Bioorganic & Medicinal Chemistry 17 (2009) 2852–2858

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Discovery and structure–activity relationships of (2-(arylthio)-benzylideneamino)guanidines as a novel series of potent apoptosis inducers

Han-Zhong Zhang, Candace Crogan-Grundy, Chris May, John Drewe, Ben Tseng, Sui Xiong Cai *

EpiCept Corporation, 6650 Nancy Ridge Drive, San Diego, CA 92121, USA

ARTICLE INFO

Article history:
Received 6 January 2009
Revised 11 February 2009
Accepted 14 February 2009
Available online 21 February 2009

Keywords:
Apoptosis inducers
Cell growth inhibition
Anticancer agents
Tubulin inhibitors
In vivo activity
HTS

ABSTRACT

1-(2-(2,5-Dimethoxyphenylthio)benzylidene)semicarbazide (**2a**) was discovered as a potent apoptosis inducer through our cell based HTS assay. SAR study led to the discovery of a more aqueous soluble analog (2-(2,5-dimethoxyphenylthio)-6-methoxybenzylideneamino)guanidine (**5e**) with EC_{50} value of 60 nM in the caspase activation assay and GI_{50} value of 62 nM in the growth inhibition assay in T47D cells. Compound **5e** was found to be an inhibitor of tubulin polymerization and efficacious in a MX-1 breast tumor model.

1. Introduction

Many traditional anticancer agents have been discovered through tumor cell growth inhibition assays in neoplastic cells. Agents active in the screening fall into two categories, cytostatic and cytotoxic, with the latter classified into two modes of action, necrotic and apoptotic. Apoptotic cell death is controlled and executed by a group of precisely regulated proteins that are frequently altered in tumor cells.[1] The mechanism of apoptosis involves a caspase cascade that is activated sequentially.[2,3] Within the caspase family, caspase-3 has been identified as one of the key effector caspases that cleave multiple protein substrates in cells, and leading to irreversible cell death.[4] In addition, several clinically used cytotoxic agents, including paclitaxel, docetaxel and vinca alkaloids, are known to primarily act by inducing apoptosis in cancer cells.[5]

In our effort to discover and develop apoptosis inducers as new anticancer agents, we have developed a cell-based, anticancer screening apoptosis platform (ASAP) HTS technology to identify apoptosis inducers using the activation of caspase-3 as a readout.[6] One of the major advantages of this technology is that by monitoring the activation of downstream caspase-3 in a cell-based assay we can discover compounds that initiate the apoptosis signal pathway through either a known mechanism or molecular target, or a novel mechanism or molecular target.[7]

Applying this ASAP HTS assay, we have discovered several classes of small molecules as novel apoptosis inducers. 4-Aryl-4*H*-chromenes, such as compound **1a**, were found to be potent apoptosis inducers[8] and several analogs have been found to have vascular targeting activity.[9] Gambogic acid (**1b**) was discovered as a fast apoptosis inducer[10] and its molecular target has been identified as transferrin receptor.[11] The 3-aryl-5-aryl-1,2,4-oxadiazoles such as **1c** were found to induce apoptosis selectively in certain tumor cells,[12] and through interaction with TIP47, an insulin growth

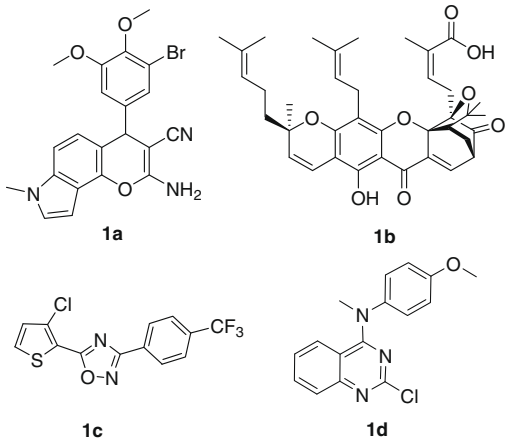


Chart 1.

* Corresponding author. Tel.: +1 858 202 4006; fax: +1 858 202 4000.
E-mail address: scai@epicept.com (S.X. Cai).

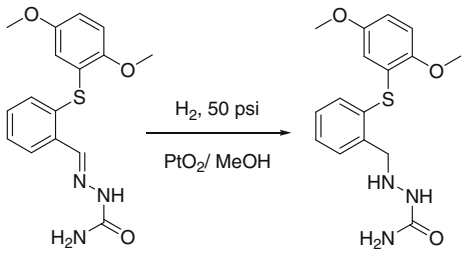
0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.02.029

factor II (IGF II) receptor binding protein.[13] 4-Anilinoquinazilines such as **1d** were discovered as highly potent apoptosis inducers with excellent in vivo anticancer activity (Chart 1).[14] Herein, we report the discovery of (2-(arylthio)benzylideneamino)guanidines as a novel series of apoptosis inducers as well as their structure–activity relationship (SAR) studies using our ASAP program.

2. Results and discussion

2.1. Chemistry

Substituted (benzylidene)semicarbazides **2a** and **2b** (Table 1) were purchased from a commercial library and the structures were confirmed by ^{1}H NMR and MS. Semicarbazide **3** was prepared from hydrogenation of **2a** (Scheme 1).[15] Compounds **5a–i** were prepared as illustrated in Scheme 2. Reaction of substituted *ortho*-fluoro- or chloro-benzaldehyde with substituted benzenethiol in the presence of sodium carbonate in heated DMF produced various substituted 2-(phenylthio)benzaldehyde **4a–i**,[16] which was refluxed with aminoguanidine and sodium acetate to produce **5a–i**. The *N*-methyl substituted analog **5j** was prepared by via condensation of *N*-Me-aminoguanidine with **4a** (Table 1). Substituted (2-phenoxybenzylideneamino)guanidines **7a** and **7b** were prepared similarly to **5** via replacing the substituted benzenethiol with substituted phenol to generate substituted 2-phenoxybenzaldehydes **6a** and **6b**, followed by condensation with aminoguanidine (Scheme 3). Similarly, compound **9** was prepared through oxidation of the thiobenzaldehyde **4a** with sodium periodate to produce



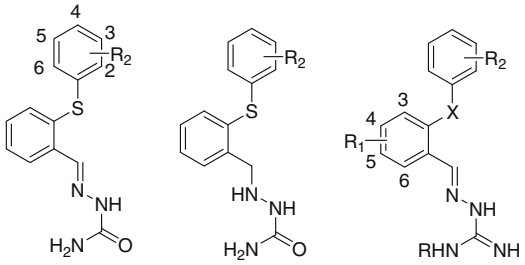
Scheme 1.

sulfinylbenzaldehyde **8**, followed by condensation of **8** with aminoguanidine (Scheme 3).

