Hydrophilic, Pro-Drug Analogues of T138067 Are Efficacious in Controlling Tumor Growth In Vivo and Show a Decreased Ability To Cross the Blood Brain Barrier

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The novel anticancer compound T138067 is an irreversible inhibitor of tubulin polymerization. Amides **3**−**6** were synthesized using standard methodologies and determined to be significantly less lipophilic than T138067 based on logP calculations. Tubulin polymerization and [^{3}H]-T138067 competition assays revealed that these amides are pro-drugs for parent aniline **2**. Amides **3**−**5** showed no detectable signs of crossing the blood brain barrier, while amide **6** was found in extremely small amounts (12 ng/g of brain tissue). Aniline **2**, which was formed in vivo from these amides, was found in significantly smaller amounts (approximately 20 to >5000 times) in the brain than when **2** was administered directly. The in vivo efficacy of amide **6** approached that of T138067 and was better tolerated when administered to athymic nude mice bearing MX-1 human mammary tumor xenografts.

Introduction

Antimitotic drugs have emerged as an effective treatment for a variety of cancers.[1] Our group has recently described the discovery of a new antimitotic agent, T138067 (Figure 1), which is currently in phase II clinical trials.[2−4] This compound has been shown to covalently bind to cysteine-239 on β-tubulin isoforms 1, 2, and 4 by way of a nucleophilic aromatic substitution reaction.[2] The covalent modification of β-tubulin inhibits the polymerization of the α,β-tubulin heterodimers into microtubules. This leads to cell arrest at the G2/M cell cycle boundary followed by apoptosis.[2] This compound and its analogues are effective against a variety of tumors, including those that express the multidrug resistant (MDR) phenotype (IC_{50} = 11−165 nM).[2−4]

Although T138067 has the ability to penetrate the blood brain barrier (BBB), to date, clinical studies have not identified central nervous system (CNS) toxicity as the main dose-limiting side effect.[5] However, dose-limiting CNS effects have been observed with other lipophilic drugs.[6] While compounds that have the ability to penetrate the BBB are promising candidates for treating brain tumors, we felt that an analogue of T138067 that does not penetrate the BBB would possess fewer potential problems with central neurotoxicity and an increased therapeutic window relative to other members of this class of compounds.

The ability of T138067 to penetrate the BBB could be due, at least in part, to its lipophilicity (ClogP = 3.26), and thus we were interested in identifying an analogue that was significantly less lipophilic. This report describes our results toward developing such compounds while retaining the same mechanism of action as T138067.

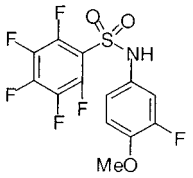

Figure 1. Structure of T138067.

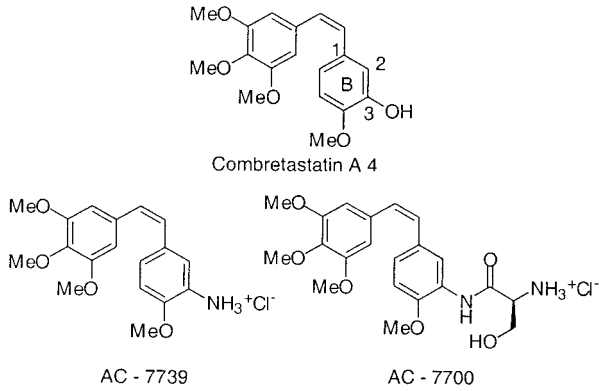

Figure 2. AC-7739 and AC-7700 are hydrophillic analogues of Combretastatin A4.

Chemistry

Combretastatin A4 is a potent antimitotic agent in vitro (Figure 2). However, this compound showed no antineoplastic effects when tested in vivo.[7] The authors hypothesized that the lack of efficacy observed was due to a poor pharmacokinetic profile originating from the compound's intrinsically low aqueous solubility. To increase its aqueous solubility, they replaced the C-3 hydroxyl group in ring B with an amine substituent and formed the HCl salt (AC-7739) as well as the L-serine amide HCl salt (AC-7700) (Figure 2). Both compounds showed enhanced aqueous solubility over Combretastatin A4 and were efficacious in vivo.[7] On the basis of

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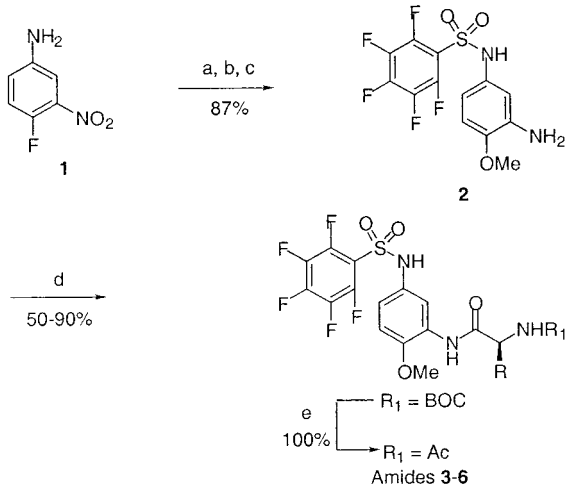
Scheme 1. General Synthesis of Amide Analogues[a]



[a] Reagents: (a) NaOMe, MeOH; (b) pentafluorobenzenesulfonyl chloride, MeOH; (c) 10% Pd/C, H_2(g), EtOAc; (d) *N*-methyl morpholine, 60 °C, protected amino acid, coupling agents used: HBTU/HOBT, HATU/HOAT, or EDCI/HOBT; (e) if R_1 = BOC then (1) TFA, CH_2Cl_2, (2) Ac_2O, 2,6-lutidine.

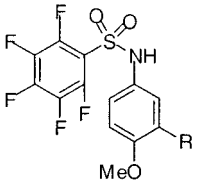
these results, we decided to synthesize a variety of amide analogues of T138067.

