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Graphic Abstract

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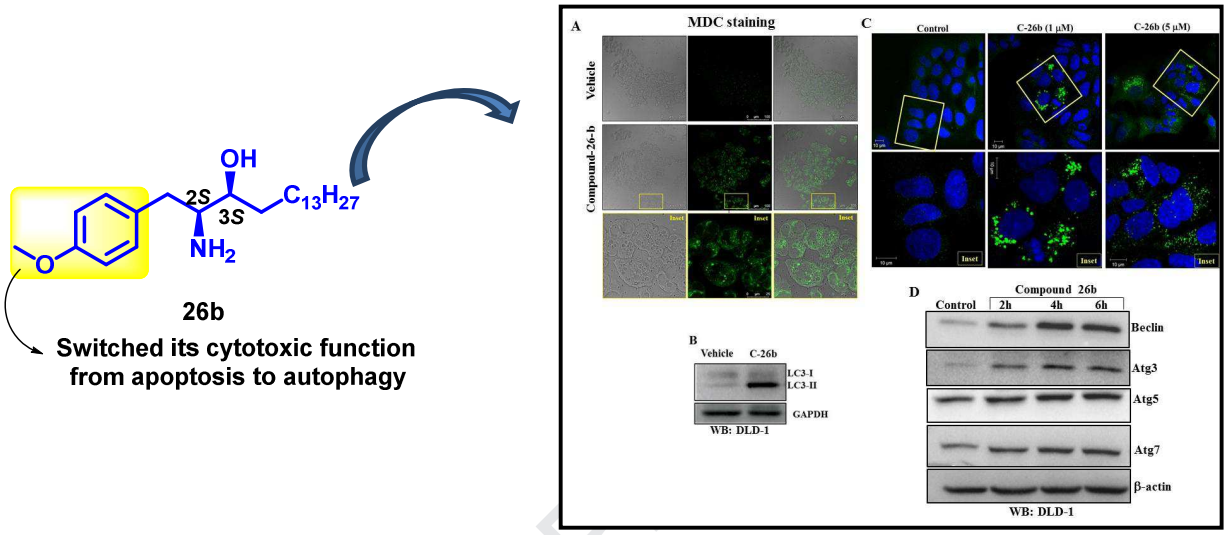


Compound 26b promotes Autophagic cell death

New Spisulosine Derivative Promotes Robust Autophagic Response to Cancer Cells

Asha Ganesher[⊥,a], Priyank Chaturvedi[⊥,b], Rohit Sahai[c], Sanjeev Meena[b], Kalyan Mitra[c], Dipak Datta*[,b], Gautam Panda*[,a]

[a] Medicinal and Process Chemistry Division, CSIR-Central Drug Research Institute, Sitapur Road, Jankipuram Extension, Lucknow 226031, UP, India
[b] Division of Cancer Biology, CSIR-Central Drug Research Institute, Sitapur Road, Jankipuram Extension, Lucknow 226031, UP, India
[c] Electron Microscopy Unit, Sophisticated Analytical Instrument Facility, CSIR-Central Drug Research Institute, Sector-10, Jankipuram Extension, Lucknow, 226 031, UP, India

[⊥] equal contribution

Corresponding author
*Gautam Panda, Phone: 915222772450, ext 4659, Fax: 915222771941;
E-mail: gautam.panda@gmail.com; gautam_panda@cdri.res.in
*Dipak Datta, Phone 915222772450, ext 4347, Fax: 915222771941;
E-mail: dipak.datta@cdri.res.in

KEYWORDS: Sphingoid, chemotherapy, autophagy, resistance, structure-activity relationship

ABBREVIATIONS
br s, broad signal; rt, room temperature; mp, melting point; THF, tetrahydrofuran; DCM, dichloromethane; MeOH, methanol; K_2CO_3, potassium carbonate; $NaHCO_3$, sodium bicarbonate; MOM-Cl, chloromethyl methyl ether; MsCl, methanesulfonyl chloride; Et_3N, triethylamine; Boc anhydride, di-tert-butyl dicarbonate; $LiAlH_4$, lithium alumininumtetrahydride; TBDPS-Cl, tert-Butyldiphenylchlorosilane

ABSTRACT
Therapy resistance by evasion of apoptosis could be one of the hallmarks of human cancer. Therefore, restoration of cell death by non-apoptotic mechanisms is critical to successfully overcome therapy resistance in cancer. By rational drug design approach, here we try to provide evidence that subtle changes in the chemical structure of spisulosine completely switched its cytotoxic function from apoptosis to autophagy. Our most potent molecule (**26b**) in a series of 16 synthesized derivatives showed robust autophagic cell death in diverse cancer cells sparing normal counterpart. Compound **26b** mediated lethal autophagy induction was confirmed by formation of characteristic autophagic vacuoles, LC3 puncta formation, upregulation of signature autophagy markers like Beclin and ATG family proteins. Altogether, we have detected novel autophagy inducer small molecule which can be tested further for drug discovery research.

1. INTRODUCTION

Cancer is one of the most serious life-threatening diseases in the world. During past several years, huge efforts have been made by researchers but the search of effective clinical approaches for the treatment of cancer is still a tough challenge. Chemotherapy using anticancer agents is another valuable option for the cancer treatment apart from surgery, immunotherapy, and radiotherapy.[1]In the last few years, it is now very common in the developed countries.[2] So, the development of novel anticancer agents is a highly active research field and has achieved considerable attention from chemists.[3] Presently, most of the drugs in clinical trials for cancer treatment are natural products or pharmacophores derived from natural product sowing to their fewer side effects, thus the impact of natural products upon anticancer drug discovery and design is very inspiring.[4,5] However, in the initial phase of these active natural or synthetic compounds, poor solubility is one of the major issues.[6] The poor prognosis of colon cancer and poor sensitivity to current therapeutics, associated with resistant to apoptosis, urge the search of new drugs which induce cancer cell death by some other mechanisms.[7]

Long-chain α-amino-alcohols i.e. sphingoid-type bases are considered as the principal backbone of the more complex sphingolipids and ceramides[8,9,10](**Fig1)**. Ceramides show its mechanism of action through cellular signaling and activating various protein kinase cascades whereas sphingolipids show diverse biological activities such as antitumor,[11,12,13] immunostimulatory, and immunosuppressive,[14]neuronal proliferation[15]and protein kinase activity variation. Sphingosine is also a sphingoid base, which is a derivative of sphinganine **2**.[16] Over the past decade, 1-deoxysphingoid bases have received increased attention from synthetic chemists due to their important biological activity.[17] Among them, spisulosine **3** has received the most attention.

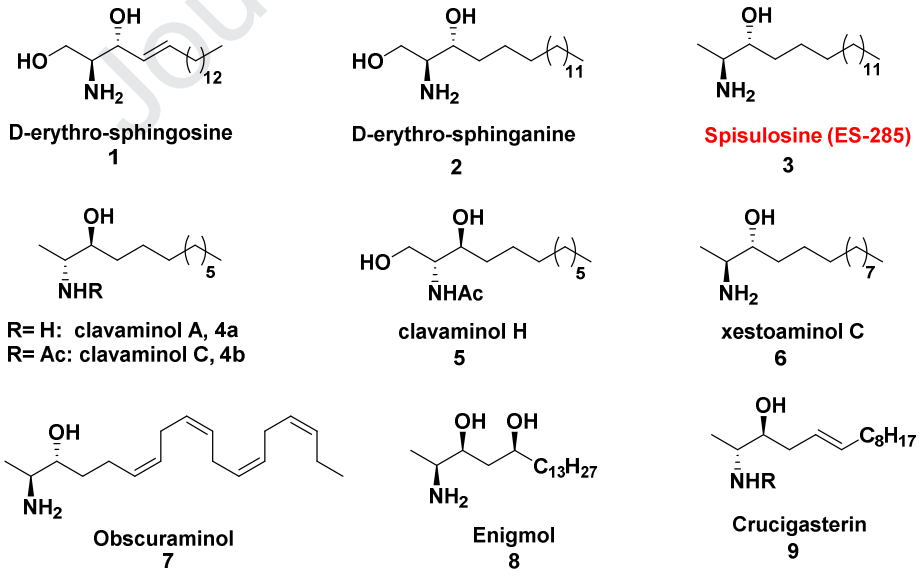


Fig. 1 Structures of several natural sphingoid bases

(*2S,3R*)-2-Amino-3-octadecanol (spisulosine or ES-285) is a marine derived bioactive compound isolated from the North Arctic clam *Spisula polynyma*[18] by Rinehart et al. This compound is known for its biological activity to inhibit cell proliferation with an IC_{50} of 1µM in the prostate tumor PC-3 and LNCaP cell lines[19]and is responsible for loss of actin stress fibres.[20]The mechanism of action of spisulosine is still hypothetical. Ceramide signaling and GTP binding protein (RHO protein) regulating actin stress fibers have been proposed as a potential molecular target.[21]Although,spisulosine was primarily developed as a novel anticancer agent but due to its poor clinical outcomes it was discontinued from phase I in 2008.[22,23]ES-285 showed a tolerable safety profile at doses up to 128 mg/m^2. In a number of clinical studies of various compounds of natural origin and especially with some marine agents, drug-related neurotoxicity has been a common problem.[24,25,26] In the phase I program of ES-285, the neurological disorders like dizziness, headache, sensory or motor neuropathy, neuropathic pain, aphasia, and decreased level of consciousness was detected. One patient died after the first administration of the ES-285 when treated at 200 mg/m^{2}for 24-h i.v. infusion following drug related central neurotoxicity. Other toxicities included phlebitis, nausea, fatigue, and fever. The pharmacokinetic studies revealed high volume of distribution and long elimination half-life, which led to discontinuation of this compound. Due to an unfavorable risk/profit balance and absence of clinically significant antitumor efficacy in the whole clinical program, recruitment of patients to spisulosine clinical trials was discontinued. The seriousness of the neurological toxicity experienced by one patient supported this decision.[27]

However, this compound is structurally similar to other related 1-deoxysphingoids with remarkable cytotoxic properties, such as obscuraminols,[28] clavaminols,[29]crucigasterins,[30] and xestoaminols,[31] (**Fig.1**). This close structural relationship makes this type of compound as a prominent lead for anticancer oriented drug discovery programs.In literature, various methods are reported for the total synthesis of this natural product,[32,33,34,35,36,37,38,39,40,41] in which chiral amino acid, carbohydrate, garner aldehyde and various achiral substrates like palmityl alcohol, palmitaldehyde, pentadec-1-yne etc have been used as an starting material (**Fig. 2**).

