Journal of Medicinal Chemistry

Article

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Synthesis and Structure–Activity Relationship Study of Chemical Probes as Hypoxia Induced Factor-1α/Malate Dehydrogenase 2 Inhibitors

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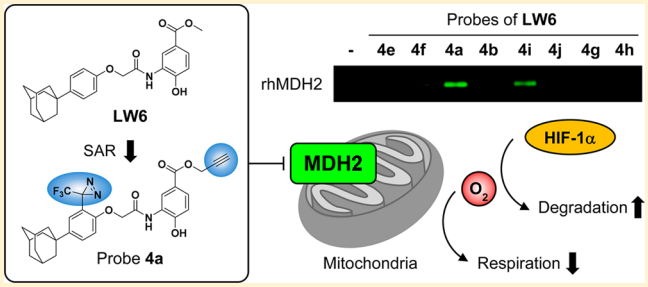
Supporting Information

ABSTRACT: A structure–activity relationship study of hypoxia inducible factor-1α inhibitor 3-aminobenzoic acid-based chemical probes, which were previously identified to bind to mitochondrial malate dehydrogenase 2, was performed to provide a better understanding of the pharmacological effects of LW6 and its relation to hypoxia inducible factor-1α (HIF-1α) and malate dehydrogenase 2 (MDH2). A variety of multifunctional probes including the benzophenone or the trifluoromethyl diazirine for photoaffinity labeling and click reaction were prepared and evaluated for their biological activity using a cell-based HRE-luciferase assay as well as a MDH2 assay in human colorectal cancer HCT116 cells. Among them, the diazirine probe **4a** showed strong inhibitory activity against both HIF-1α and MDH2. Significantly, the inhibitory effect of the probes on HIF-1α activity was consistent with that of the MDH2 enzyme assay, which was further confirmed by the effect on *in vitro* binding activity to recombinant human MDH2, oxygen consumption, ATP production, and AMP activated protein kinase (AMPK) activation. Competitive binding modes of LW6 and probe **4a** to MDH2 were also demonstrated.



INTRODUCTION

Target identification of drugs is crucial for understanding their molecular modes of action and to search for clinical biomarkers in therapeutics. The target identification process of bioactive small molecules has become a bottleneck in drug discovery, since many novel therapeutic agents have been developed by cell-based phenotypic screening, not by target-based *in vitro* assay. Thus far, various chemical and biological technologies, such as affinity chromatography, fluorescent imaging, proteomic analysis, phase display biopanning, drug affinity responsive target stability, and phenotype analysis using mutated or overexpressed yeast and zebrafish, have been developed.[1,2] On the basis of recent significant developments in fluorescent imaging of wide spectra and real-time observation of living cells, chemical biology approach-driven probes have attracted much attention in the field of drug discovery. Chemical probes of small, bioactive molecules that can react rapidly with target proteins are directly applicable for target identification in most living cells without genetic manipulation. Especially, chemical probes for biotin–streptavidin pull-down assay, activity-based assay (ABPs), photoaffinity labeling, and fluorescence imaging are the most frequently employed for target identification studies of small molecules.[3,4]

Generally, affinity chromatography using biotinylated drugs and the subsequent proteomic analysis of isolated proteins is considered to be a common and powerful method. Although the biotinylation of active compounds is relatively simple and many drug targets have been successfully identified,[1,2] this method still has limitations due to the nonspecific protein binding of hydrophobic molecules.

Of note, photoaffinity labeling can be applicable for covalent linkages between target proteins and small molecules, resulting in the selective isolation of target proteins.[5] Click chemistry for fluorescent imaging is also useful when visualizing the intracellular localization of the small molecules and to track target proteins.[5] On the basis of these techniques, a differential fluorescence approach in two-dimensional gel electrophoresis (2-DE) was employed for target identification using multifunctional chemical probes containing functionalities for click reaction and photolabeling.[5−8] Figure 1 represents the structures of the multifunctional chemical probes for target identification, including **1a–b**, **2**, **3**, and **4a–b**.[5−8]

Hypoxia inducible factor 1 (HIF-1) is a heterodimeric transcription factor that functions as a mast regulator in the

Received: August 13, 2014
Published: October 30, 2014

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522–9538

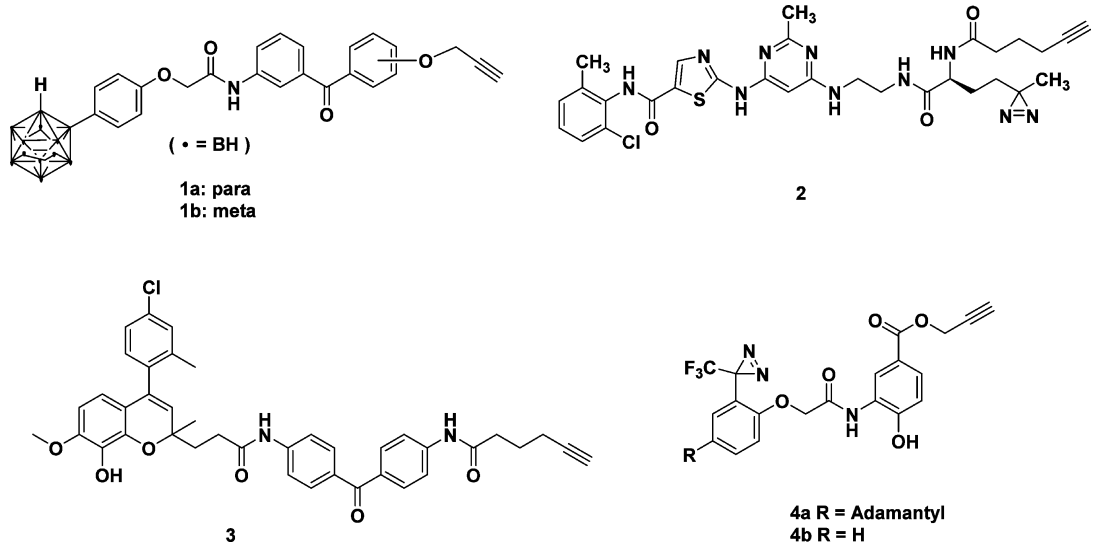
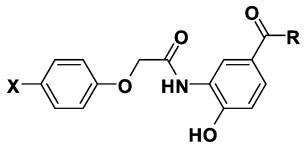


Figure 1. Chemical probes used for target identification.

Table 1. *In Vitro* HIF-1α and MDH2 Inhibitory Activity of LW6 and Acetylene Chemical Probes 4e and 4f[a]



Cmpd	Structure		HRE Luc IC_{50}(μM)	MDH2 IC_{50} (μM)
	X	R		
LW6		OCH_3	4.4 ± 1.1	6.3 ± 0.6
4e			4.3 ± 1.3	7.1 ± 0.8
4f	H		>30	>20

[a]Values are the means of three experiments.

response of tumor growth to hypoxia.[9] A high level of HIF-1α subunit correlates with aggressive tumor growth, resistance to radiation and chemotherapy, and poor clinical outcomes.[10−13] We previously reported the synthesis and biological evaluation of novel HIF-1α inhibitors.[14−18] LW6, an aryloxyacetylamino benzoic acid analogue, exhibited a potent inhibitory effect on HIF-1α accumulation and target gene expression under hypoxia (Table 1).[14,19]

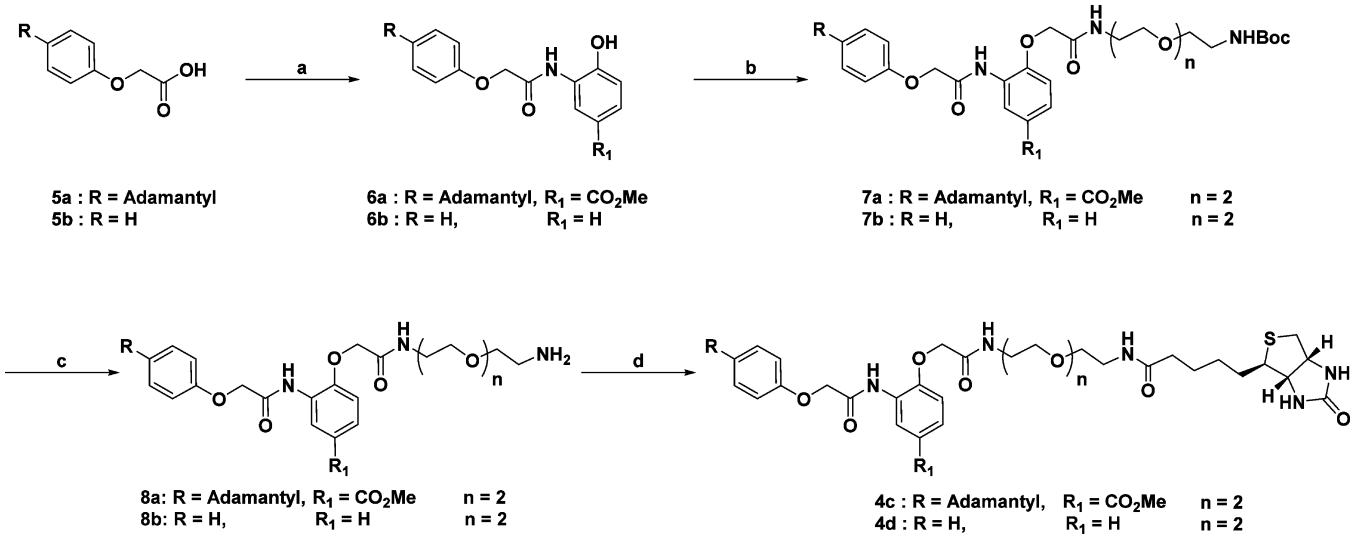
Since the regulation pathway of HIF-1α expression is complicated,[20] it is challenging to understand the mechanism of LW6 without identifying its direct target protein. Recently, we have demonstrated that the target molecule of LW6 could be mitochondrial malate dehydrogenase 2 (MDH2) in the TCA cycle using chemical probes.[7] Herein, we report the synthesis and biological evaluation of a series of multifunctional chemical probes containing functional groups including acetylene, benzophenone, or trifluoromethyl diazirine, at various sites of the parent compound LW6. The relevance of HIF-1α inhibition to MDH2 inhibition and oxygen consumption was also investigated using the probes to understand their structure−activity relationships. In addition, ATP production, AMPK signaling, and MDH2 kinetic assay were carried out for the representative compounds.

RESULTS AND DISCUSSION

Chemistry. A series of chemical probes of LW6 were synthesized as shown in Schemes 1−6. Chemical probes **4c** and **4d** carrying structural units, such as biotin reporter groups, could be readily prepared from **6a** or **6b**, whose syntheses were previously reported from **5a** and **5b**.[14] Compound **6a** was then O-alkylated with (2-{2-[2-(2-chloro-acetylamino)-ethoxy]-ethoxy}-ethyl)-carbamic acid *tert*-butyl ester under basic conditions, to yield an adamantyl derivative with linker **7a**. This derivative was further subjected for Boc deprotection to afford **8a**. Biotinylation of **8a** with (+)-biotin *N*-hydroxysuccinimide ester resulted in the desired biotin probe **4c**. The

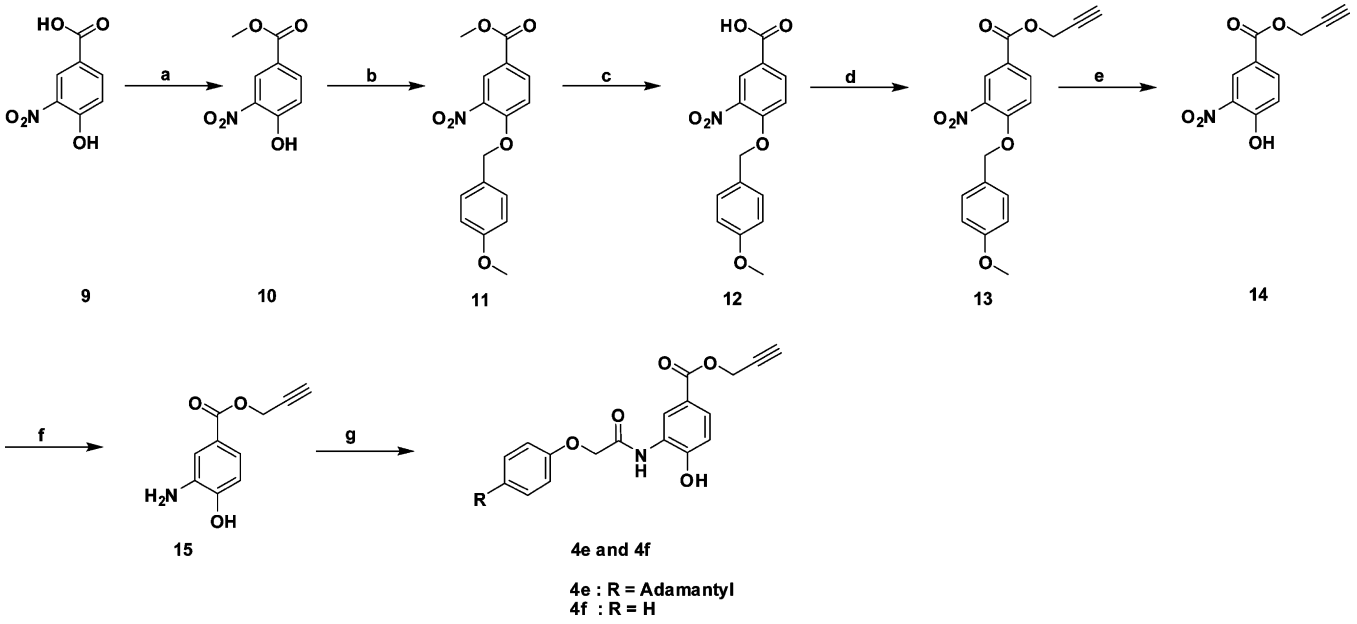
dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

Scheme 1. Synthesis of Biotin Chemical Probes 4c and 4d[a]



[a]Reagents and Conditions: (a) Methyl-3-amino-4-hydroxybenzoate, HBTU, DIPEA, DMF, rt, 12 h for **6a**, 78.9%; 2-aminophenol, HBTU, DIPEA, DMF, rt, 12 h for **6b**, 76%; (b) K_2CO_3, Cs_2CO_3, KI, acetone/DMF, (2-{2-[2-(2-chloro-acetylamino)-ethoxy]-ethoxy}-ethyl)-carbamic acid *tert*-butyl ester, 60 °C, 36 h, 43.8% for **7a** and 58.4% for **7b**; (c) TFA, DCM, rt, 1 h; (d) TEA, DMF, (+)-biotin *N*-hydroxysuccinimide ester, rt, 12 h, 33.3% for **4c** and 66.6% for **4d**.

Scheme 2. Synthesis of Acetylene Chemical Probes 4e and 4f[a]



[a]Reagents and Conditions: (a) Thionyl chloride, MeOH, 0 to 60 °C, 4 h, 93%; (b) *p*-methoxy benzyl chloride, K_2CO_3, KI, acetone, 0 to 60 °C, 12 h, 88.5%; (c) LiOH·H_2O, THF/H_2O, rt, 12 h, 92.1%; (d) propargyl bromide, K_2CO_3, DMF, 0 °C to rt, 12 h, 93.1%; (e) TFA, DCM, 0 °C to rt, 4 h, 90.2%; (f) NH_4Cl, Zn, THF/H_2O/MeOH, 70 °C, 3 h, 88.4%; (g) EDC·HCl, HOBt, DIPEA, DMF, 4-(1-adamantyl)-phenoxy acetic acid (**5a**) for **4e**, rt, 12 h, 68.7%; and phenoxyacetic acid (**5b**) for **4f**, rt, 12 h, 76.2%.