2.2. HTS and SAR studies

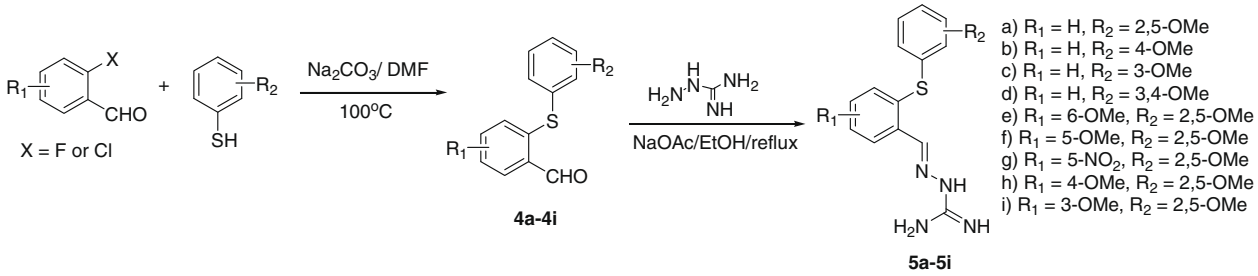
1-(2-(2,5-Dimethoxyphenylthio)benzylidene)semicarbazide **2a** was identified as an inducer of apoptosis using our cell- and caspase 3-based ASAP assays as described previously.[8] Compound **2a** was then tested in the same ASAP assay over a range of 5–10,000 nM to provide the dose–response curves for the determination of EC_{50}. The ASAP assay was also used for the testing of all compounds for SAR studies. Compound **2a** was found to have an EC_{50} of 52 nM for T47D breast cancer cells. It also demonstrated potent caspase activation activity for HCT116 colon cancer cells

Table 1
Caspase activation activity of [2-(arylthio)benzylideneamino]guanidines and related compounds

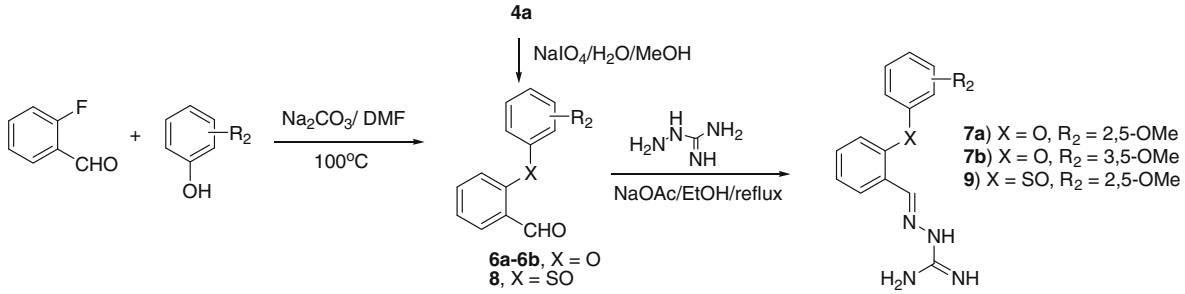


Entry	R_1	R_2	R	X	EC_{50}[a] (μM)		
					T47D	HCT116	SNU398
2a		2,5-OMe			0.052 ± 0.003	0.073 ± 0.004	0.062 ± 0.002
2b		2-OMe			>10	>10	>10
3		2,5-OMe			0.15 ± 0.01	0.29 ± 0.01	0.21 ± 0.02
5a	H	2,5-OMe	H	S	0.063 ± 0.003	0.14 ± 0.02	0.060 ± 0.006
5b	H	3-OMe	H	S	0.29 ± 0.01	0.41 ± 0.05	0.31 ± 0.01
5c	H	4-OMe	H	S	>10	>10	>10
5d	H	3,4-OMe	H	S	0.62 ± 0.06	0.86 ± 0.12	0.61 ± 0.02
5e	6-OMe	2,5-OMe	H	S	0.060 ± 0.003	0.11 ± 0.01	0.051 ± 0.018
5f	5-OMe	2,5-OMe	H	S	6.9 ± 0.5	>10	4.2 ± 1.4
5g	5-NO_2	2,5-OMe	H	S	0.20 ± 0.03	0.56 ± 0.11	0.18 ± 0.03
5h	4-OMe	2,5-OMe	H	S	0.13 ± 0.01	0.27 ± 0.03	0.12 ± 0.04
5i	3-OMe	2,5-OMe	H	S	>10	>10	>10
5j	H	2,5-OMe	Me	S	0.070 ± 0.002	0.16 ± 0.04	0.071 ± 0.025
7a	H	2,5-OMe	H	O	0.35 ± 0.02	0.59 ± 0.02	0.40 ± 0.07
7b	H	3,5-OMe	H	O	0.23 ± 0.01	0.36 ± 0.04	0.27 ± 0.01
9	H	2,5-OMe	H	SO	>10	>10	>10
Doxorubicin	NA[b]	NA	NA	NA	0.57 ± 0.023	0.29 ± 0.042	0.39 ± 0.064
Taxol	NA	NA	NA	NA	0.035 ± 0.003	0.023 ± 0.006	0.010 ± 0.001

[a] Data are the mean of three or more independent dose–response experiments and are reported as mean ± standard error of the mean (SEM).
[b] NA, not applied.



