



Synthesis and antiproliferative properties of a new ceramide analog of varacin



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ABSTRACT

A benzopentasulfane was synthesized in 8 steps with a ceramide attached through an amide bond to the 7-position of the heterocycle structure. The anticancer activity of this synthetic ceramide–benzopolysulfane drug conjugate was analyzed against five human cancer cell lines MDA-MB-231 (breast), DU145 (prostate), MIA PaCa-2 (pancreas), HeLa (cervix), and U251 (glioblastoma). The ceramide–benzopolysulfane conjugate had IC₅₀ values ranging from 10 to >20 μM with complete cell killing at 12.5 μM for MDA-MB-231 and 20 μM for DU145 and HeLa cells. The ceramide–benzopolysulfane conjugate had IC₅₀ values 1.8 and 4.0 times lower than a PEG benzopolysulfane, *N*-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)benzo[*f*][1,2,3,4,5]-pentathiepine-7-carboxamide, for MDA-MB-231 and DU145 cells, respectively. The parent “unsubstituted” benzopolysulfane, *o*-C₆H₄S₅, had IC₅₀ values 4.2 times lower and 2.7 times higher than the ceramide benzopolysulfane for MDA-MB-231 and DU145 cells, respectively. The results indicate that the polysulfur linkage is needed for activity since benzenedithiol, *o*-C₆H₄(SH)₂, had IC₅₀ values greater than 30 μM with little effect on MDA-MB-231 and DU145 cells. Thus, to account for the bioactivity, a bimolecular reaction of cellular thiol with the ceramide benzopolysulfane is a proposed followed by thiozone (S₃) extrusion.

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1. Introduction

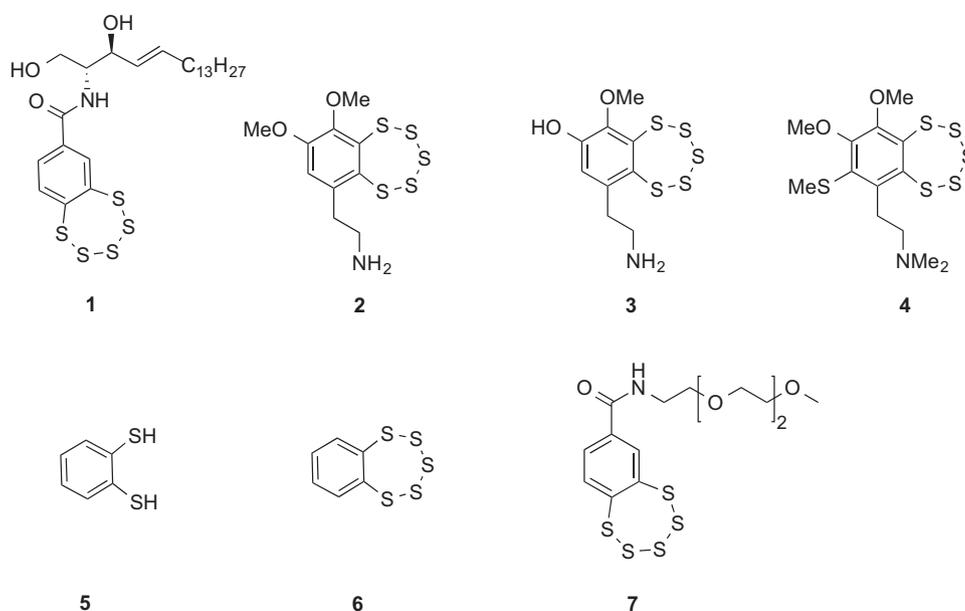
This manuscript describes a synthetic approach to ceramide–benzopolysulfane drug conjugate **1** as a new anticancer compound (Scheme 1). Benzopolysulfanes are a unique class of compounds, some isolated from marine tunicates or their associated microorganisms, e.g., varacin (**2**), lissoclinotoxin A (**3**), and *N,N*-dimethyl-5-(methylthio)varacin (**4**) (Davidson et al., 1991; Litaudon and Guyot, 1991; Compagnone et al., 1994; Searle and Molinski, 1994; Makarieva et al., 1995; Liu et al., 2004a,b; Bentley, 2005; Liu et al., 2005; Jiang et al., 2012). Common to these natural polysulfane structures is a dopamine core (Ford et al., 1994; Toste and Still, 1995; Molinski, 2004), although synthetic analogs have been prepared (Konstantinova et al., 2004, 2012; Okuma et al., 2012; Zubair et al., 2013; Koyioni et al., 2014).