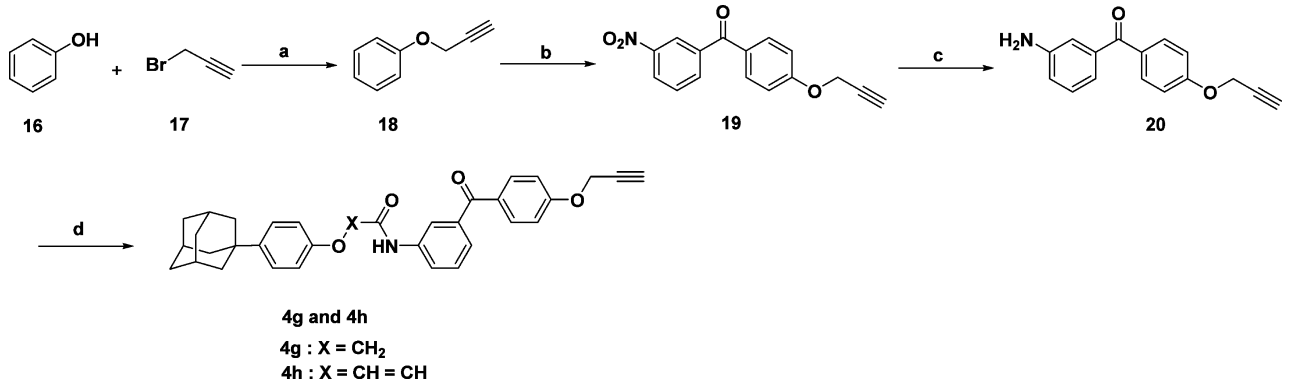
negative analogue **4d** was prepared starting from **6b** in a similar manner (Scheme 1).

For the cellular localization study, chemical probes **4e** and **4f**, in which a carboxylic acid functional group was replaced by an acetylene moiety for click conjugation, were synthesized as shown in Scheme 2. 3-Nitro-4-hydroxy benzoic acid was esterified and protected with *p*-methoxybenzyl chloride to give an ester derivative **11**. Hydrolysis and O-alkylation with propargyl bromide of **11** afforded a propargyl ester **13**. Subsequent *p*-methoxybenzyl deprotection and metal catalyzed reduction of the ester **13** gave a key intermediate **15**. Coupling **15** with 4-(1-adamantyl)-phenoxy acetic acid **5a**, which was readily prepared by our previously reported method, provided probe **4e**. Similarly, the corresponding negative control probe **4f** was prepared by coupling **15** with a commercially available phenoxyacetic acid (Scheme 2).[14]

As shown in Scheme 3, benzophenone-based multifunctional probes were also prepared in four steps. O-Alkylation of phenol with propargyl bromide afforded a phenyl propargyl ether derivative **18**. Friedel–Crafts acylation of **18** using *m*-

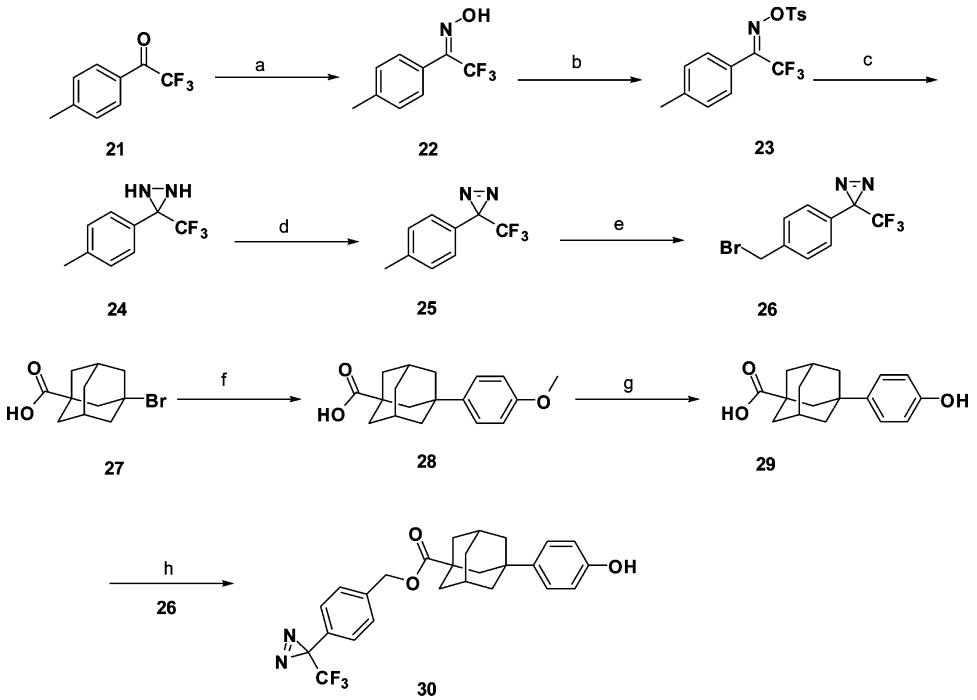
dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522–9538

Scheme 3. Synthesis of Benzophenone Chemical Probes 4g and 4h[a]



[a]Reagents and Conditions: (a) K_2CO_3, DMF, rt, 12 h, 71.4%; (b) $AlCl_3$, *m*-nitrobenzoyl chloride, DCM, −10 °C to rt, 12 h, 46.7%; (c) NH_4Cl, Fe, THF/H_2O/MeOH, 70 °C, 2 h, 75.4%; (d) EDC, HOBt, DIPEA, DMF, 4-(1-adamantyl)-phenoxy acetic acid for **4g**, rt, 12 h, 61.1%; and 4-(1-adamantyl)-phenoxyacrylic acid for **4h**, rt, 12 h, 61.8%.

Scheme 4. Synthesis of Key Intermediate 30[a]



[a]Reagents and Conditions: (a) $NH_2OH{\cdot}HCl$, pyridine, 115 °C, 4 h, 80.3%; (b) *p*-TsCl, DMAP, Et_3N, DCM, 0 °C to rt, 8 h, 84%; (c) NH_3, Et_2O, −78 °C to rt, 12 h, 82.8%; (d) MnO_2, CCl_4, 0 °C to rt, 1 h, 50.5%; (e) NBS, benzoyl peroxide, CCl_4, 77 °C, 2 h, 45.4%; (f) $AlCl_3$, anisole, −10 °C to rt, 24 h, 86%; (g) BBr_3, DCM, 0 °C to rt, 2 h, 90%; (h) K_2CO_3, DMF, 40 °C, 4 h, 25.7%.

nitrobenzoyl chloride yielded (3-nitrophenyl)(4-(prop-2-ynyloxy)phenyl)methanone **19**.[21] Selective reduction of the nitro group in the presence of iron powder and acetic acid led to the formation of a key intermediate **20**. Coupling **20** with 4-(1-adamantyl)-phenoxy acetic acid or 4-(1-adamantyl)-phenoxyacrylic acid, which were readily prepared by our previously reported method, gave benzophenone chemical probes **4g** and **4h**, respectively.[14,18]

For the synthesis of the trifluoromethyl diazirine probes **4i** and **4j**, a key intermediate **30** was prepared by multistep synthesis, as described in Scheme 4. Bromotrifluoromethyl diazirine **26** was synthesized starting from commercially available 2,2,2-trifluoro-1-*p*-tolylethanone by slightly modifying the reported procedure.[22−24] Compound **21** was converted to an oxime derivative **22** by condensation with hydroxylamine HCl in pyridine. O-Tosylation of **22** with *p*-toluenesulfonyl chloride led to the formation of a tosylated oxime **23**, which later reacted with liquid ammonia in a sealed tube to yield a diaziridine derivative **24** via elimination and subsequent cyclization. Oxidation of **24** with MnO_2 in CCl_4 afforded a diazirine **25**. A key precursor **26** was then obtained by bromination of **25** with *N*-bromosuccinimide (NBS) using radical initiator benzoyl peroxide as a catalyst. Selective O-alkylation of **26** with 3-(4-hydroxyphenyl)-adamantane-1-carboxylic acid (**29**), which was readily prepared by our previously reported method, furnished the key intermediate **30** (Scheme 4).[25] The key intermediate **30** was subsequently alkylated with ethyl chloroacetate to produce **31** in an excellent

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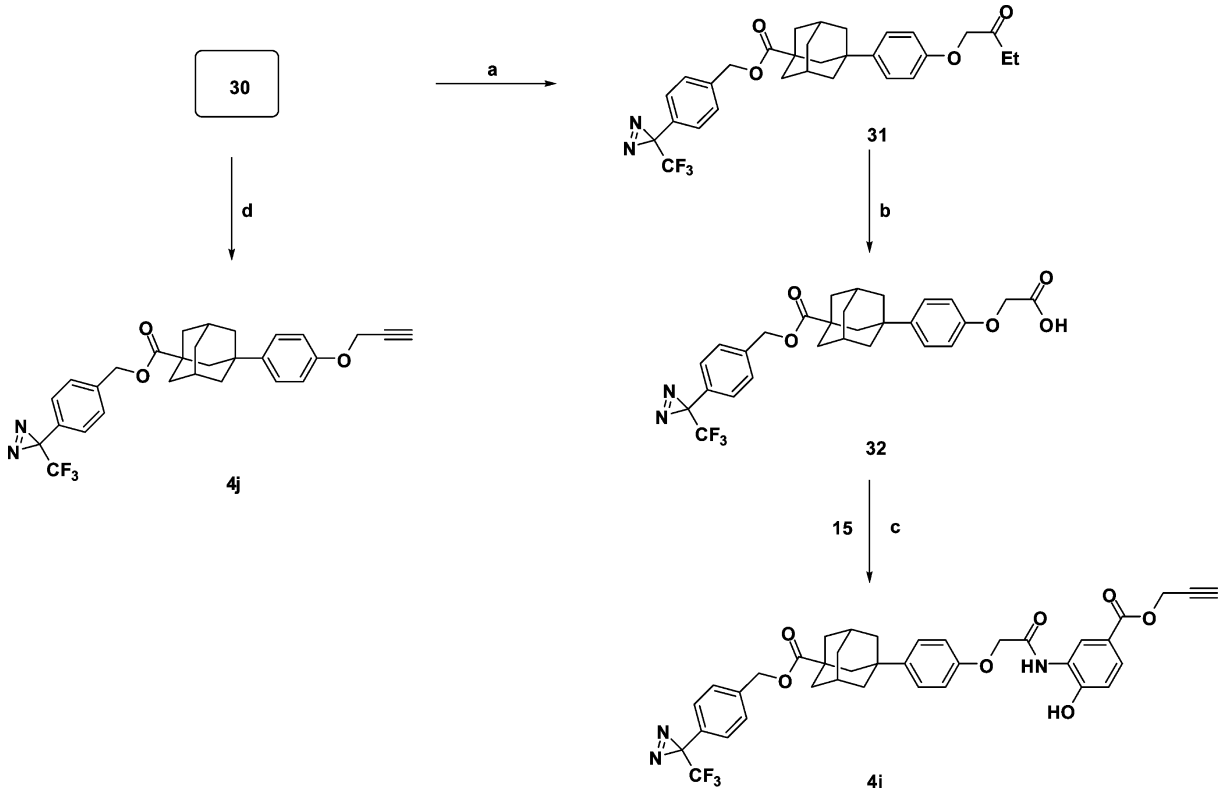
Scheme 5. Synthesis of Diazirine Chemical Probes 4i and 4j[a]



[a]Reagents and Conditions: (a) K_2CO_3, ethyl chloroacetate, DMF, rt, 12 h, 62.8%; (b) LiOH, THF/H_2O, rt, 12 h, 80%; (c) HBTU, DIPEA, DMF, rt, 12 h, 19.8%; (d) K_2CO_3, acetone, propargyl bromide, 60 °C to rt, 12 h, 62.1%.

yield. Saponification of **31** under aq. LiOH conditions gave a carboxylic acid derivative **32** and the subsequent coupling with methyl prop-2-ynyl 3-amino-4-hydroxybenzoate (**15**) produced the photoaffinity trimodular chemical probe **4i**. The negative control probe **4j** was obtained by alkylation of **30** with propargyl bromide under basic conditions, as shown in Scheme 5.

Another type of multifunctional diazirine probe **4a** and its negative analogue **4b**, which can be photolabeled and click-conjugated, were synthesized in 11 steps (Scheme 6). Commercially available 4-(1-adamntyl) phenol was subjected to iodination with potassium iodide to yield monoiodo derivative **34**, which, following methylation, gave **35a**. Reaction of 1-trifluoroacetylpiperidine with an anion derived from **35a** provided trifluoroacetyl derivative **36a**. Further, treatment of **36a** with hydroxylamine hydrochloride gave oxime derivative **37a**, which was tosylated with *p*-toluenesulfonyl chloride in order to provide a good leaving group to obtain **38a**. Reaction of **38a** with liquid ammonia in a sealed tube led to the formation of diaziridine derivative **39a** via elimination and subsequent cyclization. Diaziridine **39a** was further oxidized in the presence of iodine to give **40a**. Demethylation of **40a** by Lewis acid BBr_3 formed **41a**, which was further alkylated with ethyl chloroacetate and subjected to hydrolysis with LiOH to provide **43a**. Coupling **43a** with key intermediate **15** led to the desired multifunctional probe **4a**. Meanwhile, the negative analogue, **4b**, was also prepared by the same method, starting from commercially available 2-iodo anisole **35b**, as shown in Scheme 6.

Biological Evaluation. Biotinylated probes are often used in the affinity-based isolation of target proteins using streptoavidin beads. In our initial study, the biotin probe of LW6, **4c**, was designed as the first choice to identify the target protein for LW6 in human colorectal cancer HCT116 cells under hypoxic conditions. The biotin probe **4c** was prepared along with the negative control **4d** featuring an adamantyl-free form. When the pull-down assay was performed, followed by SDS-PAGE[26] of copurified proteins with LW6, a number of protein bands appeared with **4c**, while few bands appeared with the negative control **4d**. This result was consistent with our previous reports, which emphasized the importance of an adamantyl group for HIF-1α inhibitory activity.[14,18] Non-specific protein or indirect bindings to **4c** occurred due to a hydrophobic group of LW6 under the experimental conditions (Figure S1, Supporting Information). Even though target proteins were purified by specific binding with LW6, they were not selectable due to the limitations of the probes and methodology. Therefore, we prepared multifunctional probes by introducing a clickable group and photoreactive moiety at different sites of LW6.

