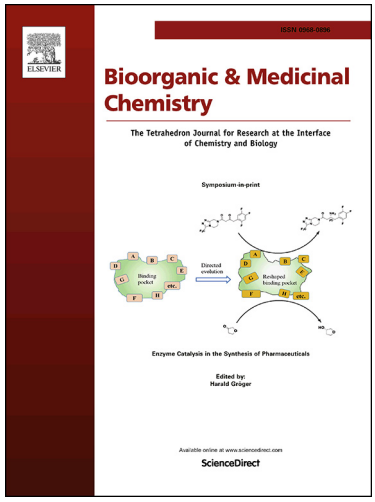
Accepted Manuscript



Chemical Synthesis and Functional Characterization of A New Class of Ceramide Analogues as Anti-Cancer Agents

Qianqian Liu, Xia Li, Yong-Sheng Bao, Jingxin Lu, Hua Li, Zhizhen Huang, Feiyan Liu

PII: S0968-0896(18)32085-6
DOI: https://doi.org/10.1016/j.bmc.2019.02.030
Reference: BMC 14766

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 12 December 2018
Revised Date: 5 February 2019
Accepted Date: 15 February 2019

Please cite this article as: Liu, Q., Li, X., Bao, Y-S., Lu, J., Li, H., Huang, Z., Liu, F., Chemical Synthesis and Functional Characterization of A New Class of Ceramide Analogues as Anti-Cancer Agents, *Bioorganic & Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.bmc.2019.02.030

Chemical Synthesis and Functional Characterization of A New Class of Ceramide Analogues as Anti-Cancer Agents

Qianqian Liu[1#], Xia Li[2#], Yong-Sheng Bao[3#], Jingxin Lu[2], Hua Li[2], Zhizhen Huang[3,4], and Feiyan Liu[2*]

[1]Zhejiang University Hospital, Zhejiang University, Hangzhou 310058, P. R. China. [2]Research Centre of Siyuan Natural Pharmacy and Biotoxicology, College of Life Sciences, Zhejiang University, Hangzhou 310058, P. R. China. [3]School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, P. R. China. [4]Department of Chemistry, Zhejiang University, Hangzhou 310028, P. R. China.

# equal contributions

Running title: Synthesis and function of anti-cancer ceramide analogueues

*Correspondence: Feiyan Liu, College of Life Sciences, Zhejiang University, Hangzhou 310058, P. R. China, e-mail: liuf64@zju.edu.cn;

Abstract

Deregulation of ceramide metabolism is a hallmark of human cancer. Ceramide analogues thereby represent a new class of anti-cancer agents. We aimed at developing effective and low toxic ceramide analogues and synthesized a new class of ceramide analogues starting from L-threonine. Several analogues exhibit potent cytotoxicity against human cancer cells *in vitro* with IC_{50} as low as 4.8 μM. These ceramide analogues decreased xIAP and Bcl-xL level and exhibited significant sensitization activity to overcome human cancer cell resistance to TRAIL, a cancer-selective agent that are being tested in human clinical trials. Furthermore, we determined that these ceramide analogues effectively suppress human cancer xenograft growth *in vivo* with no significant toxicity at the efficacious dose. Therefore, we have developed a simple and effective method to synthesize functional ceramide analogues using L-threonine as starting material and these analogues have the great potential to be further developed as anti-cancer agents in human cancer therapy.

Key words: Anti-tumor agents; Ceramide Analogues; xIAP; L-threonine; TRAIL.

1. Introduction

Sphingolipids are a family of ubiquitous membrane components that are the most structurally diverse and complex lipids owing to their numerous variations in the sphingosine bases, N-acyl linked fatty acids and head groups. Sphingolipids were originally thought to be solely the cellular energy source and membrane structure molecules. The revelation that sphingolipids are key bioeffectors that regulate a broad range of cellular processes, including cell proliferation, apoptosis, motility, differentiation, stress responses, protein synthesis, carbohydrate metabolism, immunity, and angiogenesis , is widely regarded as one of the major advances in modern biology in the past two decades [1, 2]. The two major sphingolipid metabolites, ceramide and sphingosine-1-phosphate (S1P), are the central mediators of the sphingolipid metabolism pathways. Interestingly, ceramide and S1P exhibit opposing bioactivities with ceramide being pro-apoptotic and S1P generally promoting cell survival [3,4].

Compelling experimental data from mouse models and human patients have shown that sphingolipid deregulation, namely the unbalance between ceramide and S1P, is a key factor in tumor pathogenesis, progression and cancer cell resistance to chemotherapeutic agents and radiation [5, 6]. The crucial roles of ceramide and S1P in tumor development and cancer cell responses to chemotherapy and radiation have led to extensive efforts to target the ceramide-S1P metabolism signaling pathways for anticancer therapy. For the last two decades, extensive efforts have be devoted to

develop ceramide analogues to mimic natural ceramide; small molecular inhibitors for enzymes that catalize ceramide catabolism or its conversion; and S1P receptor antagonists [7, 8]. Among these various approaches, development of ceramide analogues by far is the most commonly one. Numerous ceramide analogues with different chemical and biological properties have been developed [7, 9-20], and various ceramide analogues have been tested in human clinical trials against cancer. However, the facts that ceramide is one of the most structurally diverse and complex lipids and that ceramide analogues can potentially interact with numerous cellular targets in the sphingolipid signaling pathways make development of effective and yet low toxic ceramide analogues a challenging one and greatly limit its clinical use in human cancer therapy. Therefore, development of new ceramide analogues with effective anticancer activity and yet low toxicity are still in urgent need.

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily. Ever since its discovery in 1995, TRAIL has been under intense study since it preferentially induces apoptosis in tumor cells but not in normal cells. However, the success of TRAIL-based cancer therapy so far is limited because cancer cells, especially metastatic cancer cells, often exhibit a TRAIL-resistance phenotype. Therefore, identifying novel TRAIL sensitizers with low toxicity and high sensitization activity is also in urgent need.

For the synthesis of ceramide analogues, L-serine has been widely used as a starting material [10,11]. L-threonine, one of essential amino acids, has one more methyl and one more chiral carbon as compared to L-serine. Based on the medicinal

chemistry phenomenon of "magic methyl" effect that links the dramatic change in affinity to the addition of a single methyl group in just the right place, we hypothesized that L-threonine is a better substrate for synthesis of ceramide analogues with higher solubility and greater bioactivity. To test this hypothesis, we made use of L-threonine as a starting material for the synthesis of novel ceramide analogues. Functional assays determined that these ceramide analogues exhibit potent cytotoxicity against multiple types of human cancer cells with IC_{50} as low as 4.8 μM *in vitro*. Furthermore, sublethal doses of these ceramide analogues effective overcame human cancer cell chemoresistance. Biochemical analysis validated that these new ceramide analogues effectively decrease anti-apoptosis IAP and Bcl-xL proteins in human cancer cells. Most importantly, several of these ceramide analogues exhibited effective tumor suppressive activity at doses that cause no significant toxicity *in vivo*. Therefore, these ceramide analogues represent new candidates that have the potential to be developed as effective and yet low toxic anticancer agents.

2. Materials and Methods

2.1 Chemistry: All chemicals were purchased from Aladdin Chemical Co. All reactions were carried out under anhydrous conditions. Chromatography was performed on HPTLC silica gel 60 F254. HNMR spectra were recorded on a Bruker AVANCE 300MHz spectrometer, and CNMR spectra were recorded on Bruker AVANCE 400MHz spectrometer. Mass spectral data were recorded on LCMS-2020 (SHIMADZU) mass spectrometer.