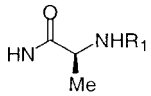
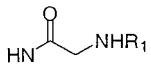
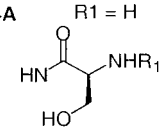
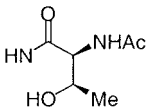
Compound **2** was previously synthesized by us and exhibited potent cytotoxic effects in vitro.[8] Scheme 1 outlines the synthesis of **2** and the reaction conditions used to synthesize amide analogues of T138067. 4-Fluoro-3-nitroaniline **1** was allowed to react with sodium methoxide followed by pentafluorobenzenesulfonyl chloride. The resulting product was then reduced over palladium on carbon to furnish aniline **2**. Due to the unreactive nature of **2**, the more active coupling reagents HBTU/HOBT and HATU/HOAT were frequently used.[9] In addition, elevated temperatures were required to form the amide bond in a reasonable amount of time and yield.[10] The unacetylated amides **3A**−**5A** were synthesized using *N*-BOC protected L-alanine, glycine, and L-serine as starting materials, respectively, followed by acidic deprotection, Table 1. The synthesis of serine amide **5A** required a hydrogenolysis of the benzyl group before BOC deprotection. Amides **3**−**5** were synthesized using N-acetylated L-alanine, glycine, and L-serine, respectively, Table 1. The synthesis of serine amide **5** required the deprotection of a *tert*-butyl group under acidic conditions, following the initial coupling reaction. Amide **6** was synthesized from *N*-BOC-*O*-benzyl-L-threonine. Acidic deprotection of the BOC group followed by acetylation and hydrogenolysis of the benzyl group gave the desired product.

Biological Results and Discussion

A variety of amides were synthesized and tested for their cytotoxicty against three tumor human cell lines. The biological activity and ClogP values of the most potent amides synthesized are summarized in Table 1. The N-acetylated amino acid amides **3**−**6** are 100 to 550 times less lipophilic than T138067 as predicted based on Ohsumi's results.[7] In addition, these amides were observed to be 2 to 7 times more potent than T138067 in all three cell lines tested, suggesting that these compounds would also be effective in treating tumors

Table 1. ClogP and Cell Based Assay Results



	R	ClogP[b,c]	Cell Line(IC_{50}, nM)[a] HeLa[d]	MCF-7[e]	MCF-7 /ADR[f]
	F (T138067)	3.26	101±11	46±9	87±14
2	NH_2	2.3	27±7	10±4.5	33±5.6
3	 R1 = Ac	1.36	43±3.3	10±4.3	35±12
3A	R1 = H	1.42	860±60	N.D.[g]	1520±210
4	 R1 = Ac	0.86	26±1.3	7.7±4.1	13±2.7
4A	R1 = H	0.93	7480±130	N.D.[g]	6730±1020
5	 R_1 = Ac	0.5	18±0.9	6.2±3.7	18±7.4
5A	R_1 = H	0.57	1230±190	44±130	2050±150
6		0.82	27±7	7.4±2.9	24±7.5

[a] Calculated using the LSW data analysis software, MDL Information Systems, 14600 Catalina St., San Leandro, CA, 94577. [b] Calculated using Crippen's method as found in CS Chem Draw Ultra Version 5.0, Cambridge Soft Corporation, 100 Cambridge Park Drive, Cambridge, MA, 02140. [c] The standard deviation is ±0.47. [d] Human cervical carcinoma cell line. [e] Human mammary carcinoma cell line. [f] Human mammary carcinoma cell line that exhibits the MDR phenotype. [g] Not determined.

expressing the MDR phenotype.[11] The unacetylated amides **3A**−**5A** were also tested and were observed to be significantly less active, Table 1. Also, the unnatural antipode of amides **3** and **5** were also synthesized and observed to be less cytotoxic.[12] This lack of efficacy and potency led to suspicions regarding the in vivo stability of these compounds and whether their cytotoxic effects were originating from parent aniline **2**.[13] To directly address this question, glycine amide **4** was synthesized in radiolabeled form (by introducing tritium via reductive dehalogenation on the nonfluorinated aromatic ring) and incubated with HepG2 cells in vitro. The $t_{1/2}$ for disappearance of radiolabeled amide **4** was approximately 150 min. Aniline **2** was the main degradation product as determined by radioisotope HPLC analysis.[12] To further test the pro-drug hypothesis, aniline **2** and amide **4** were tested for their ability to inhibit tubulin polymerization in vitro. Aniline **2** and T138067 exhibited a concentration-dependent inhibition of tubulin polymerization based on turbidometric analysis (panels C and A of Figure 3, respectively). In con-