First, the probes **4e** and **4f**, in which an acetylene moiety was installed for click conjugation with the azide linked molecule (e.g., fluorescent tag), were evaluated for their ability to inhibit HIF-1α using a cell-based HRE (hypoxia responsive element)-luciferase assay and Western blot analysis in HCT116 cells under hypoxia (Tables 1−3, Figure 2). The probe **4e** retained HIF-1α inhibitory activity (IC_{50} = 4.3 μM) of LW6, whereas the adamantyl-free probe **4f** as a negative control (>30 μM) completely lost the inhibitory activity, as shown in Table 1 and Figure 2A. Accordingly, probe **4e** was used for the intracellular localization study of LW6 through fluorescent tagging.[7]

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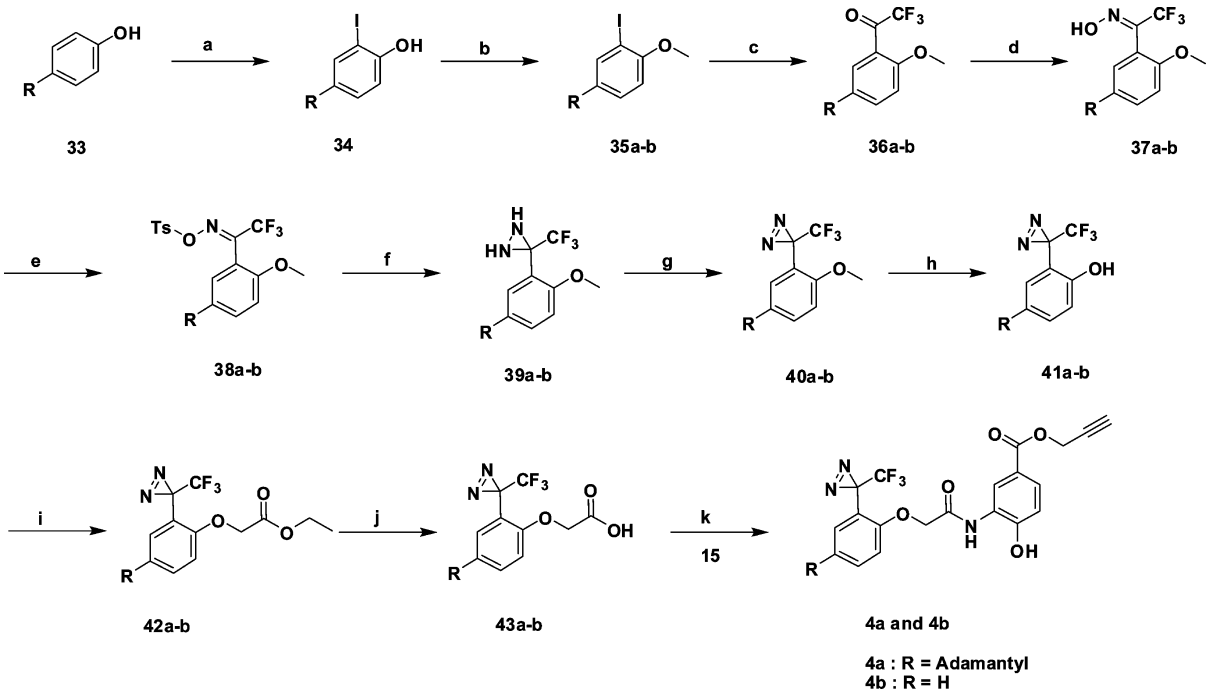
Scheme 6. Synthesis of Diazirine Chemical Probes 4a and 4b[a]



[a]Reagents and Conditions: (a) Con:H_2SO_4, KI, H_2O_2 (30 wt % solution in water), MeOH, 0 °C to rt, 12 h, 83.8%; (b) K_2CO_3, MeI, acetone, 0 °C to rt, 8 h, 97.3%; (c) *n*-BuLi, 1-trifluoroacetyl piperidine, THF; −78 °C, 12 h, 75.2% for **36a** and 72.4% for **36b**; (d) NH_2OH·HCl, pyridine, 115 °C, 4 h, 70.6% for **37a** and 90% for **37b**; (e) *p*-TsCl, DMAP, Et_3N, DCM, 0 °C to rt, 8 h, 79.6% for **38a** and 84.5% for **38b**; (f) NH_3, Et_2O, −78 °C to rt, 16 h, 81.7% for **39a** and 85.7% for **39b**; (g) I_2, Et_3N, MeOH, 0 °C to rt, 3 h, 94.3% for **40a** and 86.9% for **40b**; (h) BBr_3, DCM, −10 °C to rt, 2 h, 44.7% for **41a** and 37.4% for **41b**; (i) ethyl chloroacetate, K_2CO_3, DMF, rt, 12 h, 48.4% for **42a** and 33.3% for **42b**; (j) LiOH·H_2O, THF/H_2O, rt, 12 h, 55.5% for **43a** and 74% for **43b**; (k) EDC·HCl, HOBt, DIPEA, DMF, rt, 12 h, 18.2% for **4a** and 22.2% for **4b**.

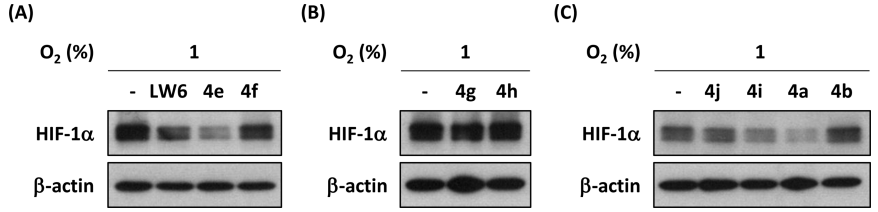


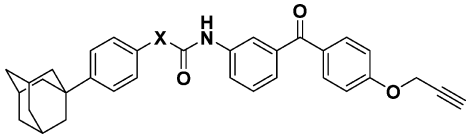
Figure 2. Effects of probes on hypoxia-induced HIF-1α accumulation. HCT116 cells were incubated for 6 h in the presence of probes (10 μM). (A) Acetylene chemical probes. (B) Benzophenone chemical probes. (C) Diazirine chemical probes. The protein levels of HIF-1α and β-actin were detected by immunoblot analysis with specific antibodies.

In addition to an acetylene moiety, compounds **4g** and **4h** bearing a benzophenone moiety for covalent attachment to the target protein were synthesized as the multifunctional chemical probes. Since benzophenone generates highly reactive radical intermediates upon photolysis, it is often used for photoaffinity labeling.[5,6] However, inhibition of HIF-1α activities of **4g** and **4h** were barely detectable in both the HRE-luciferase assay (Table 2) and the Western blot analysis (Figure 2B). The introduction of a benzophenone group to LW6 (**4g**) resulted in complete loss of HIF-1α inhibitory activity. Replacement of the oxyacetylamide linker portion with an oxyacrylic amide linker (**4h**) also failed to retain activity (Table 2). We observed that elongation of the molecule or the addition of both steric bulkiness and rigidity led to the loss of HIF-1α inhibitory activity.

In order to explore the feasibility of different photoaffinity groups, we utilized a photoreactive diazirine moiety which generates highly reactive carbene species upon photolysis. Trifluoro diazirine has several valuable characteristics, including photoactivity at long wavelengths, good chemical stability, rapid photolysis, and a smaller size in comparison with benzophenone. For example, a trifluoromethyldiazirine group was introduced to the adamantyl group with a benzyl ester linkage to provide **4i** (Table 3). Although probe **4i** showed moderate HIF-1α inhibitory activity (IC_{50} = 11.4 μM) in comparison with LW6, this result proved the efficiency of the diazirine moiety over benzophenone for LW6. However, probe **4j**, in which the amide linkage was removed and keeps the acetylene moiety on the phenoxy group, showed no effect on inhibitory activity. This result led to the understanding that a NH-phenyl group is crucial for the inhibition of HIF-1α (Table 3 and Figure 2C). In contrast, compound **4a** wherein trifluoromethyl diazirine was introduced directly to the phenyl ring of LW6 was found to maintain the potency (IC_{50} = 4.7 μM), as shown in the HRE-luciferase assay (Table 3) and Western blot of HIF-1α (Figure 2C). The negative control, adamantyl-free probe **4b**, did not show inhibitory activity.

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

Table 2. *In Vitro* HIF-1α and MDH2 Inhibitory Activity of Benzophenone Chemical Probes 4g and 4h[a]



Cpd	Structure X	HRE IC_{50} (μM)	MDH2 IC_{50} (μM)
4g		>30	>20
4h		>30	>20

[a]Values are the means of three experiments.

Encouraged by these results, we performed a target identification experiment using **4e** and **4a**.[7] The compound **4e** conjugated with a fluorescent tag, alexa 488, was localized in mitochondria, suggesting that LW6 may bind to mitochondrial protein. Finally, malate dehydrogenase 2 (MDH2) of the mitochondrial TCA cycle was identified as a target of LW6 using multifunctional probe **4a** through photoaffinity conjugation and separation of target proteins by 2D PAGE.[7]

Next, we confirmed the relation between HIF-1α and MDH2 inhibitory activities of the chemical probes derived from LW6 in the study. The MDH2 inhibitory activities of the probes were determined using isolated MDH2 from HCT116 cells treated with the chemical probes, as shown in Tables 1−3. LW6 (IC_{50} = 6.3 μM) was used as a reference compound for a comparison. As expected, **4e** (IC_{50} = 7.1 μM) and **4a** (IC_{50} = 5.5 μM) showed similar MDH2 inhibitory activities. Compound **4i** (IC_{50} = 10.2 μM) exhibited moderate MDH2 inhibition. However, the negative controls (**4b**, **4f**, and **4j**) showed negligible MDH2 inhibition activities (IC_{50} >20 μM). Of note, the probes containing benzophenone (**4g** and **4h**) did not show any significant effect on MDH2 activities.

We further examined *in vitro* binding activities of the probes to recombinant human MDH2 (rhMDH2) through photoaffinity labeling and click reaction (Figure 3). The fluorescent

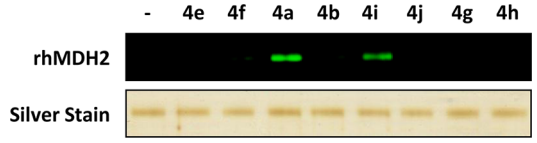
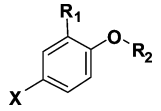
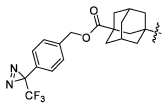
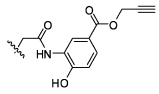
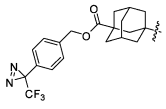
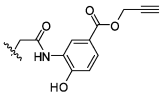
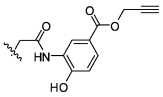


Figure 3. *In vitro* binding of chemical probes to recombinant human MDH2 (rhMDH2, 2 μg). The rhMDH2 was incubated with probes (10 μM) and then irradiated with UV (360 nm) for photoaffinity labeling of MDH2. Click reactions of probes with azide-Cy3 were performed to visualize probe−MDH2 binding.

Table 3. *In Vitro* HIF-1α and MDH2 Inhibitory Activity for Diazirine Chemical Probes 4i, 4j, 4a, and 4b[a]



Cpd	Structure X	R_1	R_2	HRE Luc IC_{50}(μM)	MDH2 IC_{50} (μM)
4i		H		11.4 ± 1.2	10.2 ± 0.7
4j		H		>30	>20
4a				4.7 ± 0.2	5.5 ± 0.5
4b	H			>30	>20

[a]Values are the means of three experiments.

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

bands, complexes of rhMDH2−probe−Cy3, were detected in the lane of the probes **4a** and **4i**. However, the probes devoid of a photoreactive moiety (**4e** and **4f**) and lacking MDH2 inhibitory activity (**4b** and **4j**) did not bind to rhMDH2. These results indicate that both probes **4a** and **4i** bind to MDH2 and inhibit HIF-1α activation through suppression of MDH2 activity inside cells. In the previous report, we demonstrated that a pull-down complex with biotin probe **4c** contained MDH2 protein, as demonstrated by Western blot analysis, indicating that MDH2 is a binding protein of LW6 and that biotin probe **4c** requires specificity to be used as a probe.[7]

Previously, we have shown that the inhibition of MDH2 activity by LW6 caused the suppression of mitochondrial respiration, which resulted in a decrease of oxygen consumption.[7] Therefore, the effects of chemical probes that retain MDH2 activity on oxygen consumption in HCT116 cells were investigated. In control HCT116 cells, the oxygen concentration in the medium was rapidly decreased (Figure 4). However, the representative chemical probe **4a** demonstrated apparent suppression in oxygen consumption by HCT116 cells, as LW6 did. This result suggests that probes inhibit HIF-1α accumulation through suppressing mitochondrial respiration via interfering with MDH2 activity.

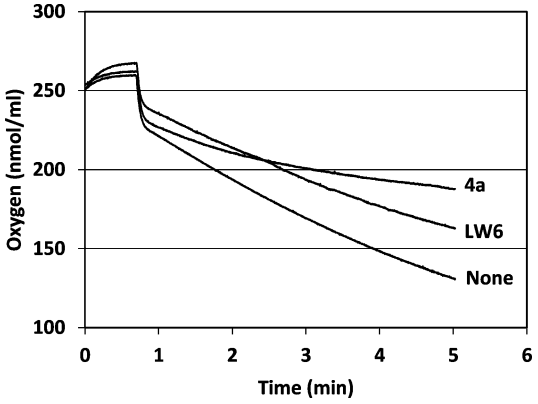


Figure 4. Effects of probes on oxygen consumption. HCT116 cells were incubated with compound LW6, diazirine chemical probe **4a** at 20 μM for 3 h, and then, oxygen consumption was measured as described in the Experimental Section.

Furthermore, we also found the inhibition of respiration by LW6 and the probe **4a** resulted in significant reduction of ATP production in HCT116 cells (Figure 5A). Then, we examined the activation of AMPK in the presence of LW6 and chemical probe **4a** because the elevation in the ratio of AMP/ATP increases phosphorylation of AMPK.[27] As shown in Figure 5B, LW6 and **4a** induced phosphorylation of AMPK and inactivation of the downstream target ACC (acetyl-CoA carboxylase), indicating that inhibition of respiration by LW6 or **4a** resulted in activation of the AMPK signaling pathway. Of significance, the design of chemical probes is as important as the structure−activity relationship study, since probes should retain the biological activity of the parent compound.

Then, we performed MDH2 kinetic assays of LW6 and chemical probes to further understand their inhibition modes of MDH2 using the purified His-MDH2 at various concentrations of NADH. LW6 inhibited MDH2 activity in a NADH-dependent manner, showing the typical intersecting line pattern for competitive inhibition with the inhibition constant (K_i) values of 1.9 μM (Figure 6). Furthermore, probe **4a** (K_i = 4.3 μM) showed competitive inhibition of MDH2 activity with NADH, suggesting LW6 and its chemical probe **4a** act as direct competitors of NADH in binding to MDH2.

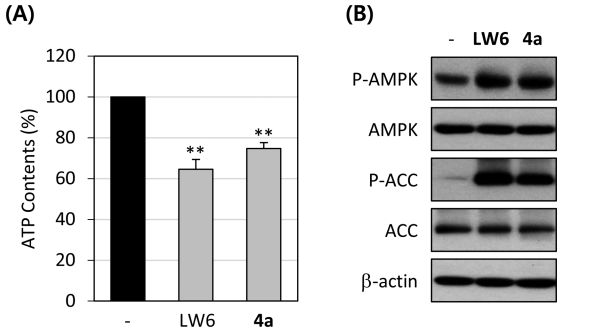


Figure 5. Effects of LW6 and chemical probe **4a** on ATP content and ATP-related signaling in HCT116 cells under hypoxic conditions. (A) ATP content in LW6 (10 μM), **4a** (10 μM)-treated cells was determined using a luciferase-based assay system. Statistical significance: $^{**}P < 0.01$, compared with untreated control. (B) Each protein level was detected by immunoblot analysis with specific antibodies.

Collectively, the introduction of a clickable acetylene moiety and photoactivatable trifluoromethyl diazirine to LW6 facilitated fluorescence imaging and conjugation to target molecules. The introduction of trifluoromethyl diazirine to LW6 did not have any significant effect on the activities of either HIF-1α or MDH2, as with LW6 alone. Accordingly, the application of these chemical probes to identify target molecules of LW6 overcame limitations of conventional methods, requiring availability of detectable amounts of target molecules and a high affinity of target molecules without inactivation or structural distortion during experiments. The structure−activity relationship of the compounds synthesized in this study using HRE-luciferase activity was consistent with that of the MDH2 enzyme assay, confirming that MDH2 is the direct target protein of LW6. These results also provide more information for better understanding of HIF-1α inhibition by targeting MDH2.