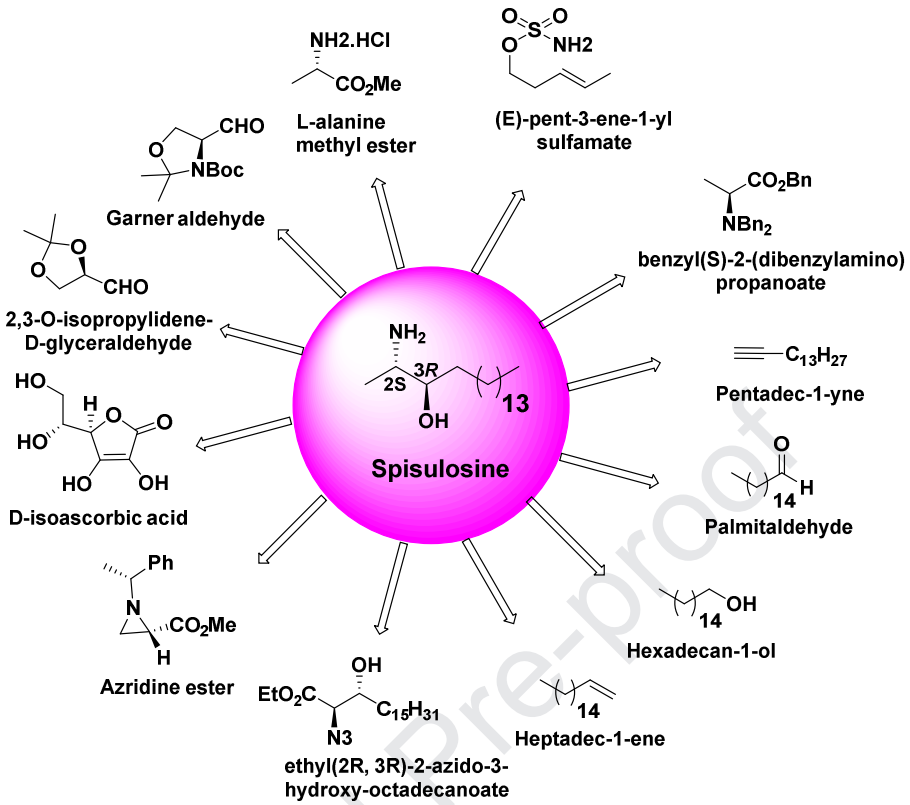


Fig. 2 Known approaches for total synthesis of spisulosine (ES-285)

However, only few reports of analogs of spisulosine are present in the literature. So far, only Delgado et al.,[42]Bittman et al.[43] and Dauban et al.[44] have synthesized spisulosine analogs **(10-19)** and evaluated for biological activity in order to evaluate the ceramides synthase activity in whole cells (**Fig.3**).[45]Bittman et al. found that analogue **10** shows IC_{50}= 27.8±3.2 µM against sphingosine kinase (SphK1 isoform) and analogue**11** was found to be a nonselective SphKs inhibitor.Delgado et al. have synthesized **3** and its analogues such as stereoisomers **12**, **13** and **14**, and dehydrospisulosines**15**, **16**, **17** and **18** in order to analyse the ceramides synthase activity in whole cells (**Fig.3**). Only three compounds (spisulosine **3,** 3-epimer **14** and 4,5-dehydrospisulosine **18)** were found to be most active for further screening. Recently, Dauban et al. have synthesized spisulosine **3** and its 3-fluoro derivative **19**and screened for biological evaluation in three malignant cells (KB, HCT-116, HL-60). As expected, only compound **3** exhibited cytotoxicity (IC_{50} in the 100 nM range).

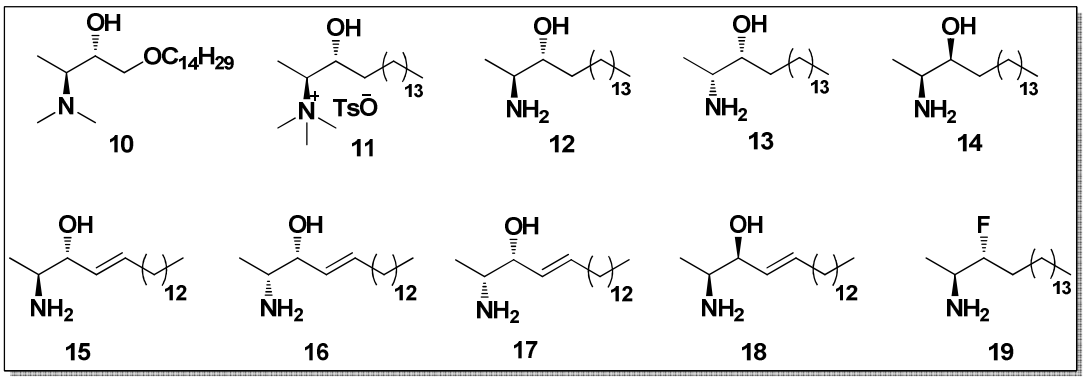


Fig. 3 Several analogs of spisulosine **3**

Although much synthetic and biological effort in the field of 1-deoxysphingoid bases and their related compounds have been done, but many challenges in this research still remain. All of these natural compounds are an interesting source of inspiration for both the organic community and biomedical research, and further intensive studies in this area could provide a new insight into the design and mechanism of actionof the sphingolipid related anticancer agents. Inspired from these analogs and their biological activity, we have synthesized a new derivative of spisulosine which induces cancer cell death by autophagy. Although the role of autophagy in cancer is very complex, various pharmacological agents with different antineoplastic properties have been shown to induce autophagic activity resulting in massive death of cells in some cancer types.For example in renal cell, carcinomas are refractory to standard therapies, but are sensitive for autophagy induced cell death.[46] Apoptosis resistance is one of the major causes of chemoresistance, and an important challenge in the treatment of cancer is the development of therapies that overcome chemoresistance. Thus activation of autophagy in apoptosis resistant cancer could potentially provide a way to induce cell death and impede malignant growth.

Rationale of the design

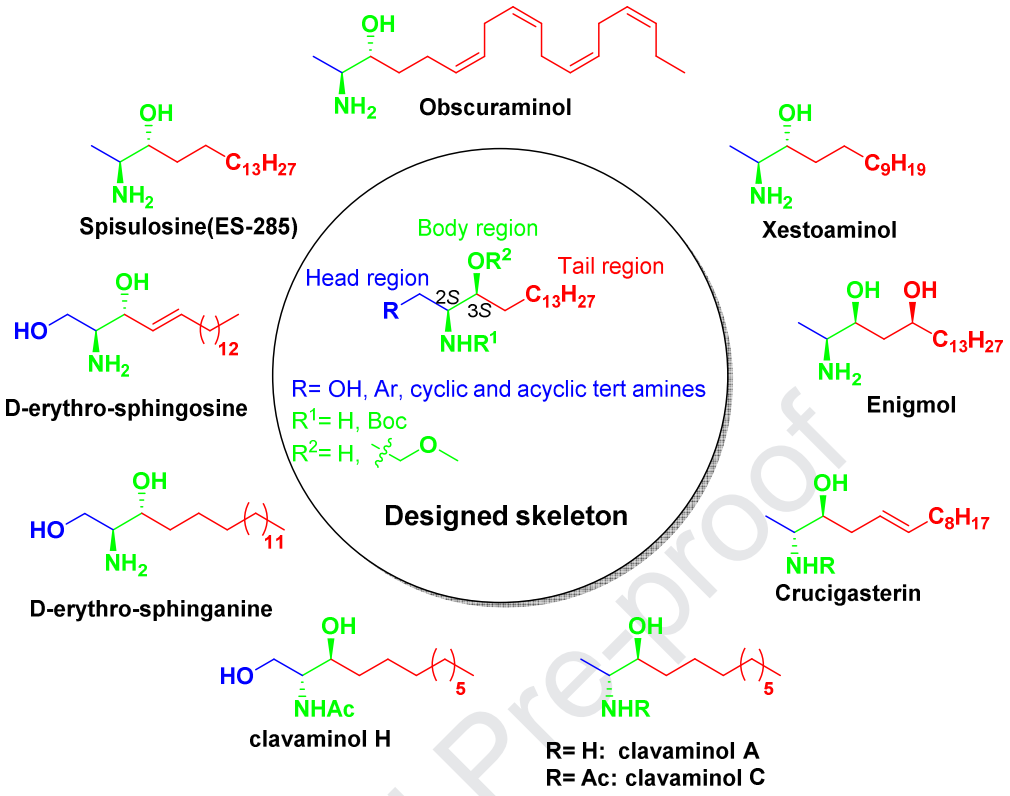


Fig. 4 Approach towards designing of spisulosine and sphinganine analogs.

