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Discovery of a potent tubulin polymerization inhibitor: Synthesis and evaluation of water-soluble prodrugs of benzophenone analog

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ABSTRACT

Prodrugs have proven to be very useful in enhancing aqueous solubility of sparingly water-soluble drugs, thereby increasing in vivo efficacy without a need of special excipients. In vitro and in vivo evaluations of a number of amino acid prodrugs of **1**, a previously identified potent tubulin polymerization inhibitor and cytotoxic against various cancer cell lines led to the discovery of **3**·**HCl** (L-valine attached) which is highly efficacious in mouse xenografts bearing human cancer. Pharmacokinetic analysis in rats revealed that compound **1** was released immediately upon administration of **3**·**HCl** intravenously, with rapid clearance of **3**·**HCl** indicating the effective cleavage of prodrug. Compound **3**·**HCl** (CKD-516) has now been progressed to phase 1 clinical trial.

Microtubules, a major type of cytoskeletal filament in cells, are formed from tubulin subunits, that is, α- and β-tubulin. Their importance in mitosis and cell division makes microtubules an attractive target for anticancer drug discovery.[1] A number of naturally occurring compounds such as paclitaxel, epothilones, vinblastin, combretastatin A-4, and colchicines exert their effect by changing dynamics of tubulin polymerization and depolymerization.[2] In addition to their direct effect on cell division, another mechanism of anticancer activity of tubulin polymerization inhibitors was revealed as VDA (vascular disrupting agent).[3] By affecting the microtubule cytoskeleton, they cause morphological and functional changes in endothelia cells that lead to rapid vasculature collapse, reduction of blood flow, and ultimately to central tumor necrosis. Representative tubulin polymerization inhibitors such as CA-4P[4] and AVE-8062[5] are believed to be more efficient, less toxic, and several of them are currently undergoing clinical trials. AVE-8062 is the serine prodrug of AVE-8063, which was found to have more potent cytotoxicity and antivascular activities compared with CA-4P and is currently undergoing several phase 3 clinical trials for the treatment of solid tumors.

One of major problem in small molecule drug discovery is its low aqueous solubility that limits its therapeutic application due to incomplete and variable absorption after administration.[6] Although sparingly water-soluble drugs can be formulated with suitable excipients for parenteral use, most formulation techniques suffer from disadvantages such as chemical and physical stability and safety issue (e.g., hypersensitivity caused by Cremophor EL used in paclitaxel).[7] Thus, prodrugs often find the high chance of success in dealing with solubility issues.[8] An approach to increase water-solubility, especially in parenteral drug delivery, is the preparation of prodrugs, in which a polar group is attached to the drug, releasing the parent drug by cleaving off in vivo via enzymatic reactions. The polar groups that are most frequently used are phosphate and amino acid. Indeed, there are numerous examples of prodrugs in which phosphate group was attached either directly linked to parent drug or through a linker group such as formaldehyde, for example, fosfluconazole,[9] fosphenytoin,[10] fludarabine phosphate,[11] and CA-4P (combretastatin A-4 phosphate).[4] Amino acid prodrugs are also found to be useful in enhancing water-solubility of parent drugs which bear either amine or alcohol function and some examples[12] are shown in Figure 1.

We have recently found **1** and its prodrug (**3**·**HCl**) as potent tubulin polymerization inhibitors.[13] Compound **1** is not only highly cytotoxic against various cancer cell lines including P-gp overexpressing cell lines with the cellular mechanism of G_2/M arrest, but also caused a significant vascular disruption leading to central tumor necrosis. However, its low water-solubility would hamper further development as a parenteral drug. This was circumvented by the preparation of (L)-valine prodrug (**3**·**HCl**) demonstrating excellent in vivo efficacy in various human tumor xenografts model due partly to enhanced water-solubility. In this communication,