CONCLUSION

The affinity-based pull-down assay using biotinylated LW6 was not successful in identifying the target protein of LW6 due to lack of selectivity. Therefore, a series of multifunctional chemical probes derived from LW6 were synthesized and evaluated for their HIF-1α inhibition activity. The chemical probe **4e**, designed for intracellular imaging through click chemistry, retained the activity in HIF-1α inhibition. On the other hand, addition of benzophenone (**4g** and **4h**), which reacts with the receptor through a radical intermediate for photoaffinity labeling, did not retain HIF-1α inhibitory activity, presumably due to a rigid conformation or steric bulkiness. Another test of a photoaffinity moiety, the diazirine moiety, as a precursor for reaction with proteins was performed through a highly reactive carbene intermediate. When a small labile photoreactive group, like the trifluoromethyl diazirine moiety, was attached to an adamantyl moiety through a benzyl ester linkage, probe **4i** exhibited HIF-1α inhibition activity, though not as high as that of LW6. It is likely that this bulkier group at the adamantyl ring may hinder efficient binding to its target protein. Finally, introduction of a trifluoromethyl diazirine moiety directly into the phenyl ring of adamantyl derivative **4a** exhibited the high potency in HIF-1α inhibitory activity among

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

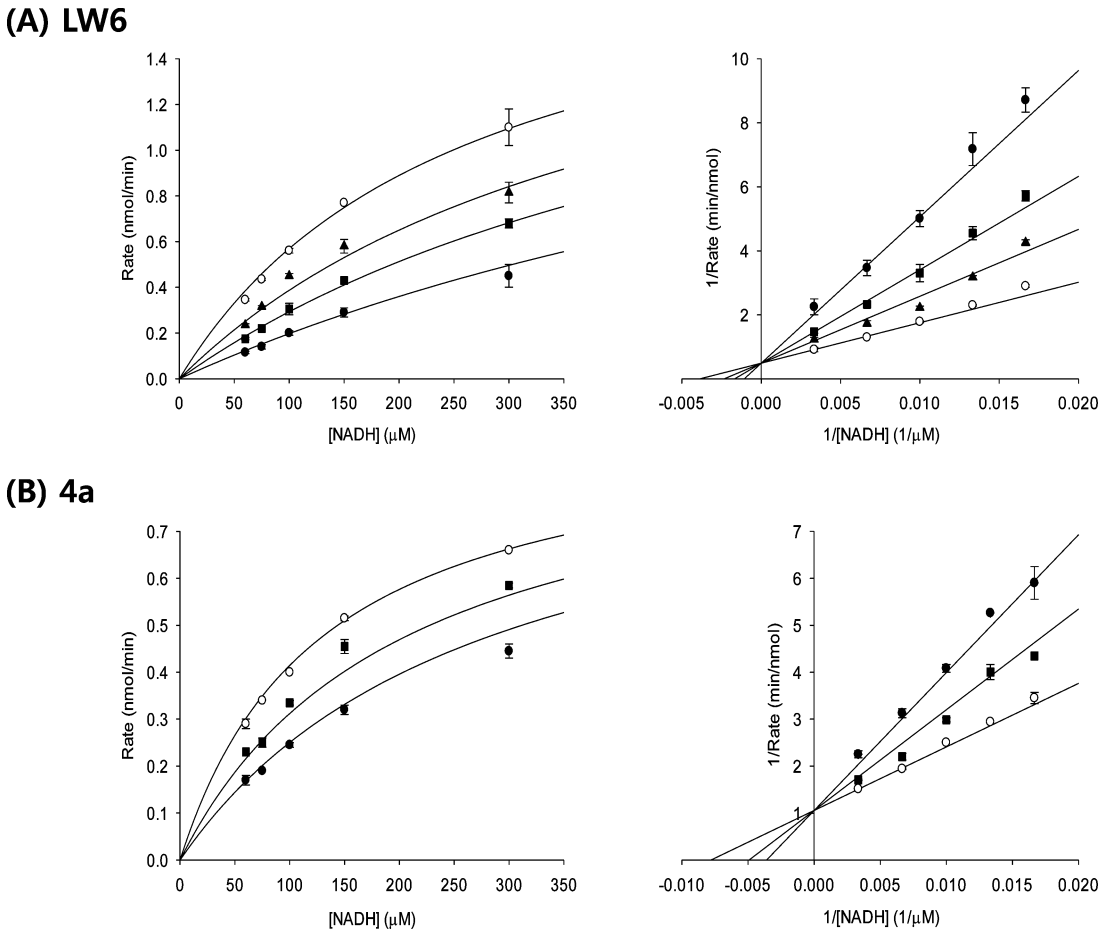


Figure 6. Kinetic study of MDH2 inhibition by LW6 and chemical probe **4a**. Double-reciprocal plot of the effect of LW6 (A) or chemical probe **4a** (B) on NADH-dependent MDH2 activity. Concentrations of LW6 and chemical probe (μM): 0 (○), 1.25 (▲), 2.5 (■), and 5 (●).

the synthesized derivatives. Then, *in vitro* binding of the probe **4a** to recombinant human MDH2 (rhMDH2) through photoaffinity labeling and click reaction was confirmed. The representative probe **4a** showed an inhibitory effect on MDH2 activity, oxygen consumption, and AMPK activation similar to that of LW6, indicating the structure−activity relationships of the probes in HIF-1α inhibitory activity were in accordance with that in the MDH2 assay. Furthermore, **4a** also showed competitive inhibition of MDH2 activity with NADH like LW6. These results suggest that well designed chemical probes based on a study of structure−activity relationships provide a reliable platform for the identification of direct target protein in drug discovery.

■ EXPERIMENTAL SECTION

All the commercial chemicals were of reagent grade and were used without further purification. Solvents were dried with standard procedures. All the reactions were carried out under an atmosphere of dried argon in flame-dried glassware. The proton nuclear magnetic resonance (^{1}H NMR) spectra were determined on a Varian (300, 400, or 500 MHz) spectrometer (Varian Medical Systems, Inc., Palo Alto, CA, USA). ^{13}C NMR spectra were recorded on a Varian (100 MHz) spectrometer. The chemical shifts are provided in parts per million (ppm) downfield with coupling constants in hertz (Hz). The mass spectra were recorded using high-resolution mass spectrometry (HRMS) (electron ionization MS) obtained on a JMS-700 mass spectrometer (Jeol, Japan) or using HRMS (electrospray ionization MS) obtained on a G2 QTOF mass spectrometer. The products from all of the reactions were purified by flash column chromatography using silica gel 60 (230−400 mesh Kieselgel 60). Additionally, thin-layer chromatography on 0.25 mm silica plates (E. Merck; silica gel 60 F254) was used to monitor reactions. The purity of the final products was checked by reversed phase high-pressure liquid chromatography (RP-HPLC), which was performed on a Waters Corp. HPLC system equipped with an ultraviolet (UV) detector set at 254 nm. The mobile phases used were (A) H_2O containing 0.05% trifluoroacetic acid and (B) CH_3CN. HPLC employed a YMC Hydrosphere C18 (HS-302) column (5 μm particle size, 12 nm pore size) that was 4.6 mm in diameter × 150 mm in size with a flow rate of 1.0 mL/min. The compound purity was assessed either using (method A) a gradient of 20% B to 100% B in 35 min or (method B) a gradient of 25% B to 100% B in 35 min. All biologically evaluated compounds' purity were >95% in both method A and method B.

Methyl 3-(2-(4-Adamantan-1-yl-phenoxy)acetamido)-4-hydroxybenzoate (6a).[14] To the mixture of 4-(1-adamantyl)-phenoxy acetic acid (**5a**) (0.3 g, 1.05 mmol), methyl-3-amino-4-hydroxybenzoate (0.16 g, 0.94 mmol), and HBTU (0.48 g, 1.26 mmol) in DMF (10 mL) was added DIPEA (0.27 mL, 1.57 mmol). The reaction mixture was stirred at room temperature overnight and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated under a vacuum. The resulting residue was purified by silica gel column chromatography (*n*-hexane:EtOAc = 3:7) to give methyl 3-(2-(4-adamantan-1-yl-phenoxy)acetamido)-4-hydroxybenzoate as a white solid (0.36 g, 78.9% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.10 (s, 1H), 9.24 (s, 1H), 8.69 (m, 1H), 7.60−7.64 (m, 1H), 7.30 (d, J = 8.4 Hz, 2H), 6.94−6.99 (m, 3H), 4.74 (s, 2H), 3.79 (s, 3H), 2.04 (m, 3H), 1.83 (m, 6H), 1.72 (m, 6H); MS (ESI) m/z 434 $(M - H)^-$; HRMS (ESI) m/z calcd for $C_{26}H_{29}O_5NNa$ $[(M + Na)^+]$, 458.1943;

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

found, 458.1942; purity 100% (as determined by RP-HPLC, method A, t_R = 22.583 min; method B, t_R = 25.333 min).

***N*-(2-Hydroxyphenyl)-2-phenoxyacetamide (6b).** To the mixture of phenoxyacetic acid (**5b**) (0.3 g, 1.97 mmol), 2-aminophenol (0.32 g, 2.96 mmol), and HBTU (1.12 g, 2.96 mmol) in DMF (10 mL) was added DIPEA (0.52 mL, 2.96 mmol). The reaction mixture was stirred at room temperature overnight and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated under a vacuum. The resulting residue was purified by silica gel column chromatography (*n*-hexane:EtOAc = 4:6) to form *N*-(2-hydroxyphenyl)-2-phenoxyacetamide as a yellow solid (0.364 g, 76.0% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.02 (brs, 1H), 9.16 (s, 1H), 7.99 (d, J = 7.8 Hz, 1H), 7.31−7.36 (m, 2H), 6.86−7.04 (m, 5H), 6.78 (dt, J = 1.8 Hz, 7.8 Hz, 1H), 4.74 (s, 2H); MS (EI) m/z 243 (M^+).

(2-{2-[2-(2-Chloro-acetylamino)-ethoxy]-ethoxy}-ethyl)-carbamic acid *tert*-butyl ester. To a solution of 1,2-bis(2-amino-ethoxy) ethane (0.3 g, 2.03 mmol) and triethylamine (0.56 g, 4.06 mmol) in methanol (4.0 mL) was added di-*tert*-butyl dicarbonate (0.55 mL, 2.43 mmol). The reaction mixture was stirred at room temperature overnight, and the methanol and TEA were removed *in vacuo* to yield oily residue, which was dissolved in CH_2Cl_2 and washed with a solution of sodium carbonate. The combined extracts were dried on anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (*n*-hexane:EtOAc = 2:8) to form monoprotected diamine as yellow oil (0.1 g, 66.7% yield). The TLC control showed almost no bis-protected diamine. The yellow oil was used without further purification. A solution of chloroacetyl chloride (0.17 g, 1.58 mmol) in CH_2Cl_2 (3 mL) was added dropwise over 20 min to a solution of monoprotected diamine (0.32 g, 1.32 mmol) and TEA in CH_2Cl_2 (3 mL) at −20 °C. The resulting brown solution was left to stir at room temperature for 24 h. The reaction solution was removed *in vacuo*, and the residue was dissolved in CH_2Cl_2 and washed with a solution of sodium carbonate. The combined extracts were dried on anhydrous $MgSO_4$, filtered, and concentrated to give a brown oil (0.3 g, 70.4% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.03 (s, 1H), 5.06 (s, 1H), 3.95 (s, 2H), 3.38−3.51 (m, 10H), 3.20 (m, 2H), 1.33 (s, 9H).

Methyl 3-[2-(4-Adamantan-1-yl-phenoxy)-acetylamino]-4-(2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazahexadecan-16-yloxy)benzoate (7a). To the solution of (2-{2-[2-(2-chloro-acetylamino)-ethoxy]-ethoxy}-ethyl)-carbamic acid *tert*-butyl ester (0.12 g, 0.38 mmol) in anhydrous acetone (10 mL) and DMF (3 mL) was subsequently added compound **6a** (0.082 g, 0.19 mmol), K_2CO_3 (0.052 g, 0.38 mmol), Cs_2CO_3 (0.030 g, 0.09 mmol), and KI (0.016 g, 0.09 mmol). The reaction mixture was heated at 60 °C for 36 h and cooled to room temperature. The mixture was evaporated under reduced pressure, and the residue was washed with water. The solution was extracted with ethyl acetate, and the combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by preparative TLC (*n*-hexane:EtOAc = 1:1) to obtain methyl 3-[2-(4-adamantan-1-yl-phenoxy)-acetylamino]-4-(2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazahexadecan-16-yloxy)benzoate as a yellow oil (0.06 g, yield 43.8%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.74 (brs, 1H), 7.83−7.86 (m, 1H), 7.32 (d, J = 9.0 Hz, 2H), 6.95 (d, J = 9.0 Hz, 2H), 6.91 (d, J = 9.0 Hz, 1H), 4.65 (s, 2H), 4.64 (s, 2H), 3.89 (s, 3H), 3.43−3.51 (m, 10H), 3.23 (m, 2H), 2.09 (brs, 3H), 1.88−1.86 (m, 6H), 1.76−1.74 (m, 6H), 1.41 (s, 9H); MS (EI) m/z 723 (M^+).

***tert*-Butyl 2-[2-(2-{2-[2-(2-Phenoxyacetamido) phenoxy] acetamido} ethoxy)ethoxy]ethylcarbamate (7b).** To the solution of (2-{2-[2-(2-chloro-acetylamino)-ethoxy]-ethoxy}-ethyl)-carbamic acid *tert*-butyl ester (0.13 g, 0.40 mmol) in anhydrous acetone (10 mL) was subsequently added compound **6b** (0.048 g, 0.2 mmol), K_2CO_3 (0.069 g, 0.50 mmol), Cs_2CO_3 (0.055 g, 0.17 mmol), and KI (0.04 g, 0.02 mmol). The reaction mixture was heated at 60 °C for 36 h and cooled to room temperature. The mixture was evaporated under reduced pressure, and the residue was washed with water. The solution was extracted with ethyl acetate, and the combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by preparative TLC (*n*-hexane:EtOAc:-MeOH = 6:3:1) to obtain *tert*-butyl 2-[2-(2-{2-[2-(2-phenoxyacetamido) phenoxy] acetamido} ethoxy)ethoxy]ethylcarbamate as a yellow oil (0.1 g, yield 58.4%). ^{1}H NMR (500 MHz, CD_3OD) δ 7.94 (d, J = 7.5 Hz, 1H), 7.32 (t, J = 8.0 Hz, 2H), 7.15 (t, J = 8.0 Hz, 1H), 7.07 (d, J = 8.5 Hz, 2H), 6.98−7.03 (m, 3H), 4.71 (s, 2H), 4.61 (s, 2H), 3.51−3.54 (m, 6H), 3.44 (q, J = 5.5 Hz, 4H), 3.17 (t, J = 5.5 Hz, 2H), 1.41 (s, 9H); MS (EI) m/z 531 (M^+).

