Bioorganic & Medicinal Chemistry Letters 22 (2012) 5169–5173

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Discovery of 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivatives as non-peptidic selective SUMO-sentrin specific protease (SENP)1 inhibitors

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ARTICLE INFO

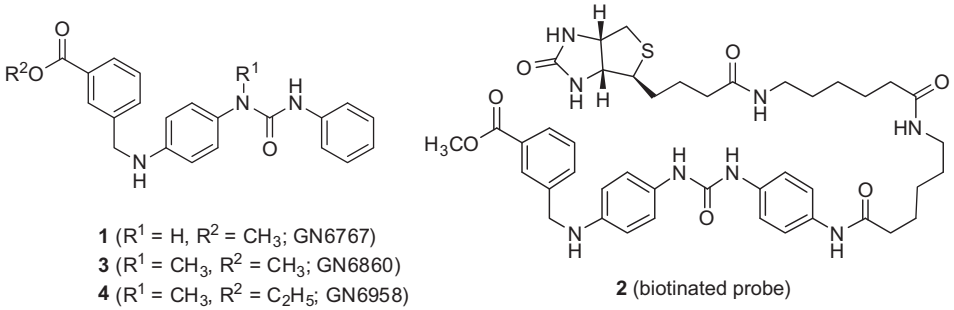
Article history:
Received 27 May 2012
Revised 22 June 2012
Accepted 26 June 2012
Available online 3 July 2012

Keywords:
SENP1
Non-peptidic inhibitors
HIF-1
deSUMOylation
Selective inhibition

ABSTRACT

We developed 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivative **4** (GN6958) as a non-peptidic selective SUMO-sentrin specific protease (SENP)1 protease inhibitor based on the hypoxia inducible factor (HIF)-1α inhibitor **1** (GN6767). The direct interaction of compound **1** with SENP1 protein in cells was observed by the pull-down experiments using the biotin-tagged compound **2** coated on the streptavidin affinity column. Among the various 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivatives tested, compounds **3** and **4** suppressed HIF-1α accumulation in a concentration-dependent manner without affecting the expression level of tubulin protein in HeLa cells. Both compounds inhibited SENP1 protease activity in a concentration-dependent manner, and compound **4** exhibited more potent inhibition than compound **3**. Compound **4** exhibited selective inhibition against SENP1 protease activity without inhibiting other protease enzyme activities in vitro.

Small ubiquitin-like modifier (SUMO), a protein that shares about 18% sequence identity with ubiquitin, modulates many biological processes including nuclear transport, transcription, replication, recombination, and chromosome segregation.[1,2] Modification of proteins by SUMO is a dynamic and reversible process and controlled by a series of on/off enzymes. 'SUMOylation', the covalent interaction between the C-terminus of SUMO and the ε-amino group of a lysine residue in the target protein, is mediated by activating (E1),[3,4] conjugating (E2),[3,5] and ligating (E3) enzymes;[6–8] however these are entirely distinct from ubiquitin E1, E2, and E3.[9,10] On the contrary, the 'deSUMOylation' is promoted by a family of SUMO/sentrin specific proteases (SENPs).[11] In the mammalian system, six SENPs (SENPs 1–3 and 5–7) have been reported and, in particular, SENP1, a nuclear protease, deconjugates a large number of SUMOylated proteins.[12] For example, SENP1 has been shown to regulate androgen receptor transactivation by targeting histone deacetylase 1 and induce c-Jun activity through deSUMOylation of p300.[13,14] Moreover, SENP1 is overexpressed in human prostate cancer specimens.[9]



1 (R^1 = H, R^2 = CH_3; GN6767)
3 (R^1 = CH_3, R^2 = CH_3; GN6860)
4 (R^1 = CH_3, R^2 = C_2H_5; GN6958)

2 (biotinated probe)

Figure 1. Structures of 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivatives **1**–**4**.