2.1.1 Synthetic procedure for 4a-d: To a solution of *N*-Boc-L-threonine **2** (2.19g, 10mmol) in anhydrous THF (60mL) 1-hydroxybenzotriazole hydrate (HOBt) (1.35g, 10mmol) was added at 0 ºC. The pH value of the solution was adjusted to 8-9 with 4-methylmorpholine. After the reaction mixture was stirred for 5 min, 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) (2.20g, 11mmol) and a solution of alkyl amine in anhydrous THF (5mL) was added. The reaction mixture was stirred at 0 ºC for 2 h and at 20ºC overnight. After evaporation of the mixture *in vacuo*, the residue was dissolved in ethyl acetate (60mL), and the solution was washed successively with saturated $NaHCO_3$, 5% $KHSO_4$, and saturated NaCl. The organic phase was separated and dried over anhydrous $MgSO_4$. After filtration and evaporation *in vacuo*, desired (2*S*,3*R*)-*tert*-butyl-3-hydroxy-1-oxo-1-(alkyl)butan-2-ylcarbamate.**3a-d** was obtained. To a solution of (2*S*,3*R*)-*tert*-butyl-3-hydroxy-1-oxo-1-(alkyl)butan-2-ylcarbamate **3** in CH_2Cl_2 (70mL) trifluoroacetic acid (10.0mL) was added at 0 ºC. The reaction mixture was stirred at 20ºC for 4 h. After evaporation *in vacuo*, the residue was dripped into the 1N NaOH (100mL), and generate precipitation. Filtration and dried *in vacuo* led to desired L-threoninamide **4a-d**.

2.1.2 Synthetic procedure for 5xa-xj: To a solution of carboxylic acid (1.0mmol) in anhydrous THF (20mL) were added (2*S*,3*R*)-2-amino-3-hydroxy-*N*-alkylbutanamide **4a-d** (1.0mmol) and HOBt (1.0mmol, 0.135g) at 0ºC. After the reaction mixture was stirred for 5 min, EDC·HCl (0.220g, 1.1mmol) was added. The

pH value of the solution was adjusted to 8-9 with 4-methylmorpholine. The reaction mixture was stirred at 0 ºC for 2 h and overnight at room temperature. After evaporation of the mixture *in vacuo*, the residue was dissolved in ethyl acetate (50mL). The solution was washed successively with saturated $NaHCO_3$, 5% $KHSO_4$, and saturated NaCl. The organic phase was separated and dried over anhydrous $MgSO_4$. After filtration and evaporation *in vacuo*, residue was purified by recrystallization in petroleum ester/ethyl acetate to give the desired ceramide analogues **5xa-xj**.

2.1.3 Synthetic procedure for 5xk-xm: At 0ºC, to a solution of 1.0mmol of (2S,3R)-2-amino-3-hydroxy-N-alkylbutanamide (4a-4d) and 2.0mmol of pyridine in anhydrous CH_2Cl_2 (20mL), 10mL CH_2Cl_2 solution of heterocyclic sulfonyl chloride or aryl sulfonyl chloride was dripped added. The mixture was stirred at 0 ºC for 2 h and overnight at room temperature. After evaporation *in vacuo*,the residue was dissolved in ethyl acetate(50ml). The solution was washed successively with saturated $NaHCO_3$, 5% $KHSO_4$, and saturated NaCl， and the organic phase was separated and dried over anhydrous $MgSO_4$ for 2 h. After filtration and evaporation *in vacuo*, residue was purified by recrystallization in petroleum ester/ethyl acetate to give the desired ceramide analogues **5xk-xm** .

2.1.4 Synthetic procedure for control compound 6： For comparing carbon skeleton of L-threoninamide with L-serinamide, control compound **6** was prepared by

the same protocol with ceramide analogues **5.**

2.2 Cell viability assay: The effect of ceramide analogues on cell viability was measured by MTT assay according to the manufacturer's instructions (ATCC, Manassas VA). Briefly, six human cancer cells (Liver Cancer HepG2，Squamous Carcinoma A431，Pancreatic Cancer SA，Breast Cancer MCF-7，Ovarian Cancer SiHa and Colon Cancer SW620) were seeded in 96-well plates ($7x10^3$ cells/well) for 24 h. 47 Ceramide analogues were then added to final concentrations of 0 to 200 μM, and the cells were cultured for another 48h, followed by MTT assay.

2.3 Apoptosis assay: Human Colon Cancer cells SW620 were treated with the indicated ceramide analogues at doses of their IC_{50} for 8 h or 24h. Cells were then fixed in ice-cold 70% ethanol for 15 min, washed with PBS and stained with Hoechst 33342 (Sigma) for 20 min in the dark. Morphologic changes were analyzed under a fluorescence microscope. DMSO used as vehicle control.

2.4 TRAIL-mediated apoptosis: Five human cancer cells(Liver Cancer HepG2，Colon Cancer SW620、LS411N，Breast Cancer MCF-7，Cervical Cancer Hela) were seeded in 96-well plates and cultured for 24 h. Ceramide analogues, TRAIL (100 ng/ml) or both ceramide analogue and TRAIL were added to the culture for 48 h. Cell death was measured by MTT assay.

2.5 Western blotting analysis: Western blotting analysis was performed essentially as previously described [32]. Anti-xIAP antibody was obtained from Cell signaling, anti-Bcl-xL was obtained from BD Biosciences, and anti-β-actin was from Sigma.

2.6 Human cancer xenograft growth inhibition: The metastatic human colon carcinoma SW620 cells were injected to athymic mice subcultaneously. Tumor-bearing mice were randomly grouped into 7 groups with 6 mice in each group. The control group was injected with 200 μL 1% polyoxythylenated castor oil, and the rest four groups of mice were treated with ceramide analogues **4c**, **5ch**, **5ck**, and **5cm**, respectively, at a dose of 100 mg/kg body weight on alternate days for 15. Tumor sizes were measured with a micrometer caliper. Serum samples were collected at days 15 and analyzed for creatine kinase level using the creatine kinase ELISA kit (Shangshai Biological Technology Co. LTD).

Statistical analysis. Where indicated, data were represented as the means ± SD. Statistical analysis was performed using two-sided *t* test, with *p*-values<0.05 considered statistically significant.

3. Results

3.1 Chemical Synthesis of a group of new ceramide analogues

The general synthetic procedure is outlined in Figure 1. Initially, L-threonine **1** was reacted with $(Boc)_2O$ in the mixed solvent of H_2O-dioxane, affording

N-Boc protected threonine **2** in 94% yield. Because long-chain ceramide analogues often exhibit greater antitumor activity due to optimum lipid solubility [10, 21], we employed long-chain amines to react with Boc-L-threonine **2** in the presence of EDCl/HOBt, giving corresponding Boc-L-threoninamide **3** in 82-88% yields [22]. After the deprotection of Boc group with trifluoroacetic acid in dichloromethane [23], four types of L-threoninamides **4a-d** (Fig. 1B**)** were obtained in 75-85% yields. Under the catalysis of EDCl/HOBt, L-threoninamides **4a-d** was carboxylated in THF to give desired ceramide analogues **5xa-xj** in 64-85% yields. We synthesized 37 ceramide analogues **5xa-xj** with each bearing two amide groups (Fig. 1B). X representatives 4 types of long-chains containing 8, 12, 14, or 18 carbons in its nitrogen. L-Threoninamides **4a-d,** on the other hand, reacted with aryl or heteroaryl sulfonyl chloride in the presence of pyridine to give ceramide analogues **5xk-xm** with each bearing one amide group and one sufonamide group. (Fig.1B). Therefore, a total of 45 ceramide analogues **5** were synthesized (Table S1).