Methyl 3-[2-(4-Adamantan-1-ylphenoxy)acetamido]-4-{2,13-dioxo-17-[(3aS,4R,6aR)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-6,9-dioxa-3,12-diazaheptadecyloxy}benzoate (4c). The compound **7a** (0.06 g, 0.08 mmol) was dissolved in the 2 mL mixture of TFA and CH_2Cl_2 (1:3), and the solution was stirred at room temperature for 1 h. Then, the solvents were removed and coevaporated with toluene three times to obtain crude free amine product **8a**. The amine product **8a** without further purification was dissolved in 1 mL of DMF, to which 0.5 mL of TEA was added. After that, to the solution was added (+)-biotin *N*-hydroxysuccinimide ester (0.034 g, 0.1 mmol), and it was stirred at room temperature overnight. The reaction was quenched by water and extracted with ethyl acetate. The organic layers were dried over anhydrous $MgSO_4$ and filtered, and then concentrated under reduced pressure. The crude product was purified by preparative TLC (CH_2Cl_2:MeOH = 10:1) to give the compound methyl 3-[2-(4-adamantan-1-ylphenoxy)acetamido]-4-{2,13-dioxo-17-[(3aS,4R,6aR)-2-oxohexahydro-1*H*-thieno[3,4-*d*]-imidazol-4-yl]-6,9-dioxa-3,12-diazaheptadecyloxy}benzoate as a white solid (0.023 g, 33.3% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.55 (d, J = 1.8 Hz, 1H), 7.76−7.75 (m,1H), 7.23 (d, J =8.0 Hz, 2H), 6.98 (d, J = 8.0 Hz, 1H), 6.91 (d, J = 8.0 Hz, 2H), 4.61 (s, 4H), 4.37−4.34 (m, 1H), 4.18−4.15 (m, 1H), 3.78 (s, 3H), 3.47−3.34 (m, 12H), 3.07−3.05 (m, 1H), 2.82−2.78 (m, 1H), 2.57 (d, J = 12.0 Hz, 1H), 2.08 (t, J = 8.0 Hz, 2H), 1.97 (brs, 3H), 1.82−1.81 (m, 6H), 1.75−1.67 (m, 6H), 1.57−1.41 (m, 4H), 1.33−1.29 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 174.7, 168.8, 168.3, 166.4, 155.4, 152.3, 145.0, 134.4, 127.4, 126.4, 125.7, 123.8, 123.4, 114.2, 111.6, 69.8, 69.2, 69.0, 67.3, 61.9, 60.2, 55.6, 43.1, 39.6, 38.8, 38.7, 36.4, 35.4, 35.3, 29.0, 28.3, 28.1, 25.4; MS (FAB) m/z 850 (MH^+); HRMS (FAB) m/z calcd for $C_{44}H_{60}N_5O_{10}S$ [MH^+], 850.4061; found, 850.4061; purity 100% (as determined by RP-HPLC, method A, t_R = 12.3 min; method B, t_R = 18.4 min).

5-[(3aS,4R,6aR)-2-Oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-*N*-{2-[2-(2-{2-[2-(2-phenoxyacetamido)phenoxy]-acetamido}ethoxy)ethoxy]ethyl}pentanamide (4d). The compound **7b** (0.058 g, 0.11 mmol) was dissolved in the 2 mL mixture of TFA and CH_2Cl_2 (1:3), and the solution was stirred at room temperature for 1 h. Then, the solvents were removed and coevaporated with toluene three times to obtain crude free amine product **8b**. The amine product **8b** without further purification was dissolved in 1 mL of DMF, to which 0.5 mL of TEA was added. After that, to the solution was added (+)-biotin *N*-hydroxysuccinimide ester (0.043 g, 0.13 mmol), and it was stirred at room temperature overnight. The reaction was quenched by water and extracted with 10% MeOH/MC. The organic layers were dried over anhydrous $MgSO_4$ and filtered, and then concentrated under reduced pressure. The crude product was purified by preparative TLC (CH_2Cl_2:MeOH = 10:1) to give compound 5-[(3aS,4R,6aR)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-*N*-{2-[2-(2-{2-[2-(2-phenoxyacetamido)-phenoxy]acetamido}ethoxy)ethoxy]ethyl}pentanamide as a pale yellow solid (0.048 g, 66.6% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.61 (s, 1H), 8.25 (t, J = 5.5 Hz, 1H), 7.96 (d, J = 7.5 Hz, 1H), 7.80 (t, J = 5.5 Hz, 1H), 7.33 (t, J = 8.0 Hz, 1H), 7.10 (t, J = 8.0 Hz, 1H), 7.06 (d, J = 8.0 Hz, 2H), 7.02−6.97 (m, 3H), 6.40 (s, 1H), 6.34 (s, 1H), 4.74 (s, 2H), 4.59 (s, 2H), 4.29 (t, J = 7.0 Hz, 1H), 4.11 (t, J = 6.0 Hz, 1H), 3.43 (t, J = 6.0 Hz, 4H), 3.36 (t, J = 5.5 Hz, 2H), 3.16 (q, J = 6.0 Hz, 2H), 3.10−3.06 (m, 1H), 2.81 (dd, J = 5.0 Hz, 12.5 Hz, 1H), 2.57 (d, J = 12.5 Hz, 1H), 2.05 (t, J = 7.5 Hz, 2H), 1.62−1.58 (m, 1H), 1.52−1.48 (m, 3H), 1.50−1.43 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.6, 168.2, 166.9, 163.1, 157.9, 148.8, 130.0, 127.3, 125.4, 122.0, 121.9, 121.8, 115.3, 113.6, 70.0, 69.9, 69.6, 68.3, 67.5,

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

61.5, 59.6, 55.9, 49.0, 38.9, 35.6, 28.6, 25.7; MS (FAB) m/z 658 (MH^+); HRMS (FAB) m/z calcd for $C_{32}H_{44}N_5O_8S$ [MH^+], 658.2911; found, 658.2911; purity 100% (as determined by RP-HPLC, method A, t_R = 10.2 min; method B, t_R = 8.5 min).

Methyl 4-Hydroxy-3-nitrobenzoate (10). To a solution of 4-hydroxy-3-nitro-benzoic acid (**9**) (2.0 g, 10.9 mmol) in methyl alcohol (25 mL) was added $SOCl_2$ (1.18 mL, 16.3 mmol) dropwise at 0 °C, and then, the mixture was refluxed for 4 h. After completion of the reaction, the reaction mixture was concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 8:2) to give methyl 4-hydroxy-3-nitrobenzoate as a yellow solid (2.0 g, 93.0% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.88 (s, 1H), 8.82 (s, 1H), 8.24 (d, J = 8.0 Hz, 1H), 7.22 (d, J = 8.0 Hz, 1H), 3.94 (s, 3H); MS (ESI) m/z 196 (M − H).

Methyl 4-(4-Methoxybenzyloxy)-3-nitrobenzoate (11). To a mixture of methyl 4-hydroxy-3-nitrobenzoate (**10**) (1.8 g, 9.1 mmol), K_2CO_3 (3.78 g, 27.3 mmol), and KI (3.03 g, 18.2 mmol) in acetone (18 mL) was added *p*-methoxy benzyl chloride (1.49 mL, 10.9 mmol) dropwise at 0 °C, and then, the mixture was refluxed for 12 h. After completion of the reaction, the reaction mixture was concentrated under reduced pressure and then partitioned between EtOAc and brine. The organic layer was separated, washed with water, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 9:1) to give methyl 4-(4-methoxybenzyloxy)-3-nitrobenzoate as a brown solid (2.56 g, 88.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (s, 1H), 8.16 (d, J = 8.0 Hz, 1H), 7.36 (d, J = 12.0 Hz, 2H), 7.16 (d, J = 12.0 Hz, 1H), 6.92 (d, J = 8.0 Hz, 2H), 5.17 (s, 2H), 3.92 (s, 3H), 3.81 (s, 3H); MS (ESI) m/z 340 (M + Na).

4-(4-Methoxybenzyloxy)-3-nitrobenzoic Acid (12). A solution of methyl 4-(4-methoxybenzyloxy)-3-nitrobenzoate (**11**) (2.0 g, 6.3 mmol) in THF/H_2O (3:1 20 mL) was treated with lithium hydroxide monohydrate (1.05 g, 25.2 mmol) and stirred at room temperature until the reaction was complete as judged by TLC. The reaction mixture was then acidified with 10% HCl to pH 4 and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (CH_2Cl_2:MeOH = 9:1) to give 4-(4-methoxybenzyloxy)-3-nitrobenzoic acid as a pale yellow solid (1.76 g, 92.1% yield). ^{1}H NMR (400 MHz, MeOD) δ 8.38 (s, 1H), 8.18 (d, J = 8.0 Hz, 1H), 7.42 (d, J = 8.0 Hz, 1H), 7.38 (d, J = 4.0 Hz, 2H), 6.93 (d, J = 8.0 Hz, 2H), 5.26 (s, 2H), 3.79 (s, 3H); MS (ESI) m/z 302 (M − H).

Prop-2-ynyl 4-(4-Methoxybenzyloxy)-3-nitrobenzoate (13). To a mixture of 4-(4-methoxybenzyloxy)-3-nitrobenzoic acid (**12**) (1.7 g, 5.6 mmol) and K_2CO_3 (2.0 g, 16.8 mmol) in DMF (17 mL) was added propargyl bromide (1.0 mL, 11.2 mmol) dropwise at 0 °C, and then, the mixture was stirred at room temperature for 12 h. After completion of the reaction, the reaction mixture was partitioned between EtOAc and brine. The organic layer was separated, washed with water, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 3:7) to give prop-2-ynyl 4-(4-methoxybenzyloxy)-3-nitrobenzoate as a yellow solid (1.78 g, 93.1% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (s, 1H), 8.19 (d, J = 8.0 Hz, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.17 (d, J = 8.0 Hz, 1H), 6.92 (d, J = 8.0 Hz, 2H), 5.24 (s, 2H), 5.05 (s, 2H), 3.81 (s, 3H), 2.52 (t, J = 4.0 Hz, 1H); MS (ESI) m/z 340 (M − H).

Prop-2-ynyl 4-Hydroxy-3-nitrobenzoate (14). To a solution of prop-2-ynyl 4-(4-methoxybenzyloxy)-3-nitrobenzoate (**13**) (1.6 g, 4.6 mmol) in CH_2Cl_2 (16 mL) was added TFA (1.43 mL, 18.7 mmol) dropwise at 0 °C, and then, the mixture was stirred at room temperature for 4 h. After completion of the reaction, the reaction mixture was concentrated and coevaporated with toluene three times under reduced pressure. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 1:1) to give prop-2-ynyl 4-hydroxy-3-nitrobenzoate as a yellow solid (0.93 g, 90.2% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.58 (s, 1H), 8.09 (d, J = 8.0 Hz, 1H), 7.14 (d, J = 8.0 Hz, 1H), 4.83 (s, 2H), 2.89 (t, J = 4.0 Hz, 1H); MS (ESI) m/z 220 (M − H).

Prop-2-ynyl 3-Amino-4-hydroxybenzoate (15). To a mixture of prop-2-ynyl 4-hydroxy-3-nitrobenzoate (**14**) (0.8 g, 3.6 mmol) and NH_4Cl (1.93 g, 36.0 mmol) in THF/MeOH/H_2O (10:5:3) (20 mL) was added Zn dust (2.36 g, 36.0 mmol) at 60 °C, and then, the mixture was refluxed for 3 h. After completion of the reaction, the reaction mixture was filtered through Celite and partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 4:6) to give prop-2-ynyl 3-amino-4-hydroxybenzoate as a yellow solid (0.61 g, 88.4% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.24 (s, 1H), 7.11 (d, J = 8.0 Hz, 1H), 6.72 (d, J = 8.0 Hz, 1H), 4.84 (s, 2H), 3.53 (t, J = 4.0 Hz, 1H); MS (ESI) m/z 190 (M − H).

(4-Adamantan-1-yl-phenoxy)acetic Acid (5a). This synthetic procedure followed our previous method.[14] White solid (1.76 g, 92.1% yield). ^{1}H NMR (400 MHz, MeOD) δ 7.26 (d, J = 7.2 Hz, 2H), 6.85 (d, J = 8.0 Hz, 2H), 4.60 (s, 2H), 2.08 (brs, 3H), 1.90−1.86 (m, 6H), 1.76 (m, 6H); MS (EI) m/z 286 (M^+).

Prop-2-ynyl 3-(2-(4-Adamantan-1-yl-phenoxy)acetamido)-4-hydroxybenzoate (4e). To a solution of (4-adamantan-1-yl-phenoxy)acetic acid (**5a**) (0.1 g, 0.35 mmol) and prop-2-ynyl 3-amino-4-hydroxybenzoate (**15**) (0.06 g, 0.35 mmol) in DMF (5.0 mL) were added (EDC·HCl) (0.08 g, 0.42 mmol), HOBt (0.056 g, 0.42 mmol), and DIPEA (0.15 mL, 0.87 mmol). The reaction mixture was stirred at room temperature overnight and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 6:4) to give prop-2-ynyl 3-(2-(4-adamantan-1-yl-phenoxy)acetamido)-4-hydroxybenzoate as a white solid (0.11 g, 68.7% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.21 (s, 1H), 9.31 (s, 1H), 8.75 (s, 1H), 7.70 (d, J = 12.0 Hz, 1H), 7.35 (d, J = 8.0 Hz, 2H), 7.06 (d, J = 4.0 Hz, 1H), 7.03 (d, J = 8.0 Hz, 2H), 4.95 (s, 2H), 4.80 (s, 2H), 3.63 (t, J = 4.0 Hz, 1H), 2.10 (brs, 3H), 2.05−1.89 (m, 6H), 1.81−1.75 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.3, 165.2, 155.7, 152.4, 144.6, 127.2, 126.3, 126.2, 122.3, 120.1, 115.3, 114.8, 79.1, 78.2, 67.6, 52.5, 43.2, 36.6, 35.6, 28.8; HRMS [M + H] calcd [$C_{28}H_{30}NO_5$], 460.2124; found, 460.2112; purity >99.9% (as determined by RP-HPLC, method A, t_R = 26.74 min; method B, t_R = 26.14 min).

Prop-2-ynyl 4-Hydroxy-3-(2-phenoxyacetamido) Benzoate (4f). To a solution of phenoxyacetic acid (**5b**) (0.1 g, 0.65 mmol) and prop-2-ynyl 3-amino-4-hydroxybenzoate (**15**) (0.12 g, 0.65 mmol) in DMF (5.0 mL) were added EDC·HCl (0.15 g, 0.78 mmol), HOBt (0.11 g, 0.78 mmol), and DIPEA (0.29 mL, 1.64 mmol). The reaction mixture was stirred at room temperature overnight and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (*n*-hexane:EtOAc = 7:3) to form prop-2-ynyl 4-hydroxy-3-(2-phenoxyacetamido) benzoate as a white solid (0.16 g, 76.2% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.20 (s, 1H), 9.33 (s, 1H), 8.74 (s, 1H), 7.69 (d, J = 12.0 Hz, 1H), 7.39 (t, J = 8.0 Hz, 2H), 7.10−7.04 (m, 4H), 4.95 (s, 2H), 4.82 (s, 2H), 3.63 (t, J = 4.0 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.1, 165.2, 157.9, 152.5, 130.1, 127.2, 126.1, 122.5, 122.0, 120.0, 115.3, 115.2, 79.1, 78.2, 67.5, 52.5; HRMS [M + H] calcd [$C_{18}H_{16}NO_5$], 326.1028; found, 326.1027; purity >99.9% (as determined by RP-HPLC, method A, t_R = 16.12 min; method B, t_R = 14.83 min).

Phenyl Propargyl Ether (18). A suspension of phenol (**16**) (1.0 g, 10.6 mmol), anhydrous potassium carbonate (4.4 g, 31.8 mmol), and propargyl bromide (**17**) (1.6 mL, 21.2 mmol) in DMF (10 mL) was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate and subsequently washed with aqueous sodium bicarbonate, brine, and water. The organic layer was dried over anhydrous $MgSO_4$. The solvent was filtered and evaporated under reduced pressure to afford a crude product, which was purified by silica gel column chromatography (hexane:EtOAc = 9:1) to give phenyl propargyl ether as a colorless oil (1.0 g, 71.4% yield). ^{1}H NMR (500

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

MHz, $CDCl_3$) δ 7.32−7.28 (m, 2H), 7.01−6.97 (m, 2H), 4.69 (s, 2H), 2.51 (t, J = 5.0 Hz, 1H); MS (ESI) m/z 133 (M + H).