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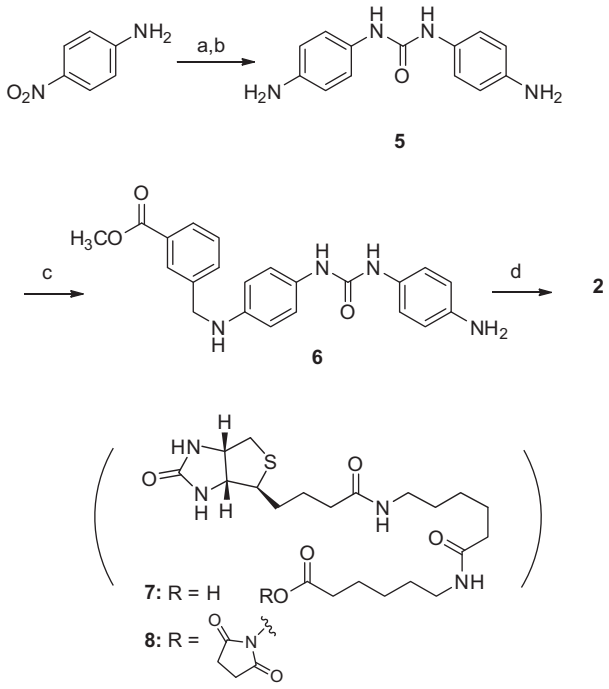
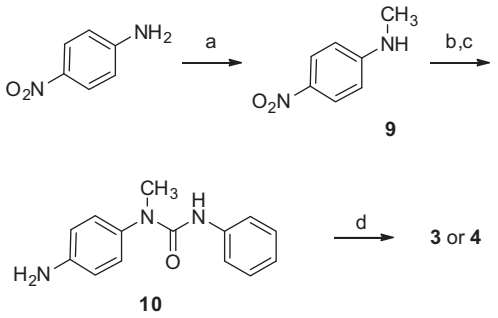
http://dx.doi.org/10.1016/j.bmcl.2012.06.084



Scheme 1. Synthesis of the biotin-conjugated probe **2**. Reagents: (a) (i) triphosgene, toluene; (ii) 4-nitroaniline, toluene, reflux; (b) H_2, Pd/C, MeOH, 2 steps 63%. (c) methyl benzaldehyde-3-carboxylate, $NaCNBH_3$, MeOH, 10%; (d) **8**, cat. DMAP, $CHCl_3$, 43%.



Scheme 2. Synthesis of the compounds **3** and **4**. Reagents: (a) (i) 1-hydroxymethylbenzotriazole, EtOH; (ii) $NaBH_4$, THF; (b) (i) triphosgene, toluene; (ii) aniline, toluene, reflux; (c) H_2, Pd/C, MeOH, 2 steps 76%; (d) methyl 3-formylbenzoate or ethyl 3-formylbenzoate, $NaCNBH_3$, MeOH.

We have focused our efforts on the development of hypoxia-inducible factor (HIF)-1 inhibitors as pathological angiogenesis inhibitors. HIF-1 is known as a heterodimeric complex consisting of a hypoxically inducible subunit, HIF-1α, and a constitutively expressed subunit, HIF-1β. Under normoxic conditions, HIF-1α protein is subject to oxygen-dependent prolyl hydroxylation, leading to rapid degradation by von Hippel–Lindau tumor suppressor protein (pVHL)-mediated ubiquitin-proteasome system (UPS).[15] Under hypoxic conditions, HIF-1α is not degraded by UPS due to the limited oxygen supply for prolyl hydroxylase (PHD) activity. The stabilized HIF-1α binds to HIF-1β to form a heterodimeric complex, which binds to the hypoxia response element (HRE) DNA sequence with co-activators to activate various genes including angiogenesis factors, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO).[16] HIF-1α is found at increased levels in a wide variety of human primary cancers compared with corresponding normal tissue. Therefore, HIF-1 has been considered an important target for the development of anticancer agents.[17–21]

We recently reported 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivative GN6767 as a new class of HIF-1α inhibitor (com-