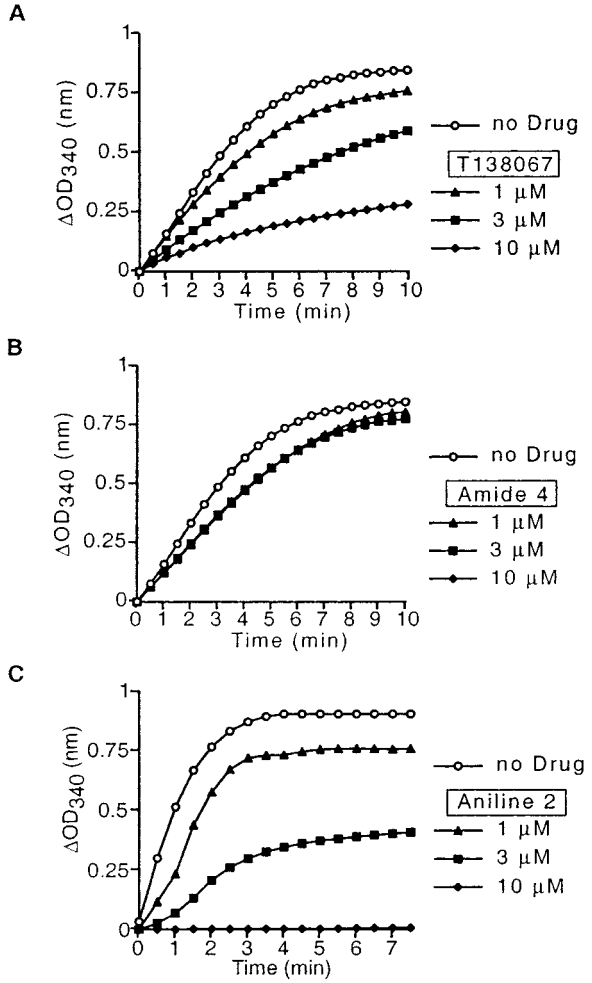


Figure 3. In vitro effect of aniline **2** and amide **4** on microtubule formation. (A−C) Polymerization reactions (40 mM tubulin) were performed at 37 °C in the absence of drug (no Drug) or at the indicated concentrations (1, 3, and 10 mM) of T138067 (panel A), amide 4 (panel B), and aniline 2 (panel C). Shown is a graphical representation of changes in the optical density at 340 nm over time (min) in the absence (no drug) or presence of the indicated compounds.

trast, amide **4** (Figure 3B) showed no significant inhibition. Amides **3**, **5**, and **6** showed a profile similar to amide **4** (see Supporting Information). Figure 4 shows the results of a competition experiment that also supports the pro-drug hypothesis. The competition between radiolabeled and cold T138067 for covalently modifying β-tubulin is shown by SDS−PAGE analysis in Figure 4A. High concentrations of unlabeled T138067 effectively compete with radiolabeled T138067. Aniline **2** also has the ability to compete with radiolabeled T138067 (Figure 4B). However, unlabeled amides **4** and **5** did not compete with radiolabeled T138067 in covalently modifying β-tubulin (panels C and D of Figure 4, respectively). The tubulin polymerization results, in addition to the competition experiments, as well as the HePG2 cell incubation experiment support the hypothesis that these amides are behaving as prodrugs and their cytotoxic effects are originating from aniline **2** in the whole cell assay.

As stated earlier, we wanted to synthesize analogues that were significantly less lipophilic than T138067, and on the basis of the ClogP values outlined in Table 1, this was achieved. However, experiments were performed that support the hypothesis that aniline **2**, with a ClogP comparable to that of T138067, was responsible for the cytotoxicity observed in the whole cell assay. In fact, convulsions were observed when aniline **2** was administered to male CD-1 mice via bolus injection, Table 2. We suspected that the central neurotoxicity observed was a result of the compound's ability to enter the brain. Surprisingly, bolus injections of amides **3**, **5**, and **6** were well tolerated at doses 2 to 4 times above those of aniline **2**, Table 2. Convulsions were only observed when administering amide **4** to CD-1 mice at three times (100 mg/Kg) the level of aniline **2**, while the other amides (**3**, **5**, **6**) did not induce convulsions even at the highest dose administered (≥100 mg/kg). Table 3 outlines the amount of compound that was found in the brain (ng/g of brain tissue) and plasma (ng/mL of plasma) of CD-1 mice exposed for 5 min to 30−40 mg/kg of T138067, aniline **2**, and amides **3**−**6** via bolus injection. None of the amides, except **6**, showed any detectable levels in the brain while aniline **2** was found in comparable amounts to T138067. More importantly, the aniline **2** formed from the in vivo hydrolysis of the various amides was found in significantly less amounts (approximately 20 to >5000 times) than when aniline **2** itself was directly administered. This was most likely the main reason amides **3**−**6** were better tolerated than aniline **2** by the CD-1 mice.

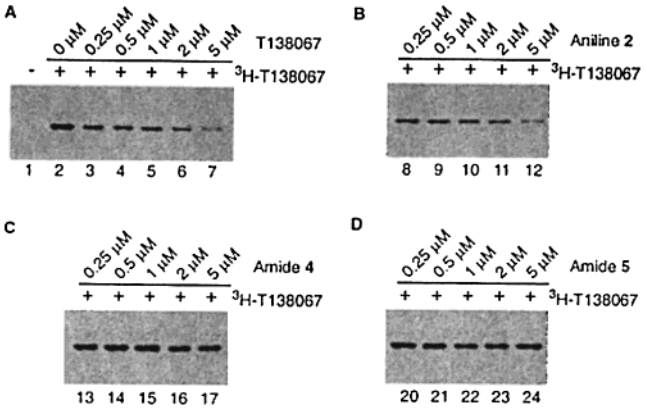


Figure 4. Amides **4** and **5** do not compete with ^{3}H-T138067 for β-tubulin binding. (A−D) Purified brain tubulin (0.5 μM) was incubated in the absence of 3H-T138067 (lane 1) or in the presence of 500 nM of ^{3}H-T138067 (lanes 2−24) and the indicated concentration of T138067 (panel A, lanes 2−7), aniline **2** (panel B, lanes 8−12), amide **4** (panel C, lanes 13−17), and amide **5** (panel D, lanes 20−24). After a 2 h incubation at 37 °C, β-tubulin-modification was analyzed by SDS−PAGE. Shown is autoradiogram of SDS−PAGE.

Table 2. Maximum Tolerated Dose (MTD) and Convulsion Threshold

compd	MTD (mg/kg)[a]	convulsion[b] threshold (mg/kg)
2	30	35
3	75	>100
4	40	100
5	125	>100
6	100	>100

[a] The number represents the maximum dose administered to CD-1 male mice that resulted in one or two deaths per cohort. This dose would be administered to an appropriate mouse model to test for in vivo efficacy. [b] CD-1 male mice were administered the compound by bolus iv injection. The number represents the minimum dose required to induce convulsions.