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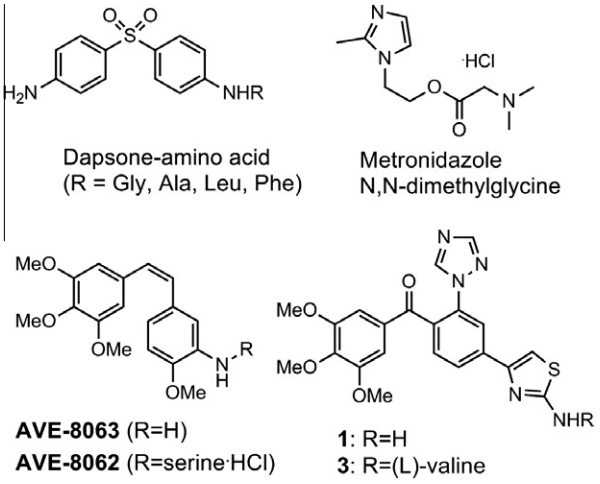


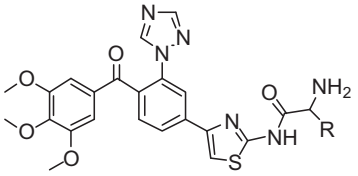
Figure 1. Examples of amino acid prodrugs and structure of title compounds (**1** and **3**).

we describe the detailed amino acid prodrug study of compound **1**, that is, synthesis of various prodrugs, in vitro activity, in vivo efficacy, and PK (pharmacokinetics) studies leading to the discovery of clinical candidate (**3·HCl**, CKD-516).

The synthesis of parent compound (**1**) and its amino acid prodrugs were prepared according to Scheme 1. 1,2,4-Triazole was reacted with compound **15**[13] to provide **16**, then its C-4 bromo group was replaced with 1-ethoxyvinyl group (**17**) by Stille coupling ($Pd(PPh_3)_2Cl_2$, THF, reflux). Bromination of **17** with NBS afforded compound **18** which was condensed with thiourea (EtOH, reflux) to give parent compound (**1**). Since several attempts of coupling reactions of amino acid with compound **1** (e.g., DCC, EDC, and HBTU) were unsuccessful, Fmoc-amino acids were first converted to acid chloride, then reacted with **1** under basic conditions at low temperature. Final compounds (**2**–**13**) were prepared by Fmoc deprotection followed by salt formation with HCl.[14] For **14** (L-Ser), coupling reaction (BOP, DIPEA) of **1** with (L)-Fmoc-Ser(OtBu)-OH proceeded in good yield, then followed by sequential deprotection (TFA then piperidine) gave desired compound.

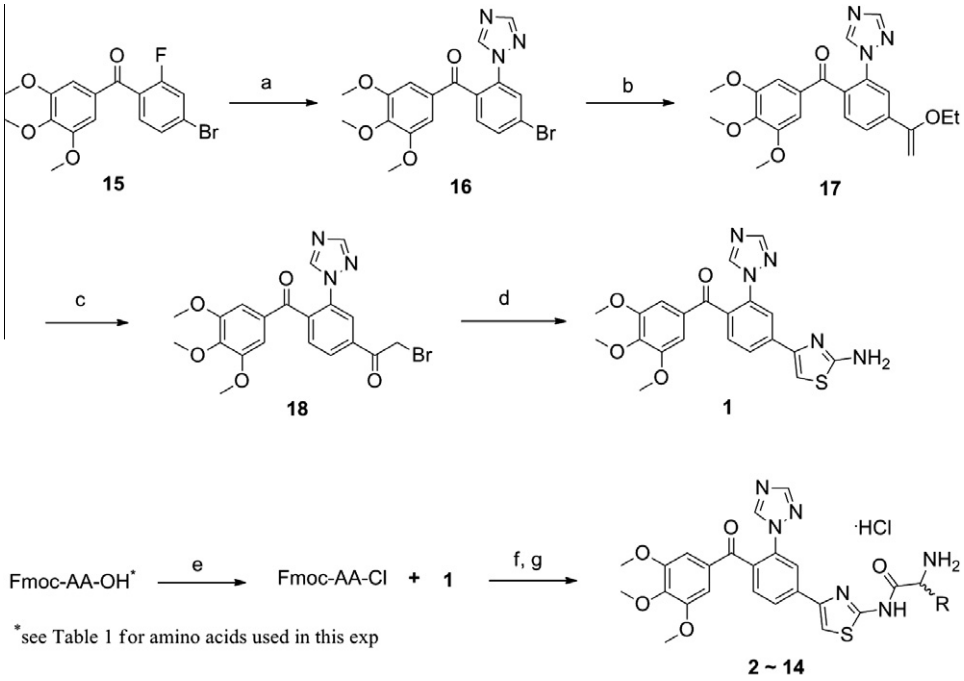
The antiproliferation potencies (IC_{50}) of parent compound and its prodrugs against human leukemia cell line (HL60) using MTT assay[15] are shown in Table 1. The IC_{50} values represent the compound concentrations requiring in 50% decrease in cell growth after 3 days of incubation. As already mentioned, compound **1** demonstrated potent cytotoxicity against HL60 (IC_{50} = 4.8 nM) as