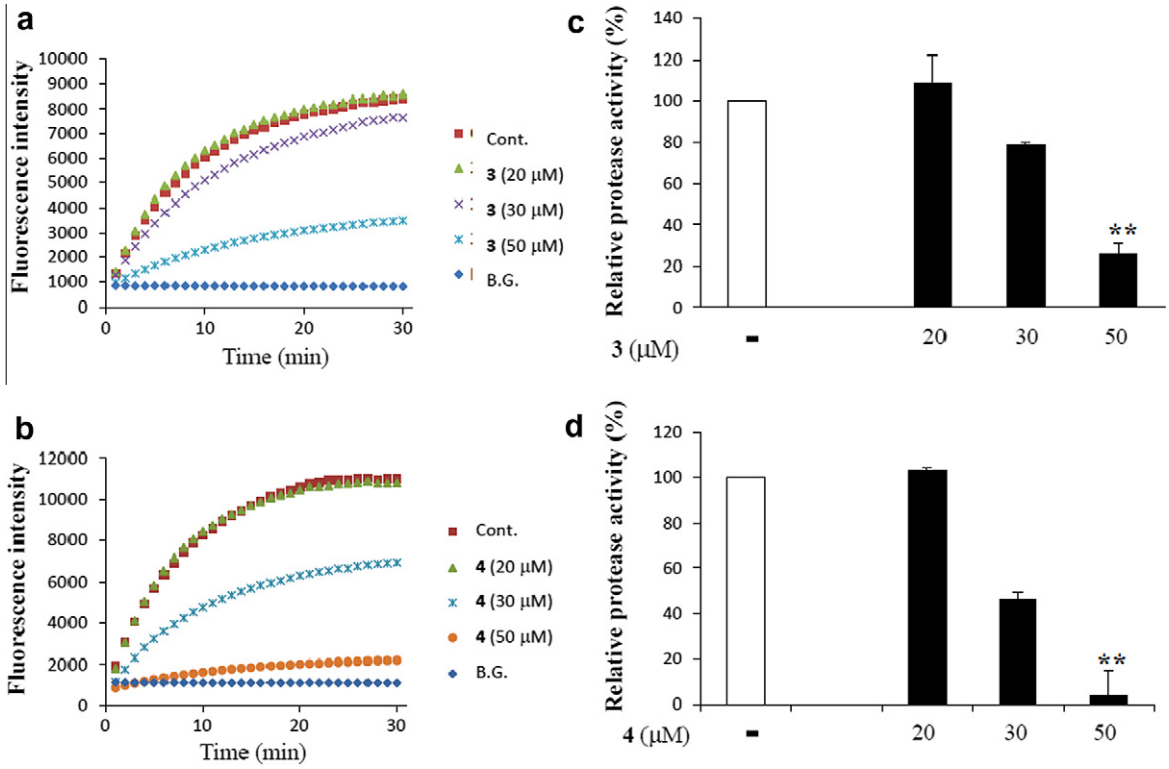


Figure 2. Inhibition of SENP1 catalytic domain (SENP1-CD) endopeptidase activity by compounds **3** and **4**. Compounds were titrated in HEPES buffer (50 mM HEPES, 0.1 mM EDTA, pH 7.9), combined with SENP1-CD (3 nM), and incubated for 10 min in 96-well plates before assaying with the SUMO-1-AMC (300 nM). Fluorescence intensity was plotted on a fluorescence plate reader (excitation/emission wavelengths 380/460 nm; Infinite F200; Tecan) for 30 min after adding the SUMO-1-AMC (a and b). Enzymatic activity was determined as the relative protease activity 5 min after adding the SUMO-1-AMC (c and d). Statistical significance: $^{**}P$ <0.01, compared with control (−).