Scheme 2.



Scheme 3.

and SNU398 liver cancer cells (Table 1). In comparison, the EC_{50} values of taxol and doxorubicin for T47D cells were 35 and 570 nM, respectively. Compound **2b** was found to be inactive in the assay up to 10 μM, >200-fold less active than that of **2a**, indicating that an OMe group at the *meta*-position of the top ring is critical for activity. Reduction of the C=N double bond resulted in compound **3**, which was threefold less potent than compound **2a**, indicating that the rigid C=N double bond is preferred over the flexible single C–N bond. Since compounds **2a** and **2b** had limited aqueous solubility due to the semicarbazide moiety, we decided to explore the replacement of the semicarbazide group with a basic aminoguanidine group to increase solubility.

Compound **5a** was found to have activity similar to compound **2a** in the three cell lines tested, indicating that the semicarbazide group can be replaced by an aminoguanidine group. In addition, compound **5a** can be prepared as a hydrochloride salt and the salt showed good aqueous solubility (10 mg/mL in Peg 400:Tween 80:0.03 M tartaric acid water = 7:9:84), for a formulation amenable to in vivo studies. Similarly, compound **5j**, with the semicarbazide moiety replaced by a *N*-methyl-aminoguanidine group, also had activity equivalent to **2a**.

The SAR of substitutions in the top ring was explored first. The 3-OMe analog **5b** was about fivefold less active than the 2,5-di-OMe analog **5a**. The 4-OMe analog **5c** was found to be inactive up to 10 μM, which was >150-fold and >30-fold less active than **5a** and **5b**, respectively, confirming the importance of a *meta*-OMe group for activity. The 3,4-di-OMe analog **5d** was slightly less active than **5b**, indicating that the 4-OMe group is not contributing to activity.

We then explored the replacement of the sulfur linker by other groups. Compound **7a**, with an oxy linker replacing the thio linker in **5a**, was fivefold less active than **5a**, indicating that the thio linker is preferred over the oxy linker. The 3,5-di-OMe oxy linker analog **7b** was about as active as **7a**, confirming the importance of the OMe group at the *meta*-position of top ring for activity. Interestingly, oxidation of the thiol linker to a sulfinyl linker led to compound **9** which was not active up to 10 μM.

We also explored substitutions at various positions of the center benzylidene ring. The 6-OMe analog **5e** was about as active as **5a**, indicating a substitution at this position is well tolerated. The 4-OMe analog **5h** was about twofold less active than **5a**, indicating a substitution at this position is also well tolerated. The 5-OMe analog **5f** was about 100-fold less active than **5a**, while the 5-NO_2 analog **5g** was only threefold less active than **5a**, suggesting that an electron withdrawing group at the 5-postion is much more preferred than an electron donating group. The 3-OMe analog **5i** was not active up to 10 μM, which is >150-fold less active than **5a**.

The caspase activation activity of these (2-(arylthio)benzylideneamino)guanidines and related compounds in the human colorectal cancer cell line HCT116 and liver cancer cell line SNU398 roughly paralleled the activity with T47D cells. The differences in EC_{50} values between the different cell lines in general are within twofold. These data suggested that these compounds most probably should have similar activity against many different cancer cell lines.

Representative compounds were also tested by the traditional cell growth inhibition (GI_{50}) assay to confirm that the active compounds can inhibit tumor cell growth. The growth inhibition assays in T47D, HCT116 and SNU398 cells were run in a 96-well microtiter plate as described previously.[9,10] Compound **2a** was found to be a potent inhibitor of cancer cell growth with GI_{50} value of 45 nM in T47D cells. Compounds **5a** and **5e** also were highly active with GI_{50} values of 82 and 62 nM in T47D cells, respectively. In comparison, the GI_{50} values of vinblastine and colchicine for T47D cells were both 7 nM.

2.3. Additional characterization of compound 5e and related compounds

The apoptosis-inducing activity of compound **5e** was also characterized by flow cytometry. T47D cells were treated with 50 nM of compound **5e** for 48 h, then stained with propidium iodide and analyzed by flow cytometry. As shown in Figure 1, control cells (Fig 1A) have a normal cell cycle profile. Cells treated with compound **5e** for 48 h showed a shift of the population into G_2/M phase as well as the apoptotic sub-G_1 phase (Fig 1B).

Since many compounds that arrest cells at G_2/M followed by apoptosis are tubulin inhibitors, we suspected that this series of

Entry	Sub (%)	G_1 (%)	S (%)	G_2M (%)
1A	10	60	13	16
1B	28	13	18	41

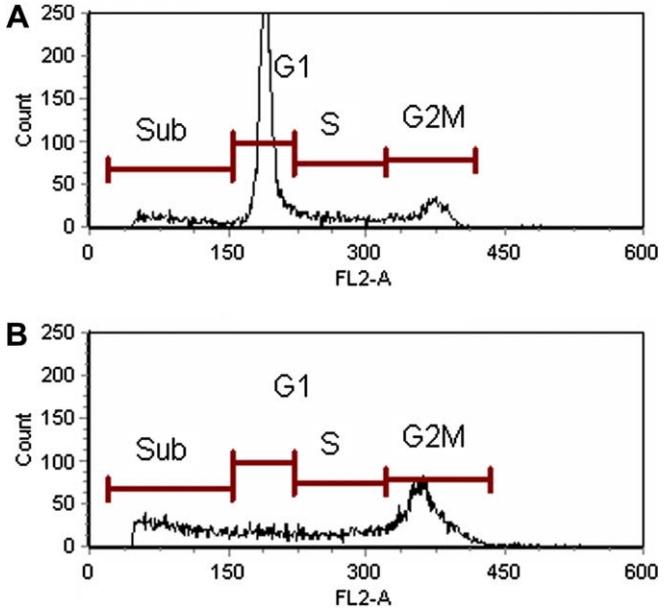


Figure 1. Drug-induced apoptosis in T47D cells as measured by flow cytometric analysis. The *x*-axis is the fluorescence intensity and the *y*-axis is the number of cells with that fluorescence intensity. (A) Control cells showing most of the cells in G_1 phase of the cell cycle. (B) Cells treated with 50 nM of compound **5e** for 48 h showing a shift to G_2/M as well as cells with sub-diploid DNA content, which are apoptotic cells with fragmented nuclei.