Table 3. Brain-Plasma Comparison Using CD-1 Mice[a]

compd	concentration	
	brain (ng/g)	plasma (ng/mL)
T138067	864 ± 210[b]	6272 ± 1425
2	1169 ± 836	8926 ± 2194
3	0	62 ± 34
3 → **2**[c]	0.2 ± 0.2	156 ± 26.8
4	0	112.8 ± 7
4 → **2**[c]	13.2 ± 5.6	729 ± 232
5	0	4.2 ± 1.5
5 → **2**[c]	54.4 ± 14.85	695 ± 120
6	12.6 ± 0.67	52.2 ± 15.7
6 → **2**[c]	1 ± 0.4	113 ± 38

[a] CD-1 mice (n = 5) were exposed for 5 min to the desired compound by volus injection. [b] The standard deviation was calculated using the STDEVP program in Microsoft Excel 5.0. [c] This line represents the amount of aniline **2** formed by the in vivo hydrolysis of the parent amide.

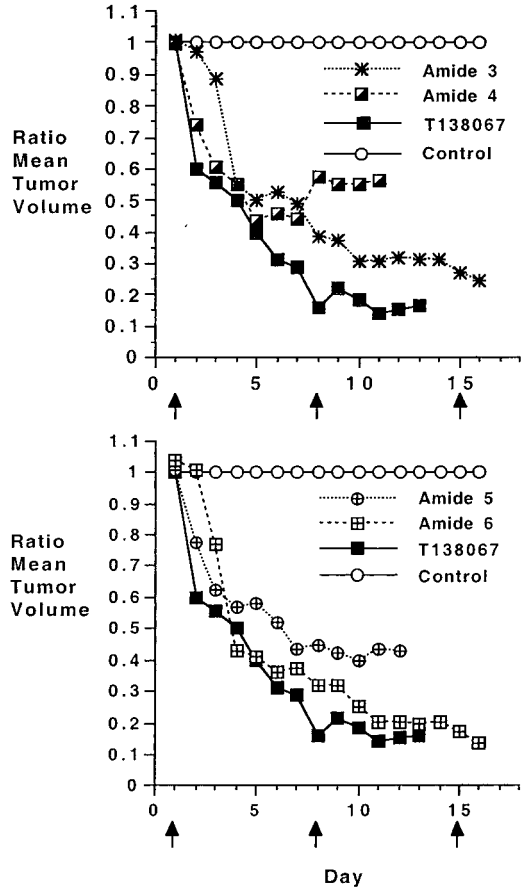


Figure 5. Effect of amides **3**−**6** and T138067 on tumor growth of mice bearing MX-1 tumor xenografts. The maximum tolerated dose was administered for each test compound: amide **3** (75 mg/kg), amide **4** (40 mg/kg), amide **5** (125 mg/kg), amide **6** (100 mg/kg). T138067 was infused at a dose of 30 mg/kg/h for 4 h. Results are expressed as the daily ratio of the mean tumor volume of treated animals to saline treated control mice (n = 7−12 for each treatment group). Arrows indicate treatment.

The in vivo efficacies of T138067 and amides **3**−**6** are outlined in Figure 5.[14] The athymic nude mice were treated intravenously (iv) with maximum tolerated doses of each compound on days 1, 8, and 15. Amides **3**−**6** were administered as bolus iv injections while T138067 was infused over 4 h.[15] Suppression of tumor growth by T138067 was significant (Figure 5). Both amides **3** and **6** were able to suppress tumor growth effectively, particularly amide **6** which was able to reduce tumor size to levels similar to T138067 at day 13 (Figure 5). Amides **4** and **5** were less efficacious than amides **3** and **6** at suppressing tumor growth (Figure 5). A certain percentage of animals from each of these groups had to be euthanized during the course of the experiments due to excessive weight loss, ≥20% of their original weight, or ulcerating tumors. In addition, the last of the control animals for T138067, amide **4**, and amide **5** either died or were euthanized (due to an ulcerating tumor or excessive tumor size) on day 13, 11, and 12, respectively. The lack of control animals in these experiments precluded the ratio of mean tumor volume from being calculated.

Conclusions

Amides **3**−**6** can be readily synthesized using standard methodologies. The calculated logP values suggest that these amides are significantly less lipophilic than T138067, and this is manifested in their inability to penetrate the BBB, Table 3. It is possible that the amides cytotoxic effects are originating from another mechanism(s). However, the HEpG2 cell and polymerization experiments (Figures 3 and 4) support the hypothesis that the less lipophilic amides are not the constitutively active agents but rather are acting as prodrugs for the more lipophilic aniline **2**. Due to the acute toxic nature of **2**, this compound was never evaluated for its efficacy in vivo. However, when amides **3**−**6** are dosed at their maximally tolerated doses, these compounds are efficacious at suppressing tumor growth with no signs of CNS toxicity, especially amides **3** and **6**. This lack of CNS toxicity is understandable given that significantly smaller amounts of aniline **2** enter the brain via the in vivo hydrolysis of the amide bond, Table 3. The data described supports the idea that the amide side chains are actively or passively facilitating the distribution of efficacious quantities of the pro-drugs to the intracellular site of action before a significant amount of amide hydrolysis can take place in the plasma. Once inside the cell, amide cleavage takes place releasing the active component, aniline **2**.