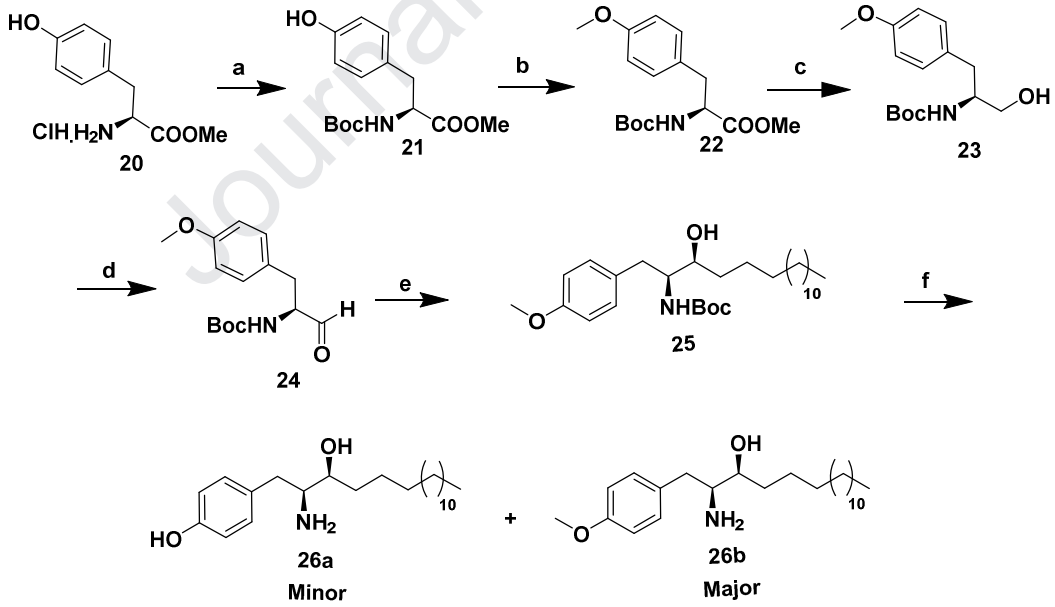
The simple 1,2-amino alcohols have been observed to show a rare type of bioactivity such as anti-proliferative and cytotoxic activities along with their ability to influence the sphingolipids biosynthesis and metabolism. Because of their wide applications in biological field, some of these sphingosine related natural products have been selected as lead molecule for the design of new anticancer agents.[47]

Structural analysis of all the sphingoid type bases revealed that 1,2 amino alcohol (the body region) is highly preserved. From literature survey, we hypothesized that the poor pharmacokinetic profile perhaps arises from the strong intra-molecular hydrogen bonding between two adjacent amino and hydroxyl functionalities present in spisulosine skeleton which may be responsible for its long elimination half-life. Thus, the free amino and hydroxyl group were protected to observe the effect of substitution on water solubility as well as activity (**Fig.4**). Further, the head region of spisulosine was modified with substituted phenyl groups, cyclic and acyclic tertiary amines in order to see the effect of hydrophobicity/hydrophilicity on their activity. Learned from the earlier investigations[34-37], the tail region was kept intact with 14 carbon atoms.

2. RESULTS AND DISCUSSION

Chemistry

The designed spisulosine derivatives **25** and **26b** were synthesized according to the procedure reported in **scheme 1**. Naturally abundant L-tyrosine amino acid was taken as starting material. Firstly, L-tyrosine methyl ester hydrochloride **20** was protected in presence of Boc anhydride to furnish Boc protected tyrosine methyl ester **21** in good yield (86%). In the next step, the free OH of compound **21** was methylated in presence of methyl iodide by using K_2CO_3 as a base in acetone to provide compound **22** in very good yield (88%), which was subjected to $LiAlH_4$mediated reduction of the ester group to afford alcohol **23** in excellent yield (93%). Dess-Martin oxidation in DCM-H_2O afforded aldehyde **24** within 1h whose immediate treatment with tetradecylmagnesium chloride Grignard reagentafforded the *syn*-diastereomer **25** in good yield (73% over two steps) according to the known chelation controlled addition of Grignard reagents to the Boc protected amino aldehydes to produce the corresponding β-amino alcohol with high *syn*-selectivity.[48] The formation of the syn adduct **25** was explained with the Cram model having a chelation-controlled transition state (**Fig.5**).[49] Finally, removal of the protecting group under acidic condition (4N HCl in dioxane) afforded diastereomerically pure (2*S*,3*S*)-2-amino-1-(4-methoxyphenyl)octan-3-ol **26b** $\{[\alpha]_D^{25}$ -10.0 (c 0.05, MeOH) in 65% yield but equal amount of demethylated product **26a** was also observed. To reduce the minor product **26a**, Boc deprotection with Dowex® 50Wx8 resin in MeOH was done and **26b** as the major product was formed and only 5% of minor **26a** was observed.



Scheme 1. Reagents: a) Boc anhydride, $NaHCO_3$, MeOH, 0 °C- RT, 4 h, 86 % b) CH_3I, K_2CO_3, acetone, 0 °C- RT, 4-5 h, 88 % c) $LiAlH_4$, dry THF, 0 °C- RT, 1 h, 93 % d) Dess-Martin Periodinane, DCM-H_2O, 0 °C- RT, 1 h e) $C_{14}H_{29}MgCl$, dry THF, 0 °C- RT, 4-5 h, 73 % over

two steps f) 4N HCl in dioxane, RT, 0.5 h, 65 % (50% conversion) or Dowex® 50Wx8 resin, MeOH, 50 °C, 5 h, 85 % (95 % conversion).

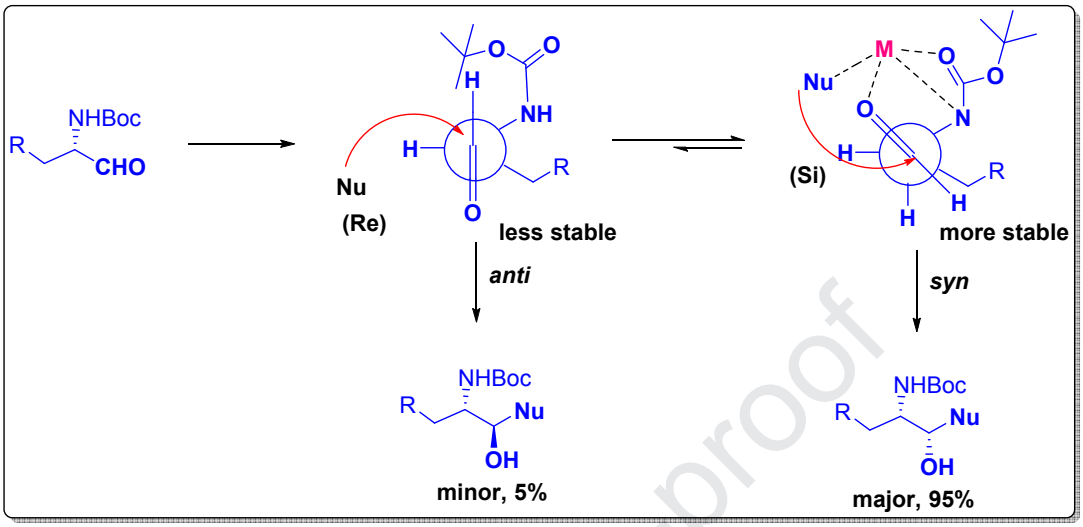
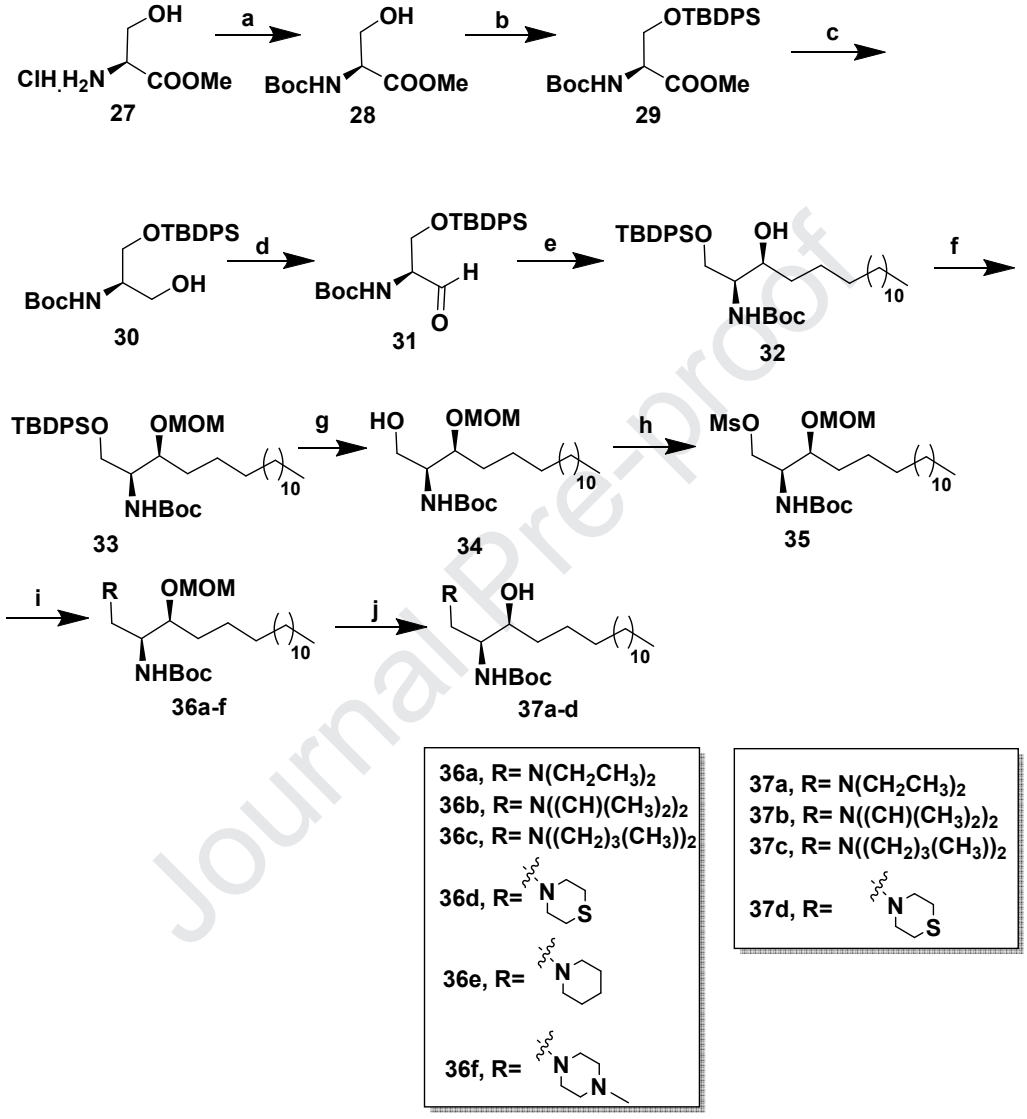