apoptosis inducers might also be tubulin inhibitors. The original hit **2a** was found to be active in the tubulin polymerization assay,[19] with an IC_{50} value of 8 μM. Similarly, compounds **3**, **5a**, **5e** and **5h** were found to inhibit tubulin polymerization with IC_{50} values of 3–9 μM. In comparison, the IC_{50} values for vinblastine and colchicine were 0.5 μM. While not definitive these data suggest that inhibition of tubulin polymerization probably is the mechanism of action for the apoptosis inducing activity of these compounds. Since the original hit semicarbazide **2a** and the aminoguanidine analogs such as **5a** and **5e** have similar activity profiles in both the caspase activation assay and growth inhibition assay, it is reasonable that they should have the same mechanism of action.

Due to its good solubility profile, the HCl salt of compound **5e** was selected for in vivo studies. Compound **5e** was tested in the MX-1 breast cancer model, a model that is not specific for certain agents and is widely used for testing common chemotherapeutics such as camptothecin derivatives and paclitaxel derivatives.[17] The MX-1 in vivo experiment was performed as described previously.[18] Compound **5e** inhibited tumor growth dose dependently and produced 50% tumor growth inhibition when dosed once a day for 5 days for two weeks at a dose of 25 mg/kg (Figure 2), and is well tolerated with maximum body weight decrease of <10%. In comparison, taxol dosed daily IP for 5 days at 8 mg/kg, or a single intravenous dose of doxorubicin at 10 mg/kg, both the MTD, resulted in 50–80% reduction of tumor growth.

3. Conclusion

In conclusion, 1-(2-(2,5-dimethoxyphenylthio)benzylidene) semicarbazide (**2a**) was identified as a potent apoptosis inducer through our ASAP assay. Exploration of the SAR led to the discovery of a series of (2-(arylthio)benzylideneamino)guanidines, compounds such as **5a** and **5e**, as potent apoptosis inducers with good aqueous solubility. These compounds also were found to have good activity in the cell growth inhibition assay. The ability of compound **5e** to induce apoptosis was confirmed in a flow cytometry assay in T47D cells. Compounds **2a**, **5a** and **5e** were found to inhibit the polymerization of tubulin, which most probably is the mechanism of action for the apoptosis inducing activity of these compounds. Compound **5e** also was found to have good activity in a MX-1 breast cancer in vivo model, suggesting that **5e** or related compounds could be developed as potential anticancer agents.

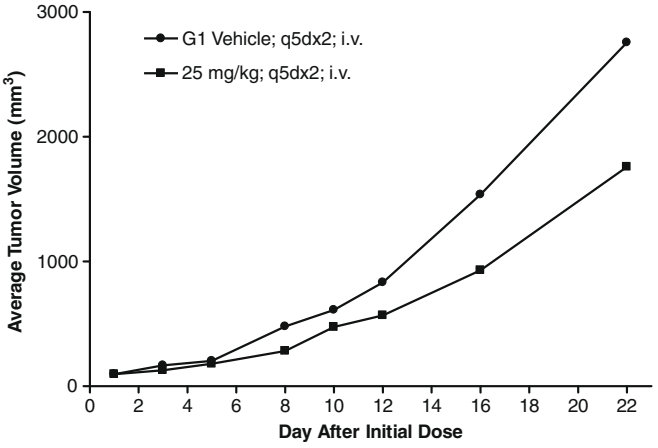


Figure 2. Compound **5e** inhibited the growth of established (~100 mm^3) MX-1 tumor xenografts in Crl:Nu/Nu-nuBR mice. Compound **5e** was dosed iv at 25 mg/kg on days 1–5 and 8–12. *P*-value as calculated by Student's *t*-test is 0.03.

4. Experimental

4.1. General methods and materials

Commercial-grade reagents and solvents were obtained from Acros, Aldrich, Lancaster, or Butt Park and were used without further purification. Thin-layer chromatography (TLC), usually using ethyl acetate/hexane as the solvent system, was used to monitor reactions. The ^{1}H NMR spectra were recorded at 300 MHz. Elemental analyses were performed by Numega Resonance Labs, Inc. (San Diego, CA). Human breast cancer cells T47D, human colorectal cancer cells HCT116 and liver cancer cells SNU398 were obtained from American Type Culture Collection (Manassas, VA). Compounds **4a**–**i** were synthesized according to reported procedure via reaction of the corresponding substituted 2-chlorobenzaldehyde or 2-fluorobenzaldehyde with substituted benzenethiol.[16]

4.1.1. 2-(3,5-Dimethoxyphenoxy)benzaldehyde (6b)

A mixture of 2-fluorobenzaldehyde (2.48 g, 20 mmol), 3,5-dimethoxyphenol (3.08 g, 20 mmol) and potassium carbonate (2.76 g, 20 mmol) in DMF (20 ml) was heated to 150 °C under argon for 8 h. The reaction mixture was then allowed to cool to room temperature and partitioned between ethyl acetate and water. The organic layer was washed with saline, dried over Na_2SO_4 and concentrated to give solid, which was purified by column chromatography (hexane/EtOAc 10:1) to give 2.9 g (56%) of **6b** as white solid. ^{1}H NMR ($CDCl_3$): 10.47 (s, 1H), 7.92 (q, J_1 = 7.5 Hz, J_2 = 2.1 Hz, 1H), 7.53 (t, J = 7.5 Hz, 1H), 7.20 (t, J = 7.8 Hz, 1H), 6.99 (d, J = 8.1 Hz, 1H), 6.28 (t, J = 1.8 Hz, 1H), 6.21 (d, J = 2.1 Hz, 2H), 3.77 (s, 6H).

