the addition of 100 μ M **1a** (blue dashed line) or 100 μ M probe **3** (black dotted line) when compared to buffer (purple dotted line) or 100 μ M **1a** (solid green line).

Scheme 1.

Structures of the spirohexenolide A (**1a**), spirohexenolide B (**1b**), acetate **1c**, probe **2** and probe **3**. Spirohexenolide A (**1a**) is converted to probes **2** and **3** through a two-step process.