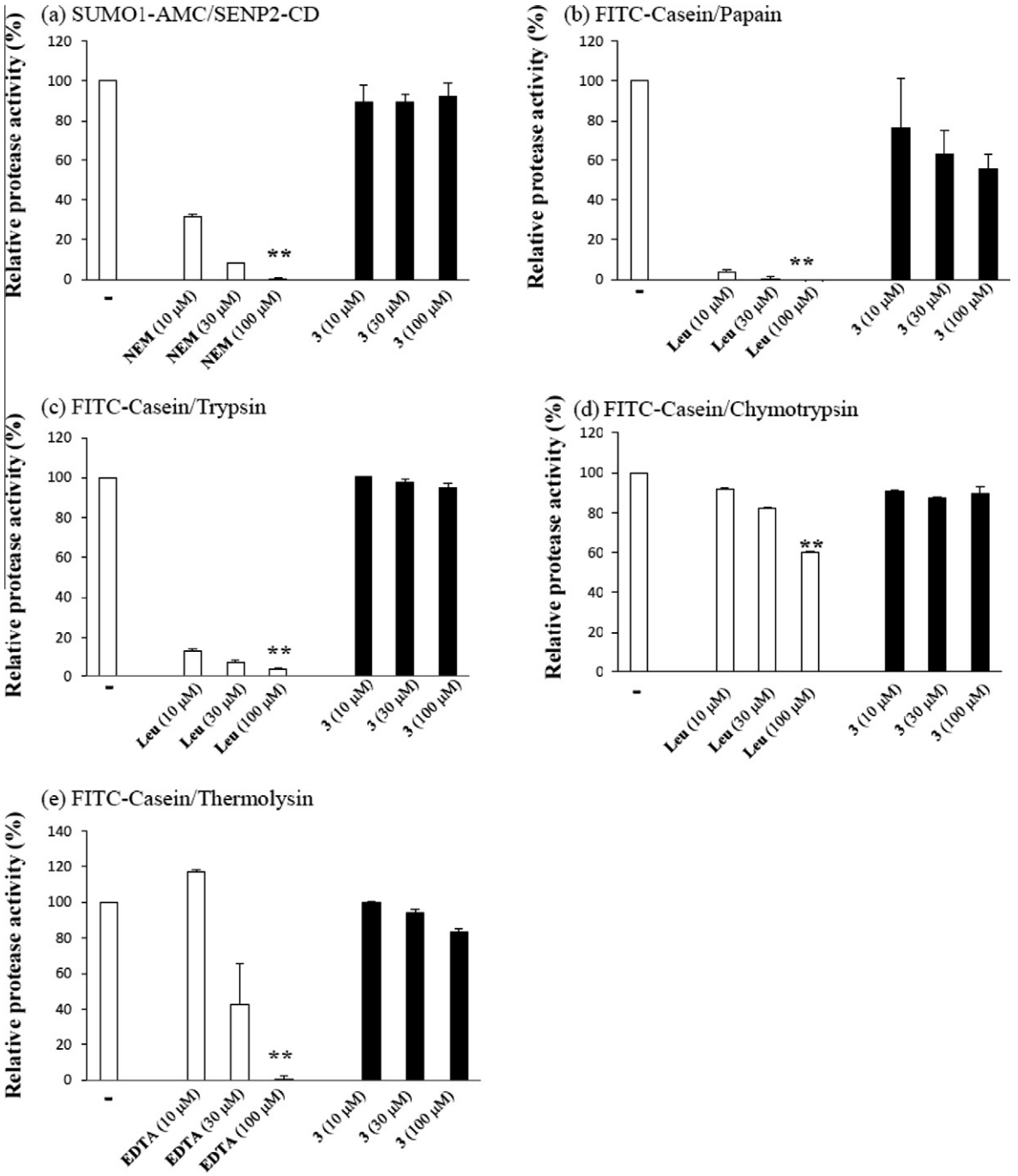


Figure 3. Titration of various proteases assayed in 96-well plates using the SUMO-1-AMC or FITC-casein substrates. (a) Recombinant human SENP2 catalytic domain (SENP2-CD) was serially diluted in HEPES buffer (50 mM HEPES, 0.1 mM EDTA, pH 7.9), or (b) papain (from papaya latex), (c) trypsin (from bovine pancreas), (d) chymotrypsin (from bovine pancreas), and (e) thermolysin (from bacillus thermoproteolyticus rokko) were serially diluted in TBS buffer (50 mM TBS, pH 7.2). Enzymatic activity was determined as the relative protease activity 5 min after adding SUMO-1-AMC substrate (a) or 10 min after adding FITC-casein substrate (b–e) on a fluorescence plate reader. Statistical significance: $^{*}P$ <0.05 and $^{**}P$ <0.01, compared with control (−).

pound **1** in Fig. 1).[20] Although GN6767 exhibited potent inhibition against HIF-1α accumulation under hypoxic condition and inhibited the hypoxia-induced HIF-1 transcriptional activity in KEK293 cells (IC_{50} = 7.2 μM), the inhibition mechanism has not been observed yet.[21]

In 2007, Yeh and co-workers investigated the way in which SENP1 controls EPO production by regulating the stability of HIF-1α under hypoxia. SUMOylation of HIF-1α induced by hypoxia promotes its binding to pVHL, which results in degradation of HIF-1α through a proline hydroxylation-independent UPS. Thus, SENP1 plays a key role in regulation of HIF-1α stability under hypoxia.[22]