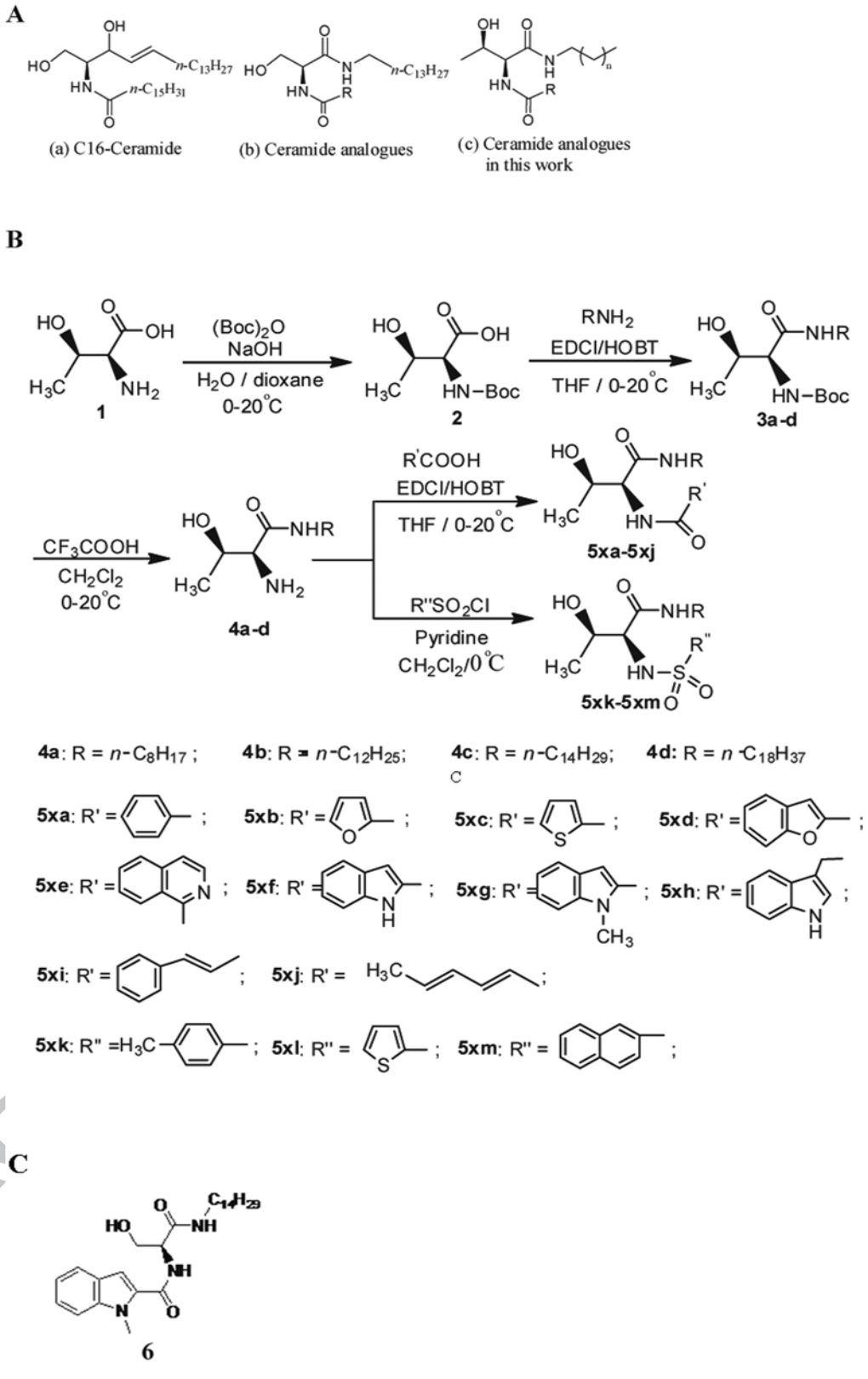


Figure 1

Figure1. Ceramide analogue synthesis outline. A. Natural C16-ceramide (a), ceramide analogue (b) and ceramide analogues synthesized in this study (c). B. Summary of ceramide synthesis. C. The structure of control compound **6**. The different functional groups are indicated in the bottom.

3.2 Ceramide analogues exhibits potent cytotoxicity against human cancer cells *in vitro*

To functional determine the bioactivities of these new ceramide analogues in suppression of human cancer cell growth, we performed cancer cell growth inhibition assays using six types of human cancer cells. It is clear that most of the newly synthesized analogues exhibit potent anti-cancer activity against all six types of human cancer cells. Interestingly, it seems that these analogues are relatively more cytotoxic to breast cancer cell MCF-7 cells (Table 1). A structure-activity-relation was observed between length of the alkyl chain and cytotoxicity of these analogues. Because of various kinds of acyl groups coupled with four different length carbon chain cores, these analogues didn't exhibit alkyl chain length dependent on cytotoxicity. For example, **5bd** has most cytotoxicity in benzofuran-2-carboxamides, but **5dd**, **5dc**, **5dh** are less cytotoxic than analogues with shorter alkyl chains (Table 1). However in general, '**b'** serial (N-dodecyl) and '**c'** serial (N-tetradecyl) have higher cytotoxicity against all tested cancer cell lines than 'a' serial (N-octyl) and 'd' serial (N-octadecyl) . For instance, 5bd, 5cd have higher cytotoxicity than 5ad, 5dd. These associations thus indicate that only L-threoninamide coupled with middle long carbon chain in the amide group rather than original amino group have greater anti-cancer cytotoxicity (Table 1, Table S1).

A structure-activity-relation was also found that heteroaryl carbonyl groups substituted L-threoninamides were more potent than aromatic carbonyl groups or

aliphatic carbonyl groups substituted L-threoninamides. For example, **5cd, 5ce, 5cf, 5cg, 5ch** are superior to **5ca, 5ci, 5cj** in all six types of (HepG2, A431, MES-SA, Siha, MCF-7, SW620) human cancer cell lines. Above all, **5ch** containing indole ring emerged as one of the most potent analogue tested in this study with lowest IC_{50} of 6.81μM in MCF-7 and 4.80μM in SiHa, respectively (Table1). These results indicate that heteroaryl carbonyl group could significantly increase anti-cancer cytotoxicity of ceramide analogues. In addition, **5cg** has one more methyl than L-serinamide derivative **6**, and its cytotoxicity is higher than L-serinamide derivative **6** for six types of human cancer cells, suggesting that carbon skeleton of L-threoninamide is superior to L-serinamide after coupling with the same heteroaryl carbonyl group (Table1).

Table 1.Cytotoxicity of 47 novel ceramide analogues against human cancer cells in vitro