4.1.2. 2-(2,5-Dimethoxyphenoxy)benzaldehyde (6a)

47%, solid. ^{1}H NMR ($CDCl_3$): 10.62 (s, 1H), 7.91 (q, J_1 = 7.5 Hz, J_2 = 2.1 Hz, 1H), 7.45 (t, J = 6.9 Hz, 1H), 7.13 (t, J = 7.8 Hz, 1H), 6.95 (d, J = 9.0 Hz, 1H), 6.78–6.65 (m, 3H), 3.76 (s, 6H).

4.1.3. 2-(2,5-Dimethoxyphenylsulfinyl)benzaldehyde (8)

To a 5 °C solution of sodium periodate (449 mg, 2.1 mmol) in water (20 mL) and methanol (10 mL) was added 2-(2,5-dimethoxyphenylthio)benzaldehyde (**4a**, 0.55 g, 2 mmol). It was then warmed to room temperature and stirred for 2 days. The mixture was poured into water (30 mL) and extracted with ethyl acetate (3 × 10 mL). The organic layer was dried over Na_2SO_4, concentrated to give crude product, which was purified by column chromatography to give 380 mg (66%) of **8** as solid. ^{1}H NMR ($CDCl_3$): 10.65 (s, 1H), 8.06 (d, J = 8.1 Hz, 1H), 7.97–7.93 (dd, J = 7.8, 1.5 Hz, 1H), 7.76–7.71 (dt, J = 7.8, 1.2 Hz, 1H), 7.60–7.55 (t, J = 6.6 Hz, 1H), 7.39 (d, J = 3.0 Hz, 1H), 6.95–6.91 (m, 1H), 6.77–6.74 (d, J = 8.7 Hz, 1H), 3.83 (s, 6H), 3.69 (s, 3H).

4.1.4. 1-(2-(2,5-Dimethoxyphenylthio)benzyl)semicarbazide (3)

A mixture of 1-(2-(2,5-dimethoxyphenylthio)benzylidene)-semicarbazide (**2a**, 120 mg, 0.36 mmol), and platinum(IV) oxide in methanol (100 mL) and 1.5 N hydrochloride in ethanol (five drops) was hydrogenated under 50 psi for 20 h. It was filtered and evaporated and the residue was purified by column chromatography (EtOAc/MeOH/Et_3N 10:1:1) to give 30 mg (25%) of compound **3** as white solid. ^{1}H NMR (DMSO-d_6): 7.62 (d, J = 7.2 Hz, 1H), 7.48–7.219 (m, 3H), 7.21 (br s, 1H), 7.07 (d, J = 8.7 Hz, 1H), 6.91–6.87 (m, 1H), 6.37 (d, J = 2.7 Hz, 1H), 5.90 (br s, 2H), 5.07 (br s, 1H), 4.00 (s, 2H), 3.84 (s, 3H), 3.60 (s, 3H). Anal. Calcd for $C_{16}H_{19}N_3O_3S{\cdot}0.5H_2O$: C, 56.12; H, 5.89; N, 12.27. Found: C, 56.79; H, 6.09; N, 12.08.

4.1.5. (2-(2,5-Dimethoxyphenylthio)benzylideneamino)-guanidine (5a)

A mixture of 2-(2,5-dimethoxyphenylthio)benzaldehyde (**4a**, 1.09 g, 4 mmol), aminoguanidine bicarbonate (0.55 g, 4 mmol) and sodium acetate (0.33 g, 4 mmol) in ethanol (30 mL) was refluxed overnight. The reaction mixture was then allowed to cool to room temperature and partitioned between ethyl acetate and water. The organic layer was washed with saline, dried over Na_2SO_4 and concentrated to give 1.31 g (99%) of **5a** as white solid. ^{1}H NMR ($CDCl_3$): 8.60 (s, 1H), 7.87 (d, J = 7.20 Hz, 1H), 7.31–7.18 (m, 3H), 6.84–6.70 (m, 2H), 6.55 (d, J = 3.0 Hz, 1H), 5.25 (br s, 2H), 4.25 (br s, 1H), 3.82 (s, 3H), 3.65 (s, 3H). Anal. Calcd for $C_{16}H_{18}N_4O_2S$: C, 58.16; H, 5.49; N, 16.96. Found: C, 58.19; H, 5.68; N, 16.93.

The following compounds were prepared from the corresponding substituted 2-phenylthiobenzaldehyde **4b–i**, 2-phenoxybenzaldehyde **6a–b**, or 2-(phenylsulfinyl)benzaldehyde **8** and aminoguanidine, or N^1-methylaminoguanidine by a procedure similar to that described for the preparation of compound **5a**.

4.1.6. (2-(3-Methoxyphenylthio)benzylideneamino)guanidine (5b)

Yield: 74%, solid. ^{1}H NMR ($CDCl_3$): 8.62 (s, 1H), 7.85 (d, J = 7.8 Hz, 1H), 7.29–7.19 (m, 5H), 6.91–6.77 (m, 2H), 5.25 (s, 2H), 4.25 (s, 2H), 3.76 (s, 3H). Anal. Calcd for $C_{15}H_{16}N_4OS$: C, 59.98; H, 5.37; N, 18.65. Found: C, 59.83; H, 5.66; N, 18.90.

4.1.7. (2-(4-Methoxyphenylthio)benzylideneamino)guanidine (5c)

Yield: 34%, solid. ^{1}H NMR ($CDCl_3$): 8.58 (s, 1H), 7.66 (q, J_1= 7.5 Hz, J_2= 1.8 Hz, 1H), 7.42 (d, J = 6.6 Hz, 2H), 7.17–7.07 (m, 2H), 6.94–6.89 (m, 3H), 5.38 (s, 2H), 4.38 (s, 2H), 3.83 (s, 3H). Anal. Calcd for $C_{15}H_{16}N_4OS$: C, 59.98; H, 5.37; N, 18.65. Found: C, 59.77; H, 5.59; N, 18.91.