Fig. 5 *Syn*-productformation bynucleophilic additions via chelation-controlled Cram model.

Another explanation of this *syn*-stereochemistry was explained by Ichihashi and Mori in 2003[50] by converting diastereomeric mixture of Boc protected amino alcohol to the corresponding oxazolidines. By conducting NOE experiment on this mixture of oxazolidines exposed that *syn*-adduct was the major isomer.

Spisulosine derivatives **30, 32, 33, 34, 36a-f, 37a-d,** were synthesized according to the **scheme 2**. Almost same procedure was used as mentioned in **scheme 1**. Instead of using L-tyrosine, L-serine amino acid was used as a starting material in this scheme. Firstly, L-serine methyl ester hydrochloride **27**was protected by using Boc anhydride and $NaHCO_3$ as a base to provide Boc protected serine methyl ester **28** in excellent yields (94%). The free -OH group of compound **28** was then protected with TBDPS-Cl in presence of imidazole to give compound **29** in very good yield (89%), which was then subjected to $LiAlH_4$ mediated reduction of ester to afford alcohol **30** in good yield (88%). Further, the alcohol **30** was oxidized through Dess-Martin oxidation in DCM-H_2O to furnish aldehyde **31**, which was then subjected to Grignard reaction by using Grignard reagent tetradecylmagnesium chloride in THF solvent to provide long chain *syn*-diastereomer **32** in good yield (81% over two steps) according to the above chelation controlled Cram model (**Fig.5**). Compound **32** was then reacted with MOM-Cl by using DIPEA as a base to give compound **33**in 79 % yield which was further reacted with TBAF to remove the TBDPS group to furnish compound **34** with good yield (78%), the free –OH of compound **34** was then reacted with MsCl by using Et_3N as a base to make it a good living group (compound **35**). Substitution reaction was done by using different secondary amines in acetonitrile under reflux condition on compound **35** to furnish compounds **36a-f** in good yields (64-72%) with retention

of *syn*-stereochemistry. In the last step, the MOM group was removed by using ethane dithiol in presence of $BF_3.OEt_2$ in dry DCM to afford compounds **37a-d** in good yields (69-72%).



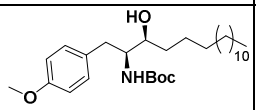
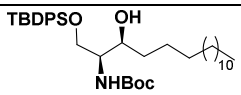
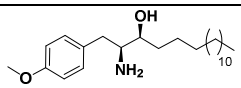
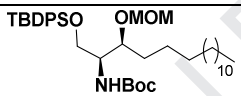
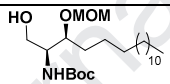
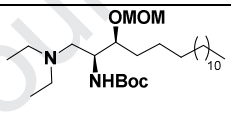
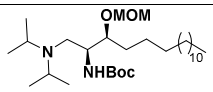
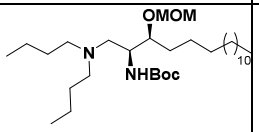
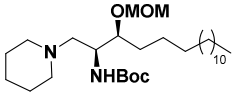
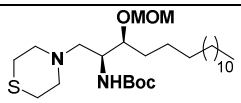
Scheme 2. Reagents: a) Boc anhydride, $NaHCO_3$, MeOH, 0 °C- RT, 4 h, 94 % b) TBDPS-Cl, imidazole, dry DCM, 0 °C- RT, 2 h, 89 % c) $LiAlH_4$, dry THF, 0 °C- RT, 1h, 88 % d) Dess-Martin Periodinane, DCM-H_2O, 0 °C- RT, 1 h e) $C_{14}H_{29}MgCl$, dry THF, 0 °C- RT, 4-5 h, 81 % over two steps f) MOM-Cl, DIPEA, dry DCM, 0 °C- RT, 2-3 h, 79 % g) TBAF, dry THF, 0 °C- RT, 4-5 h, 78 % h) MsCl, Et_3N, dry DCM, 0 °C- RT, 4-5 h, 84 % i) RH, CH_3CN, 0 °C- reflux, 5-6 h, 64-72 % j) Ethane dithiol, $BF_3.OEt_2$, dry DCM, 0 °C- RT, 2 h, 69- 72 %.

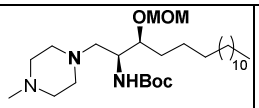
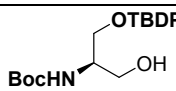
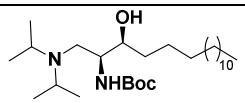
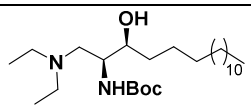
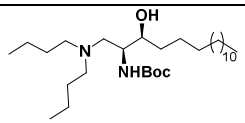
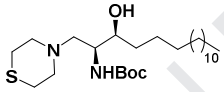
Biological Evaluation:

Novel spisulosine analogs are cytotoxic against cancer cells

We screened a series of 16 compounds for their cytotoxic activity against six different cancer cell lines representing three cancer types by using well established SRB assay as described in method section. Representative cell lines from different cancer types are DLD-1, HT-29 (Colorectal adenocarcinoma), MCF-7, MDA-MB231 (Breast adenocarcinoma), A549, NCI-H358 (Lung carcinoma). Detailed results of these investigations in terms of percentage inhibitions of cell growth at 10 μM of each compound are given in **Table 1**. Here, we observed compound **26b** was found to be potentially active against all the tested cell lines at a 10 μM dose, having maximum effect in colon cancer cell lines (more than 95% cytotoxicity in both the cell lines). Next, we determined its IC_{50} in a panel of colon cancer cell lines as mentioned in the bottom panel which was turned out to be less than 5 μM in all the colon cancer cells (**Fig.6**).

Table 1: Percentage (%) cell growth inhibition at 10μM dose in diverse cancer cell lines

			Breast		Colon		Lung	
Sr. No.	**Compound Code**	**Structure**	**MCF-7**	**MDA-MB-231**	**DLD-1**	**HT-29**	**A549**	**NCI-H358**
1	**25**		8.77	-16.50	27.95	-7.41	23.41	6.37
2	**32**		2.79	-37.45	10.42	-14.40	14.57	3.11
3	**26b**		89.28	96.84	97.30	97.66	94.85	90.40
4	**33**		-7.95	-27.98	-14.62	-18.76	15.13	-2.89
5	**34**		10.15	-5.67	54.84	22.02	25.91	18.43
6	**36a**		25.25	0.84	46.23	-3.21	27.47	14.95
7	**36b**		26.91	30.98	53.90	19.92	22.63	26.58
8	**36c**		27.06	22.26	56.05	14.98	42.23	25.12
9	**36e**		27.34	9.84	43.70	14.19	18.98	15.22
10	**36d**		23.99	4.73	58.55	8.57	29.92	22.68

11	**36f**		28.01	24.00	31.95	19.34	18.25	18.86
12	**30**		28.26	32.29	73.50	46.25	25.45	53.43
13	**37b**		25.28	-1.17	34.83	11.62	22.93	11.26
14	**37a**		18.71	-1.45	14.73	15.30	18.52	8.37
15	**37c**		21.54	-9.61	23.42	9.83	29.97	9.40
16	**37d**		30.25	6.84	32.37	12.04	31.96	10.81
	Doxorubicin (10µM)		88.50	80.16	76.68	78.40	80.25	77.46

Structure-activity-relationship studies

Based on the biological data reported in **Table 1**, many structure activity relationships (SAR) could be deduced. According to the results, we found that the compound **26b** with 4-methoxyphenyl in the head region and free amine and hydroxyl functionality in the body region was found to be more potent and showed better inhibitory activities against all the tested cell lines at a 10 µM dose, showing maximum effect in colon cancer cell lines (more than 95% cytotoxicity in both the cell lines) whereas its protected analog **25** (Boc protected amine) was 3 to 100-fold less potent than compound **26b**.