Table 1
IC_{50} values of various amino acid prodrugs of compound **1** against HL60



Compd	R	IC_{50}[a] (μM)
1	H	0.0048
2	Glycine	0.027
3	(L)-Valine	0.110
4	(L)-Phenylalanine	0.089
5	Dimethylglycine	0.005
6	(L)-Proline	0.098
7	(L)-Leucine	0.076
8	(L)-Cyclohexylalanine	0.086
9	(D)-4-Fluorophenylalanine	0.74
10	(D)-4-Chlorophenylalanine	0.92
11	(D)-Phenylglycine	0.28
12	(L)-Alanine	0.013
13	(D)-4-CF_3-phenylalanine	>2
14	(L)-Serine	0.081
AVE-8063	—	0.012

[a] IC_{50} values are the means of at least two determinations. See the text for detailed experimental conditions.



Scheme 1. Reagents and conditions: (a) 1,2,4-triazole, K_2CO_3, DMF, 130 °C, 12 h; (b) tributyl (1-ethoxy-vinyl)tin, $Pd(PPh_3)_2Cl_2$, THF, reflux, 6 h; (c) NBS, THF/H_2O v/v 3:1, 2 h; (d) thiourea, EtOH, reflux, 2 h; (e) DMF (cat.), $SOCl_2$, CH_2Cl_2, reflux, 2 h; (f) DIPEA, pyridine, CH_2Cl_2, 0 °C to rt, overnight; (g) piperidine, CH_3CN, rt, 12 h, then, HCl.

well as several other cancer cell lines such as MDA-MB-231 (IC_{50} = 9.8 nM), a P-gp overexpressing MDR (multidrug resistant) positive cell line. The cytotoxicity of **1** were consistently better (three to sixfold) than AVE-8063 in all cell lines including MDA-MB-231 (IC_{50} = 45 nM for AVE-8063) that was highly resistant to doxorubicin (IC_{50} >600 nM). It is interesting to observe that cytotoxicity of synthesized prodrugs were quite variable ranging from single nanomolar to micromolar, indicating the rate of hydrolysis of prodrugs were different from each other under assay conditions.[8a] Compounds **2** (Gly), **5** (dimethyl-Gly), and **12** (L-Ala) were found to have higher cytotoxicity, implying that smaller amino acids seemed to be hydrolyzed more rapidly relative to bulkier analogs. The same degree of antiproliferation activity with **1** was noted in the case of dimethylglycine (**5**), which gave us a high expectation in animal efficacy study. Other prodrugs, whether hydrophobic (**3**, **4**, **6**, **7**, and **8**) or hydrophilic (**14**) were in the very close range of cytotoxicity (70–110 nM) and this suggested the steric factor was involved in enzymatic hydrolysis of amino acid prodrugs in this specific case. The low cytotoxicity of prodrugs of D-amino acids (**9**, **10**, **11**, and **13**) indicated that they are not good substrates of hydrolytic enzymes as observed in other reports.[16]