Benzopolysulfanes have been reported to possess antiproliferative activity with IC₅₀ values in the low micromolar range; for example, ethylaminobenzopentathiepin yielded an IC₅₀ value of 0.26 μg/mL against HeLa cells (Sato et al., 1995). It has been suggested that the bioactivity of varacin **2** derives from DNA damage because of an observed difference in toxicity toward the CHO cell line EM9 (chloro deoxyuridine sensitive) compared to BR1 (Davidson et al., 1991). While benzopolysulfanes show promising bioactivity, they remain relatively understudied as therapeutic agents due to the lability of the polysulfur ring and their low water solubility.

To ameliorate the low water solubility problem, in 2010, synthesized benzopolysulfanes 4-CH₃(OCH₂CH₂)₃NHC(O)-C₆H₄-1,2-S_{*x*} (*x*=3–7 and 9) with a PEG group attached through an amide bond, where the pentasulfane **7** was the major constituent (Mahendran et al., 2010; Bittman et al., 2010). Antiproliferative activity of the PEGylated benzopolysulfanes in cancer cells increased in comparison to the parent-unsubstituted benzopolysulfane (**6**) due to the PEG substitution. Somewhat relatedly, Bittman reported on a ceramide-disulfane conjugate *N*-(4',5v-dithiaheptanoyl)-D-erythro-ceramide that had greater antiproliferative activity than ceramide itself in BT549, A549, and DU145

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Scheme 1. Sulfur compounds.

cancer cells (Bittman et al., 2010). Because sphingolipid ceramides (Delgado et al., 2012, 2013) are known to play a role in antiproliferative activity (Flowers et al., 2012) as well as cell signaling pathways, e.g., apoptosis, cell proliferation and angiogenesis (Gangoiti et al., 2010; Merrill, 2011), we sought to design and test a new ceramide-polysulfane compound, **1**.

Here, we describe our efforts to (i) synthesize ceramide-benzopolysulfane drug conjugate **1**, (ii) identify the number of sulfur linked atoms in the major cyclic product, (iii) examine the anticancer activity of **1** compared to 1,2-benzenedithiol **5** and benzopolysulfanes **6** and **7**, and (iv) propose a mechanism for its bioactivity. As we will see, the pentasulfur linkage is the key moiety found in ceramide conjugate **1**, while other forms may exist, e.g., tri- and heptasulfanes, as will be discussed.

2. Results and discussion

2.1. Synthesis

The synthesis of 4-nitrophenyl benzo[*f*][1,2,3,4,5]pentathiepine-7-carboxylate **9** was carried out as described before (Mahendran et al., 2010), using 3,4-dihydroxybenzoic acid (**8**) as a starting material, as well as the use of thiostannole and disulfur dichloride chemistry (Lienard et al., 2007; Ogawa et al., 1994; Konstantinova and Rakitin, 2014) (Scheme 2). Briefly, compound **9** was synthesized in seven steps in 2.8% yield, and was reacted with *D*-erythro-sphingosine (0.035 mmol) in THF to form conjugate **1**. THF was evaporated leaving a residue of **1**, which was diluted with CH_2Cl_2 and washed with saturated aqueous NaHCO_3 , then 1 M HCl, and then water, and purified by chromatography on silica. The yield of the reaction to form **1** from **9** was 21%. Our ^1H NMR and ^{13}C NMR

spectra provide evidence that the amine group in *D*-erythro-sphingosine forms an amide to couple to the 7-position of the heterocycle structure (Figs. S1 and S2, Supplementary material). In the ^{13}C NMR spectrum, the quaternary peaks are weak and carbonyl peak at 161.2 ppm is barely visible due to low concentration of the sample. It is possible that other cyclic polysulfanes related to benzopentasulfane **1** were formed in low yields, but this was not determined.