We thought that SENP1 inhibitor has potential for development as an anti-tumor agent. No inhibitor of SENP1 was reported when we started the current study.[23,24] We actually synthesized biotin-conjugated chemical probe of compound **1** and tried to identify the target molecules of compound **1**. Fortunately, SENP1 protein was detected from the affinity column coated with a biotin molecule **2** (Scheme 1). Thus we screened compounds, synthesized as HIF-1α inhibitors in our laboratory,[20,21] for the inhibition of SENP1 activity using in vitro SENP protease assay, and discovered that compound **1** displayed SENP1 protease inhibition (Fig. 1). We tested various 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea deriva-

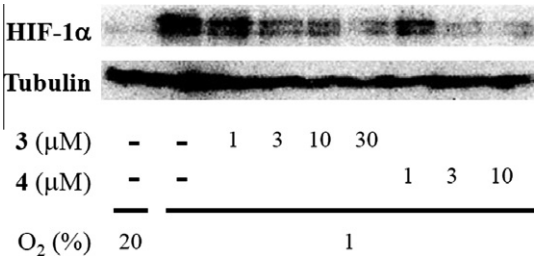


Figure 4. Effect of compounds **3** and **4** on hypoxia-induced accumulation of HIF-1α protein in HeLa cells. HeLa cells were incubated for 6 h with the indicated concentrations of compounds under hypoxic condition. The levels of HIF-1α protein were detected by immunoblot analysis with the protein specific antibody. Tubulin was used as load control.

tives including the compounds reported previously,[20] and found that the methyl- (compound **3**, $R^1 = CH_3$, $R^2 = CH_3$) and the ethyl- (compound **4**, $R^1 = CH_3$, $R^2 = C_2H_5$) substituents at the urea moiety possess more efficient for the SENP1 protease inhibition. (See Scheme 2)

Synthesis of the biotin-conjugated chemical probe of compound **1** is shown in Scheme 1. The reaction of 4-nitroaniline with triphosgene in toluene afforded the corresponding isocyanate, which reacted with 4-nitroaniline and the resulting 1,3-bis(4-nitrophenyl)urea was hydrogenated to give 1,3-bis(4-aminophenyl)urea **5** in 63% yield in two steps. Reductive amination of **5** with methyl 3-formylbenzoate gave compound **6**, which reacted with activated biotin-conjugated carboxylic acid **8** to afford the biotinylated probe **2**.

Synthesis of compounds **3** and **4** is shown in Scheme 1. *N*-Methyl-4-nitroaniline **9**, derived from 4-nitroaniline,[25] was treated with triphosgene to afford the corresponding isocyanate, which reacted with aniline to give urea derivative. The nitro group was hydrogenated under palladium catalyzed condition to give compound **10** in 76% yield in two steps. Reductive amination of compound **10** with methyl 3-formylbenzoate or ethyl 3-formylbenzoate gave compound **3** and **4** in 46% and 61% yields, respectively.

We first examined the direct interaction of 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivatives with SENP1 protein in cells. The *streptavidin HP SpinTrap affinity column* was coated with the biotin-tagged compound **2** and HeLa cell lysate was passed through this column. The proteins trapped by the affinity column were eluted with 2% SDS solution and the eluted materials were analyzed by western blots using a SENP1 antibody. SENP1 protein was retained on the affinity column in the presence of biotin conjugated **2**, whereas no SENP1 protein was detected from the affinity column coated with a biotin molecule **7**, revealing that biotin conjugated **2** interact with SENP1 in the cells (Fig. S1 in the Supplementary data).