Although the synthesized prodrugs showed variable cytotoxicity resulting from different hydrolysis rate, it is unlikely that this trend would translate to in vivo system because of complications arisen from numerous factors (dissolution rate, hepatic and plasma clearance, and hydrolysis rate, etc.). Thus, as many analogs were subjected to in vivo efficacy studies excluding some analogs that are difficult to synthesize in large amount (e.g., **6** and **14**). Nude mice bearing two human colon cancer cell line (CX-1 and HCT-116) were dosed ip with vehicle or test compounds on Q4D×4 schedules[17] (preliminary results showed that ip administration of test compounds in every 4 days (Q4D) gave the best results compared to other schedules such as Qd, Q2D, or 2×/wk). AVE-8062 was included as a comparator and the results are shown in Table 2. AVE-8062 induced modest tumor growth inhibition (IR = 34% at 100 mg/kg) in CX-1 which is considered somewhat aggressive form of cancer, however significantly inhibited tumor growth in HCT116 (IR = 60% at 80 mg/kg). Although compound **2** showed better efficacy than AVE-8062 in CX-1, overt toxicity was observed at higher doses (two deaths at 10 mg/kg). Significant and dose-dependent tumor growth inhibition was demonstrated with compound **3**·**HCl** (IR = 28% at 5 mg/kg and 64% at 10 mg/kg) which is much better than AVE-8062 in CX-1. Compound **4** induced

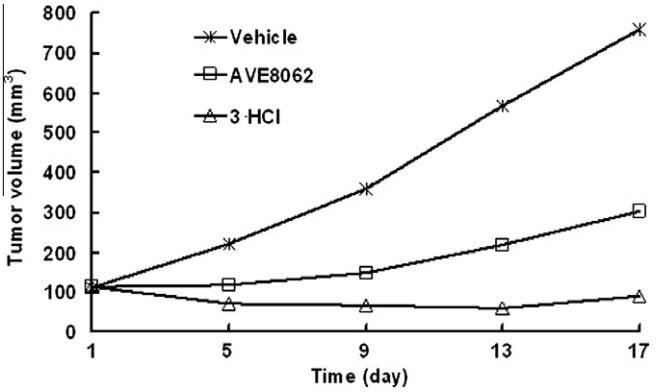


Figure 2. Antitumor efficacy of **3**·**HCl** in mouse xenografts. Nude mice bearing human colon HCT116 were treated with vehicle control or test compound (10 mg/kg, ip, Q4D×4). AVE-8062 was used a comparator (80 mg/kg, ip, Q4D×4). Data are means of tumor volume (mm^3) at each time point (n = 6 per group). Compounds were administered on days 2nd, 6th, 10th and 14th.

dose-dependent tumor growth inhibition which is comparable to AVE-8062, although the efficacy is modest. Interestingly, no significant efficacy (IR ~20%) was observed with compound **5** in CX-1 despite its strong in vitro cytotoxicity (Table 1) demonstrating the importance of suitable pharmacokinetics. For HCT116 xenografts, although AVE-8062 was shown to have significant antitumor activity, compound **3** displayed impressive in vivo efficacy (Fig. 2, IR = 88% at 10 mg/kg, p <0.001). The (L)-alanine prodrug (**12**) was found to induce significant tumor growth inhibition, however, it is inferior to compound **3** considering its apparent toxicity (one death at 10 mg/kg) at higher dose.

It was evident that starting from the same parent molecule (**1**), distinct profiles were achieved by attaching different amino acids. Based on promising antitumor efficacy, compound **3**·**HCl** was selected for further profiling. The apparent aqueous solubility was substantially improved (<100 μg/ml for **1** vs 930 mg/ml for **3**·**HCl** in deionized water) as anticipated, which is quite acceptable as parenteral administration in human use. When incubated in liver microsomes[18] from different species, **1** and **3**·**HCl** was shown to have somewhat different stability. Thus, when **3**·**HCl** was incubated for 1 h in human and dog liver microsomes (at 5 μM), only a fraction of compound was remained (15%, and 10%, respectively),

Table 2
Antitumor activities of selected compounds in BALB/c mice[a]

Compd	CX-1				HCT116			
	Doses (mg/kg)	B.W. change (%)	IR[b] (%)	Survival number	Doses (mg/kg)	B.W. change (%)	IR[b] (%)	Survival number
Control	—	+24.5	—	—	—	−5.7	—	—
AVE-8062	100	+22.8	34	6/6	80	0	60**	6/6
2	5	+22.2	40*	6/6	N.D.			
	10	+21.0	44*	4/6				
3	5	+25.7	28	6/6	10	+5.1	88***	6/6
	10	+14.7	64**	6/6				
4	5	+28.9	21	6/6	N.D.			
	10	+24.5	43	6/6				
5	5	+24.8	23	6/6	N.D.			
	10	+26.7	20	6/6				
12	N.D.				5	−1.7	62**	6/6
					10	+4.7	74***	5/6