Experimental Section

^{1}H NMR spectra were obtained on a Bruker DRX 400 MHz or a Varian Gemini 400 MHz spectrometer. Chemical shifts were reported in parts per million (ppm) with DMSO-d_6 as the internal standard. The abbreviations s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, and b = broad are used throughout. Melting points were determined in open-ended capillary tubes using a FP62 Mettler Toledo melting point apparatus and are uncorrected. Elemental analysis was performed by Atlantic Microlab Inc, Norcross, GA, and are within ±0.4% of the calculated values. Low-resolution mass spectra were determined at Tularik Inc. using a Hewlett-Packard Series 1100 MSD ES-mass spectrometer. Thin-layer chromatography (TLC) was performed on 0.25 mm Analtech Inc. precoated silica gel glass plates. Visualization on TLC was achieved using ultraviolet light, an I_2 developing chamber, and/or heating of TLC plates previously submerged in a 7% solution of phosphomolybdic acid in 95% ethanol. Flash column chromatography was performed using silica gel 60 (230−400 mesh) obtained from EM Science. Reagents and solvents were commercial grades and were used as supplied, unless otherwise stated. All moisture-sensitive reactions were carried out in glassware that was flame dried under high vacuum (0.5−2.0 mmHg) and then purged with nitrogen. The term "concentrated" refers to the removal of volatile solvents

using a rotary evaporator connected to a KNF Neurberger vacuum pump.

Abbreviations: *N*-hydroxybenzotriazole (HOBT), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*-methylmorpholine (NMM), 1-hydroxy-7-azabenzotriazole (HOAT), *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), trifluoracetic acid (TFA), acetic acid (AcOH).

4-Methoxy-3-nitroaniline. To a 1 M solution of 3-nitro-4-fluoroaniline (**1**) (16.7 g, 107 mmol) in anhydrous methanol at ambient temperature was added sodium methoxide (23.1 g, 428 mmol), and the resulting solution was heated to reflux with stirring for 21 h. The reaction mixture was then cooled to 0 °C, and a 12 M solution of HCl (13.4 mL) was added dropwise followed by water (250 mL). The crude mixture was extracted three times with Et_2O (200 mL). The organic layers were combined, washed with brine (300 mL), dried over Na_2SO_4, and concentrated under vacuum to yield 17.5 g (97%) of product as a dark brown solid which was used without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.09 (d, J = 9 Hz, 1H), 7.01 (dd, J = 2.8, 1.3 Hz, 1H), 6.85 (ddd, J = 9, 2.8, 1.4 Hz, 1H), 5.2 (s, 2H), 3.75 (s, 3H).

2,3,4,5,6-Pentafluoro-*N*-(4-methoxy-3-nitrophenyl)benzenesulfonamide. To a 0.4 M solution of 4-methoxy-3-nitroaniline (17.5 g, 104 mmol) in anhydrous methanol was added dropwise pentafluorobenzenesulfonyl chloride (7.7 mL, 52 mmol), and the resulting mixture was stirred at ambient temperature for 1 h. The reaction mixture was concentrated under vacuum and purified by column chromatography (10−30% EtOAc in hexane) to yield 18.1 g (87%) of product as an orange solid, mp 95−97 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (d, J = 2.7 Hz, 1H), 7.51 (dd, J = 9, 2.7 Hz, 1H), 7.09 (d, J = 9.0 Hz, 1H), 3.95 (s, 3H). MS (EI): *m*/*z* 817 (30, 2M+Na-2H), 398 (30, M+), 397 (100, M−H).

***N*-(3-Amino-4-methoxy-phenyl)-2,3,4,5,6-pentafluorobenzenesulfonamide (2).** To a 0.15 M solution of 2,3,4,5,6-pentafluoro-*N*-(4-methoxy-3-nitrophenyl)benzenesulfonamide (18.1 g, 45.5 mmol) in 100% anhydrous ethanol was added 10% Pd/C (4.84 g, 4.55 mmol). Hydrogen gas was bubbled through the solution for 1 min, and the resulting mixture was stirred for 24 h under 1 atm of hydrogen. The crude reaction mixture was filtered through a pad of Celite, and the Celite was washed with ethanol (500 mL). The filtrate was concentrated under vacuum to yield 16.5 g (99%) of product as an off white solid which was used without further purification, mp 142−143 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.64 (s, 1H), 6.68 (d, J = 8.4 Hz, 1H), 6.44 (d, J = 2.1 Hz, 1H), 6.3 (dd, J = 8.4, 2.1 Hz, 1H), 4.88 (bs, 2H), 3.69 (s, 3H). MS(EI) *m*/*z* 369 (100, M+H).

(2*S*)-2-Acetylamino-*N*-(2-methoxy-5-pentafluorobenzenesulfonylamino-phenyl)-propionamide (3). To aniline **2** (500 mg, 1.36 mmol) was added *N*-acetyl-L-alanine (356 mg, 2.72 mmol), HOAT (370 mg, 2.72 mmol), and HATU (1.034 g, 2.72 mmol). DMF (7 mL) was then added, followed by NMM (149 μL, 0.36 mmol), and the resulting mixture was heated to 65 °C with stirring for 22 h. The crude reaction mixture was cooled, followed by addition of 1 M solution of HCl (50 mL) and EtOAc (50 mL). The aqueous phase was extracted three times with EtOAc (50 mL). The organic phase was washed with brine (50 mL), dried over Na_2SO_4, and concentrated under vacuum. The crude oil was purified by column chromatography (1−5% MeOH in CH_2Cl_2) to yield 468 mg (72%) of product as a pale yellow solid, mp 189−190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.89 (s, 1H), 9.1 (s, 1H), 8.28 (d, J = 6.5 Hz, 1H), 7.89 (s, 1H), 7.0 (d, J = 8.9 Hz, 1H), 6.88 (dd, J = 8.8, 2.7 Hz, 1H), 4.55−4.35 (m, 1H), 3.8 (s, 3H), 1.85 (s, 3H), 1.23 (d, J = 7.2 Hz, 3H). Anal. Calcd for $C_{18}H_{16}F_5N_3O_5S$: C, 44.91; H, 3.35; N, 8.73. Found: C, 44.69; H, 3.29; N, 8.67. MS(EI) *m*/*z* 481 (25, M+), 480 (100, M−H).