To further optimize the lead compound **26b**, we performed a SAR study on the head and body region by introducing different tertiary amines in the head region and protecting the free amine and hydroxyl group of the body region (compound no- **36a** to **36f**). As shown in **Table 1**, most

of the compounds showed only moderate activity in DLD-1 cell line. Further exploration of SAR at the body region was conducted by protecting only the amine group and keeping the hydroxyl group free (compound no- **37a** to **37d**) but the results were not satisfactory, there was tremendous decrease in activity. Hence, from the SAR studies it was clear that the free amine and hydroxyl group was crucial for the activity. With this structure-activity-relationship analysis, analogue **26b** was selected for further evaluation.

	Breast		Lung		Colon	
Compound Code	MCF-7	MDAMB-231	A549	NCI-H358	DLD-1	HT-29
25	8.77	-16.5	23.41	6.37	27.95	-7.41
32	2.79	-37.45	14.57	3.11	10.42	-14.4
26b	**89.28**	**96.84**	**94.85**	**90.4**	**97.3**	**97.66**
33	-7.95	-27.98	15.13	-2.89	-14.62	-18.76
34	10.15	-5.67	25.91	18.43	54.84	22.02
36a	25.25	0.84	27.47	14.95	46.23	-3.21
36b	26.91	30.98	22.63	26.58	53.9	19.92
36c	27.06	22.26	42.23	25.12	56.05	14.98
36e	27.34	9.84	18.98	15.22	43.7	14.19
36d	23.99	4.73	29.92	22.68	58.55	8.57
36f	28.01	24	18.25	18.86	31.95	19.34
30	28.26	32.29	25.45	53.43	73.5	46.25
37b	25.28	-1.17	22.93	11.26	34.83	11.62
37a	18.71	-1.45	18.52	8.37	14.73	15.3
37c	21.54	-9.61	29.97	9.4	23.42	9.83
37d	30.25	6.84	31.96	10.81	32.37	12.04

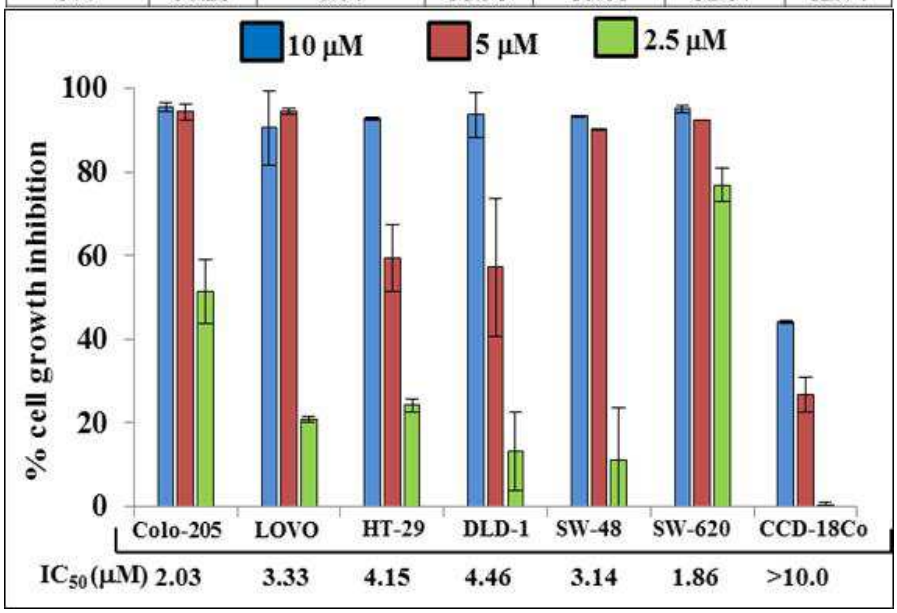


Fig. 6 Novel Spisulosine analogs are cytotoxic against colon cancer cells. Different human cancer cells (MCF-7, MDA-MB-231, A549, NCI-H358, DLD-1, and HT-29) were treated with 10 μM of different Spisulosine derivatives for 48 hours and cytotoxicity was measured by SRB assay as described in Materials and Methods. Percent growth inhibitions were tabulated (Top Panel). Various human colon cancer cells (COLO-205, LOVO, HT-29, DLD-1, SW48, and SW-620) and normal human colon fibroblast (CCD-18Co) cells were treated with varying concentrations of compound **26b** for 48 h and the half maximal proliferation inhibitory concentration (IC_{50}) values were determined (Bottom Panel) by SRB assay. Columns, average of triplicate readings of samples; *error bars*, ± S.D.

Compound 26b promotes non apoptotic cell death

Potent cytotoxic action of compound **26b** prompted us to determine its primary mode of action. To our great surprise, we observed that compound **26b** did not pose its cytotoxic effects through classical apoptosis (**Fig. 7A-D**). It did not result in neither generation of early apoptotic cells as detected by Annexin-V-PI staining nor cleavage of hallmark apoptotic features like PARP protein. Moreover, it promotes a robust increase in cellular granularity through vacuole formation (**Fig. 7B**). In contrast, parent spisulosine treatment has been shown to increase Annexin-V positive cells as well as PARP cleavage indicating an active apoptotic process.

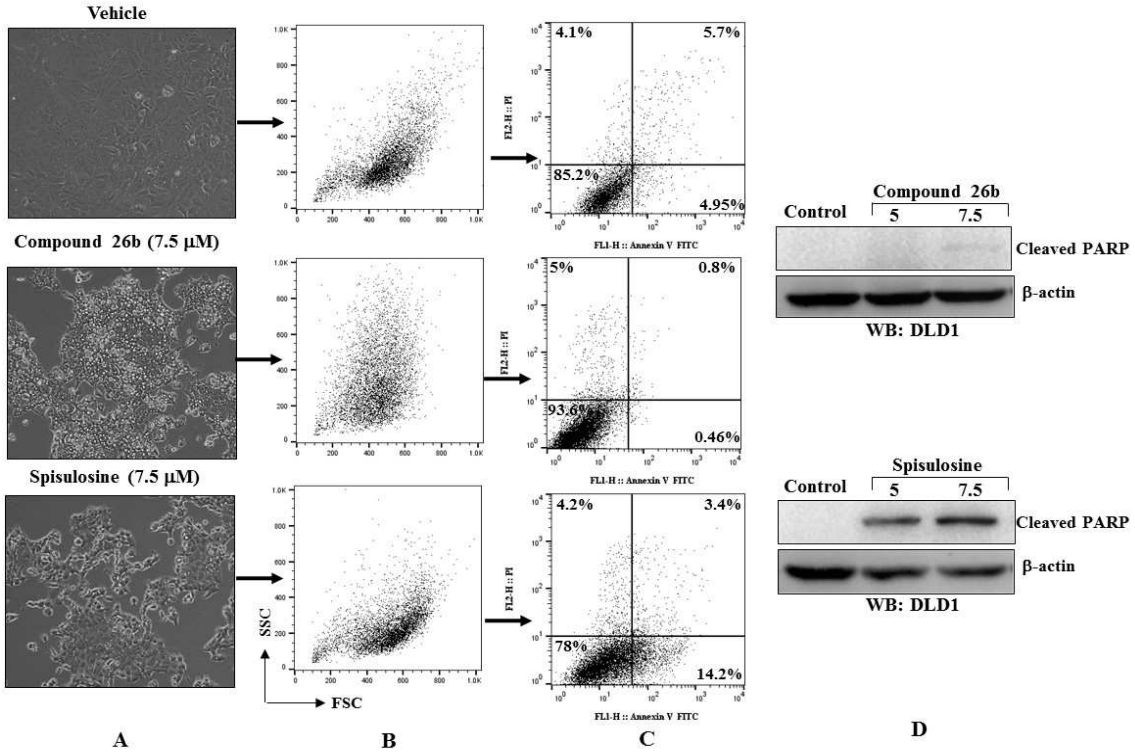


Fig.7 Compound 26b promotes non-apoptotic cell death. Panel A-D, DLD-1 cells were treated with either vehicle or Compound **26b** or Spisulosine at 7.5 µM for 18 hours and analyzed for early apoptosis (Annexin-V staining by FACS) and Cleaved PARP expression (Western Blot). Representative photo micrographs were shown in panel A. Cell size (FSC) and cellular Granularity (SSC) were shown by FACS in panel B. In panel C, representative FACS plots show Annexin-V-FITC and PI stained cells. Immunoblot analysis (panel D) shows the expression of cleaved PARP (Apoptotic marker protein) following protein isolation of vehicle and treated cells.

Compound 26b promotes Autophagic cell death

As we found, the cytotoxic cell death induced by compound **26b** was not due to apoptosis, and also observed that compound **26b** treatment promotes intracytoplasmic vacuole accumulation in colon cancer (DLD-1) cells, which is characteristic feature of cells undergoing autophagy. To confirm the formation of autophagic vesicles, we stained cells with a fluorescent compound MDC (monodansylcadaverine) which is an autophagy marker and act as lysomotrophic agent and labels the acidic compartments of fused lysosomes[51,52]. Under confocal microscopy analysis,

we observed vacuoles were positively stained by MDC and appeared as distinct dot like structures distributing in cytoplasm of DLD-1 cells treated by compound **26b**; the fluorescent density of MDC-labeled particles in DLD-1 cells were higher in compound **26b** treated group than in control group (**Fig**. **8A**) suggesting autophagy induction upon compound treatment. LC3 lipidation is a classical biomarker of cellular autophagy levels. During autophagy, the 16-kDa cytosolic (LC3-I) form is post-translationally modified to form 14kDa (LC3-II) and translocated to autophagosome**.** The western blot analysis results (**Fig. 8B**) shown an increase in conversion of LC3B-I to LC3B-II. Further, we have confirmed our observation by determining the LC-3B-II puncta formation following compound treatments at different doses (**Fig. 8C**).