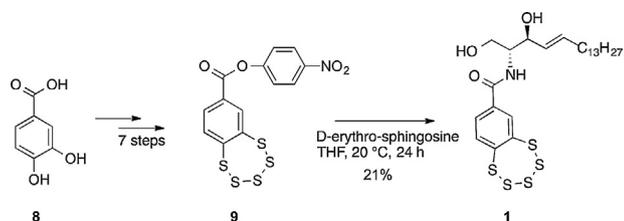
2.2. Mass spectrometry

In addition to NMR, mass spectrometry was used to assist in the characterization of **1** as a pentasulfane. Mass spectrometry has been useful for determining the number of sulfur atoms in polysulfanes (Block et al., 2010; Block, 2013). High-resolution mass spectroscopy data indicated that the number of sulfur present in the polysulfur ring is five. Mass spectrometry data were obtained in electron spray ionization (ESI) method in both positive and negative modes. Positive and negative adducts of the molecule found within a 2-ppm error limit. Calculated mass m/z for $\text{C}_{25}\text{H}_{39}\text{NO}_3\text{S}_5$ (**M**) is 561.1533 and found 561.1536. In ESI positive mode, the calculated m/z mass for $[\text{M}+\text{H}]^+$ adduct is 562.16063 found 562.16198, calculated m/z mass for $[\text{M}+\text{Na}]^+$ adduct is 584.14257 and found 584.14247. In negative mode calculated m/z mass for $[\text{M}-\text{H}]^-$ adduct is 560.14607 and found 560.14594, calculated m/z mass for $[\text{M}+\text{Cl}]^-$ adduct is 596.12275 found 596.12254, calculated m/z mass for $[\text{M}+\text{CF}_3\text{CO}_2]^-$ adduct is 674.13894 found 674.13890.

2.3. Antiproliferative activity in a new polysulfane

Fig. 1 and Table 1 show the antiproliferative effects of the ceramide-benzopolysulfane conjugate **1** with MDA-MB-231 (breast), DU145 (prostate), MIA PaCa-2 (pancreas), HeLa (cervix), and U251 (glioblastoma) cancer cells.

Cells were incubated with **1** in varying concentrations (0–20 μM) for 48–72 h followed by determination of cell viability using MTT assay and cell proliferation with a CyQUANT assay (Bittman et al., 2010). Fig. 1 shows that compound **1** inhibited the proliferation of all the cell lines with the exception of the glioblastoma U251 cell line. At the highest concentration tested,

Scheme 2. Synthesis of ceramide-benzopolysulfane drug conjugate **1**.

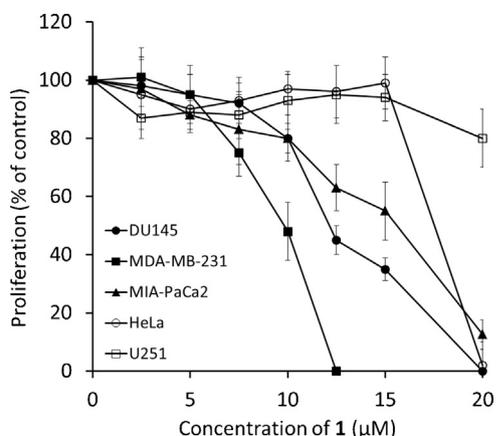


Fig. 1. Effect of the ceramide-benzopolysulfane conjugate **1** on the growth of DU145 (●), MDA-MB-231 (■), MIA PaCa-2 (▲), HeLa (○) and U251 (□) cancer cells determined by CyQuant and MTT assay as described in Section 4. The values were expressed as the decrease in cell numbers relative to controls without any compound. Data are the mean \pm SE ($n = 6-8$).

20 μ M, proliferation of U251 cells was inhibited by only 20% relative to controls incubated with the vehicle. The MDA-MB-231 breast cancer cell line, which is negative for estrogen receptors, progesterone receptors and HER-2 receptors (triple negative) was the most sensitive to compound **1**, with an IC_{50} of 10 μ M and complete loss of proliferation at a concentration of 12.5 μ M. Triple negative breast tumors do not respond to hormonal therapy or herceptin. Furthermore, IC_{50} values of 12 and 16 μ M were obtained with DU145 and MIA PaCa-2 cells, respectively. However, up to 15 μ M, compound **1** had little effect on the proliferation of HeLa cells, but a concentration of 20 μ M resulted in complete loss of proliferation. This may indicate the existence of a threshold in the cellular levels of the compound beyond which rapid loss of proliferative ability of the cells occurs.