4.1.8. (2-(3,4-Dimethoxyphenylthio)benzylideneamino) guanidine (5d)

Yield: 46%, solid. ^{1}H NMR ($CDCl_3$): 8.56 (s, 1H), 7.66 (q, J_1 = 7.5 Hz, J_2 = 1.8 Hz, 1H), 7.20–6.90 (m, 6H), 5.34 (s, 2H), 4.35 (s, 2H), 3.91 (s, 3H), 3.83 (s, 3H). Anal. Calcd for $C_{16}H_{18}N_4O_2S$: C, 58.16; H, 5.49; N, 16.96. Found: C, 58.07; H, 5.81; N, 16.91.

4.1.9. (2-(2,5-Dimethoxyphenylthio)-6-methoxy benzylideneamino)guanidine (5e)

Yield: 98%, solid. ^{1}H NMR ($CDCl_3$): 8.80 (s, 1H), 7.12–6.90 (m, 4H), 6.67 (d, J = 8.4 Hz, 1H), 6.33 (d, J = 8.1 Hz, 1H), 3.91 (s, 3H), 3.77 (s, 3H), 3.75 (s, 3H). Anal. Calcd for $C_{17}H_{20}N_4O_3S$: C, 56.65; H, 5.59; N, 15.54. Found: C, 56.26; H, 5.34; N, 15.48.

4.1.10. (2-(2,5-Dimethoxyphenylthio)-5-methoxy benzylideneamino)guanidine (5f)

Yield: 72%, solid. ^{1}H NMR ($CDCl_3$): 8.62 (s, 1H), 7.61 (d, J = 3.0 Hz, 1H), 7.41 (d, J = 8.4 Hz, 1H), 6.90 (q, J_1 = 8.4 Hz, J_2 = 2.7 Hz, 1H), 6.74 (d, J = 9.0 Hz, 1H), 6.57 (q, J_1 = 8.4 Hz, J_2 = 2.7 Hz, 1H), 6.17 (d, J = 3.0 Hz, 1H), 3.87 (s, 3H), 3.85 (s, 3H), 3.59 (s, 3H). Anal. Calcd for $C_{17}H_{20}N_4O_3S$: C, 56.65; H, 5.59; N, 15.54. Found: C, 56.53; H, 5.72; N, 15.54.

4.1.11. (2-(2,5-Dimethoxyphenylthio)-5-nitrobenzylideneamino)guanidine (5g)

Yield: 93%, solid. ^{1}H NMR ($CDCl_3$): 8.56 (s, 1H), 8.49 (d, J = 2.7 Hz, 1H), 7.88–7.84 (m, 1H), 7.06–6.88 (m, 4H), 5.35 (br s, 2H), 4.32(br s, 2H), 3.78 (s, 3H), 3.76 (s, 3H). Anal. Calcd for $C_{16}H_{17}N_5O_4S$: C, 51.19; H, 4.56; N, 18.66. Found: C, 51.44; H, 4.87; N, 18.35.

4.1.12. (2-(2,5-Dimethoxyphenylthio)-4-methoxybenzylideneamino)guanidine (5h)

Yield: 54%, solid. ^{1}H NMR ($CDCl_3$): 8.80 (s, 1H), 7.77 (d, J = 7.8 Hz, 1H), 7.40 (t, J = 8.1 Hz, 1H), 6.95 (d, J = 8.1 Hz, 1H), 6.71 (d, J = 9.0 Hz, 1H), 6.54–6.50 (m, 1H), 6.03 (d, J = 2.7 Hz, 1H), 5.15 (br s, 2H), 4.11 (br s, 2H), 3.85 (s, 3H), 3.80 (s, 3H), 3.56 (s, 3H). Anal. Calcd for $C_{17}H_{20}N_4O_3S$: C, 56.65; H, 5.59; N, 15.54. Found: C, 56.58; H, 5.86; N, 14.90.

4.1.13. (2-(2,5-Dimethoxyphenylthio)-3-methoxybenzylideneamino)guanidine (5i)

Yield: 36%, solid. ^{1}H NMR ($CDCl_3$): 8.55 (s, 1H), 7.82 (d, J = 9.0 Hz, 1H), 6.85–6.72 (m, 4H), 6.59 (d, J = 3.0 Hz, 1H), 5.16 (br s, 2H), 4.06 (br s, 2H), 3.82 (s, 3H), 3.72 (s, 3H), 3.66 (s, 3H). Anal. Calcd for $C_{17}H_{20}N_4O_3S$: C, 56.65; H, 5.59; N, 15.54. Found: C, 56.70; H, 5.76; N, 15.54.

4.1.14. (2-(2,5-Dimethoxyphenylthio)benzylideneamino)-*N*-3-methylguanidine (5j)

Yield: 46%, solid. ^{1}H NMR ($CDCl_3$): 8.62 and 8.55 (2br s, 1H), 7.88 (d, J = 8.1 Hz, 1H), 7.30–7.15 (m, 3H), 6.83–6.70 (m, 2H), 6.55 (br s, 1H), 6.30 (br s, 1H), 3.81 (s, 3H), 3.65 (s, 3H), 3.56 (s, 3H), 2.92 and 2.86 (2 br s, 3H). Anal. Calcd for $C_{17}H_{20}N_4O_2S$: C, 59.28; H, 5.85; N, 16.27. Found: C, 59.43; H, 5.76; N, 15.96.

4.1.15. (2-(2,5-Dimethoxyphenoxy)benzylideneamino)-guanidine (7a)

Yield 84%, white solid. ^{1}H NMR ($CDCl_3$): 8.53 (s, 1H), 7.96 (q, J_1 = 7.8 Hz, J_2 = 1.8 Hz, 1H), 7.24 (t, J = 7.2 Hz, 1H), 7.08 (t, J = 7.8 Hz, 1H), 6.90 (d, J = 9.0 Hz, 1H), 6.81 (d, J = 7.8 Hz, 1H), 6.57 (q, J_1 = 8.7 Hz, J_2 = 3.0 Hz, 1H), 6.38 (d, J = 3.0 Hz, 1H), 4.80 (br s, 4H), 3.79 (s, 3H), 3.68 (s, 3H). Anal. Calcd for $C_{16}H_{18}N_4O_3{\cdot}H_2O$: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.83; H, 6.07; N, 16.86.