Cancer cells	Live Cancer	Squamous Cancer	Pancreatic Cancer	Breast Cancer	Ovarian Cancer	Colon Cancer
	HepG(μM)	A431(μM)	SA(μM)	MCF-7(μM)	SiHa(μM)	SW620(μM)
4c	N/A	14.58±0.76	10.98±1.39	10.69±0.98	10.98±1.39	18.74±3.81
4d	44.52±3.49	21.32±1.72	>200	27.42±1.48	39.54±0.88	21.37±1.24
5aa	136.48±5.92	61.33±4.09	34.62±4.17	111.47±4.66	112.45±2.34	200
5ba	>400	41.18±0.69	75.45±6.24	36.57±8.86	63.92±6.01	200
5ca	54.21±3.95	118.49±9.09	56.04±7.43	24.77±6.75	>200	200
5da	>400	113.05±7.87	>400	31.75±6.55	38.22±2.52	200
5ab	192.16±11.09	64.5±0.78	64.65±0.5	91.84±4.21	124.01±8.78	200
5bb	146.86±22.28	92.44±2.64	93.49±3.13	67.49±4.88	64.32±1.68	87.5±7.58
5cb	>400	190.28±6.86	148±11.63	104.63±5.03	127.1±7.41	200
5db	42.29±3.73	19.93±0.45	52.55±6.02	21.38±2.4	13.35±1.05	>200
5ac	149.26±8.7	32.15±4.6	60.93±4.29	72.46±3.37	91.85±0.59	136.32±42.57
5bc	30.1±3.84	16.61±2.12	23.28±3.33	30.43±2.8	23.85±3.65	29.19±0.57
5cc	88.4±15.53	26.45±4.03	21.22±3.78	36.07±0.27	63±0.25	78.44±11.128
5dc	91.96±7.76	33.3±3.32	53.49±5.32	25.73±0.47	116.6±3.9	200
5ad	81.26±9.13	52.74±3.82	40.78±3.63	47.22±7.3	66.48±2.5	200
5bd	22.85±1.18	20.04±0.9	10.49±1.31	20.89±0.54	12.85±2.07	21.32±1.28
5cd	23.38±1.19	20.09±0.16	9.61±0.56	23.38±1.19	14.23±1.84	26.7±2.48
5dd	>200	78.79±6.99	161.96±4.55	126.36±7.19	>200	>200
5ae	88.19±1.92	26.87±2.81	22.91±2.59	63.21±4.73	65.99±5.48	67.75±0.099
5be	65.69±2.63	62.76±7.56	51.93±0.42	50.53±5.65	56.62±4.91	65.79±4.14
5ce	28.56±3.77	23.04±3.72	24.41±3.60	22.79±3.94	21.6±2.26	30.4±2.09
5de	96.94±6.77	44.13±2.02	49.14±4.72	30.78±0.67	51.17±3.46	136.26±13.29
5bf	11.97±2.38	10.31±2.78	12.28±2.75	12.28±2.75	21.25±0.01	18.75±1.2
5cf	10.51±0.35	12.25±1.90	12.11±2.77	15.11±2.97	14.20±1.80	15.20±1.86
5df	89.42±0.27	80.97±11.42	35.65±0.27	35.65±0.27	29.56±1.48	23.56±1.18
5bg	>200	168.74±14.66	31.3±1.14	31.3±1.14	>200	>200
5cg	34.45±2.34	13.68±1.73	25.65±0.42	25.65±0.42	26.52±2.33	21.52±3.33
5dg	>200	60.98±1.82	36.37±0.19	36.37±0.19	112.98±12.02	123.98±13.82
5bh	22.61±2.89	23.41±3.48	19.97±0.74	15.5±2.3	13.6±1.02	22.02±2.86
5ch	21.11±1.92	15.47±2.95	11.52±0.97	6.81±1.73	4.8±0.29	21.965±0.71
5dh	57.57±2.69	31.78±1.93	54.91±3.90	37.33±1.82	65.85±6.45	103±7.48
5ai	55.55±0.64	36.04±3.36	25.59±4.41	35.06±0.2	41.41±5.96	61.35±0.27
5bi	>200	>200	154.82±1.16	>200	>200	200
5ci	150.18±4.37	64.86±4.87	80.87±1.9	64.57±4.54	35.82±0.68	200
5di	127.61±9.04	177.08±7.17	47.09±0.54	39.22±2.21	35.94±2.64	200
5aj	101.68±4.07	58.24±0.11	40.44±2.16	148.86±3.24	123.22±2.5	200
5bj	>400	171.45±12.08	189.28±12.18	37.1±4.11	>200	200
5cj	>400	>200	184.37±1.9	27.76±0.14	>200	200
5dj	284.22±8.30	187.13±2.41	>200	79.63±2.34	>200	200
5bk	25.78±0.52	25.19±0.99	27.23±0.99	31.12±1.99	31.59±4.56	25.59±3.56
5ck	10.92±0.54	11.99±1.87	10.89±1.25	12.89±1.5	19.23±2.14	15.23±3.16
5dk	75.76±9.46	34.03±1.03	47.88±5.2	60.12±3.2	29.78±2.23	32.48±1.23
5bl	25.88±1.21	21.99±0.86	20.1±1.65	19.1±1.65	30.94±1.28	27.94±1.08
5cl	21.57±2.05	21.97±0.56	11.56±2.2	18.66±1.2	20.37±2.47	18.36±1.4
5dl	>200	>200	>200	>200	>200	>200
5cm	11.38±1.11	19.57±2.16	10.21±0.3	12.21±1.2	11.10±1.39	9.36±0.69
5dm	19.83±3.12	23.66±1.46	21.23±0.81	27.76±1.81	19.90±3.31	24.63±2.33
Compond 6	45.23±0.53	28.12±1.08	35.12±0.45	45.12±2.46	38.34±3.56	40.36±2.5

Table 1. Cytotoxicity of 47 novel ceramide analogues against six human cancer cells *in vitro* .Cells ($1x10^4$ ml^{-1}) were incubated with various concentrations of 47 novel ceramide analogues for 48h. Cell viability was measured by MTT assay. IC_{50} represents concentration causing a 50% growth inhibition. Data are mean ± SD, $N = 4$

To determine whether the cytotoxicity observed above is due to apoptosis induction, we next selected four ceramide analogues which have higher cytotoxicity and tested their apoptosis-inducing activity in SW620 cells. Morphologic changes of apoptotic cells were analyzed by staining cells with Hoechst 33342 and observed under a fluorescence microscope. Shining cells were considered as apoptotic cells. It is clear that **5ch**， **5ck, 5cm, 5cg** especially **5ch** show greater pro-apoptotic activity with more shining cells as compared to control. Moreover, **5cg which** has one more methyl than L-serinamide derivative **6** shows greater pro-apoptotic activity with more shining cells as compared to control compound 6 (Fig.2). Therefore, these observations validate the above cytotoxicity assays that the pro-apoptotic action of L-threoninamide is greater than L-serinamide after coupling with same heteroaryl carbonyl group.

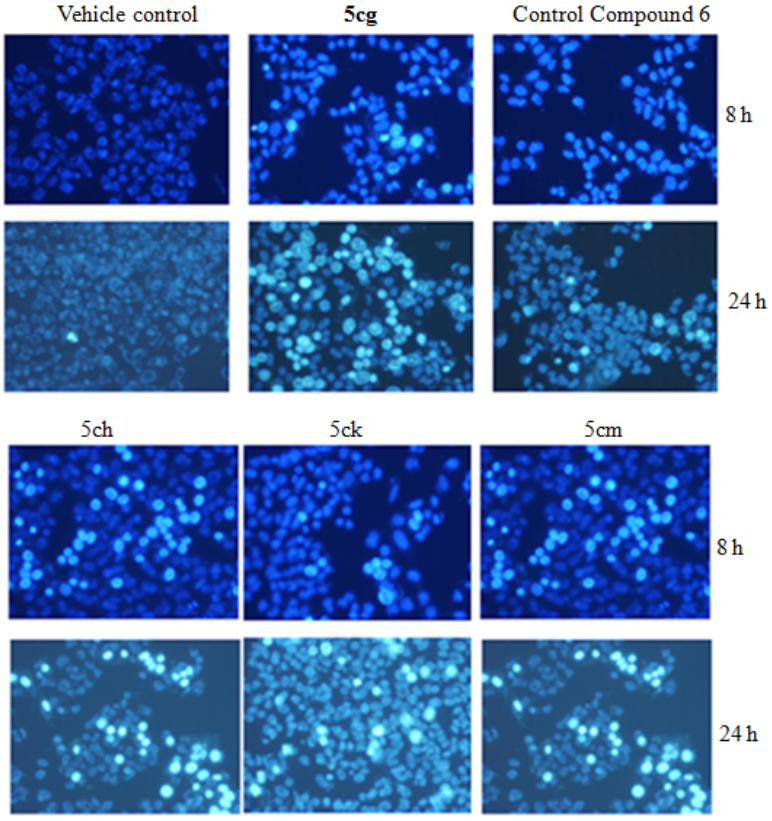


Figure 2. Ceramide analogues induce human cancer cell apoptosis. SW620 cells were treated with the indicated ceramide analogues at doses of their IC_{50}. Cells analyzed for apoptosis at 8 and 24 h. Cells were fixed and stained with Hoechst 33342. Shown are representative images of one of three experiments.

3.3 Ceramide analogues decrease Bcl-xL and xIAP levels in human cancer cells

Ceramide regulates cellular proliferation and apoptosis through various signaling pathways. However, compelling experimental data indicate that ceramide promotes apoptosis through modulating key apoptosis regulators in both the extrinsic and intrinsic apoptosis signaling pathways [24-29]. Specifically, ceramide targets the Bcl-xL and xIAP to directly induce apoptosis or to increase tumor cell sensitivity to apoptosis induction [24, 30, 31]. Therefore, we examined the effects of these new ceramide analogues on Bcl-xL and xIAP as biochemical markers of these ceramide analogues' bioactivity. Indeed, all four ceramide analogues tested which has lower IC_{50} decreased Bcl-xL and/or xIAP protein level in the metastatic human colon carcinoma cells (Fig. 3). However, although all four analogues decreased Bcl-xL protein level, only two of the four analogues (**4c** and **5ch)** decreased xIAP protein level (Fig. 3).