Our data on **1** were compared to literature data on antiproliferative effects of 1,2-benzenedithiol **5**, benzopolysulfanes **6** and **7** against DU145 and MDA-MB-231 cells (Mahendran et al., 2010). The comparisons are shown in Fig. 2 for DU145 cells and in Fig. 3 for MDA-MB-231 cells. Benzenedithiol **5** had very little effect on the growth of both cell lines and IC_{50} values were greater than 30 μ M. The ceramide conjugate **1** was more active than the parent unsubstituted benzopolysulfane **6** against MDA-MB-231 (breast cancer) cells with an IC_{50} of 10 μ M compared with 27 μ M. On the other hand, the ceramide conjugate **1** (IC_{50} of 12 μ M) was less active than the parent benzopolysulfane **6** (IC_{50} of 4.9 μ M) in DU145 (prostate cancer) cells. The reason for this difference is unclear as solubility issues would be expected to impact both cell lines similarly.

The ceramide conjugate **1** and PEG conjugate **7** were able to completely inhibit the proliferation of both the DU145 and MDA-MB-231 cell lines. IC_{50} values show that PEG conjugate is more effective than the ceramide conjugate. Thus, the ceramide substituent on benzopolysulfane plays a role in enhanced activity

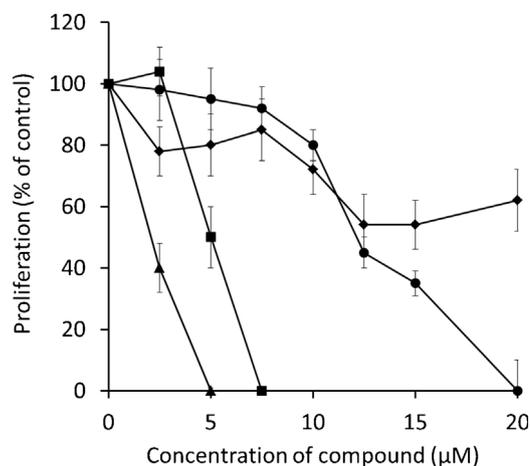


Fig. 2. Effect of ceramide-benzopolysulfane conjugate **1** (●), benzenedithiol **5** (◆), parent-benzopolysulfane **6** (■), PEG-benzopolysulfane conjugate **7** (▲) on the growth of DU145 (prostate) cancer cells. Data for **5-7** were taken from Mahendran et al., 2010.

in some cells, but not others. The hydrophilicity conferred by the PEG conjugate may be contrasted with the lipophilicity of the ceramide conjugate, where both can influence the localization of the polysulfanes in the cells. To estimate lipophilicity, we have computed (C) the octanol-water partition coefficients ($\log P$) for the ceramide **1** and PEG **7** drug conjugates using ChemBioDraw (version 14.0). As would be expected, **1** is more lipophilic ($C \log P = 9.3$) than **7** ($C \log P = 3.8$). The effects of **1, 6-7** on human normal epithelial cells were not investigated and therefore no conclusions can be made regarding their in vitro selectivity. Any future clinical utility of the compounds will depend on their ability to be effective against cancer cells at concentrations that are not toxic to normal cells in the body. This will require determination of maximum tolerable levels

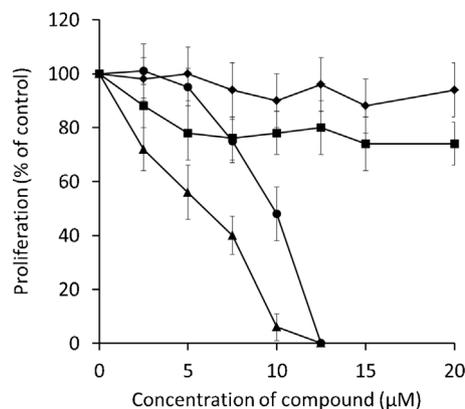


Fig. 3. Effect of ceramide-benzopolysulfane conjugate **1** (●), benzenedithiol **5** (◆), parent-benzopolysulfane **6** (■), PEG-benzopolysulfane conjugate **7** (▲) on the growth of MDA-MB-231 (breast) cancer cells. Data for **5-7** were taken from Mahendran et al., 2010.