N.D.: not determined.
[a] Nude mice bearing human CX-1 or HCT116 cancer were dosed ip with vehicle or tested compounds on a Q4d×4 schedule. All data are expressed as mean values (n = 6 per group).
[b] IR (%) = $(1 - T/C) \times 100$; T, tumor volume (treated); C, tumor volume (untreated). IRs' were measured on the 4th day after last dosing.
* p <0.05.
** p <0.01.
*** p <0.001.

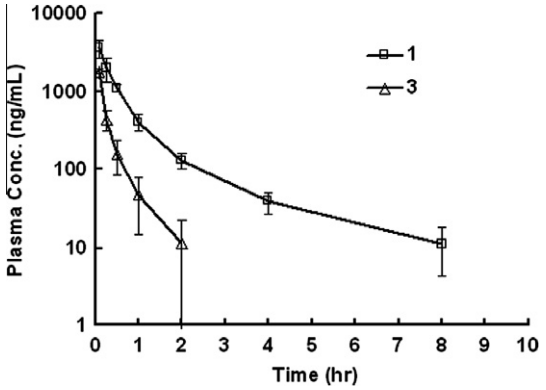


Figure 3. Time versus plasma concentration curves of **1** and **3** after iv administration (10 mg/kg) of **3**·**HCl** to fasted male Sprague-Dawley rats. LLQ = 10 ng/ml.

presumably hydrolyzed to **1** by the action of various hydrolytic enzymes present in microsomes. In contrast, about 60% remained in the case of **1** both in human and dog liver microsomes. Because the active component is compound **1** upon administration of amino acid prodrugs to mouse, their hydrolysis rate in vivo seemed to play a key role in maximizing its antitumor efficacy. In order to assure that parent compound was liberated in vivo, PK studies were performed, that is, plasma concentration of **1** was measured after intravenous administration of **3**·**HCl** to the rats (10 mg/kg in saline). Figure 3 shows the time versus plasma concentration of both **1** and **3** where compound **1** was detected right after administration with the half-life of 1.65 h while compound **3** disappeared rapidly from the plasma ($t_{1/2}$ = 0.41 h) and fell down below the LLQ (lower limit of quantitation) after 2 h. This result clearly indicated that prodrug (**3**·**HCl**) was capable of being cleaved efficiently in vivo as anticipated delivering active component, **1**. Similar pharmacokinetic profile was observed in dogs (data not shown).

In this communication, we described the discovery of highly water-soluble prodrug (**3**·**HCl**) of potent cytotoxic compound **1** by incorporating L-valine into the amino group of thiazole ring in **1**. Various amino acid prodrugs (L- or D-form) were prepared, and their in vitro antiproliferation activity against HL60 then in vivo efficacy was evaluated. Distinct efficacy profiles were observed in mouse xenografts bearing human tumor (CX-1 and HCT-116) depending on the amino acids attached. Among them, **3**·**HCl** with its aqueous solubility significantly improved showed marked antitumor efficacy and it was evident from pharmacokinetic analysis that upon administration of **3**·**HCl** intravenously, it was rapidly cleared from plasma with appearance of parent compound (**1**). Phase 1 clinical trial of compound **3**·**HCl** (CKD-516) is currently underway.