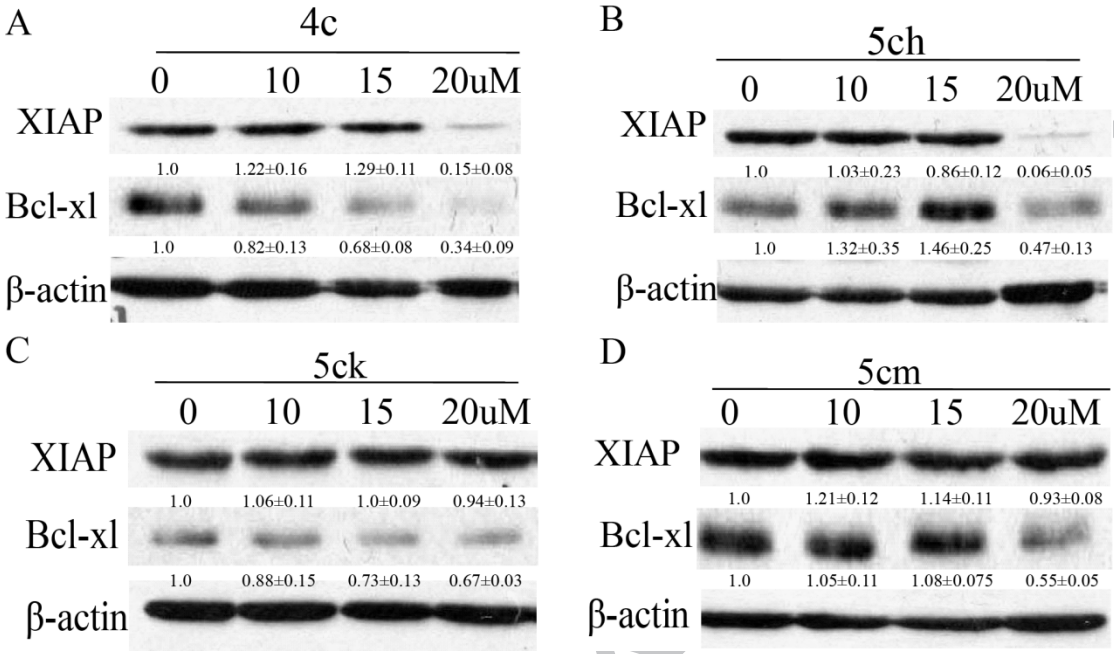


Figure 3. Ceramide analogues decrease Bcl-xL and xIAP in human colon carcinoma cells. The metastatic human colon carcinoma SW620 cells were treated with the indicated ceramide analogues at the indicated concentrations for 48 h, and analyzed for Bcl-xL and xIAP protein levels by Western blotting analysis.

3.4 Ceramide analogues effectively overcome human cancer cell resistance to TRAIL

TRAIL is a cancer-selective agent that has been extensively tested in clinical trials as an anti-cancer agent. However, the clinical outcome so far is poor, which is diminishing the hope to develop TRAIL or TRAIL receptor agonists as anti-cancer agents. This outcome, however, is not totally surprising since it is known that human cancer cells are often resistant to TRAIL-induced apoptosis [32, 33]. Because TRAIL-induced apoptosis depends on the intrinsic apoptosis pathway [34], and ceramide analogues decreases Bcl-xL and xIAP [24, 30, 31], we reasoned that these ceramide analogues should be effective in sensitization of human cancer cells to TRAIL-induced apoptosis, and screened some of these new ceramide analogues with IC_{50}

greater than 50μM. This is because we classify these new ceramide analogues into two groups: 1) highly cytotoxic compounds (HCC) and low cytotoxic compounds (LCC). For HCC the objective is to determine their mechanism of action as potential monotherapeutic agent for cancer therapy. For LCC, the objective is to develop their agents as adjuvant agents to enhance the efficacy of approved anti-cancer agents. Human cancer LS411N, SW620, MCF-7, HeLa and HepG2 cells were cultured in the presence of TRAIL, ceramide analogue at a dose of their IC_{50}, or both TRAIL and ceramide analogue. Cell growth inhibition was then measured. As expected, human cancer cells are resistant to TRAIL (Fig. 4). Interestingly, seven ceramide analogues tested significantly increased the sensitivity of these 5 types of human cancer cells to TRAIL-mediated growth inhibition (Fig. 4).

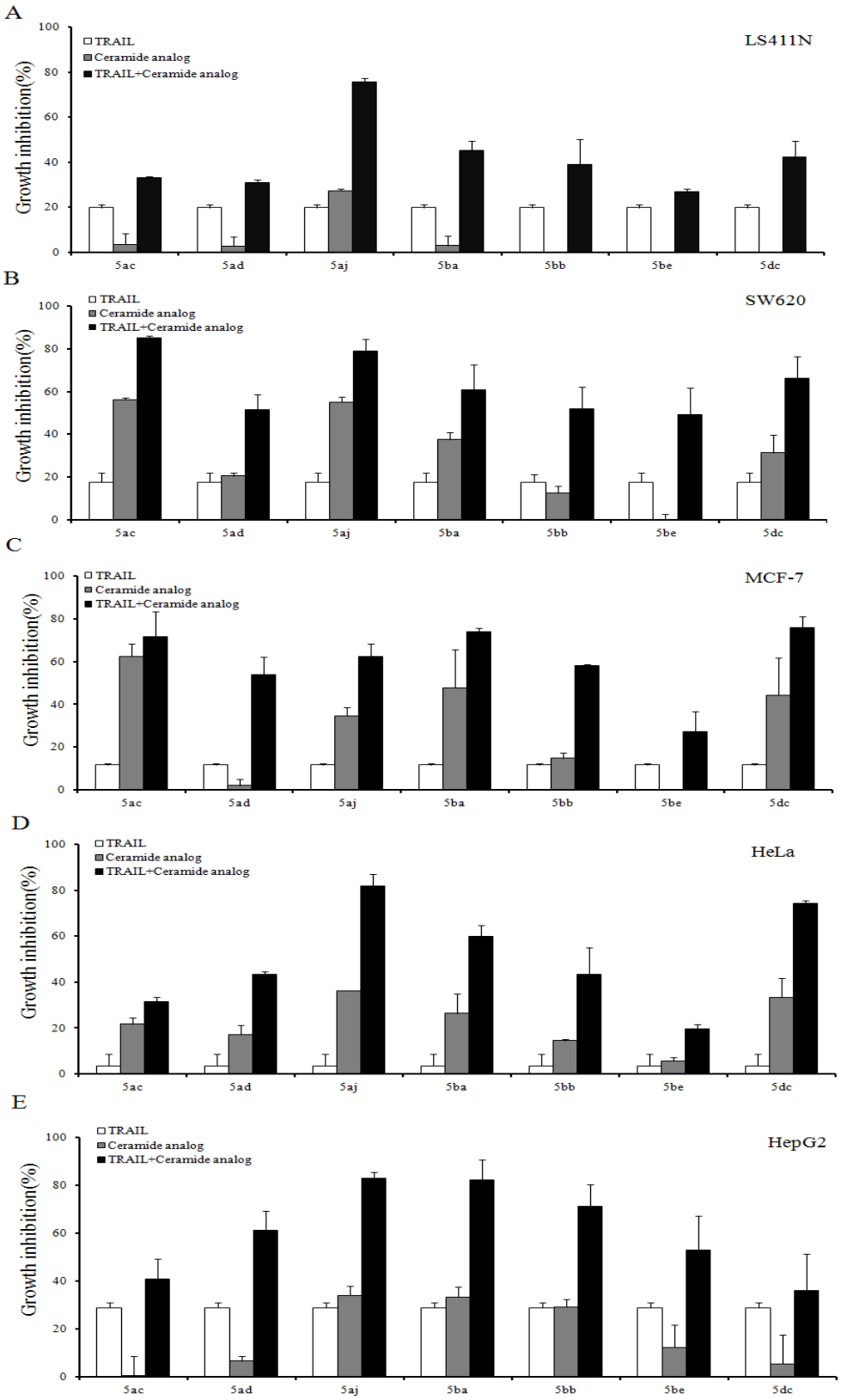


Figure 4. Ceramide analogues overcame human cancer cell resistance to TRAIL. The indicated human cancer cells were seeded in 96-well plate for 24 h. Cells were then treated with TRAIL (100 ng/ml), ceramide analogue at a dose of its IC_{50}, or both TRAIL and ceramide analogue for another 48 h. Cell viability was measured by MTT assay. Growth inhibition was

calculated by the formula: OD570 of untreated cells – OD570 of treated cells. Column: mean, bar:SD.