Table 1

IC_{50} of the sulfur compounds against cancer cells (in μ M).

Cell line	Ceramide-benzopolysulfane conjugate 1	Benzene dithiol 5 ^a	Parent-benzopolysulfane 6 ^a	PEG-benzopolysulfane conjugate 7 ^a
MDA-MB-231	10 (12.5) ^b	>30	27	5.5
DU145	12 (20) ^b	>30	4.9	3
MIA PaCa-2	16 (>20) ^b	-	-	-
HeLa	18 (20) ^b	-	-	-
U251	>20	-	-	-

^a IC_{50} values from Mahendran et al., 2010.

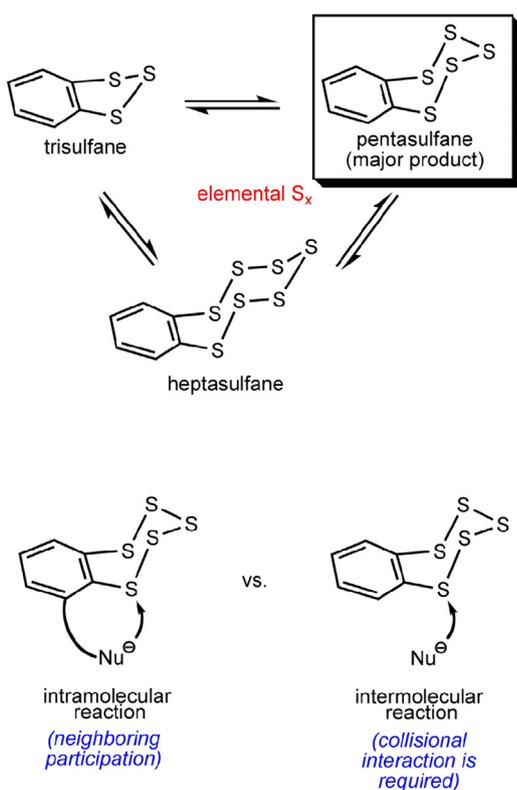
^b Concentrations that resulted in 100% cell kill are shown in parentheses.

of the compounds in an animal model in future studies, and its subsequent utilization in efficacy studies in a cancer xenograft model.

2.4. Mechanistic evaluation

Here, we postulate on the factors underlying the bioactivity of benzopolysulfanes **1**, **6** and **7** (Scheme 3). Two mechanistic aspects were considered. One emanates from polysulfane linkage RSS_xSR equilibria, and the second from nucleophilic substitution reactions.

First, a facet of polysulfanes is that even after purification, mixtures of polysulfanes can arise in solution (Schroll and Barany, 1986; Compagnone et al., 1994). Dimers can also arise such as tetrathiocins (Liu et al., 2004a,b; Asquith et al., 2015). A facile equilibration has been reported to take place between the tri-, penta-, and hepta-sulfanes (*o*-C₆H₄S₃, *o*-C₆H₄S₅, and *o*-C₆H₄S₇) (Brzostowska et al., 2007). A previous density functional theory (DFT) study showed an alternating stability pattern in benzopolysulfanes where odd membered rings are more stable except the 3-membered ring thiirene (Brzostowska and Greer, 2004). The stability was due to staggered sulfur long-pair electrons in the odd-membered rings, but not in the even-membered rings. Although the smallest even-membered ring (dithiete) was also unstable; it suffers from ring strain (Castillo et al., 2008). The range of the polysulfane linkages is probably solvent dependent, since elemental sulfur is structurally similar to benzopolysulfanes and also tends to equilibrate (Tebbe et al., 1982). What structures are favored in the highest proportions in polysulfane equilibria are probably determined by the amount of available elemental sulfur. As an aside, elemental S_x is found in significant amounts in marine environments, which may serve as a source for sulfur in biomolecules (Mopper and Taylor, 1986; Passier et al., 1999), to transform insoluble S₈ into a more soluble form in aromatic molecules.