We next examined the effect of compounds on in vitro SENP1 endopeptidase activity. Each compound was combined with a SENP1 catalytic domain and incubated with fluorogenic SUMO-1-AMC (7-amino-4-methylcoumarin). The enzymatic activity was determined by the release of fluorescent AMC. Although GN6767 displayed 40% inhibition of the SENP1 endopeptidase activity at 100 μM concentration, significant inhibition was not observed at 50 μM (Fig. S2). A methyl substituent on the urea nitrogen ($R^1 = CH_3$) in the urea derivatives increased inhibitory potency of SENP1 endopeptidase activity. As shown in Figure 2, both methyl-substituted urea derivatives, **3** and **4**, inhibited SENP1 endopeptidase activity in a concentration-dependent manner. Compound **3** displayed 74% inhibition of the SENP1 endopeptidase activity at a 50 μM concentration and its ethyl ester derivative **4** displayed 97% inhibition at the same concentration. The IC_{50} values of compounds **3** and **4** were calculated to be 39.5 ± 0.8 μM and 29.6 ± 0.5 μM, respectively. During our study of SENP1 inhibitors, first SENP1 inhibitors were reported by Bogyo and the co-workers.[23] They developed the peptide-based inhibitors, however, those compounds also inhibited SENP2 activity.

Since compound **4** showed significant inhibition toward SENP1 endopeptidase activity, we further investigated the selectivity of compound **4** against other proteases.[26] We chose SENP2 and papain as cysteine protease, trypsin and chymotrypsin as serine protease, and thermolysin as metallo protease, and examined inhibition of compound **4** toward these protease activities. The fluorogenic substrate SUMO-1-AMC was employed for measurement of the enzymatic activities of SENP2. *N*-Ethylmaleimide (NEM), known as a cysteine protease inhibitor, was used as a positive control.[27] As shown in Figure 3a, NEM (10-100 μM) inhibited SENP2 endopeptidase activity markedly. However, compound **4** did not exhibit significant inhibition of SENP2 at a range of 10–100 μM concentrations. The fluorogenic substrate fluorescein isothiocyanate (FITC)-Casein[28] was employed for measurement of the other enzymatic activities (Fig. 3b–e). Leupeptin hydrochloride (Leu), known as serine/cysteine inhibitor,[29] was used as a positive control for the experiments of papain and trypsin (Fig. 3b and c), and *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)[30] and EDTA[31] were used as positive controls for the experiments of chymotripsin and thermolysin (Fig. 3d and e), respectively. Although weak inhibitions of compound **4** against papain were observed in a concentration-dependent manner (Fig. 3b), compound **4** did not exhibit significant inhibition toward trypsin, chymotripsin, and thermolysin at a range of 10–100 μM concentrations (Fig. 3c–e). These results indicate that compound **4** possesses selective inhibitory potency toward SENP1 enzymatic activity.

We also demonstrated the effect of the synthesized compounds **3** and **4** on the hypoxia-induced HIF-1α accumulation in HeLa human cervical cancer cells by western blot analysis. The results are shown in Figure 4. Both compounds **3** and **4** suppressed HIF-1α accumulation in a concentration-dependent manner without affecting the expression level of tubulin protein and compound **4** displayed higher inhibitory potency against HIF-1α accumulation than compound **3** in HeLa cells. Actually, significant suppression of HIF-1α accumulation was observed at a 3 μM concentration of compound **3** under hypoxic condition.

In conclusion, we developed 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivative **4** as a SENP1 protease inhibitor. Inhibition of compound **4** against SENP1 protease activity is not as high as that against HIF-1α accumulation (Fig. 2d vs Fig. 4), suggesting that suppression of HIF-1α accumulation could be independent of SENP1 inhibition by compound **4**. Even in these cases, as far as we know, there are only two reports of SENP1 protease inhibitors; the peptidic SENPs protease inhibitors[23] and benzodiazepine-based non-peptidic inhibitors.[24] Therefore, the current investigation is a great potential not only as tools for the study of SUMO-related action mechanisms but also as a new type of anticancer drug design.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.06.084.

References and notes

1. Yeh, E. T. H.; Gong, L.; Kamitani, T. *Gene* **2000**, *248*, 1.
2. Johnson, E. S. *Annu. Rev. Biochem.* **2004**, *73*, 355.
3. Johnson, E. S.; Schwienhorst, I.; Dohmen, R. J.; Blobel, G. *EMBO J.* **1997**, *16*, 5509.
4. Gong, L.; Li, B.; Millas, S.; Yeh, E. T. *FEBS Lett.* **1999**, *448*, 185.
5. Gong, L.; Kamitani, T.; Fujise, K.; Caskey, L. S.; Yeh, E. T. *J. Biol. Chem.* **1997**, *272*, 28198.