(3-Nitrophenyl)(4-(prop-2-ynyloxy)phenyl)methanone (19).[21] The *m*-nitrobenzoyl chloride (1.0 g, 5.38 mmol) was gradually added to a mixture of phenyl propargyl ether (**18**) (0.71 g, 5.38 mmol) and anhydrous aluminum chloride (1.07 g, 8.08 mmol) in dichloromethane at −10 °C with continuous stirring. After addition, the mixture was stirred overnight at room temperature and quenched with 10% hydrochloric acid. The mixture was diluted with dichloromethane and dried over anhydrous magnesium sulfate. The filtrate was concentrated under reduced pressure and purified by column chromatography on silica gel to give (3-nitrophenyl)(4-(prop-2-ynyloxy)phenyl)methanone as a pale yellow solid (0.7 g, 46.7% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.58 (s, 1H), 8.43−8.41 (m, 1H), 8.09 (d, J = 5.0 Hz, 1H), 7.82 (d, J = 10.0 Hz, 2H), 7.69 (t, J = 10.0 Hz, 1H), 7.09 (d, J = 10.0 Hz, 2H), 4.80 (s, 2H), 2.57 (t, J = 5.0 Hz, 1H); MS (EI) m/z 282 (M + H).

(3-Aminophenyl)(4-(prop-2-ynyloxy)phenyl)methanone (20). To a mixture of (3-nitrophenyl)(4-(prop-2-ynyloxy)phenyl)-methanone (**19**) (0.6 g, 2.13 mmol) and NH_4Cl (1.14 g, 21.3 mmol) in THF/MeOH/H_2O (10:5:3) (20 mL) was added iron powder (1.19 g, 21.3 mmol) at 60 °C, and then, the mixture was refluxed for 2 h. After completion of the reaction, the reaction mixture was filtered through Celite and partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 4:6) to give (3-aminophenyl)(4-(prop-2-ynyloxy)phenyl)methanone as a yellow semisolid (0.4 g, 75.4% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, J = 8.0 Hz, 2H), 7.24−7.22 (m, 1H), 7.10−7.07 (m, 2H), 7.04 (d, J = 8.0 Hz, 2H), 6.88−6.86 (m, 1H), 4.78 (s, 2H), 3.80 (brs, 2H), 2.56 (t, J = 4.0 Hz, 1H); MS (ESI) m/z 252 (M + H).

2-(4-Adamantan-1-yl-phenoxy)-*N*-[3-(4-prop-2-ynyloxybenzoyl)phenyl] Acetamide (4g). To a solution of (4-adamantan-1-yl-phenoxy)acetic acid (**5a**) (0.1 g, 0.35 mmol) and (3-aminophenyl)(4-(prop-2-ynyloxy)phenyl)methanone (**20**) (0.08 g, 0.35 mmol) in DMF (5.0 mL) were added (EDC·HCl) (0.08 g, 0.42 mmol), HOBt (0.056 g, 0.42 mmol), and DIPEA (0.15 mL, 0.87 mmol). The reaction mixture was stirred at room temperature overnight and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 6:4) to give 2-(4-adamantan-1-yl-phenoxy)-*N*-[3-(4-prop-2-ynyloxybenzoyl)phenyl] acetamide as a white solid (0.11 g, 61.1% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (brs, 1H), 8.00−7.98 (m, 1H), 7.85 (d, J = 8.0 Hz, 2H), 7.83 (t, J = 4.0 Hz, 1H) 7.53−7.45 (m, 2H), 7.33 (d, J = 8.0 Hz, 2H), 7.05 (d, J = 8.0 Hz, 2H), 6.94 (d, J = 8.0 Hz, 2H), 4.78 (s, 2H), 4.61 (s, 2H), 2.57 (t, J = 4.0 Hz, 1H), 2.09 (brs, 3H), 1.88−1.80 (m, 6H), 1.75−1.72 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.8, 166.8, 161.2, 154.7, 145.7, 138.9, 137.0, 132.5, 130.6, 129.1, 126.3, 126.1, 123.6, 121.0, 114.5, 114.4, 77.8, 76.2, 67.7, 55.9, 43.3, 36.7, 35.7, 28.9; HRMS [M + H] calcd [$C_{34}H_{34}NO_4$], 520.2463; found, 520.2461; purity >99.9% (as determined by RP-HPLC, method A, t_R = 28.65 min; method B, t_R = 28.35 min).

(*E*)-3-(4-Adamantan-1-ylphenoxy)-*N*-(3-(4-(prop-2-ynyloxy)-benzoyl)phenyl)acrylamide (4h). To a solution of 4-(1-adamantyl)-phenoxyacrylic acid (0.1 g, 0.33 mmol) and (3-aminophenyl)(4-(prop-2-ynyloxy)phenyl)methanone (**20**) (0.08 g, 0.33 mmol) in DMF (5.0 mL) were added (EDC·HCl) (0.076 g, 0.40 mmol), HOBt (0.054 g, 0.40 mmol), and DIPEA (0.14 mL, 0.82 mmol). The reaction mixture was stirred at room temperature overnight and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 6:4) to give (*E*)-3-(4-adamantan-1-ylphenoxy)-*N*-(3-(4-(prop-2-ynyloxy)benzoyl)phenyl)acrylamide as a white solid (0.11 g, 61.8% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.90 (d, J = 10.0 Hz, 2H), 7.84 (d, J = 5.0 Hz, 2H), 7.77 (s, 1H), 7.46−7.42 (m, 2H), 7.36 (d, J = 10.0 Hz, 2H), 7.06−7.03 (m, 5H), 5.63 (d, J = 11.4 Hz, 2H), 4.77 (s, 2H), 2.55 (t, J = 2.5 Hz, 1H), 2.10 (brs, 3H), 1.90−1.89 (m, 6H), 1.78−1.73 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.2, 164.8, 161.1, 158.3, 153.7, 148.1, 138.7, 138.3, 132.5, 130.6, 129.0, 126.4, 125.4, 123.6, 120.9, 117.3, 114.5, 103.9, 77.8, 76.2, 55.9, 43.2, 36.7, 35.9, 28.9; MS (EI) m/z 532 (M^+); HRMS (EI) m/z calcd for $C_{35}H_{34}NO_4$ [M + H], 532.2410; found, 532.2488; purity 100% (as determined by RP-HPLC, method A, t_R = 29.61 min; method B, t_R = 29.7 min).

2,2,2-Trifluoro-1-(4-methylphenyl)-1-ethanone Oxime (22). To a stirred solution of 2,2,2-trifluoro-1-(2-methoxyphenyl)ethanone (**21**) (3.0 g, 15.94 mmol) in pyridine (30.0 mL) was added hydroxylamine hydrochloride (3.3 g, 47.83 mmol). Then, the mixture was refluxed for 4 h. The pyridine was evaporated, 50 mL of an aqueous solution of citric acid (10%) and CH_2Cl_2 were added to the residue, and the organic layer was extracted. The organic layer was separated, washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 2:8) to give 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone oxime as a white solid (2.6 g, 80.3% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.40 (d, J = 10.0 Hz, 2H), 7.28 (d, J = 10.0 Hz, 2H), 2.40 (s, 3H); MS (EI) m/z 203 (M^+).

2,2,2-Trifluoro-1-(4-methylphenyl)-1-ethanone *O*-(*p*-Toluenesulfonyl) Oxime (23). To a mixture of 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone oxime (**22**) (2.5 g, 12.30 mmol), 4-dimethylaminopyridine (0.75 g, 6.15 mmol), and triethylamine (2.57 mL, 18.45 mmol) in CH_2Cl_2 (25.0 mL) was added *p*-toluene sulfonyl chloride (2.81 g, 14.76 mmol) at 0 °C, and then, the mixture was stirred at room temperature for 8 h. After completion of the reaction, the reaction mixture was partitioned between CH_2Cl_2 and brine. The organic layer was separated, washed with water, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 1:9) to give 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone *O*-(*p*-toluenesulfonyl) oxime as a white solid (3.6 g, 84% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, J = 8.0 Hz, 2H), 7.38 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 7.27 (d, J = 8.0 Hz, 2H), 2.47 (s, 3H), 2.40 (s, 3H); MS (EI) m/z 357 (M^+).

3-(4-Methylphenyl)-3-trifluoromethyldiaziridine (24). Liquid ammonia (6.0 mL) was added at −78 °C to an ether (30.0 mL) solution of 2,2,2-trifluoro-1-(4-methylphenyl)-1-ethanone *O*-(*p*-toluenesulfonyl) oxime (**23**) (3.0 g, 8.39 mmol) in a sealed tube. The solution was stirred at room temperature for 16 h. The mixture was carefully cooled to −78 °C, and the sealed tube was opened. The ammonia was evaporated, and white precipitate was filtered and washed with ether. The filtrate was concentrated *in vacuo* to give 3-(4-methylphenyl)-3-trifluoromethyldiaziridine as a white solid (1.4 g, 82.8% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (d, J = 10.0 Hz, 2H), 7.22 (d, J = 10.0 Hz, 2H), 2.75 (d, J = 10.0 Hz, 1H), 2.37 (s, 3H), 2.17 (d, J = 10.0 Hz, 1H); MS (EI) m/z 202 (M^+).

3-(4-Methylphenyl)-3-trifluoromethyldiazirine (25). To the compound 3-(4-methylphenyl)-3-trifluoromethyldiaziridine (**24**) (1.0 g, 4.94 mmol) in CCl_4 (10 mL) was added MnO_2 (1.29 g, 14.83 mmol) at 0 °C, and then, the mixture was stirred at room temperature for 1 h. After completion of the reaction, CCl_4 was concentrated under reduced pressure at low temperature due to volatility of the product. Flash chromatography of the residue over silica gel (hexane:EtOAc = 9.5:0.5) afforded 3-(4-methylphenyl)-3-trifluoromethyldiazirine as a colorless solid (0.5 g, 50.5% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19 (d, J = 10.0 Hz, 2H), 7.08 (d, J = 10.0 Hz, 2H), 2.35 (s, 3H); MS (EI) m/z 200 (M^+).

3-(4-Bromomethylphenyl)-3-trifluoromethyldiazirine (26). A solution of 3-(4-methylphenyl)-3-trifluoromethyldiazirine (**25**) (0.4 g, 2.0 mmol) in CCl_4 (4 mL) was heated to 70 °C, powdered NBS (0.66 g, 3.75 mmol) was added and stirred for 10 min and then benzoyl peroxide (15 mg) was added, and the reaction was refluxed for 2 h. The precipitate was filtered, the solvent was removed *in vacuo* at 20 °C, and the crude product was purified by column chromatography eluting with hexane/CH_2Cl_2 (20:1) to give 3-(4-bromomethylphenyl)-3-trifluoromethyldiazirine as a yellow oil (0.25 g, 45.4% yield). ^{1}H

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

NMR (500 MHz, $CDCl_3$) δ 7.19 (d, J = 10.0 Hz, 2H), 7.08 (d, J = 10.0 Hz, 2H), 4.50 (s, 3H); MS (EI) m/z 277 (M^+).

3-(4-Hydroxyphenyl)-adamantane-1-carboxylic Acid (29). The synthetic procedure was followed as per our previous method.[25]

4-[3-({4-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}-carbonyl)adamantan-1-y]phenol (30).[23] The mixture of 3-(4-hydroxyphenyl)-adamantane-1-carboxylic acid (**29**) (0.34 g, 1.25 mmol) and 3-(4-bromomethylphenyl)-3-trifluoromethyl-3*H*-diazirine (**26**) (0.52 g, 1.91 mmol) was dissolved in 10 mL of anhydrous DMF, and $KHCO_3$ (0.19 g, 1.91 mmol) was added. The reaction mixture was heated at 40 °C for 4 h and quenched by aqueous $NaHCO_3$. The solution was extracted with EA, and the organic layers were dried over $MgSO_4$. The filtrate was concentrated and purified by column chromatography on silica gel to form 4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}carbonyl)adamantan-1-y]phenol as a white solid (0.15 g, 25.7% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, J = 8.4 Hz, 2H), 7.17−7.23 (m, 4H), 6.79 (d, J = 8.0 Hz, 2H), 5.15 (s, 2H), 2.23 (m, 2H), 2.04 (m, 2H), 1.96 (m, 4H), 1.89 (m, 4H), 1.72 (m, 2H); MS (EI) m/z 470 (M^+).

Ethyl-2-{4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]-benzyloxy}carbonyl)adamantan-1-y]phenoxy}acetate (31). The 4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}-carbonyl)adamantan-1-y]phenol (**30**) (0.3 g, 0.63 mmol) and ethyl chloroacetate (0.15 g, 1.27 mmol) were dissolved in 5 mL of anhydrous DMF. K_2CO_3 (0.26 g, 1.91 mmol) was added and stirred at room temperature overnight. The excess solvent was evaporated and washed with water. The mixture was extracted with ethyl acetate and dried over $MgSO_4$. The filtrate was concentrated under reduced pressure and the residue was purified by MPLC, which afforded ethyl-2-{4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}-carbonyl)adamantan-1-y]phenoxy}acetate as a colorless oil (0.22 g, 62.8% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 (d, J = 8.0 Hz, 2H), 7.26 (d, J = 8.5 Hz, 2H), 7.18 (d, J = 8.0 Hz, 2H), 6.89 (d, J = 8.5 Hz, 2H), 5.10 (s, 2H), 4.60 (s, 2H), 4.27 (q, J = 7.0 Hz, 2H), 2.22 (m, 2H), 2.01 (m, 2H), 1.93 (m, 4H), 1.86 (m, 4H), 1.72 (m, 2H), 1.30 (t, J = 7.0 Hz, 3H); MS (FAB^+) m/z 556 (M^+).

2-{4-[3-({4-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}-carbonyl)adamantan-1-y]phenoxy}acetic Acid (32). The ethyl-2-{4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}carbonyl)-adamantan-1-y]phenoxy}acetate (**31**) (0.2 mg, 0.36 mmol) was dissolved in 20 mL of THF, and $LiOH{\cdot}H_2O$ was added and stirred at room temperature, which was monitored by TLC. The excess solvent was evaporated and washed with 10% HCl. The mixture was extracted with EA and dried over $MgSO_4$. The filtrate was concentrated to give crude product 2-{4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}carbonyl)adamantan-1-y]phenoxy}acetic acid without further purification.

Prop-2-ynyl-4-hydroxy-3-(2-{4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}carbonyl)adamantan-1-yl]-phenoxy}acetamido)benzoate (4i). The mixture of 2-{4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}carbonyl)adamantan-1-y]phenoxy}acetic acid (**32**) (96.6 mg, 0.18 mmol), HBTU (104 mg, 0.27 mmol), and DIPEA (35 mg, 0.25 mmol) was dissolved in 4 mL of DMF and MC. The solution was stirred at room temperature for 30 min, prop-2-ynyl 3-amino-4-hydroxybenzoate (**15**) (52 mg, 0.27 mmol) was added, and the mixture was stirred overnight. The excess solvent was removed under reduced pressure, and the residue was washed with water and 10% HCl. The mixture was extracted with EA and dried over $MgSO_4$. The filtrate was concentrated and purified by column chromatography, which gave prop-2-ynyl-4-hydroxy-3-(2-{4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}carbonyl)-adamantan-1-yl]phenoxy}acetamido)benzoate as a pale yellow solid (25 mg, 19.8% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.80 (s, 1H), 8.58 (s, 1H), 7.88 (dd, J = 2.0 Hz, 8.5 Hz, 1H), 7.76 (d, J = 2.0 Hz, 1H), 7.34−7.37 (m, 3H), 7.19 (d, J = 8.0 Hz, 2H), 7.08 (d, J = 8.5 Hz, 2H), 6.97 (d, J = 8.5 Hz, 2H), 5.12 (s, 2H), 4.90 (d, J = 2.5 Hz, 2H), 4.69 (s, 2H), 2.50 (t, J = 2.5 Hz, 1H), 2.03 (m, 2H), 1.94 (m, 6H), 1.88 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.8, 168.8, 164.9, 154.6, 153.7, 144.7, 138.2, 129.6, 128.0, 126.7, 126.5, 124.6, 124.5, 121.6, 120.2, 114.6, 75.0, 67.2, 65.1, 52.5, 44.2, 42.2, 41.9, 38.1, 36.1, 35.5, 31.9, 29.7, 29.4, 28.6; HRMS [M + H] calcd [$C_{38}H_{35}F_3N_3O_7$], 702.2378; found, 702.2378; purity >98% (as determined by RP-HPLC, method A, t_R = 28.8 min; method B, t_R = 29.3 min).