Scheme 3. Equilibration and nucleophilic attack.

Second, reactions of nucleophiles assist in ring-opening of polysulfanes (Steudel, 2002; Sato, 2002) and may play a role in the bioactivity. Thiols are often present in biological media, and thiolate ions are known nucleophiles for attacking pentathiepin heterocycles (Tonika and Gates, 2003; Chatterji and Gates, 2003). A near neighbor amine group confers an enhanced bioactivity in natural pentathiepins, where trapping studies suggested thiozone, S₃, as a reactive intermediate (Greer, 2001; Brzostowska and Greer, 2003). For ceramide **1**, we suggest that S₃ is the reactive intermediate underlying bioactivity. Reactive sulfur species (Sato et al., 1987; Lee et al., 2002; Lee, 2009; Münchberg et al., 2007; Anwar et al., 2008; Nielsen et al., 2011; Czepukojc et al., 2013a,b) such as diatomic sulfur, S₂, can arise in other polysulfanes (Abu-Yousef and Harpp, 1998; Abu-Yousef, 2006; Zysman-Colman and Harpp, 2007; Rys et al., 2008; Startsev et al., 2015). Relatedly, the antiproliferative activity of Bittman's ceramide-disulfane conjugate, *N*-(4',5'-dithiaheptanoyl)-D-erythro-ceramide, was correlated to a reduction in cellular glutathione (GSH) levels in cancer cells (Bittman et al., 2010). For ceramide **1**, the consumption of GSH may be important, as well as the modification of cysteine sites as a cellular thiolstat. Lastly, the benefit of combining ceramide with other drugs, such as tamoxifen, has enhanced the anticancer activity in breast cancer cells (Morad et al., 2012), where further design and anticancer studies of ceramide-polysulfanes would likely prove useful.

3. Conclusion

A polysulfane conjugated to a ceramide **1** was synthesized and tested for antiproliferative activity in vitro. It was found to have IC₅₀ values ranging from 10 to 12 μM for MDA-MB-231 and DU145 cells, and ≥16 μM for MIA PaCa-2, HeLa and U251 cells. For the bioactivity, intermolecular nucleophilic activation and thiozone (S₃) release are likely key reactions. Unlike natural product polysulfanes with near neighbor amine substituents (e.g., **2–4**), ceramide **1** and PEG **7** drug conjugates would require an intermolecular nucleophilic activation. Because key cellular cysteine proteins may be one target of the varacin derivatives, future work could focus on modification of cysteine sites and intramolecular activation control with polysulfane side-groups other than amines as found in the natural products, **2–4**.

4. Experimental

4.1. Chemicals and reagents

Sodium hydroxide, potassium hydroxide, sodium sulfate (anhydrous), magnesium sulfate (anhydrous), NaCl, NaHCO₃, THF, CHCl₃, CH₂Cl₂, hydrochloric acid (12 M), acetone-d₆, CDCl₃, and hexanes were purchased from Sigma-Aldrich USA (St. Louis, MO, USA) or Fisher Scientific USA (Pittsburgh, PA, USA) and used as received without further purification. D-erythro-sphingosine was obtained from Avanti Polar Lipids (Alabaster, AL). Compound **9** was synthesized as described in our previous paper (Mahendran et al., 2010). Purification of the product mixtures was carried out by column chromatography using silica gel with particle sizes of 40–60 Å. TLC was carried out using silica gel 60F 254 TLC-plates (Sigma-Aldrich, St. Louis, MO, USA). Nuclear magnetic resonance (NMR) data were acquired using Bruker 400 MHz instrument (Bruker BioSpin Corporation, Billerica, MA, USA). Proton NMR data were acquired at 400 MHz and ¹³C NMR data were acquired at 100.6 MHz. HRMS data were collected using Agilent 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