Acknowledgment

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References and notes

1. Jordan, M. A.; Wilson, L. *Nat. Rev. Cancer* **2004**, *4*, 253. and references therein.
2. (a) Romagnoli, R.; Baraldi, P. G.; Carrion, M. D.; Cruz-Lopez, O.; Cara, C. L.; Basso, G.; Viola, G.; Khedr, M.; Balzarini, J.; Mahboobi, S.; Sellmer, A.; Bramcale, A.; Hamel, E. *J. Med. Chem.* **2009**, *52*, 5551; (b) Sirisoma, N.; Kasib, S.; Pervin, A.; Zhang, H.; Jiang, S.; Willardsen, A.; Anderson, M.; Baichwal, V.; Mather, G. G.; Jessing, K.; Hussain, R.; Hoang, K.; Pleiman, C. M.; Tseng, B.; Drewe, J.; Cai, S. X. *J. Med. Chem.* **2008**, *51*, 4771; (c) Duan, J.-X.; Cai, X.; Meng, F.; Lan, L.; Hart, C.; Matteucci, M. *J. Med. Chem.* **2007**, *50*, 1001; (d) Wang, L.; Woods, K. W.; Li, Q.; Frost, D.; Rosenberg, S. H.; Sham, H. L. *J. Med. Chem.* **2002**, *45*, 1697; (e) Morris, P. G.; Fornier, M. N. *Expert Rev. Anticancer Ther.* **2008**, *9*, 175; (f) Meng, F.; Cai, X.; Duan, J.; Matteucci, M. G.; Hart, C. P. *Cancer Chemother. Pharmacol.* **2008**, *61*, 953; (g) Wu, Y.-S.; Coumar, M. S.; Chang, J.-Y.; Sun, H.-Y.; Kuo, F.-M.; Kuo, C.-C.; Chen, Y.-J.; Chang, C.-Y.; Hsiao, C.-L.; Liou, J.-P.; Chen, C.-P.; Yao, H.-T.; Chiang, Y.-K.; Tan, U.-K.; Chen, C.-T.; Chu, C.-Y.; Wu, S.-Y.; Yeh, T.-K.; Lin, C.-Y.; Hsieh, H.-P. *J. Med. Chem.* **2009**, *52*, 4941.
3. (a) Hinnen, P.; Eskens, F. *Br. J. Cancer* **2007**, *96*, 1159; (b) Kasibhatla, S.; Gourdeau, H.; Meerovitch, K.; Drewe, J.; Reddy, S.; Qiu, L.; Zhang, H.; Bergeron, F.; Bouffard, D.; Yang, Q.; Herich, J.; Lamothe, S.; Cai, S. X.; Tseng, B. *Mol. Cancer Ther.* **2004**, *3*, 1365; (c) Patterson, D. M.; Rustin, G. J. S. *Clin. Oncol.* **2007**, *19*, 443; (d) Gridelli, C.; Rossi, A.; Maione, P.; Rossi, M.; Castaldo, V.; Sacco, P. C.; Volantuoni, G. *Oncologist* **2009**, *14*, 612.
4. Griggs, J.; Metcalfe, J. C.; Hesketh, R. *Lancet Oncol.* **2001**, *2*, 82.
5. Kim, T. J.; Ravoori, M.; Landen, C. N.; Kamat, A. A.; Han, L. Y.; Lu, C.; Lin, Y. G.; Merritt, W. M.; Jennings, N.; Spannuth, W. A.; Langley, R.; Gershenson, D. M.; Coleman, R. L.; Kundra, V.; Sood, A. K. *Cancer Res.* **2007**, *67*, 9337.
6. Waterbeemd, H.; Lennaernas, H.; Artursson, P. Drug Bioavailability. In *Methods and Principles in Medicinal Chemistry*; Mannhold, R., Kubinyi, H., Timmerman, H., Eds.; Wiley-VCH: Weinheim, 2003; Vol. 18, pp 189–242.
7. Gelderblom, H.; Verweij, J.; Nooter, K.; Sparreboom, A. *Eur. J. Cancer* **2001**, *37*, 1590.
8. (a) Stella, V. J.; Nti-Addae, K. W. *Adv. Drug Delivery Rev.* **2007**, *59*, 677; (b) Bradshaw, T. D.; Chua, M.-S.; Trapani, V.; Sausville, E. A.; Stevens, M. F. G. *Br. J. Cancer* **2002**, *86*, 1348; (c) Wermuth, C. G. In *The Practice of Medicinal Chemistry*; Wermuth, C. G., Ed., 3rd ed.; Academic Press: Burlington, 2008; pp 721–746.