4-[3-({4-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl] benzyloxy} carbonyl) adamantan-1-y]-(prop-2-ynyloxy) Benzene (4j). The compound 4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxy}-carbonyl)adamantan-1-y]phenol (**30**) (0.15 g, 0.32 mmol) was dissolved in 8 mL of anhydrous DMF, and K_2CO_3 (0.09 g, 0.64 mmol) was added. The mixture was heated at 60 °C for 2 h and cooled to room temperature, and then, propargyl bromide (0.054 g, 0.48 mmol) was added. The reaction mixture was stirred overnight and then washed with water. The mixture was extracted with dichloromethane and then washed with brine. The organic phase was dried over $MgSO_4$ and filtered. The filtrate was evaporated under reduced pressure, and the residue was purified by column chromatography on silica gel to afford 4-[3-({4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl] benzyloxy} carbonyl) adamantan-1-y]-(prop-2-ynyloxy) benzene as a colorless oil (0.1 g, 62.1% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, J = 8.4 Hz, 2H), 7.28 (dd, J = 3.2 Hz, 5.2 Hz, 2H), 7.18 (d, J = 8.4 Hz, 2H), 6.94 (dd, J = 2.4 Hz, 6.8 Hz, 2H), 5.11 (s, 2H), 4.67 (d, J = 2.8 Hz, 2H), 2.51 (t, J = 2.4 Hz, 1H), 2.23 (m, 2H), 2.02 (m, 2H), 1.93 (m, 4H), 1.87 (m, 4H), 1.72 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.9, 155.6, 143.1, 138.2, 128.8, 128.0, 127.9, 126.7, 125.9, 114.5, 78.8, 75.4, 65.0, 55.8, 44.3, 42.2, 42.1, 41.9, 38.1, 35.9, 35.6, 28.7; HRMS [M + H] calcd [$C_{29}H_{28}F_3N_2O_3$], 509.2020; found, 509.2023; purity >98% (as determined by RP-HPLC, method A, t_R = 30.45 min; method B, t_R = 30.11 min).

4-Adamantan-1-yl-2-iodophenol (34). A solution of 4-adamantyl-phenol (**33**) (4.00 g, 17.5 mmol), potassium iodide (2.9 g, 17.5 mmol), and sulfuric acid (1.40 mL, 26.2 mmol) in methanol (400 mL) was stirred at 0 °C while hydrogen peroxide (30%) (4.0 mL, 35.0 mmol) was added dropwise. Stirring was continued for 12 h at ambient temperature. Then, the solvent was removed under reduced pressure. The residue was diluted with concentrated sodium bisulfite (100 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Flash chromatography of the residue over silica gel (hexane:EtOAc = 2:8) afforded 4-adamantan-1-yl-2-iodophenol as a white solid (5.2 g, 83.8% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (s, 1H), 7.23 (d, J = 8.0 Hz, 1H), 6.93 (d, J = 12.0 Hz, 1H), 5.10 (s, 1H), 2.08 (brs, 3H), 1.85−1.82 (m, 6H), 1.76−1.70 (m, 6H); MS (ESI) m/z 353 (M + H).

4-Adamantan-1-yl-2-iodo-1-methoxybenzene (35a). To a mixture of 4-adamantan-1-yl-2-iodophenol (**34**) (5.00 g, 14.1 mmol) and K_2CO_3 (5.8 g, 42.3 mmol) in acetone (50 mL) was added methyl iodide (1.75 mL, 28.2 mmol) dropwise at 0 °C, and then, the mixture was stirred at room temperature for 8 h. After completion of the reaction, evaporated acetone and the reaction mixture were partitioned between CH_2Cl_2 and brine. The organic layer was separated, washed with water, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 1:9) to give 4-adamantan-1-yl-2-iodo-1-methoxybenzene as a white solid (5.05 g, 97.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (s, 1H), 7.28 (d, J = 8.0 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 3.85 (s, 3H), 2.08 (brs, 3H), 1.85−1.82 (m, 6H), 1.76−1.70 (m, 6H); MS (ESI) m/z 369 (M + H).

1-(5-Adamantan-1-yl-2-methoxyphenyl)-2,2,2-trifluoroethanone (36a). To a stirred solution of 4-adamantan-1-yl-2-iodo-1-methoxybenzene (**35a**) (5.0 g, 13.57 mmol) in THF (50 mL) at −78 °C was added dropwise 1.6 M solution of *n*-butyllithium in hexane (12.7 mL, 20.35 mmol) over a 1 h period. After stirring the mixture at the same temperature for 8 h, a solution of 1-trifluoroacetyl piperidine (2.4 mL, 16.25 mmol) in THF (10 mL) was added dropwise over 1 h. Following the addition, the mixture stirred at −78 °C for a further 3 h before the reaction was quenched with an aqueous solution of NH_4Cl. Ether was added, and the organic layer was extracted three times with ether (3 × 50 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Flash chromatography of the residue over silica gel (hexane:EtOAc = 2:8) afforded 1-(5-adamantan-1-yl-2-methoxyphen-

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

yl)-2,2,2-trifluoroethanone as a pale yellow semisolid (3.45 g, 75.2% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (s, 1H), 7.61 (d, J = 8.0 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 3.89 (s, 3H), 2.17 (brs, 3H), 1.85−1.82 (m, 6H), 1.78−1.72 (m, 6H); MS (ESI) m/z 339 (M + H).

2,2,2-Trifluoro-1-(2-methoxyphenyl)ethanone (36b). To a stirred solution of 1-iodo-2-methoxybenzene (**35b**) (5.0 g, 21.35 mmol) in THF (50 mL) at −78 °C was added dropwise 1.6 M solution of *n*-butyllithium in hexane (24.8 mL, 32.0 mmol) over a 1 h period. After stirring the mixture at the same temperature for 8 h, a solution of 1-trifluoroacetyl piperidine (3.75 mL, 25.6 mmol) in THF (11 mL) was added dropwise over 1 h. Following the addition, the mixture was stirred at −78 °C for a further 3 h before the reaction was quenched with an aqueous solution of NH_4Cl. Ether was added, and the organic layer was extracted three times with ether (3 × 50 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Flash chromatography of the residue over silica gel (hexane:EtOAc = 2:8) afforded 2,2,2-trifluoro-1-(2-methoxyphenyl)ethanone as a pale yellow semisolid (3.15 g, 72.4% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (d, J = 8.0 Hz, 1H), 7.59 (t, J = 8.0 Hz, 1H), 7.07−7.03 (m, 2H), 3.98 (s, 3H); MS (ESI) m/z 205 (M + H).

1-(5-Adamantan-1-yl-2-methoxyphenyl)-2,2,2-trifluoroethanone Oxime (37a). To a stirred solution of 1-(5-adamantan-1-yl-2-methoxyphenyl)-2,2,2-trifluoroethanone (**36a**) (3.2 g, 9.45 mmol) in pyridine (32 mL) was added hydroxylamine hydrochloride (1.31 g, 18.91 mmol). Then, the mixture was refluxed for 4 h. The pyridine was evaporated, 25 mL of an aqueous solution of citric acid (10%) and CH_2Cl_2 was added to the residue, and the organic layer was extracted. The organic layer was separated, washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 7:3) to give 1-(5-adamantan-1-yl-2-methoxyphenyl)-2,2,2-trifluoroethanone oxime as a white solid (2.4 g, 70.6% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (s, 1H), 7.41 (d, J = 8.0 Hz, 1H), 7.22 (s, 1H), 6.88 (d, J = 8.0 Hz, 1H), 3.82 (s, 3H), 2.09 (brs, 3H), 1.89−1.88 (m, 6H), 1.79−1.74 (m, 6H); MS (ESI) m/z 354 (M + H).

2,2,2-Trifluoro-1-(2-methoxyphenyl)ethanone Oxime (37b). To a stirred solution of 2,2,2-trifluoro-1-(2-methoxyphenyl)ethanone (**36b**) (3.0 g, 14.69 mmol) in pyridine (30.0 mL) was added hydroxylamine hydrochloride (3.06 g, 44.08 mmol). Then, the mixture was refluxed for 4 h. The pyridine was evaporated, 50 mL of an aqueous solution of citric acid (10%) and CH_2Cl_2 was added to the residue, and the organic layer was extracted. The organic layer was separated, washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 1:9) to give 2,2,2-trifluoro-1-(2-methoxyphenyl)ethanone oxime as a white solid (2.9 g, 90.0% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 7.44 (d, J = 8.0 Hz, 1H), 7.01 (m, 2H), 3.84 (s, 3H); MS (ESI) m/z 220 (M + H).

1-(5-Adamantan-1y-l-2-methoxyphenyl)-2,2,2-trifluoroethanone *O*-Tosyl Oxime (38a). To a mixture of 1-(5-adamantan-1-yl-2-methoxyphenyl)-2,2,2-trifluoroethanone oxime (**37a**) (2.8 g, 7.92 mmol), 4-dimethylaminopyridine (0.48 g, 3.96 mmol), and triethylamine (1.65 mL, 11.88 mmol) in CH_2Cl_2 (28 mL) was added *p*-toluene sulfonyl chloride (1.81 g, 9.50 mmol) at 0 °C, and then, the mixture was stirred at room temperature for 8 h. After completion of the reaction, the reaction mixture was partitioned between CH_2Cl_2 and brine. The organic layer was separated, washed with water, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 1:9) to give 1-(5-adamantan-1y-l-2-methoxyphenyl)-2,2,2-trifluoroethanone *O*-tosyl oxime as a white solid (3.2 g, 79.6% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (d, J = 8.0 Hz, 2H), 7.44 (d, J = 8.0 Hz, 1H), 7.37 (d, J = 8.0 Hz, 2H), 7.03 (s, 1H), 6.88 (d, J = 8.0 Hz, 1H), 3.74 (s, 3H), 2.48 (s, 3H), 2.09 (brs, 3H), 1.85−1.82 (m, 6H), 1.76−1.70 (m, 6H); MS (ESI) m/z 508 (M + H).

2,2,2-Trifluoro-1-(2-methoxyphenyl)ethanone *O*-Tosyl Oxime (38b). To a mixture of 2,2,2-trifluoro-1-(2-methoxyphenyl)-ethanone oxime (**37b**) (2.5 g, 11.40 mmol), 4-dimethylaminopyridine (0.69 g, 5.7 mmol), and triethylamine (2.38 mL, 17.11 mmol) in CH_2Cl_2 (25.0 mL) was added *p*-toluene sulfonyl chloride (2.60 g, 13.68 mmol) at 0 °C, and then, the mixture was stirred at room temperature for 8 h. After completion of the reaction, the reaction mixture was partitioned between CH_2Cl_2 and brine. The organic layer was separated, washed with water, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 1:9) to give 2,2,2-trifluoro-1-(2-methoxyphenyl)ethanone *O*-tosyl oxime as a white solid (3.6 g, 84.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, J = 8.0 Hz, 2H), 7.49 (t, J = 8.0 Hz, 1H), 7.37 (d, J = 8.0 Hz, 2H), 7.09 (d, J = 8.0 Hz, 1H), 7.02−6.92 (m, 2H), 3.79 (s, 3H), 2.48 (s, 3H); MS (ESI) m/z 374 (M + H).

3-(5-Adamantan-1-yl-2-methoxyphenyl)-3-(trifluoromethyl) Diaziridine (39a). Liquid ammonia (6.0 mL) was added at −78 °C to an ether (30.0 mL) solution of 1-(5-adamantan-1y-l-2-methoxyphenyl)-2,2,2-trifluoroethanone *O*-tosyl oxime (**38a**) (3.0 g, 5.91 mmol) in a sealed tube. The solution was stirred at room temperature for 16 h. The mixture was carefully cooled to −78 °C, and the sealed tube was opened. The ammonia was evaporated, and white precipitate was filtered and washed with ether. The filtrate was concentrated *in vacuo* to give 3-(5-adamantan-1-yl-2-methoxyphenyl)-3-(trifluoromethyl) diaziridine as a pale yellow solid (1.7 g, 81.7% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 (s, 1H), 7.38 (d, J = 10.0 Hz, 1H), 6.88 (d, J = 10.0 Hz, 1H), 3.85 (s, 3H), 2.67 (d, J = 10.0 Hz, 1H), 2.47 (d, J = 10.0 Hz, 1H), 2.09 (brs, 3H), 1.88−1.86 (m, 6H), 1.77−1.72 (m, 6H); MS (ESI) m/z 353 (M + H).

3-(2-Methoxyphenyl)-3-(trifluoromethyl)diaziridine (39b). Liquid ammonia (6.0 mL) was added at −78 °C to an ether (30.0 mL) solution of 2,2,2-trifluoro-1-(2-methoxyphenyl)ethanone *O*-tosyl oxime (**38b**) (3.0 g, 8.03 mmol) in a sealed tube. The solution was stirred at room temperature for 16 h. The mixture was carefully cooled to −78 °C, and the sealed tube was opened. The ammonia was evaporated, and white precipitate was filtered and washed with ether. The filtrate was concentrated *in vacuo* to give 3-(2-methoxyphenyl)-3-(trifluoromethyl)diaziridine as a pale yellow solid (1.5 g, 85.7% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, J = 8.0 Hz, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.00 (t, J = 8.0 Hz, 1H), 6.94 (d, J = 8.0 Hz, 1H), 3.79 (s, 3H), 2.69(d, J = 10.0 Hz, 1H), 2.47(d, J = 10.0 Hz,1H); MS (ESI) m/z 219 (M + H).

3-(5-Adamantan-1-yl-2-methoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine (40a). To a mixture of 3-(5-adamantan-1-yl-2-methoxyphenyl)-3-(trifluoromethyl)diaziridine (**39a**) (1.6 g, 4.54 mmol) and triethylamine (1.90 mL, 13.62 mmol) in methanol (16 mL) was added iodine (1.37 g, 4.99 mmol) at 0 °C, and then, the mixture was stirred at room temperature for 3 h. After completion of the reaction, aqueous citric acid 10% (10 mL) and $Na_2S_2O_3$ were added. The organic layer was extracted three times with ether. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Flash chromatography of the residue over silica gel (hexane:EtOAc = 9.5:0.5) afforded 3-(5-adamantan-1-yl-2-methoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine as a pale yellow solid (1.5 g, 94.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (s, 1H), 7.33 (d, J = 8.0 Hz, 1H), 6.83 (d, J = 8.0 Hz, 1H), 3.85 (s, 3H), 2.09 (brs, 3H), 1.88−1.86 (m, 6H), 1.76−1.72 (m, 6H); MS (ESI) m/z 351 (M + H).