3.5 Inhibitory activity of ceramide analogues against human cancer xenograft *in vivo*

To determine whether the above observed in vitro anti-tumor cell cytotoxicity can be translated into tumor growth inhibition in vivo, we examined the efficacy of these newly synthesized ceramide analogues in suppression of human tumor growth in vivo. The metastatic human colon carcinoma SW620 cells were injected sc to athymic mice. Tumor-bearing mice were then treated with four of the ceramide analogues, respectively. Tumor growth was monitored over time. All 4 ceramide analogues exhibited tumor growth suppression activity. Among them, three analogues (**4c, 5ch** and **5cm**) exhibited potent suppressive efficacy against the established tumor xenograft, while the other one (**5ck**) exhibited mild tumor growth suppression activity (Fig. 5).

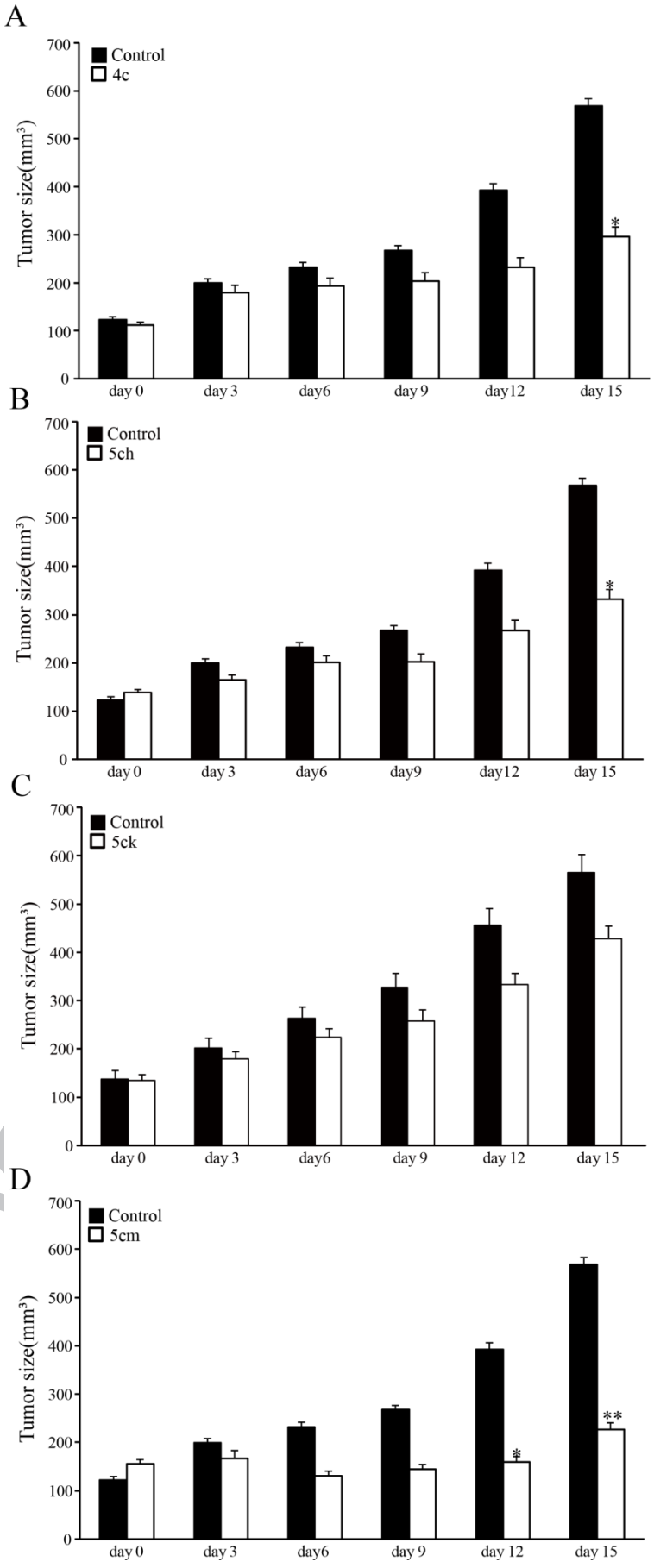


Figure 5. Ceramide analogues suppress human cancer growth in vivo. The metastatic human colon carcinoma SW620 cells ($3x10^6$ cells/mouse) were injected subcutaneously to athymic mice. Mice were randomized into experimental groups (n=6 for each group) when the tumors 60-80 mm^3. The tumor-bearing mice were treated with saline or the indicated ceramide analogues (100 mg/kg body weight) on alternate days for a total of 15 days. Tumor size was measured in three diameter with micrometer caliper at the indicate times to permit calculation of tumor volume.

3.6 Ceramide analogues exhibit no significant toxicity *in vivo*

We next sought to determine the toxicity of these ceramide analogues in mice. Three of the ceramide analogues that were tested for *in vivo* with potent anti-cancer activity were administered to mice, and serum level of creatine kinase, a commonly used biochemical marker of myocardial infarction, rhabdomyolysis, muscular dystrophy, autoimmune myositides and and acute renal failure, was measured as *in vivo* toxicity indicator. Serum was collected from tumor-bearing mice after the last treatment as in Figure 5, and measured for creatine kinase level. None of the ceramide analogues caused significant increase in serum creatine kinase level (Table 2).

Table 2. Mouse serum toxicity profiles of ceramide analogues

Treatment (100mg /kg body weight)	Serum Enzyme Level	
	CK（U/L）	Blood Collected Day
Control (n=6)	1.94±1.04	15
4c (n=6)	2.98±0.79	15
5ch (n=6)	2.44±0.61	15
5ck (n=6)	2.32±0.34	15
5cm (n=6)	2.63±1.02	15

Measurements were performed 15 days after ceramide analogues injection.
CK, creatine kinase

Table 2. Evaluation of the toxicity of four novel ceramide analogues *in vivo*. Serum samples of tumor-bearing nude mice were collected at days 15 after injection, and analyzed for creatine kinase level using the creatine kinase ELISA kit. Data are mean ± SD, *N* = 6

4. Discussion

For the synthesis of ceramide analogues, L-serine has been widely used as a starting material [10, 11]. A series of novel ceramide analogues have been synthesized by changing sphingosine backbone to a long-chain L-serinamide and introducing amide or imine substituents on the original nitrogen [14]. These analogues exhibit high efficacy as anti-proliferative agents against human cancer cell lines [15, 16]. L-threonine, one of essential amino acids, has one more methyl and one more chiral carbon as compared to L-serine. Based on the medicinal chemistry phenomenon of "magic methyl" effect that links the dramatic change in affinity to the addition of a single methyl group in just the right place, we hypothesized that L-threonine is a better substrate for synthesis of ceramide analogues with higher solubility and greater bioactivity and made use of L-threonine as a starting material for the synthesis of novel ceramide analogues. We successfully synthesized 47 novel ceramide analogues with great solubility and high yield. Therefore, we have established a simple and yet efficient synthesis procedure to chemically synthesize new ceramide analogues starting from L-threonine.