9. Sobue, S.; Tan, K.; Layton, G.; Leclerc, V.; Weil, A. *Br. J. Clin. Pharmacol.* **2004**, *57*, 773.
10. Stella, V. J. *Adv. Drug Delivery Rev.* **1996**, *19*, 311.
11. Plunkett, W.; Huang, P.; Gandhi, V. *Semin. Oncol. Nurs.* **1990**, *17*, 3.
12. (a) Pochopin, N. L.; Charman, W. N.; Stella, V. J. *Drug Metab. Dispos.* **1994**, *22*, 770; (b) Bundgaard, H.; Larsen, C.; Thorbek, P. *Int. J. Pharm.* **1984**, *18*, 67; (c) Vollmann, K.; Qurishi, R.; Hockemeyer, J.; Mueller, C. E. *Molecules* **2008**, *13*, 348.
13. (a) Detailed SAR studies of **1** will be reported in a separate publication. *J. Med. Chem.*, submitted for publication.; (b) Lee, K. J.; Kim, S. J.; Park, G. T.; Han, B. H.; Choi, J. H.; Hwang, I.-C.; Lee, J.; Choi, H.; Kim, Y. H.; Kim, D.-H.; Lee, S.; Hong, C. I. *Abstract of Papers*, 100th American Association for Cancer Research (AACR), Denver, CO, 2009; Abstract 5561.; (c) Kim, S. J.; Kim, S.-M.; Choi, J. H.; Lee, D. H.; Lee, D. W.; Park, G. T.; Lim, I.-T.; Jeon, D.-J.; Lee, S. W.; Kim, D.-H.; Lee, K. J.; Lee, S.; Hong, C. I. *Abstract of Papers*, 101th American Association for Cancer Research (AACR), Washington D.C., 2010; Abstract 4425.
14. *Selected data for* **3**·**HCl**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.00 (s, 1H), 9.07 (s, 1H), 8.70 (d, J = 4.4 Hz, 3H), 8.30 (d, J = 1.5 Hz, 1H), 8.16 (dd, J = 8.0, 1.6 Hz, 1H), 8.11 (s, 1H), 7.97 (s, 1H), 7.70 (d, J = 8.1 Hz, 1H), 6.85 (s, 2H), 3.94 (m, 1H), 3.71 (s, 6H), 3.69 (s, 3H), 2.25 (m, 1H), 0.98 (d, J = 6.9 Hz, 6H).; ^{13}C NMR (100 MHz, DMSO-d_6) δ 192.9, 168.0, 157.8, 152.9, 152.7, 147.5, 144.8, 142.5, 137.6, 135.8, 132.8, 131.8, 131.2, 126.2, 121.8, 112.5, 106.9, 60.6, 57.8, 56.4, 30.4, 18.8, 18.3; HRMS calcd for $C_{26}H_{28}N_6O_5S$ (free base) 537.1915 $[M+H]^+$, found 537.1915. For **12**: ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.92 (br, 1H, NH), 9.03 (s, 1H), 8.42 (br m, 3H), 8.28 (s, 1H), 8.17 (dd, 1H, J = 8.1, 1.4), 8.09 and 7.98 (2s, 2H), 7.72 (d, 1H, J = 8.1), 6.86 (s, 2H), 3.72 (s, 6H), 3.70 (s, 3H), 1.50 (d, 3H, J = 7.0). MS (ESI) m/z 509.0 (M^++1).
15. HL60 cells were seeded into 96-well plates and the compound diluents were added. After 72 h incubation at 37 °C in a humidified 5% CO_2 atmosphere, cell viability was determined by addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, final concentration of 0.25 mg/ml).
16. Ohsumi, K.; Hatanaka, T.; Nakagawa, R.; Fukuda, Y.; Morinaga, Y.; Suga, Y.; Nihei, Y.; Ohishi, K.; Akiyama, Y.; Tsuji, T. *Anti-Cancer Drug Des.* **1999**, *14*, 539.
17. In brief, HCT116 and CX-1 cells were implanted sc in the flanks of nude mice. After 20–25 days, tumors from several animals were excised. The viable portion of the tumor was fragmented and implanted sc in the flanks of nude mice. Therapy was started after tumor volumes reached to 100–200 mm^3.
18. Coleman, M. D. *Human Drug Metabolism*; Wiley: Bermingham, 2005. Chapter 3.