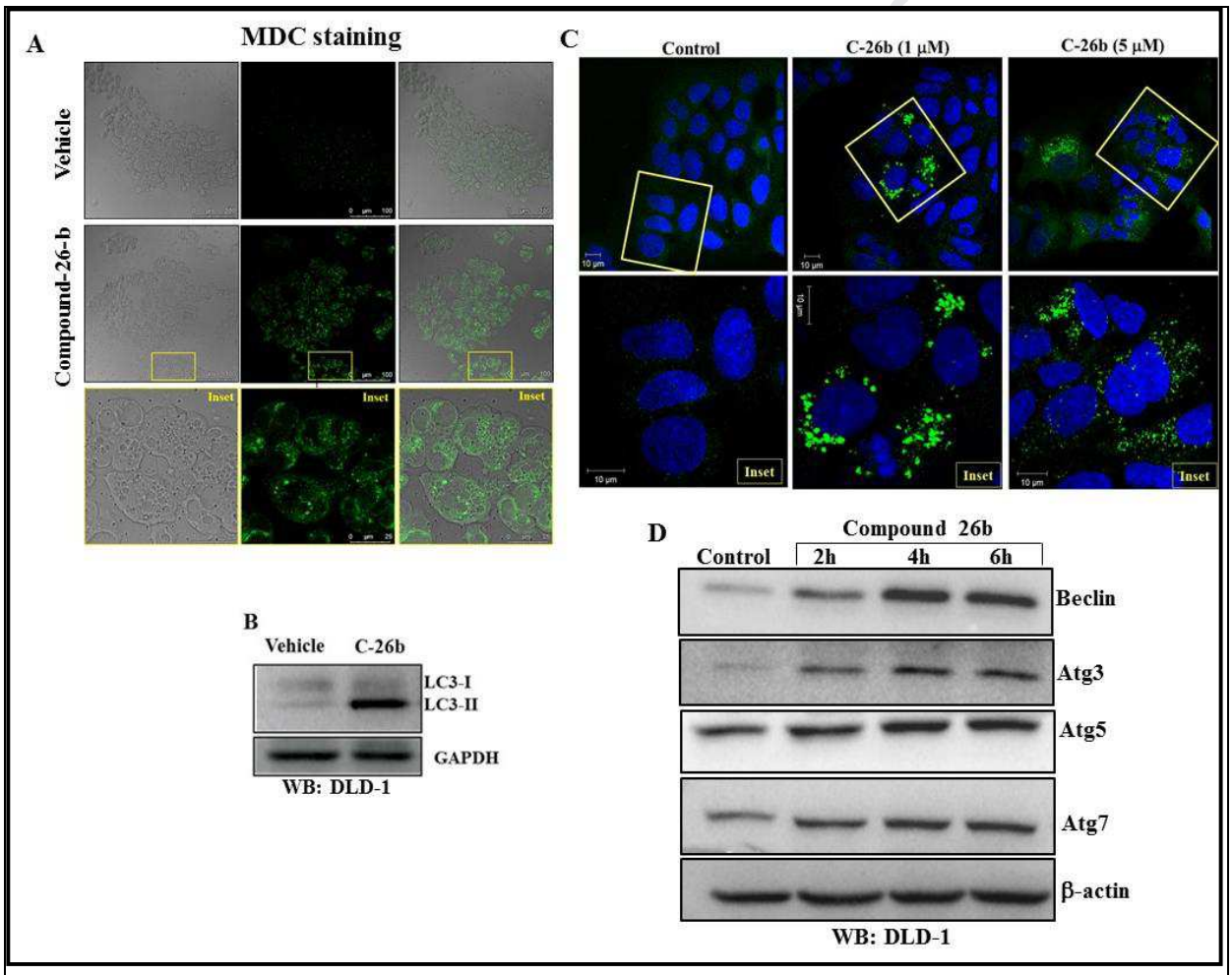


Fig.8 Compound 26b promotes Autophagic cell death. Panel A-B, DLD-1 cells were treated with either vehicle or compound **26b** at 7.5 µM for 18 hours. Cells were stained with autophagy marker MDC (monodancycadaverin) and analyzed under confocal microscope (panel A). Cells were harvested for protein isolation and subjected to immunoblot for LC-3 and GAPDH expression (panel B). DLD-1 cells were treated with either vehicle or compound **26b** for 6 hours

and subjected to confocal microscopy for determination of LC-3 puncta formation (panel C). DLD-1 cells were treated with either vehicle or compound **26b** at 10 µM for indicated hours. Cells were harvested for protein isolation and subjected to immunoblot for Beclin and Atg protein expression (panel D)

Moreover, compound **26b** robustly induces the expression of classical autophagy markers like Beclin-1, Atg3, Atg5 and Atg7, compared to control at multiple time points (**Fig. 8D**).

To further confirm our previous observations, we performed TEM analysis of control and treated cells. As observed in **Fig. 9**, compound 26b induced numerous classical autophagic vacuoles characterized by the presence of double membrane vesicles with cargo. Compared to control cells, no mitochondrial or nuclear damage were observed in the treated cells, suggesting absence of apoptosis in response to compound treatment.

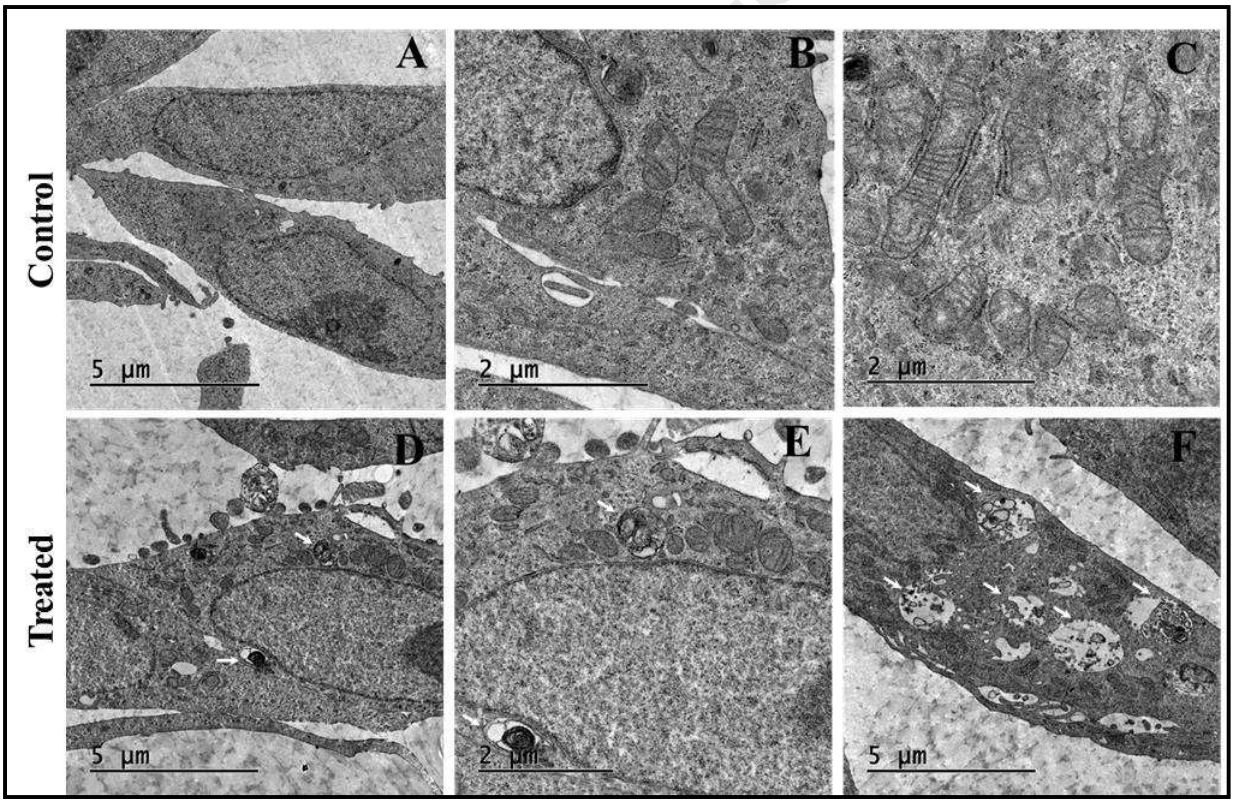


Fig. 9 Compound 26b induces autophagic vacuole formation. Representative transmission electron micrographs of control (A-C) and treated (D-F) DLD-1 colorectal adenocarcinoma cells. Untreated cells display normal ultrastructure of organelles like mitochondria, rough ER and nucleus (B, C). Autophagic vacuoles (arrows) were observed in the treated (5 µM for 6 hours) cells. No structural alterations were observed in the mitochondria in treated cells indicating autophagy selective responses.

To test whether compound **26b** induced autophagy linked with cellular cytotoxicity, we pretreated our cells with bafilomycinA1, a specific autophagy inhibitor, and observed the

To test whether compound **26b** induced autophagy linked with cellular cytotoxicity, we pretreated our cells with Bafilomycin-A1, a specific autophagy inhibitor, and observed the attenuation of compound **26b** induced autophagic vacuole formation and subsequent cell death compared with only compound **26b** treated cells. Interestingly, pretreatment of Bafilomycin in presence of parent compound spisulosine in DLD-1 cells did not inhibit cytotoxic cell death indicative of non autophagic cell death (**Fig. 10A and 10B**).

Based on this data, we concluded that the major difference between parent compound spisulosine and our potent compound **26b** was *para*-methoxyphenyl part which may be responsible for the autophagic cell death because the parent compound was going through classical apoptosis and our compound **26b** induced cell death via vacuole formation.