In vitro cytotoxicity analysis revealed that the majority of these 47 new ceramide analogues are bioactive as they can effectively suppress human cancer cell growth. Several analogues (i.e. **5ch** and **5cm**) exhibited super ant-proliferative activity with IC_{50} of 4.8-11 μM. Initial testing of four of these new ceramide analogues indicate that they are also effective in suppression of established human cancer xenograft growth *in vivo*. However, it is interesting to notice that the analogue with the most

potent *in vitro* cytotoxicity (i.e.**5ch**) does not exhibit the greatest suppressive efficacy against human xenograft growth *in vivo*, suggesting that in addition to its cytotoxicity potential, other factors, such as pharmacokinetics and pharmacodynamics of the analogues may also be important determinants of *in vivo* anti-cancer activity. More studies are apparently needed to expand the *in vivo* studies to more analogues to identify the most effective and low toxic ceramide analogues among these 47 compounds for cancer therapy.

TRAIL is a cancer-selective agent that has been extensively tested in human cancer patients for the last decade. However, so far, the test results in human cancer patients are not encouraging and the hope for developing TRAIL or TRAIL receptor agonist for human cancer therapy is fading. This outcome is not totally surprising since it is known that most human cancer cells are resistant to TRAIL [33, 34]. We screened some of these new ceramide analogues with IC_{50} greater than 50μM in five types of human cancer cells, and observed that seven analogues are effective in overcoming human cancer cell resistant to TRAIL. Our data thus indicate that these new ceramide analogues have the potential to be developed as an adjunct agent for TRAIL-based cancer therapy to improve the efficacy of TRAIL therapy.

Numerous studies have demonstrated that ceramide targets the Bcl-2 family proteins and IAPs to induce cellular apoptosis [24-29]. We observed that indeed the new ceramide analogues can significantly decrease the anti-apoptotic Bcl-xL and xIAP protein level in human cancer cells [31]. However, the although degree of decrease in xIAP and Bcl-xL protein level induced by a particular analogue is generally

associated with the *in vitro* cytotoxicity, the decrease of Bcl-xL and xIAP is only partially associated with the in vivo tumor suppression efficacy of a particular ceramide analogue. These observations suggest that these ceramide analogues suppress tumor growth only partially through inhibiting these anti-apoptotic mediators. Therefore, further studies are needed to further elucidate the molecular mechanisms underlying ceramide-mediated apoptosis and tumor suppression.

In summary, we have developed a simple and efficient new procedure to chemically synthesize ceramide analogues using L-threonine as substrate. A total of 47 novel ceramide analogues have been synthesized. Initial analysis indicates that many of these ceramide analogues are bioactive as anti-cancer agents with IC_{50} as low as 4.8 μM. Furthermore, these ceramide analogues also exhibited potent sensitization activity to overcome human cancer cell resistant to TRAIL. More significantly, several of these new ceramide analogues suppressed established human cancer xenograft growth in vivo without significant toxicity. These ceramide analogues thus hold great potential to be developed as a class of new anti-cancer agents.

Acknowledgments

This study is supported by National Natural Science Foundation of China (No. 31570811) Natural Science Foundation of Zhejiang Province (LY15C020001) and Si-Yuan Foundation.

References

[1] R.A. Wolff, R.T. Dobrowsky, A. Bielawska, L.M. Obeid, Y.A. Hannun, *J Biol Chem*, **1994,** 269, 19605-19609.

[2] B. Ogretmen, Y.A. Hannun, *Nat Rev Cancer*, **2004**, 4, 604-616.

[3]Samy A. F. Morad and Myles C. Cabot, *Nature Review/Cancer*, **2013,** 13, 51-65

[4]Sahar A. Saddoughi,, Besim Ogretmen, *Advances in Cancer Research**, 2013**, 117, 37-58*

[5] A. Apraiz, J.K. Idkowiak-Baldys, M.D. Boyano, G. Perez-Yarza, Y.A. Hannun, A. Asumendi, *BMC Cancer,* **2011**, 11, 477.

[6] J.C. Cheng, A. Bai, T.H. Beckham, S.T. Marrison, C.L. Yount, K. Young, P. Lu, A.M. Bartlett, B.X. Wu, B.J. Keane, K.E. Armeson, D.T. Marshall, T.E. Keane, M.T. Smith, E.E. Jones, R.R. Drake, Jr., A. Bielawska, J.S. Norris, X. Liu, *Clin Invest*, **2013**, 123, 4344-4358.

[7] A. Bielawska, J. Bielawski, Z.M. Szulc, N. Mayroo, X. Liu, A. Bai, S. Elojeimy, B. Rembiesa, J. Pierce, J.S. Norris, Y.A. Hannun, *Bioorg Med Chem*, **2008**, 16, 1032-1049.

[8] B. Oskouian, J.D. Saba, *Adv Exp Med Biol*, **2010**, 688, 185-205.

[9] A. Singh, H.J. Ha, J. Park, J.H. Kim, W.K. Lee, *Bioorg Med Chem*, **2011**, 19, 6174-6181.

[10] H. Niiro, H. Azuma, S. Tanago, K. Matsumura, K. Shikata, T. Tachibana, K. Ogino, *Bioorg Med Chem,* **2004**, 12, 49-51.

[11] V. Gududuru, E. Hurh, G.G. Durgam, S.S. Hong, V.M. Sardar, H. Xu, J.T. Dalton, D.D. Miller, *Bioorg Med Chem Lett,* **2004**, 14, 4919-4923.

[12] E. Bieberich, T. Kawaguchi, R.K. Yu, *J Biol Chem*, **2000**, 275, 177-181.

[13] K. Kim, J. Kang, S. Kim, S. Choi, S. Lim, C. Im, C. Yim, *Arch Pharm Res,* **2007**, 30, 570-580.

[14] J.W. Antoon, J. Liu, M.M. Gestaut, M.E. Burow, B.S. Beckman, M. Foroozesh, Design, *J Med Chem*, 2009, 52, 5748-5752.

[15] J. Liu, J.W. Antoon, A. Ponnapakkam, B.S. Beckman, M. Foroozesh, *Bioorg Med Chem*, **2010,** 18, 5316-5322.

[16] J.W. Antoon, B.S. Beckman, *Bioorg Med Chem Lett*, **2012**, 22, 2624-2628.

[17] M. Selzner, A. Bielawska, M.A. Morse, H.A. Rudiger, D. Sindram, Y.A. Hannun, P.A. Clavien, *Cancer Res,* **2001**, 61, 1233-1240.

[18]K. Perez-Labrada, I. Brouard, I. Mendez and D. G. Rivera, *J. Org. Chem.* **2012**, 77, 4660−4670.

[19]S. Barui, S. Saha, V. Yakati and A.Chaudhuri, *Mol. Pharmaceutics* **2016**, 13, 404−419

[20]S. Vudhgiri,S. R. Routhu,C. G. Kumar,R. B. N. Prasad, R. C.R. Jala, *Med Chem Res, 2017, DOI 10.1007/s00044-017-2049-9*

[21] Y.T. Chang, J. Choi, S. Ding, E.E. Prieschl, T. Baumruker, J.M. Lee, S.K. Chung, P.G. Schultz, *J Am Chem Soc,* **2002**, 124, 1856-1857.

[22] R.S. Joshi, P.G. Mandhane, P.V. Badadhe, C.H. Gill, *Ultrason Sonochem*, **2011**, 18, 735-738.

[23] Y. Kavanagh, C.M. Chaney, J. Muldoon, P. Evans, *J Org Chem*, **2008**, 73, 8601-8604.

[24] C.E. Chalfant, K. Rathman, R.L. Pinkerman, R.E. Wood, L.M. Obeid, B. Ogretmen, Y.A. Hannun, *J Biol Chem,* **2002**, 277, 12587-12595.

[25] L.J. Siskind, T.D. Mullen, K. Romero Rosales, C.J. Clarke, M.J. Hernandez-Corbacho, A.L. Edinger, L.M. Obeid, *J Biol Chem,* **2010**, 285, 11818-11826.