2(*S*)-Amino-*N*-(2-methoxy-5-pentafluoro-benzenesulfonylamino-phenyl) propionamide (3A) was synthesized from aniline **2** (210 mg, 0.57 mmol) and *tert*-butoxycarbonyl-L-alanine (140 mg, 0.74 mmol) using the procedure described for amide **3**. HBTU/HOBT were used as coupling agents. BOC deprotection was performed using the procedure described for amide **5**. The crude oil was purified by column chromatography (2−10% MeOH in CH_2Cl_2) to yield 33 mg (59%) of product as a pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.0 (s, 1H), 9.75 (s, 1H), 8.1 (s, 1H), 7.85 (d, J = 2.6 Hz, 1H), 7.03 (d, J = 8.8 Hz, 1H), 6.9 (dd, J = 8.8, 2.6 Hz, 1H), 4.15 (m, 1H), 3.8 (s, 3H),1.4 (d, J = 6.9 Hz, 3H). MS(EI) *m*/*z* 438 (100, M−H).

2-Acetylamino-*N*-(2-methoxy-5-pentafluoro-benzenesulfonylamino-phenyl) acetamide (4) was synthesized in 90% yield from aniline **2** (500 mg, 1.36 mmol) and *N*-acetylglycine (207 mg, 1.77 mmol) using the procedure described for amide **3**. HBTU/HOBT were used as coupling agents. The final product was purified by trituration with MeOH/CH_2Cl_2, mp 209−210 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.9 (s, 1H), 9.1 (s, 1H), 8.29 (t, J = 5.7 Hz, 1H), 7.88 (s, 1H), 6.99 (d, J = 8.9 Hz, 1H), 6.88 (dd, J = 8.8, 2.6 Hz, 1H), 3.85 (d, J = 5.9 Hz, 2H), 3.79 (s, 3H), 1.89 (s, 3H). Anal. Calcd for $C_{17}H_{14}F_5N_3O_5S$: C, 43.68; H, 3.0; N, 8.99. Found: C, 43.43; H, 3.14; N, 8.79. MS(EI) *m*/*z* 467 (20, M+), 466 (100, M−H).

2-Amino-*N*-(2-methoxy-5-pentafluoro-benzenesulfonylamino-phenyl) acetamide (4A) was synthesized from aniline **2** (60 mg, 0.16 mmol) and *tert*-butoxycarbonylamino-acetic acid (29 mg, 0.163 mmol) using the two-step procedure described for amide **3A**. EDCI/HOBT were used as coupling agents. The crude oil was purified by column chromatography (1−5% MeOH in CH_2Cl_2) to yield 33 mg (61%) of product as a pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.7 (s, 1H), 7.75 (s, 1H), 6.85 (d, J = 8.9 Hz, 1H), 6.72 (d, J = 8.8 Hz, 1H), 3.75 (s, 3H), 3.5 (s, 2H). MS(EI): *m*/*z* 369 (100, M-glycine), 426 (25, M+H).

(2*S*)-2-Acetylamino-3-hydroxy-*N*-(2-methoxy-5-pentafluorobenzenesulfon-ylamino-phenyl)-propionamide (5) was synthesized in two steps from aniline **2** (500 mg, 1.36 mmol) and *N*-acetyl-*O*-*tert*-butyl-L-serine (360 mg, 1.77 mmol) using the procedure for amide **3**. HBTU/HOBT were used as coupling reagents. To a 0.16 M solution of (2*S*)-2-acetylamino-3-*tert*-butoxy-*N*-(2-methoxy-5-pentafluorobenzenesulfonyl-amino-phenyl)-propionamide (700 mg, 1.26 mmol) at 0 °C was added TFA (8.4 mL, 100 mmol), and the resulting solution was allowed to stir for 8.5 h at 0 °C followed by warming to ambient temperature for an additional 5 h. The crude reaction was concentrated under vacuum with azeotropic removal of TFA with hexane and purified by column chromatography (1−5% MeOH in CH_2Cl_2). The resulting material was repurified using reverse phase HPLC chromatography (5−95% CH_3CN in a 0.1% TFA aqueous solution) to yield 507 mg of amide **5** (68%) as a white solid, mp 98−99 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.9 (s, 1H), 9.09 (s, 1H), 8.2 (d, J = 7.3 Hz, 1H), 7.99 (d, J = 2.2 Hz, 1H), 7.0 (d, J = 8.6 Hz, 1H), 6.88 (dd, J = 8.7, 2.6 Hz, 1H), 5.1 (t, J = 5.1 Hz, 1H), 4.4 (apparent q, J = 6.1 Hz, 1H), 3.8 (s, 3H), 3.7−3.55 (m, 2H), 1.9 (s, 3H). Anal. Calcd for $C_{18}H_{16}F_5N_3O_6S\cdot0.5H_2O$: C, 42.69; H, 3.38; N, 8.30. Found: C, 42.55; H, 3.32; N, 8.19. MS(EI) *m*/*z* 497 (20, M+), 496 (100, M−H).

2(*S*)-Amino-3-hydroxy-*N*-(2-methoxy-5-pentafluoro-benzenesulfonylamino-phenyl)-propionamide (5A) was synthesized from aniline **2** (203 mg, 0.55 mmol) and 3-benzyloxy-2(*S*)-*tert*-butoxycarbonylaminopropionic acid (195 mg, 0.66 mmol) using the two-step procedure described for amide **3A**. HBTU/HOBT were used as coupling agents (note: before BOC deprotection, the benzyl group was removed using the procedure for amide **6**). The crude solid was purified by column chromatography (1−10% MeOH in CH_2Cl_2) to yield 89 mg (30% for the three steps) of product as a pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.9 (bs, 1H), 7.95 (s, 1H), 6.95 (d, J = 8.5 Hz, 1H), 6.8 (d, J = 8.6 Hz, 1H), 6.9 (dd, J = 8.8, 2.6 Hz, 1H), 4.1 (bs, 1H), 3.8 (s, 3H), 3.7−3.5 (m, 3H). MS(EI) *m*/*z* 454 (100, M−H).