6. Johnson, E. S.; Gupta, A. A. *Cell* **2001**, *106*, 735.
7. Pichler, A.; Gast, A.; Seeler, J. S.; Dejean, A.; Melchior, F. *Cell* **2002**, *108*, 109.
8. Kahyo, T.; Nishida, T.; Yasuda, H. *Mol. Cell* **2001**, *8*, 713.
9. Cheng, J.; Bawa, T.; Lee, P.; Gong, L.; Yeh, E. T. *Neoplasia* **2006**, *8*, 667.
10. Yeh, E. T. *J. Biol. Chem.* **2009**, *284*, 8223.
11. Mukhopadhyay, D.; Dasso, M. *Trends Biochem. Sci.* **2007**, *32*, 286.
12. Mikolajczyk, J.; Drag, M.; Békés, M.; Cao, J. T.; Ronai, Z. E.; Salvesen, G. S. *J. Biol. Chem.* **2007**, *282*, 26217.
13. Cheng, J.; Wang, D.; Wang, Z.; Yeh, E. T. *Mol. Cell. Biol.* **2004**, *24*, 6021.
14. Cheng, J.; Perkins, N. D.; Yeh, E. T. *J. Biol. Chem.* **2005**, *280*, 14492.
15. Huang, L. E.; Gu, J.; Schau, M.; Bunn, H. F. *Proc. Nat. Acad. Sci. U.S.A.* **1998**, *95*, 7987.
16. Jiang, B.-H.; Rue, E.; Wang, G. L.; Roe, R.; Semenza, G. L. *J. Biol. Chem.* **1996**, *271*, 17771.
17. Garber, K. *J. Natl. Cancer Inst.* **2005**, *97*, 1112.
18. Yeo, E.-J.; Chun, Y.-S.; Cho, Y.-S.; Kim, J.; Lee, J.-C.; Kim, M.-S.; Park, J.-W. *J. Natl Cancer Inst.* **2003**, *95*, 516.
19. Won, M.-S.; Im, N.; Park, S.; Boovanahalli, S. K.; Jin, Y.; Jin, X.; Chung, K.-S.; Kang, M.; Lee, K.; Park, S.-K.; Kim, H. M.; Kwon, B. M.; Lee, J. J.; Lee, K. *Biochem. Biophys. Res. Commun.* **2009**, *385*, 16.
20. Uno, M.; Ban, H. S.; Nakamura, H. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3166.
21. Shimizu, K.; Maruyama, M.; Yasui, Y.; Minegishi, H.; Ban, H. S.; Nakamura, H. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1453.
22. Cheng, J.; Kang, X.; Zhang, S.; Yeh, E. T. H. *Cell* **2007**, *131*, 584.
23. Albrow, V. E.; Ponder, E. L.; Fasci, D.; Békés, M.; Deu, E.; Salvesen, G. S.; Bogyo, M. *Chem. Biol.* **2011**, *18*, 722.
24. Qiao, Z.; Wang, W.; Wang, L.; Wen, D.; Zhao, Y.; Wang, Q.; Meng, Q.; Chen, G.; Wu, Y.; Zhou, H. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6389.
25. Katritzky, A. R.; Rachwal, S.; Rachwal, B. *J. Chem. Soc., Perkin Trans. 1* **1987**.
26. Ichihara, Y.; Sogawa, K.; Takahashi, K. *J. Biochem.* **1982**, *92*, 1.
27. Nicholson, B.; Leach, C. A.; Goldenberg, S. J.; Francis, D. M.; Kodrasov, M. P.; Tian, X.; Shanks, J.; Sterner, D. E.; Bernal, A.; Mattern, M. R.; Wilkinson, K. D.; Butt, T. R. *Protein Sci.* **2008**, *17*, 1035.
28. Sally, S. T. *Anal. Biochem.* **1984**, *143*, 30.
29. Inoue, S.; Bar-Nun, S.; Roitelman, J.; Simoni, R. D. *J. Biol. Chem.* **1991**, *266*, 13311.
30. Miao, Q.; Sun, Y.; Wei, T.; Zhao, X.; Zhao, K.; Yan, L.; Zhang, X.; Shu, H.; Yang, F. *J. Biol. Chem.* **2008**, *283*, 8218.
31. Iizuka, K.; Kawaguchi, H.; Kitabatake, A. *J. Mol. Cell Cardiol.* **1993**, *25*, 1101.