4.2. N-Ceramidobenzo-1,2,3,4,5-pentathiepine-7-carboxamide (1)

Yield 2 mg (21%). A 1.5-mL solution of D-erythro-sphingosine (10.48 mg, 0.035 mmol) of THF was added to a solution of 4-nitrophenyl benzo[*f*][1,2,3,4,5]pentathiepine-7-carboxylate **9** (7 mg, 0.017 mmol) in 2.5 mL of THF. The reaction mixture was stirred under argon atmosphere overnight for 24 h. The solvent was evaporated and the residue was dissolved in 10 mL of CH₂Cl₂. The organic layer was washed with saturated aqueous NaHCO₃ solution (3 times, 10 mL ea), 1 M HCl (3 times, 10 mL ea), and water (3 times, 10 mL ea). The organic solvent was evaporated and the crude product was chromatographed (CHCl₃/CH₃OH, 10:1) to yield 2 mg of **9**, *R*_f = 0.57, ¹H NMR (CHCl₃, 400 MHz): δ 8.25 (d, *J* = 1.9 Hz, 1H), 7.92 (d, *J* = 8.0 Hz, 1H), 7.76 (dd, *J* = 7.90, 1.9 Hz, 1H), 7.00 (brs, 1H), 5.81 (dd, *J* = 12.0, 9.0 Hz, 1H), 5.63 (m, 1H), 4.90 (m, 1H), 4.11 (m, 2H), 3.65 (m, 1H), 2.45 (m, 1H), 2.08 (m, 2H), 1.27 (m, 22H), 0.88 (t, *J* = 7.2 Hz, 3H); ¹³C NMR (CHCl₃, 100 MHz): δ 161.2, 146.5, 144.2, 136.2, 135.3, 134.4, 128.7, 128.6, 74.9, 62.5, 54.5, 32.3, 31.9, 29.7–29.1, 22.7, 14.1; HRMS (+ESI) calcd for C₂₅H₃₉NO₃S₅ = 561.1533, found 561.1536, (M+H)⁺ calcd *m/z* 562.16063 found 562.16198, (M+Na)⁺ calcd *m/z* 584.14257 found 584.14247; HRMS (–ESI) calcd *m/z* (M–H)[–] calcd *m/z* 560.14607, found 560.14594, (M+Cl)[–] calcd *m/z* 596.12275, found 596.12254, (M+CF₃COO)[–] calcd *m/z* 674.13894, found 674.13890.

4.3. Cell culture

The cell lines were grown from frozen stocks originally obtained from the American Type Culture Collection (ATCC). The prostate cancer cell line DU145, breast cancer cell line MDA-MB-231, and pancreatic cancer cell line MIA PaCa-2 cells were grown in DMEM media. The brain cancer cell line U251 and cervix cancer cell line HeLa cells were grown in EMEM medium. All the media were supplemented with 10% fetal bovine serum and 1% (5000 I.U./mL) penicillin/streptomycin to make complete “growth media”. The cells were maintained in a humidified atmosphere of 5% CO₂ incubator at 37 °C.

4.4. Cell proliferation and cytotoxicity assays

Cell proliferation and cytotoxicity was assessed by cell proliferation assays as previously described (Bittman et al., 2010; Mahendran et al., 2010). Cells were seeded in a 96-well plate at 2000 cells/well density (200 μL of the growth medium per well) and maintained in 5% CO₂ incubator at 37 °C until they were in log phase growth. After 24 h incubation, the growth medium was aspirated out and 200 μL of growth medium containing the test compounds (0–20 μM concentration) was added to the cells. Cell proliferation assay: after 48 h incubation at 37 °C, the growth medium containing the test compounds was removed and the plates were maintained at –80 °C for 5–7 days. The plates were thawed to room temperature, appropriate concentration of CyQuant reagent in lysis buffer (200 μL) was added to each well and fluorescence was measured using plate reader (excitation 485 nm/emission 535 nm wavelength) to determine% cell proliferation. The CyQUANT assay measures the DNA content of the cells, which is a measure of cell numbers. The reagent contains a dye that fluoresces strongly upon binding to DNA. Cell toxicity assay: toxicity of the cells was measured by the MTT assay, which measures the metabolic activity found in viable cells. After 72 h incubation at 37 °C, the growth medium containing the test compounds was removed and cell viability was evaluated using a 96-titer solution cell proliferation MTT assay in a microplate reader (UV absorption at 490 nm).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chemphyslip.2015.07.023>.

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