(1*S*,2*R*)-[2-Benzyloxy-1-(2-methoxy-5-pentafluorobenzenesulfonylamino-phenylcarbamoyl)-propyl]carbamic acid *tert*-butyl ester was synthesized in 43% yield from aniline **2** (3.0 g, 8.2 mmol) and *N*-BOC-*O*-benzyl-L-threonine (2.3 g, 7.6 mmol) using the procedure described for amide **3**.

^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (d, J = 2.4 Hz, 1H), 7.28−7.11 (m, 5H), 7.0 (dd, J = 8.6, 2.6 Hz, 1H), 6.93 (d, J = 8.8 Hz, 1H), 4.6 (1/2ABq, J = 11.6 Hz, 1H), 4.46 (1/2ABq, J = 11.4 Hz, 1H), 4.3 (bs, 1H), 4.09 (bs, 1H), 3.7 (s, 3H), 1.49 (s, 9H), 1.21 (d, J = 3.2 Hz, 1H). MS(EI) *m/z* 682 (60, M+Na), 658 (100, M−H).

(2*S*,3*R*)-2-Acetylamino-3-hydroxy-*N*-(2-methoxy-5-pentafluorobenzene-sulfonylaminophenyl)butyramide (6). To a 0.08 M solution of fully protected amide **6** (2.14 g, 3.25 mmol) in CH_2Cl_2 at 0 °C was added TFA (20 mL), and the resulting mixture was allowed to stir for 2 h at 0 °C. The crude reaction was concentrated under vacuum with azeotropic removal of TFA with hexane and used directly in the next reaction. To a 0.06 M solution of crude amine (1.82 g, 3.25 mmol) at 0 °C was added 2,6-lutidine (760 μL, 6.5 mmol) followed by acetic anhydride (336 μL, 3.58 mmol). The resulting mixture was allowed to stir for 1 h at 0 °C. The crude reaction was acidified with 2 M aqueous HCl (100 mL) and extracted 3 times with EtOAc (100 mL). The combined organic layers were washed twice with saturated aqueous brine (150 mL), dried over Na_2SO_4, concentrated under vacuum, and used directly in the next reaction. To a 0.03 M solution of crude acetamide (1.95 g, 3.25 mmol) in AcOH was added 20% (50% by wt) palladium hydroxide (3.46 g, 3.25 mmol) followed by bubbling H_2(g) through the solution for 1 min. The resulting mixture was allowed to stir for 2 h under 1 atm of H_2(g). The crude reaction mixture was filtered through a pad of Celite, and the Celite was then washed three times with EtOAc (50 mL). The filtrate was concentrated under vacuum with azeotropic removal of AcOH with hexanes and purified by reverse phase HPLC chromatography (15−45% CH_3CN in a 0.1% TFA aqueous solution) to yield 1.28 g of amide **6** (78%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.9 (s, 1H), 9.1 (s, 1H), 8.05 (d, J = 8.3 Hz, 1H), 7.99 (d, J = 2.5 Hz, 1H), 7.0 (d, J = 8.8 Hz, 1H), 6.86 (dd, J = 8.8, 2.6 Hz, 1H), 5.2 (d, J = 4.8 Hz, 1H), 4.39 (dd, J = 8.1, 3.6 Hz, 1H), 4.1−4.0 (m, 1H), 3.8 (s, 3H), 1.95 (s, 3H), 1.08 (d, J = 6.3 Hz, 3H). Anal. Calcd for $C_{19}H_{18}F_5N_3O_6S\cdot 0.5H_2O$: C, 43.81; H, 3.68; N, 8.07. Found: C, 43.87; H, 3.56; N, 7.89. MS(EI) *m/z* 511 (20, M+), 510 (100, M−H).

Cytotoxicity Analysis. The cytotoxicity analysis studies were performed as previously described in ref 3.

In Vitro Tubulin Polymerization Reaction. Ice-cold bovine brain tubulin solution (400 μg in BRB80 buffer/10% glycerol) was mixed with 49 μL of cold BRB80 buffer, 10 μL of cold GTP solution (10 mM), and 1 μL of DMSO or 1 μL of compound solution in DMSO. The mixture was immediately transferred to a quartz cuvette equilibrated at 37 °C. Changes in the optical density at 340 nm were monitored every 30 s in a temperature-controlled photospectrometer (Hewlett-Packard) at 37 °C.

In Vitro Tubulin Binding Competition Assay. Ice-cold bovine brain tubulin solution (0.5 μg in 8.9 μL BRB80 buffer (80 mM PIPES (pH 6.8), 0.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol)) was mixed with 1 μL of DMSO or 1 μL of compound solution in DMSO and incubated at 37 °C min in the presence 0.1 μL of ^{3}H-T138067 (20 Ci/mmol; 1 mCi/ml) or 0.1 μL of DMSO. Following a 90 min incubation, proteins were analyzed by SDS−PAGE.

Brain-Plasma Concentration Determination Studies. T138067, aniline **2**, and amides **3**−**6** were separately dissolved in 0.4 M aqueous NaOH (5 mL) and titrated to a pH between 8 and 9 by dropwise addition of 1 M aqueous HCl. The final volume was adjusted to 10 mL with the addition of saline (0.9% NaCl) to furnish final concentrations between 3 and 4 mg/mL. Five CD-1 male mice per cohort were administered between 30 and 40 mg/kg of desired compound by bolus injection into the lateral tail vein within 10 s. The mice were then sacrificed 5-min post-dose administration. The animals were anesthetized with carbon dioxide, and 0.5 mL of plasma was collected from each animal by cardiac or vena cava puncture. The plasma samples were placed in tubes containing EDTA, cooled on ice until centrifugation, and stored at −20 to −70 °C until final analysis. The brains were removed, flashed frozen in liquid nitrogen, and stored at −20 to −70 °C until further analysis.