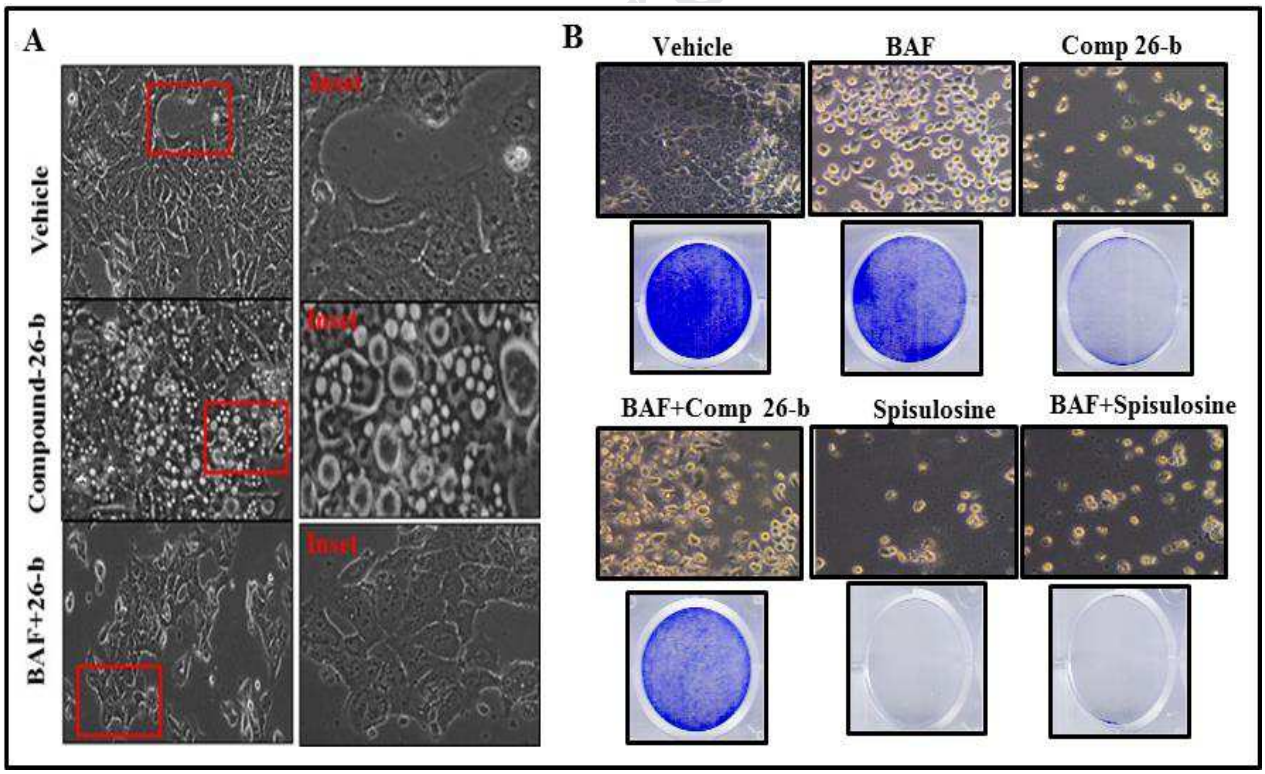


Fig. 10 Compound 26b promotes autophagic cell death. DLD1 cells were treated with either vehicle or compound 26b (7.5 µM) or autophagy inhibitor Bafilomycin A1 (100nm) plus compound 26b (7.5 µM) for 24 hours and analyzed under phase-contrast microscope (panel A). Cellular cytotoxicity was performed by crystal violet staining in control and treated (48hours) cells and representative photomicrographs were shown (panel B).

3. CONCLUSION

We have synthesized a series of spisulosine analogs based on a rational design taking inputs from various sphingoid type bases having anticancer effects. The aim was to create a novel library with better pharmacokinetic profile and to determine its mechanism of action. During our search toward its cytotoxic mode of action, we surprisingly discovered that unlike parent spisulosine, compound **26b** promoted robust autophagic cell death to cancer cells instead of classical apoptosis. Compound **26b** treatment resulted in massive vacuole formation which was found to be positive for autophagy marker expression like LC3-II puncta formation, upregulation of Atg proteins etc. Biochemically, it induced classical autophagy markers but not promoted hallmark apoptotic features like cleavage of PARP or caspases. Cytotoxic effect of compound **26b** can be effectively rescued by the autophagy inhibitor but not in case of parent spisulosine. Overall, we discovered a new small molecule that markedly induces autophagic cell death to various cancer cells.

4. EXPERIMENTAL PROTOCOLS

Materials and methods

All commercially available starting materials and reagents were used without further purification. Organic solvents were dried by standard methods. All dry reactions were carried out under argon or nitrogen atmosphere. All the compounds were characterized by ^{1}H, ^{13}C, ESI-MS and HRMS. All reactions were monitored by TLC using 2.5x5 cm plates coated with 0.25 mm thickness of silica gel. Visualization was done by using UV light and iodine vapors. Purification was done by silica gel (100-200 mess) column chromatography by using freshly distilled solvents. NMR spectra were recorded on BrukerAvance DPX 200FT, Bruker Robotics, Bruker DRX 400, 100 and 125 (400 MHz for ^{1}H and 100, 125 MHz for ^{13}C) using $CDCl_3$ and CD_3OD as solvent with TMS as an internal standard, δ in parts per million, and J in hertz. Mass spectra were recorded using electron spray ionization (ESI-MS) or Fast atom bombardment spectra (FAB-MS) using Argon/xenon as the FAB gas. HRMS spectra were recorded using ESI/Q-TOF conditions. Optical rotation was determined by a Rudolph Research Analytical Autopol III Automatic Polarimeter. Specific rotations $[\alpha]_D^{25}$ for compound **26b** is given in deg cm^3 g^{-1} dm^{-1}. The purity of the compounds was determined by high performance liquid chromatography (HPLC). Chiral HPLC of compound **26b** was performed on W2998 instrument by using Chiral-IA column; eluent A, propan-2-ol with 0.1% diethyl amine; eluent B, acetonitrile with 0.1%

diethyl amine, isocratic (10:90) with a flow rate of 0.6mL min^{-1} and detection at 280 nm; column temperature of 25 °C; injection of 10 μL of 1 mM **26b**.

General procedure for the synthesis of compound 25

To a stirred solution of intermediate aldehyde **24** (0.35 mmol) in dry THF (2 ml), tetradecylmagnesium chloride 1M (0.43 mmol) was added dropwise under ice cooled condition. After 5 min of stirring, ice was removed and the reaction mixture was stirred at rt for 4-5 h. After completion of the reaction, it was quenched by slow addition of NH_4Cl solution under ice cooled condition. The aqueous layer was extracted with EtOAc (3x30 ml) and then the combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuum. Purification of the extracted mixture was done by silica gel (100-200 mess) column chromatography (EtOAc/Hexane) afforded diastereomerically pure **25** with the *syn* configuration as a solid.

tert-butyl ((2S,3S)-3-hydroxy-1-(4-methoxyphenyl)octan-2-yl)carbamate(25)

Solid; yield 73%; mp 73 °C; R_f (30% EtOAc/Hexane) 0.71; ^{1}H NMR (400 MHz, $CDCl_3$) 7.13 (2H, J= 8.1 Hz, d, Ar-H), 6.8 (2H, J= 8.4 Hz, d, Ar-H), 3.78 (3H, s, -OCH_3), 3.69-3.61 (1H, m, -CH), 3.54 (1H, m, -CH), 2.81-2.80 (2H, m, -CH_2), 1.93 (1H, s, -OH), 1.40-1.23 (33H, m, ^{t}Bu-H, aliphatic H), 0.87 (3H, J= 6.3 Hz, t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 158.17, 156.15, 130.20, 113.91, 79.25, 71.67, 63.10, 55.24, 37.77, 34.60, 32.82, 31.92, 29.66, 29.57, 29.35, 28.35, 25.68, 22.68, 14.10, 1.01*; m/z* (ESI) 363.1 (100%), 364.1 (20%), 509.0 (3%, MCH_3OH^+); HRMS (ESI): MH^+ found 378.3382 $C_{29}H_{51}NO_4$ requires 477.3818 (because of removal of Boc group).

General procedure for the synthesis of compound 26b

To the compound **25,** 4N HCl in dioxane was added dropwise under ice cooled condition and stirred for 0.5 hr at the same temperature. After completion of the reaction, solvent was evaporated under reduced pressure. Then water was added to the residue and extracted with EtOAc thrice (3x30 ml). The combined organic layer was basified with $NaHCO_3$ two times, washed with brine and dried over anhydrous Na_2SO_4 and concentrated under reduced pressure and subjected to silica gel column chromatography to obtain the final compound **26b** as diastereomerically pure with the *syn* configuration in major amount (65 % yield) with some demethylated product **26a** (minor).To reduce the minor product **26a**, Boc deprotection with Dowex® 50Wx8 resin in MeOH was done and **26b** as the major product (85 % yield) was formed and only 5% of **26a** was observed. The obtained compound **26b** was optically pure with specific rotation {$[\alpha]_D^{25}$ -10.0 (c 0.05, MeOH).