Table 1.Cytotoxicity of 47 novel ceramide analogues against human cancer cells *in vitro*

Cancer cells	Live Cancer	Squamous Cancer	Pancreatic Cancer	Breast Cancer	Ovarian Cancer	Colon Cancer
	HepG2(μM)	A431(μM)	SA(μM)	MCF-7(μM)	SiHa(μM)	SW620(μM)
4c	N/A	14.58±0.76	10.98±1.39	10.69±0.98	10.98±1.39	18.74±3.81
4d	44.52±3.49	21.32±1.72	＞200	27.42±1.48	39.54±0.88	21.37±1.24
5aa	136.48±5.92	61.33±4.09	34.62±4.17	111.47±4.66	112.45±2.34	200
5ba	＞400	41.18±0.69	75.45±6.24	36.57±8.86	63.92±6.01	200
5ca	54.21±3.95	118.49±9.09	56.04±7.43	24.77±6.75	＞200	200
5da	＞400	113.05±7.87	＞400	31.75±6.55	38.22±2.52	200
5ab	192.16±11.09	64.5±0.78	64.65±0.5	91.84±4.21	124.01±8.78	200
5bb	146.86±22.28	92.44±2.64	93.49±3.13	67.49±4.88	64.32±1.68	87.5±7.58
5cb	＞400	190.28±6.86	148±11.63	104.63±5.03	127.1±7.41	200
5db	42.29±3.73	19.93±0.45	52.55±6.02	21.38±2.4	13.35±1.05	＞200
5ac	149.26±8.7	32.15±4.6	60.93±4.29	72.46±3.37	91.85±0.59	136.32±42.57
5bc	30.1±3.84	16.61±2.12	23.28±3.33	30.43±2.8	23.85±3.65	29.19±0.57
5cc	88.4±15.53	26.45±4.03	21.22±3.78	36.07±0.27	63±0.25	78.44±11.128
5dc	91.96±7.76	33.3±3.32	53.49±5.32	25.73±0.47	116.6±3.9	200
5ad	81.26±9.13	52.74±3.82	40.78±3.63	47.22±7.3	66.48±2.5	200
5bd	22.85±1.18	20.04±0.9	10.49±1.31	20.89±0.54	12.85±2.07	21.32±1.28
5cd	23.38±1.19	20.09±0.16	9.61±0.56	23.38±1.19	14.23±1.84	26.7±2.48
5dd	＞200	78.79±6.99	161.96±4.55	126.36±7.19	＞200	＞200
5ae	88.19±1.92	26.87±2.81	22.91±2.59	63.21±4.73	65.99±5.48	67.75±0.099

[26] H. Lee, J.A. Rotolo, J. Mesicek, T. Penate-Medina, A. Rimner, W.C. Liao, X. Yin, G. Ragupathi, D. Ehleiter, E. Gulbins, D. Zhai, J.C. Reed, A. Haimovitz-Friedman, Z. Fuks, R. Kolesnick, *PLoS One*, **2011**, 6 e19783.

[27] J.E. Chipuk, G.P. McStay, A. Bharti, T. Kuwana, C.J. Clarke, L.J. Siskind, L.M. Obeid, D.R. Green, *Cell,* **2012**, 148, 988-1000.

[28] L.J. Beverly, L.A. Howell, M. Hernandez-Corbacho, L. Casson, J.E. Chipuk, L.J. Siskind, *Biochem J*, **2013**, 492, 111-119.

[29] L. Casson, L. Howell, L.A. Mathews, M. Ferrer, N. Southall, R. Guha, J.M. Keller, C. Thomas, L.J. Siskind, L.J. Beverly, *PLoS One*, **2013**, 8, e54925.

[30] B.J. Kroesen, S. Jacobs, B.J. Pettus, H. Sietsma, J.W. Kok, Y.A. Hannun, L.F. de Leij, *J Biol Chem*, **2003**, 278, 14723-14731.

[31] A.V. Paschall, M.A. Zimmerman, C.M. Torres, D. Yang, M.R. Chen, X. Li, E. Bieberich, A. Bai, J. Bielawski, A. Bielawska, K. Liu, *BMC Cancer,* **2014**, 14, 24.

[32] A. Ashkenazi, *Nat Rev Drug Discov,* **2008**, 7, 1001-1012.

[33] C. Voelkel-Johnson, Y.A. Hannun, A. El-Zawahry, *Mol Cancer Ther,* **2005**, 4, 1320-1327.

[34] A. Ashkenazi, V.M. Dixit, *Science*, **1998**, 281, 1305-1308.

5be	65.69±2.63	62.76±7.56	51.93±0.42	50.53±5.65	56.62±4.91	65.79±4.14
5ce	28.56±3.77	23.04±3.72	24.41±3.60	22.79±3.94	21.6±2.26	30.4±2.09
5de	96.94±6.77	44.13±2.02	49.14±4.72	30.78±0.67	51.17±3.46	136.26±13.29
5bf	11.97±2.38	10.31±2.78	12.28±2.75	12.28±2.75	21.25±0.01	18.75±1.2
5cf	10.51±0.35	12.25±1.90	12.11±2.77	15.11±2.97	14.20±1.80	15.20±1.86
5df	89.42±0.27	80.97±11.42	35.65±0.27	35.65±0.27	29.56±1.48	23.56±1.18
5bg	＞200	168.74±14.66	31.3±1.14	31.3±1.14	＞200	＞200
5cg	34.45±2.34	13.68±1.73	25.65±0.42	25.65±0.42	26.52±2.33	21.52±3.33
5dg	＞200	60.98±1.82	36.37±0.19	36.37±0.19	112.98±12.02	123.98±13.82
5bh	22.61±2.89	23.41±3.48	19.97±0.74	15.5±2.3	13.6±1.02	22.02±2.86
5ch	21.11±1.92	15.47±2.95	11.52±0.97	6.81±1.73	4.8±0.29	21.965±0.71
5dh	57.57±2.69	31.78±1.93	54.91±3.90	37.33±1.82	65.85±6.45	103±7.48
5ai	55.55±0.64	36.04±3.36	25.59±4.41	35.06±0.2	41.41±5.96	61.35±0.27
5bi	＞200	＞200	154.82±1.16	＞200	＞200	200
5ci	150.18±4.37	64.86±4.87	80.87±1.9	64.57±4.54	35.82±0.68	200
5di	127.61±9.04	177.08±7.17	47.09±0.54	39.22±2.21	35.94±2.64	200
5aj	101.68±4.07	58.24±0.11	40.44±2.16	148.86±3.24	123.22±2.5	200
5bj	＞400	171.45±12.08	189.28±12.18	37.1±4.11	＞200	200
5cj	＞400	＞200	184.37±1.9	27.76±0.14	＞200	200
5dj	284.22±8.30	187.13±2.41	＞200	79.63±2.34	＞200	200
5bk	25.78±0.52	25.19±0.99	27.23±0.99	31.12±1.99	31.59±4.56	25.59±3.56
5ck	10.92±0.54	11.99±1.87	10.89±1.25	12.89±1.5	19.23±2.14	15.23±3.16
5dk	75.76±9.46	34.03±1.03	47.88±5.2	60.12±3.2	29.78±2.23	32.48±1.23
5bl	25.88±1.21	21.99±0.86	20.1±1.65	19.1±1.65	30.94±1.28	27.94±1.08
5cl	21.57±2.05	21.97±0.56	11.56±2.2	18.66±1.2	20.37±2.47	18.36±1.4
5dl	＞200	＞200	＞200	＞200	＞200	＞200
5cm	11.38±1.11	19.57±2.16	10.21±0.3	12.21±1.2	11.10±1.39	9.36±0.69
5dm	19.83±3.12	23.66±1.46	21.23±0.81	27.76±1.81	19.90±3.31	24.63±2.33
Compound 6	45.23±0.53	28.12±1.08	35.12±0.45	45.12±2.46	38.34±3.56	40.36±2.5

Table 2. Mouse serum toxicity profiles of ceramide analogues

Treatment (100mg/kg body weight)	Serum Enzyme Level	
	CK(U/L)	Blood Collected Day
Control (n=6)	1.94±1.04	15
4c (n=6)	2.98±0.79	15
5ch (n=6)	2.44±0.61	15
5ck (n=6)	2.32±0.34	15

5cm	(n=6)	2.63±1.02	15

Measurements were performed 15 days after ceramide analogues injection.

CK, creatine kinase

Graphical Abstract