4.1.16. (2-(3,5-Dimethoxyphenoxy)benzylideneamino)-guanidine (7b)

Yield 86%, solid. ^{1}H NMR ($CDCl_3$): 8.44 (s, 1H), 8.02 (q, J_1 = 7.8 Hz, J_2 = 1.5 Hz, 1H), 7.30 (t, J = 7.2 Hz, 1H), 7.17 (t, J = 7.8 Hz, 1H), 6.99 (d, J = 8.1 Hz, 1H), 6.15 (t, J = 2.1 Hz, 1H), 6.08

(d, J = 3.0 Hz, 2H), 5.20 (br s, 2H), 3.72 (s, 6H), 2.00 (br s, 2H). Anal. Calcd for $C_{16}H_{18}N_4O_3\cdot H_2O$: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.81; H, 6.20; N, 17.01.

4.1.17. (2-(2,5-Dimethoxyphenylsulfinyl)benzylideneamino) guanidine (9)

Yield 32%, white solid. ^{1}H NMR (CD_3OD): 8.43 (s, 1H), 7.89 (q, J_1 = 7.5 Hz, J_2 = 1.8 Hz, 1H), 7.74 (q, J_1 = 7.8 Hz, J_2 = 1.8 Hz, 1H), 7.57–7.44 (m, 2H), 7.10–6.98 (m, 3H), 3.73 (s, 3H), 3.31 (s, 3H). Anal. Calcd for $C_{16}H_{18}N_4O_3S\cdot H_2O$: C, 51.46; H, 5.67; N, 15.00. Found: C, 51.57; H, 5.27; N, 15.07.

4.2. Caspase activation assay (EC_{50})

The potency of compound **2a** and related compounds as inducer of apoptosis was measured by our caspase-based cell assay as previously described.[12] Human breast cancer cells T47D, colon cancer cells HCT116 and liver cancer cells SNU398 were grown according to media component mixtures designated by American Type Culture Collection in RPMI-1640 + 10% FCS in a 5% CO_2–95% humidity incubator at 37 °C. Cells were harvested using trypsin and washed at 600 g and resuspended at 0.65×10^6 cells/mL into RPMI media + 10% FCS. The cells were treated with 0.005–10 μM concentrations of compound **2a** or related compounds in RPMI-1640 and incubated at 37 °C for 48 h. The samples were then treated with the fluorogenic substrate *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110, [9] and incubated at 37 °C for 3 h. The fluorescent signal was measured using a fluorescent plate reader (Model Spectrafour Plus Tecan). The EC_{50} (μM) was determined by a sigmoidal dose–response calculation (XLFit3, IDBS), as the concentration of compound that produces the 50% maximum response. The caspase activation activity (EC_{50}) of the compounds tested in the three cancer cell lines, T47D, HCT116 and SNU398 are summarized in Table 1.

4.3. Cell growth inhibition assays (GI_{50})

The potency of compound **2a** and related compounds as inhibitors of cell proliferation was measured as previously described.[12] Briefly, T47D, HCT116 and SNU398 cells were treated with various concentrations of **2a** or related compounds. The samples were incubated at 37 °C for 48 h, then treated with CellTiter-Glo™ reagent (Promega). The samples were mixed by agitation and incubated at room temperature for 10–15 min. Plates were then read using a luminescent plate reader (Model Spectrafluor Plus Tecan Instrument). GI_{50} values were calculated from dose–response curves using XLFit3 (IDBS) software to determine the concentration for 50% reduction in proliferation, the baseline being the initial cell number. The GI_{50} for compound **2a** and related compounds are summarized in Table 2.

Table 2
Cell growth inhibition of [2-(arylthio)benzylideneamino]guanidines and related compounds

Entry	GI_{50}[a] (μM)		
	T47D	HCT116	SNU398
2a	0.045 ± 0.007	0.29 ± 0.14	0.055 ± 0.008
3	0.31 ± 0.04	0.90 ± 0.02	0.18 ± 0.02
5a	0.082 ± 0.003	0.061 ± 0.014	0.021 ± 0.005
5e	0.062 ± 0.013	0.048 ± 0.015	0.028 ± 0.01
5h	0.037 ± 0.003	0.097 ± 0.010	0.034 ± 0.009
7a	0.32 ± 0.02	0.46 ± 0.02	0.12 ± 0.01
Vinblastine	0.007 ± 0.002	ND[b]	ND
Colchicine	0.007 ± 0.001	ND	ND

[a] Data are the mean of three or more independent dose–response experiments and are reported as mean ± standard error of the mean (SEM).
[b] ND, not determined.

4.4. Measurement of apoptosis by flow cytometry

Cell cycle analysis was performed as previously described.[12] Briefly, T47D cells were treated with 50 nM of compound **5e**, and incubated for 48 h at 37 °C. Control cells were treated with the solvent (DMSO). After the 48 h incubation, cells were treated with propidium iodide, and analyzed on a flow cytometer. All flow cytometry analyses were performed on FACScalibur (Becton Dickinson) using Cell Quest analysis software. Fluorescence intensity is plotted on the *x*-axis and the number of cells with that fluorescence intensity is plotted on the *y*-axis. The sub-diploid amount of DNA is indicative of apoptotic cells that have undergone DNA degradation or fragmentation.

4.5. Tubulin inhibition assay

The tubulin inhibition assay was run as previously reported.[19] In brief, lyophilized tubulin (Cytoskeleton #ML113, 1 mg, 3 MAP-rich) was assayed for the effect of the test compound on tubulin polymerization as measured by change in fluorescence of 4,6-diamidino-2-phenylindole (DAPI). Polymerized tubulin (sample treated with DMSO and sample treated with the tubulin stabilizer Taxol) gives a higher DAPI fluorescence as compared to non-polymerized tubulin (vinblastine used to determine baseline). The IC_{50} for tubulin inhibition was the concentration found to decrease the fluorescence of DAPI by 50%.