3-(2-Methoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine (40b). To a mixture of 3-(2-methoxyphenyl)-3-(trifluoromethyl)diaziridine (**39b**) (1.4 g, 6.41 mmol) and triethylamine (2.68 mL, 19.25 mmol) in methanol (14 mL) was added iodine (1.79 g, 7.05 mmol) at 0 °C, and then, the mixture was stirred at room temperature for 3 h. After completion of the reaction, aqueous citric acid 10% (10 mL) and $Na_2S_2O_3$ were added. The organic layer was extracted three times with ether. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Flash chromatography of the residue over silica gel (hexane:EtOAc = 9.5:0.5) afforded 3-(2-methoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine as a pale yellow oil (1.2 g, 86.9% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, J = 8.0 Hz, 1H), 7.40 (t, J = 8.0 Hz, 1H), 7.00 (t, J = 8.0 Hz, 1H), 6.93 (d, J = 8.0 Hz, 1H), 3.93 (s, 3H); MS (ESI) m/z 217 (M + H).

dx.doi.org/10.1021/jm501241g | *J. Med. Chem.* 2014, 57, 9522−9538

4-Adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)-phenol (41a). To a solution of 3-(5-adamantan-1-yl-2-methoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine (**40a**) (1.4 g, 3.99 mmol) in CH_2Cl_2 (14 mL) was added BBr_3 (0.45 mL, 4.79 mmol) in CH_2Cl_2 (10 mL) through a septum using a syringe at −10 °C. After stirring for 30 min at the same temperature, the cold bath was removed, and the mixture was stirred for 1.5 h. After completion of the reaction, the mixture was partitioned between CH_2Cl_2 and water, and the organic layer was washed with brine, dried, and concentrated. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 9.5:0.5), which afforded 4-adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenol as a pale yellow semisolid (0.6 g, 44.7% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.93 (s, 1H), 7.71 (m, 2H), 7.10 (d, J = 8.0 Hz, 1H), 2.08 (brs, 3H), 1.85−1.82 (m, 6H), 1.76−1.70 (m, 6H); MS (ESI) m/z 337 (M + H).

2-(3-(Trifluoromethyl)-3*H*-diazirin-3-yl)phenol (41b). To a solution of 3-(2-methoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine (**40b**) (1.0 g, 4.62 mmol) in CH_2Cl_2 (10 mL) was added BBr_3 (0.52 mL, 5.55 mmol) in CH_2Cl_2 (10 mL) through a septum using a syringe at −10 °C. After stirring for 30 min at the same temperature, the cold bath was removed, and the mixture was stirred for 1.5 h. After completion of the reaction, the mixture was partitioned between CH_2Cl_2 and water, and the organic layer was washed with brine, dried, and concentrated. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 9.5:0.5), which afforded 2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenol as a pale yellow oil (0.35 g, 37.4% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.06 (s, 1H), 7.62 (d, J = 8.0 Hz, 1H), 7.32 (t, J = 8.0 Hz, 1H), 7.00 (t, J = 8.0 Hz, 1H), 6.93 (d, J = 8.0 Hz, 1H); MS (ESI) m/z 203 (M + H).

Ethyl 2-(4-Adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetate (42a). A suspension of 4-adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenol (**41a**) (0.5 g, 1.48 mmol), anhydrous potassium carbonate (0.61 g, 4.45 mmol), and ethyl chloroacetate (0.32 mL, 2.97 mmol) in DMF (5 mL) was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate and subsequently washed with aqueous sodium bicarbonate, brine, and water. The organic layer was dried over anhydrous $MgSO_4$. The solvent was filtered and evaporated under reduced pressure to afford a crude solid, which was purified by silica gel column chromatography (hexane:EtOAc = 3:1) to give ethyl 2-(4-adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)-acetate as a white solid (0.3 g, 48.4% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (s, 1H), 7.55 (d, J = 8.0 Hz, 1H), 6.84 (d, J = 8.0 Hz, 1H), 4.69 (s, 2H), 4.27 (q, J = 7.2 Hz, 2H), 2.10 (brs, 3H), 1.87−1.84 (m, 6H), 1.81−1.78 (m, 6H), 1.30 (t, J = 8.0 Hz, 3H); MS (ESI) m/z 423 (M + H).

Ethyl 2-(2-(3-(Trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)-acetate (42b). A suspension of 2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenol (**41b**) (0.3 g, 1.48 mmol), anhydrous potassium carbonate (0.61 g, 4.45 mmol), and ethyl chloroacetate (0.32 mL, 2.97 mmol) in DMF (3 mL) was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate and subsequently washed with aqueous sodium bicarbonate, brine, and water. The organic layer was dried over anhydrous $MgSO_4$. The solvent was filtered and evaporated under reduced pressure to afford a crude solid, which was purified by silica gel column chromatography (hexane:EtOAc = 3:1) to give ethyl 2-(2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetate as a white solid (0.14 g, 33.33% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (d, J = 8.0 Hz, 1H), 7.30 (t, J = 8.0 Hz, 1H), 7.00 (t, J = 8.0 Hz, 1H), 6.92 (d, J = 8.0 Hz, 1H), 4.60 (s, 2H), 4.24 (q, J = 7.2 Hz, 2H), 1.30 (t, J = 8.0 Hz, 3H); MS (ESI) m/z 289 (M + H).

2-(4-Adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetic Acid (43a). A solution of ethyl 2-(4-adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetate (**42a**) (0.2 g, 0.47 mmol) in THF/H_2O (3:1, 2 mL) was treated with lithium hydroxide monohydrate (0.07 g, 1.89 mmol) and stirred at room temperature until the reaction was complete as judged by TLC. The reaction mixture was then acidified with 10% HCl to pH 4 and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo* to obtain 2-(4-adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetic acid as a white solid (0.1 g, 55.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (s, 1H), 7.66 (d, J = 8.0 Hz, 1H), 6.94 (d, J = 8.0 Hz, 1H), 4.75 (s, 2H), 2.12 (brs, 3H), 1.91−1.88 (m, 6H), 1.79−1.76 (m, 6H); MS (ESI) m/z 395 (M^+).

2-(2-(3-(Trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetic Acid (43b). A solution of ethyl 2-(2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetate (**42b**) (0.12 g, 0.41 mmol) in THF/H_2O (3:1, 2 mL) was treated with lithium hydroxide monohydrate (0.07 g, 1.66 mmol) and stirred at room temperature until the reaction was complete as judged by TLC. The reaction mixture was then acidified with 10% HCl to pH 4 and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo* to obtain 2-(2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetic acid as a white solid (0.08 g, 74.0% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (d, J = 8.0 Hz, 1H), 7.35 (t, J = 8.0 Hz, 1H), 7.12 (t, J = 8.0 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 4.66 (s, 2H); MS (ESI) m/z 261 (M + H).

Prop-2-ynyl 3-(2-(4-Adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetamido)-4-hydroxybenzoate (4a). To a solution of 2-(4-adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetic acid (**43a**) (0.08 g, 0.20 mmol) and prop-2-ynyl 3-amino-4-hydroxybenzoate (**15**) (0.04 g, 0.20 mmol) in DMF (2.0 mL) were added EDC·HCl (0.046 g, 0.24 mmol), HOBt (0.032 g, 0.24 mmol), and DIPEA (0.09 mL, 0.5 mmol). The reaction mixture was stirred overnight at room temperature and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 6:4) to give prop-2-ynyl 3-(2-(4-adamantan-1-yl-2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)-acetamido)-4-hydroxybenzoate as a pale yellow solid (0.02 g, 18.2% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.10 (s, 1H), 10.00 (s, 1H), 8.08 (s, 1H), 7.94 (s, 1H), 7.89 (d, J = 8.0 Hz, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.07 (d, J = 8.0 Hz, 1H), 7.01 (d, J = 8.0 Hz, 1H), 4.91 (t, J = 4.0 Hz, 2H), 4.79 (s, 2H), 2.51 (t, J = 4.0 Hz, 1H), 2.12 (brs, 3H), 1.91−1.88 (m, 6H), 1.79−1.76 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.8, 164.9, 156.1, 154.2, 145.7, 134.4, 129.6, 129.1, 125.0, 124.9, 121.8, 120.1, 118.7, 115.0, 113.6, 77.7, 74.2, 67.3, 60.4, 52.4, 43.0, 36.5, 35.9, 29.7; HRMS [M + H] calcd [$C_{30}H29F_3N_3O_5$], 567.1915; found, 567.1909; purity >99.9% (as determined by RP-HPLC, method A, t_R = 23.83 min; method B, t_R = 22.95 min).

Prop-2-ynyl-4-hydroxy-3-(2-(2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetamido)benzoate (4b). To a solution of 2-(2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetic acid (**43b**) (0.06 g, 0.23 mmol) and prop-2-ynyl 3-amino-4-hydroxybenzoate (**15**) (0.04 g, 0.23 mmol) in DMF (2.0 mL) were added EDC·HCl (0.052 g, 0.27 mmol), HOBt (0.037 g, 0.27 mmol), and DIPEA (0.1 mL, 0.57 mmol). The reaction mixture was stirred at room temperature overnight and then partitioned between EtOAc and brine. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated in a vacuum. The resulting residue was purified by silica gel column chromatography (hexane:EtOAc = 7:3) to give prop-2-ynyl 4-hydroxy-3-(2-(2-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenoxy)acetamido)benzoate as a white solid (0.02 g, 22.2% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.80 (s, 1H), 8.62 (s, 1H), 7.89 (d, J = 8.0 Hz, 1H), 7.78 (s, 1H), 7.39 (t, J = 8.0 Hz, 2H), 7.10 (t, J = 8.0 Hz, 1H), 7.02 (d, J = 8.0 Hz, 2H), 4.90 (s, 2H), 4.71 (s, 2H), 2.58 (t, J = 4.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.8, 164.9, 156.1, 154.2, 129.6, 129.1, 125.0, 124.9, 121.8, 120.1, 115.0, 113.6, 77.7, 74.2, 67.3, 60.4, 52.4; HRMS [M + H]$^+$ calcd [$C_{20}H_{14}F_3N_3O_5$], 434.2331; found, 434.2321; purity >99.9% (as determined by RP-HPLC, method A, t_R = 26.16 min; method B, t_R = 25.38 min).

Cell Culture. Human colorectal carcinoma HCT116 cells were cultured in a 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Cells were seeded at a density of 5 × 10^5 cells/mL/well in a 12-well tissue culture plate at 37 °C for 20 h for subsequent experiments. Hypoxic conditions were achieved by

dx.doi.org/10.1021/jm501241g | J. Med. Chem. 2014, 57, 9522−9538

replacing cells with 1% O_2, 94% N_2, and 5% CO_2 in a multigas incubator (Sanyo, Osaka, Japan).

HRE-Luciferase Reporter Assay. HCT116 cells expressing both a hypoxia response element (HRE)-dependent firefly luciferase reporter and a CMV-renilla luciferase reporter were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco).[18] The cells (2×10^4 cells/0.1 mL/well) were seeded in a 96-well tissue culture plate for 20 h for subsequent experiments. The cells were incubated for 12 h with or without drugs in normoxic or hypoxic conditions (1% O_2, 94% N_2, and 5% CO_2). The luciferase activities of cells were measured using a Dual-Luciferase Assay System (Promega, Madison, WI) with a Victor X Light luminescence reader (PerkinElmer, Boston, MA).

Immunoblot Analysis. Cells were washed with PBS, lysed with RIPA buffer (20 mM HEPES (pH 7.4), 1% Triton X-100, 10% glycerol, 1 mM EDTA, 5 mM sodium fluoride, 10 μg/mL phenylmethylsulfonylfluoride (PMSF), and 1 mM sodium vanadate) for 15 min at 4 °C, and centrifuged at 13 000 rpm for 15 min. Lysates were then boiled for 5 min in 5× sample buffer (50 mM Tris (pH 7.4), 4% sodium dodecyl sulfate (SDS), 10% glycerol, 4% 2-thioethanol, and 50 μg/mL bromophenol blue) at a ratio of 4:1. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membranes (Millipore, Billerica, MA), and immunoblotted with anti-HIF-1α (BD Transduction Laboratories, San Diego, CA) or anti-β-actin (Abcam, Cambridge, U.K.). Protein expression was visualized on Kodak Biomax X-ray film (Kodak, Rochester, NY).

MDH2 Activity Assay. The activity of MDH2 was determined using a MDH2 activity assay kit (Abcam) according to the manufacturer's instructions. Cells were lysed with RIPA buffer containing protease and phosphatase inhibitors. A 100 μL portion of proteins (100 μg/mL) was incubated for 3 h at room temperature in each well. After washing three times with washing buffer, 50 μL of drug diluted in base buffer was added to the well. This was preincubated for 1 h at room temperature and then for 3 h at 4 °C. The assay reagents (the final concentrations of malate and NAD+ were 5 and 1 mM, respectively) were added to the well, and the absorbance at 450 nm was measured at 30 and 60 min.

***In Vitro* MDH2 Binding Assay.** The recombinant human MDH2 (BioVision, Mountainview, CA, USA, 2 μg) was incubated with probes, and then photoaffinity labeling was performed by irradiation with 360 nm UV radiation (UVP, Upland, CA, USA) for 30 min on ice. Click reactions of the probe and Cy3 azide (Click Chemistry Tools, Scottsdale, AZ, USA) were established with the Click-iT Protein Reaction Buffer Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After the click reaction, proteins were precipitated with methanol/chloroform/water (60/15/40, v/v) and denatured by boiling for 5 min in a sample buffer. Proteins were separated by SDS-PAGE, and fluorescence was detected with a Typhoon 9410 imaging system (GE Healthcare, Piscataway, NJ, USA).

Measurement of Oxygen Consumption. The oxygen consumption by mitochondrial respiration in HCT116 cells was measured using an Oxygraph (Hansatech Instruments, Norfolk, U.K.). After drug treatment for 3 h, HCT116 cells were harvested, and then, oxygen consumption was measured for 5 min at 37 °C with a thermoregulated controlled circulating system.[28]

Determination of ATP Content. Intracellular ATP content was determined with the ENLITEN ATP Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Purification of Recombinant Human MDH2. The pOTB7-MDH2 clone was obtained (Korea Human Gene Bank, Medical Genomics Research Center, KRIBB, Korea) and subcloned into pET28a (Merck, Germany). 6-His-hMDH2 protein was purified using Ni-NTA affinity chromatography after IPTG induction of hMDH2 in *E. coli*. N-Terminal 6-histidines were removed from the recombinant protein by the treatment with thrombin. Then, recombinant hMDH2 was purified by size-exclusion chromatography on a HiLoad 16/600 Superdex 75 pg (GE healthcare, USA).

Kinetic Assay of MDH2. The enzyme activity of MDH2 was measured by oxaloacetate-dependent NADH oxidation assays, where the NADH concentration was determined by measuring absorbance at 340 nm. The reaction was performed in 100 mM potassium phosphate buffer (pH 7.4) with 0.25 nM His-MDH2, 600 μM oxaloacetic acid, and various concentrations of NADH (60, 75, 100, 150, and 300 μM). The Vmax and Km were determined from double-reciprocal Lineweaver–Burk plots using Sigmaplot 13.0, and a graph was plotted for the velocity against the concentration of NADH.

■ ASSOCIATED CONTENT

Supporting Information

Pull-down assay with biotin probes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ACKNOWLEDGMENTS

This study was supported by National Research Foundation (NRF) grants (2012M3A9C1053532, 2012M3A9B4028787, and 2014R1A2A2A01005455) funded by Ministry of Science, ICT and Future Planning, Korea, and KRIBB Initiative of the Korea Research Council of Fundamental Science and Technology. This work was inspired by the international and interdisciplinary environments of the JSPS Asian CORE Program, "Asian Chemical Biology Initiative".

■ ABBREVIATIONS USED

HIF, hypoxia inducible factor; MDH2, malate dehydrogenase 2; 2D, two-dimensional; HRE, hypoxia-response element; AMPK, AMP activated protein kinase; ACC, acetyl-CoA carboxylase; ABPs, activity-based assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NBS, *N*-bromosuccinimide; br s, broad signal; *n*-BuLi, *n*-butyllithium; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; rt, room temperature; THF, tetrahydrofuran

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