Brain Preparation. Each brain was weighed, followed by washing with cold PBS (1 mL) and H_2O (0.5 mL). The brain was then transferred to a 5 mL homogenizer, and 40 μL (1 μg/mL) of an internal standard and 1% AcOH (1 mL) in 1:1 EtOH/CH_3CN solution were then added. The brain was homogenized and transferred to a high-speed tube. The homogenizer was washed once with 1% AcOH (1 mL) in 1:1 EtOH/CH_3CN solution and twice with CH_3CN (0.5 mL) and combined with the homogenized brain in the high-speed tube and centrifuged at 20,000 rpm for 15 min at 4 °C. The extract was transferred into a 5 mL glass tube, and the pellet was washed once with 1% AcOH (1 mL) in 1:1 EtOH/CH_3CN solution and twice with CH_3CN (0.5 mL) and added to the extract. The homogenizer was washed as described above and added to the extract. The extract was then concentrated to 100 μL in a speed vacuum chamber at ambient temperature. The concentrated extract was transferred to a 1.5 mL tube, and the original glass tube was washed once with a 1% AcOH in a 1:2 H_2O/CH_3CN solution (400 μL) followed by 1% AcOH in CH_3CN (100 μL). The washes were combined with the concentrated extract and centrifuged for 15 min at 4 °C. The supernatant was transferred to another tube and dried using a speed vacuum at ambient temperature. The resulting pellet was suspended in 1% AcOH in MeOH (100 μL) and spun briefly, and the supernatant was then analyzed.

Plasma Preparation. Plasma samples (50 μL) from each animal in the cohort were combined. Proteins from the combined aliquots were precipitated using 1% AcOH in CH_3CN (200 μL). The precipitated proteins were separated by centrifugation at 10000*g* for 5 min, and the supernatant was then analyzed.

The sample analyses were performed using a high performance liquid chromatography mass spectrometer, Perkin-Elmer Sciex (Toronto, Canada) API 365 triple quadruple mass spectrometer. The mass spectrometer was interfaced via a Sciex turbo ion spray probe to a liquid chromatograph, consisting of a Shimadzu (Columbia, MD) LC-10AD pump system and a DGU-14A degasser, and a Perkin-Elmer (Perkin-Elmer, Norfolk, CT) series 200 autosampler. Separation of the compounds and the interference was achieved using a YMC 5 μM C18 column (2 × 50 mm). Mass spectra were determined with an ion source temperature at 300 °C and nitrogen gas flow rate of 7 L/min. The ion source of the mass spectrometer was operated in the negative ionization mode. The multiple reaction monitoring (MRM) system, with a dwell time of 500 ms, was used for quantitation. The collision energy was set at 38 eV. Data were acquired by Sciex sample control and MacQuan software.

In Vivo Efficacy Studies. Animals. Male CD-1 outbred mice, 8−10 weeks of age, were obtained from Charles River (Hollister, CA, or Portage, MI). Female athymic nude mice, 5−8 weeks of age and weighing 18−20 g, were used (Simonsen Laboratories, Gilroy, CA, or Harlan Sprague Dawley Indianapolis, IN). Animals were housed in groups on irradiated corncob bedding in HEPA filtered ventilated rack housing. Irradiated PMI Picolab 5058 rodent diet (PMI Mills, Indianapolis, IN) and autoclaved, hyperchlorinated water were provided ad libitum.

Tumor Cells. MX-1, a human mammary carcinoma, was obtained from NCI, Frederick, MD. The tumor was maintained as a solid tumor tissue harvested from donor mice. Tumor from donor mice was minced thoroughly in sterile DMEM culture medium (Mediatech, Herndon, VA) containing 1% penicillin/streptomycin and 10% fetal bovine serum. A 200 μL aliquot of the slurry was inoculated sc on the right flank of each mouse. Tumor size was monitored beginning 2−3 days following inoculation until it was an appropriate size.

Maximum Tolerated Dose Determination Studies. Lethal dose assessment of test compounds was performed using the "up-and-down" method.[15]

Tumor Xenograft Efficacy Studies. Animals bearing tumors were randomly assigned to treatment and vehicle

control groups. Test compounds were administered iv at the maximum tolerated dose determined by the method described above. Mice were dosed every 7 days for a total of 2 or 3 doses. Saline treated control groups were run parallel to all treatment groups. Animals receiving T138067 or saline controls had a cannula surgically implanted in their jugular vein. All animals received similar doses of anesthesia. T138067 was administered by intravenous infusion at a dose of 30 mg/kg/h for 4 h at a rate of 2.5 mg/kg/h. A subsequent intravenous infusion was given via a lateral tail vein. Body weights and tumor volumes were measured at regular intervals posttreatment. Animals that became moribund due to drug toxicity, that is demonstrating a body weight loss greater than 20%, were euthanized. Animals with ulcerating tumors, or tumors larger than 2000 mm^3, were euthanized. Studies were terminated when the tumor of the last control animal became too large. Tumor volume was calculated by the following formula:

$$\text{volume } (mm^3) = \frac{\text{length (mm)} \times (\text{width (mm)})^2}{2}$$

Data are expressed as the mean ratio of the daily tumor volume for the compound treated group to its saline treated control group.

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Supporting Information Available: Tubulin polymerization assay results for amides **3**, **5**, and **6** (as outlined in Figure 3) and experimentally determined ratios of mean body weight for T138067 and amides **3**−**6** from the efficacy studies outlined in Figure 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Taxol (paclitaxel) has an $IC_{50} > 15000$ nM against the MCF-7/ADR cell line while its $IC_{50} = 1.1 \pm 0.2$ nM against the MCF-7 cell line. Vincristine has an $IC_{50} = 350 \pm 71$ nM against the MCF-7/ADR cell line while its $IC_{50} = 2.3 \pm 0.5$ nM against the MCF-7 cell line. The MCF-7/ADR cell line expresses the MDR phenotype; see refs 3 and 4.

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