4.6. MX-1 tumor model

MX-1 tumors were passaged serially in the mammary fat pad of female Crl:Nu/Nu-nuBR mice (Charles River Labs). For this study, tumors from several animals were excised. The viable portion of the tumor was cut into pieces (~30 mm^3) and a single piece was implanted into the right lateral mammary pad of each animal using a trocar. The tumor was allowed to grow to an average volume of 100 mm^3 and mice randomized into test groups (N = 8). The animals were dosed intravenous with 25 μL of 6% ETOH/5% Tween 80/89% D5 W formulation of compound **5e** (HCl salt) at dose of 25 mg/kg/day on days 1–5 and 8–12. The mice were observed daily for morbitity, mortality and signs of toxicity. Tumors and body weights were measured from day 1 to the end of study with the frequency measurements determined by the rate of growth of the tumor. Tumor growth was monitored using external measurements with a caliper and tumor volumes were calculated using the formula/6(width^2 × length), where width represents the smaller tumor diameter. The results are shown in Figure 2. These studies conformed to the recommendations set forth in the USPHS Policy on Humane Care and Use of Laboratory Animals.

References and notes

1. Henson, P. M.; Bratton, D. L.; Fadok, V. A. *Curr. Biol.* **2001**, *11*, R795.
2. Kasibhatla, S.; Tseng, B. *Mol. Cancer. Ther.* **2003**, *2*, 573.
3. Green, D. R.; Reed, J. C. *Science* **1998**, *281*, 1309.
4. Salvesen, G. S.; Dixit, V. M. *Cell* **1997**, *91*, 443.
5. Kolenko, V. M.; Uzzo, R. G.; Bukowski, R.; Finke, J. H. *Apoptosis* **2000**, *5*, 17.
6. Cai, S. X.; Zhang, H. Z.; Guastella, J.; Drewe, J.; Yang, W.; Weber, E. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 39.
7. Cai, S. X.; Drewe, J.; Kasibhatla, S. *Curr. Med. Chem.* **2006**, *13*, 2627.
8. Kemnitzer, W.; Drewe, J.; Jiang, S.; Zhang, H.; Crogan-Grundy, C.; Labreque, D.; Bubenick, M.; Attardo, G.; Denis, R.; Lamothe, S.; Gourdeau, H.; Tseng, B.; Kasibhatla, S.; Cai, S. X. *J. Med. Chem.* **2008**, *51*, 417.
9. 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.
10. Zhang, H. Z.; Kasibhatla, S.; Wang, Y.; Herich, J.; Guastella, J.; Tseng, B.; Drewe, J.; Cai, S. X. *Bioorg. Med. Chem.* **2004**, *12*, 309.
11. Kasibhatla, S.; Jessen, K.; Maliartchouk, S.; Wang, J.; English, N.; Drewe, J.; Qui, L.; Archer, S.; Ponce, A.; Sirisoma, N.; Jiang, S.; Zhang, H.-Z.; Gehlsen, K.; Cai, S. X.; Green, D. R.; Tseng, B. *Proc. Natl. Acad. Sci.* **2005**, *102*, 12095.

12. Zhang, H.-Z.; Kasibhatla, S.; Kuemmerle, J.; Kemnitzer, W.; Oliis-Mason, K.; Qui, L.; Crogran-Grundy, C.; Tseng, B.; Drewe, J.; Cai, S. X. *J. Med. Chem.* **2005**, *48*, 5215.
13. Jessen, K.; English, N.; Wang, J.; Qui, L.; Brand, R.; Maliartchouk, S.; Drewe, J.; Kuemmerle, J.; Zhang, H.-Z.; Gehlsen, K.; Tseng, B.; Cai, S. X.; Kasibhatla, S. *Mol. Cancer Ther.* **2005**, *4*, 761.
14. (a) Sirisoma, N.; Kasibhatla, S.; Pervin, A.; Zhang, H.; Jiang, S.; Willardsen, J. 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; (b) Kasibhatla, S.; Baichwal, V.; Cai, S. X.; Roth, B.; Skvortsova, I.; Skvortsov, S.; Lukas, P.; English, N. M.; Sirisoma, N.; Drewe, J.; Pervin, A.; Tseng, B.; Carlson, R. O.; Pleiman, C. M. *Cancer Res.* **2007**, *67*, 5865.
15. Thomas, T. L.; Fedorchuk, M.; Shetty, B. V.; Anderson, F. E. *J. Med. Chem.* **1970**, *13*, 196.
16. (a) Jilek, J.; Sindelar, K.; Pomykacek, J.; Kmonicek, V.; Sedivy, Z.; Hrubantova, M.; Holubek, J.; Svatek, E.; Ryska, M. *Collect. Czech., Chem. Commun.* **1989**, *54*, 3294; (b) Urban, J.; Sedivy, Z.; Holubek, J.; Svatek, E.; Ryska, M.; Koruna, I.; Dlabac, A.; Valchar, M.; Metysova, J. *Collect. Czech., Chem. Commun.* **1990**, *55*, 1077.
17. Sapra, P.; Zhao, H.; Mehlig, M.; Malaby, J.; Kraft, P.; Longley, C.; Greenberger, L. M.; Horak, I. D. *Clin. Cancer Res.* **2008**, *14*, 1888.
18. Gourdeau, H.; Leblond, L.; Hamelin, B.; Desputeau, C.; Dong, K.; Kianicka, I.; Custeau, D.; Bourdeau, C.; Geerts, L.; Cai, S. X.; Drewe, J.; Labrecque, D.; Kasibhatla, S.; Tseng, B. *Mol. Cancer Ther.* **2004**, *3*, 1375.
19. Zhang, H.-Z.; Drewe, J.; Tseng, B.; Kasibhatla, S.; Cai, S. X. *Bioorg. Med. Chem.* **2004**, *12*, 3649.