(2S,3S)-2-amino-1-(4-methoxyphenyl)octan-3-ol (26b)

Solid; yield 85%; mp 64 °C; R_f (5% MeOH/$CHCl_3$) 0.32; ^{1}H NMR (400 MHz, $CDCl_3$) 7.18 (2H, J= 7.8Hz, d, Ar-H), 6.80 (2H, J= 7.8Hz, d, Ar-H), 6.16 (2H, br s, -NH_2), 3.74 (3H, s, -OCH_3), 3.60-3.26 (2H, m, -$(CH)_2$), 2.94-2.87 (2H, m, -CH_2), 1.25 (26H, m, aliphatic H), 0.87 (3H, J= 6.0

Hz, t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 158.66, 130.51, 128.10, 114.28, 70.87, 57.63, 55.15, 36.00, 34.05, 31.93, 29.75, 29.69, 29.58, 29.38, 25.53, 22.68, 14.10; *m/z* (ESI) 378.1 (100%, MH^+) 379.2 (20%); HRMS (ESI): MH^+ found 378.3354 $C_{24}H_{43}NO_2$ requires 378.3372. HPLC analysis: retention time = 1.870 min; peak area, 98.63%; eluent A, propan-2-ol with 0.1% diethyl amine; eluent B, acetonitrile with 0.1% diethyl amine, isocratic (10:90) over 30 min with a flow rate of 0.6 mL min^{-1} and detection at 280 nm; column temperature of 25 °C. Specific rotation: (*2S, 3S*)-**26b**{$[\alpha]_D^{25}$ -10.0 deg cm^3 g^{-1} dm^{-1} (c 0.05 g cm^{-3}, MeOH)}

General procedure for the synthesis of compound 30

To an ice cooled solution of ester **29** (4.37 mmol) in dry THF (10 ml), $LiAlH_4$ (5.24 mmol) was added slowly, and allowed to stirred at rt for 1 h. After completion of the reaction, it was quenched by slow addition of EtOAc and water, the aqueous layer was extracted with EtOAc (3x 30 ml), the combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and solvent was removed under reduced pressure to furnish the alcohol compound **30**. The crude product was purified over 100-200 mess silica gel column chromatography by using EtOAc/Hexane as an eluent.

(R)-tert-butyl (1-((tert-butyldiphenylsilyl)oxy-3-hydroxypropan-2-yl)carbamate (30)

Solid; yield 88 %; mp 74 °C; R_f (30% EtOAc/Hexane) 0.42; ^{1}H NMR (400 MHz, $CDCl_3$) 7.64 (4H, J= 7.5 Hz, d, Ar-H), 7.42-7.34 (6H, m, Ar-H), 3.79-3.67 (5H, m, -$(CH_2)_2$, -CH), 2.86 (1H, br s, -OH), 1.42 (9H, s, ^{t}Bu-H), 1.06 (9H, s, ^{t}Bu-H); ^{13}C NMR (100 MHz, $CDCl_3$) 156.14, 135.57, 133.04, 132.99, 129.93, 127.88, 79.59, 63.87, 63.14, 53.25, 28.44, 26.93, 19.28; *m/z* (ESI) 429.6 (100%), 430.8 (22%, MH^+); HRMS (ESI): MH^+ found 430.2417 $C_{24}H_{37}NO_4Si$ requires 430.2414.

General procedure for the synthesis of compound 32

To the solution of previously prepared aldehyde **31** (0.23 mmol) in dry THF (2ml), tetradecylmagnesium chloride 1M (0.35 mmol) was added slowly at 0 °C and was allowed to stir at rt for 4-5 hr. After completion, the reaction was quenched by addition of NH_4Cl solution and water layer was extracted with EtOAc (3x30ml). The combined organic layer was washed with brine solution, dried over anhydrous Na_2SO_4 and solvent was concentrated under reduced pressure. The crude product was purified over silica gel column chromatography to furnish the diastereomerically pure compound **32** with the *syn* configuration.

tert-butyl ((2S,3S)-1-((tert-butyldiphenylsilyl)oxy)-3-hydroxyoctan-2-yl)carbamate (32)

Oil; yield 81%; R_f(30% EtOAc/Hexane) 0.7; ^{1}H NMR (400 MHz, $CDCl_3$) 7.72-7.63 (4H, m, Ar-H), 7.45-7.34 (6H, m, Ar-H), 3.93-3.81 (2H, m, -$(CH)_2$), 3.62-3.59 (2H, m, -CH_2), 2.95 (1H, br s, -OH), 1.43 (9H, s, ^{t}Bu-H), 1.25 (26H, m, aliphatic H), 1.06 (9H, s, ^{t}Bu-H), 0.87 (3H, J= 6.6 Hz,

t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 156.09, 135.58, 135.54, 134.83, 132.72, 129.97, 129.51, 127.88, 127.64, 79.27, 72.67, 66.65, 63.00, 53.97, 33.85, 32.80, 31.94, 29.70, 29.46, 29.37, 28.40, 26.89, 26.77, 26.59, 25.77, 25.59, 22.70, 19.17, 14.12; m/z (ESI) 626.0 (100%), 627.0 (30%, MH^+); HRMS (ESI): MH^+ found 626.4608 $C_{38}H_{63}NO_4Si$ requires 626.4605.

General procedure for the synthesis of compound 33

To an ice cooled solution of synthesized compound **32** (1.59 mmol) in dry DCM (15 ml), DIPEA (9.58 mmol) was added and stirred for 5 min at 0 °C. After 5 min, MOM-Cl (6.38 mmol) was added slowly and then allowed to stir at rt for 3 h. After completion of the reaction, water was added to the reaction mixture and aqueous layer was extracted with CH_2Cl_2 (3x30ml), the combined organic layer was washed with brine solution, dried over anhydrous Na_2SO_4, and solvent was removed under reduced pressure and the crude product was subjected to silica gel column chromatography to furnish the compound **33**.

tert-butyl ((5S,6S)-10,10-dimethyl-5-pentyl-9,9-diphenyl-2,4,8-trioxa-9-silaundecan-6-yl)carbamate (33)

Oil; yield 79%; R_f (10% EtOAc/Hexane) 0.5; ^{1}H NMR (400 MHz, $CDCl_3$) 7.66-7.64 (4H, m, Ar-H), 7.42-7.35 (6H, m, Ar-H), 4.56 (2H, s, -CH_2), 3.84-3.82 (2H, m, -$(CH)_2$), 3.68-3.57 (2H, m, -CH_2), 3.27 (3H, s, -CH_3), 1.41 (9H, s, ^{t}Bu-H), 1.25 (26H, m, aliphatic H), 1.05 (9H, s, ^{t}Bu-H), 0.87 (3H, J= 6.5Hz, t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 155.67, 135.61, 133.38, 129.68, 127.70, 96.44, 78.96, 63.29, 55.70, 53.63, 31.93, 29.69, 29.36, 28.38, 26.84, 25.47, 22.69, 19.22, 14.11, 1.01; m/z (ESI) 669.9 (100%), 671.0 (35%, MH^+); HRMS (ESI): MH^+ found 670.4857 $C_{40}H_{67}NO_5Si$ requires 670.4867.

General procedure for the synthesis of compound 34

To a stirred solution of compound **33** (0.37 mmol) in dry THF (2 ml), 1M TBAF (0.55 mmol) was added at 0 °C and allowed to stir at rt for 4-5 h. After completion of the reaction, water was added to the reaction mixture and aqueous layer was extracted with CH_2Cl_2 (3x30ml), the combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and solvent was concentrated under reduced pressure to afford the compound **34**. Purification was done by using 100-200 mesh silica gel column chromatography.

tert-butyl ((2S,3S)-1-hydroxy-3-(methoxymethoxy)octan-2-yl)carbamate(34)

Oil; yield 78%; R_f(30% EtOAc/Hexane) 0.4; ^{1}H NMR (400 MHz, CD_3OD) 4.68 (2H, s, -CH_2), 3.76-3.66 (2H, m, -$(CH)_2$), 3.65-3.51 (2H, m, -CH_2), 3.39 (3H, s, -CH_3), 1.46 (9H, s, ^{t}Bu-H), 1.31 (26H, m, aliphatic H), 0.92 (3H, J= 6.4Hz, t, -CH_3); ^{13}C NMR (100 MHz, CD_3OD) 156.84, 96.19, 78.77, 77.98, 76.47, 61.07, 60.75, 54.73, 54.12, 31.69, 31.01, 29.38, 29.30, 29.20, 29.09,

27.40, 25.12, 22.35, 13.08; m/z (ESI) 431.8 (100 %), 432.9 (20 %, MH^+); HRMS (ESI): MH^+ found 432.3700 $C_{24}H_{49}NO_5$ requires 432.3689.

General procedure for the synthesis of compounds 36a-f

To an ice cooled solution of intermediate **35** (0.19 mmol) in acetonitrile (3 ml), RNH (0.58 mmol, various secondary amines) were added and subjected to reflux for 5-6 h. After completion of the reaction, solvent was evaporated and water was added to the residue, the aqueous layer was extracted with EtOAc (3x30 ml). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was subjected to silica gel column chromatography to furnish the desired compounds **36a-f** as diastereomerically pure compounds with *syn*-stereochemistry in good yields 64-72%.

tert-butyl ((2S,3S)-1-(diethylamino)-3-(methoxymethoxy)octan-2-yl)carbamate (36a)

Solid; yield 72 %; mp 57 °C; R_f (30% EtOAc/Hexane) 0.15; ^{1}H NMR (400 MHz, $CDCl_3$) 6.04 (1H, s, -NH), 4.71-4.66 (3H, m, $-CH_2$, -CH), 4.45-4.39 (2H, m, $-CH_2$), 4.11-4.07 (1H, m, -CH), 3.89-3.83 (1H, m, -CH), 3.45-3.38 (6H, m, $-CH_3$, $-CH_2$, -CH), 1.25 (41 H, m, aliphatic H), 0.87 (3H, J= 6.5 Hz, t, $-CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) 159.31, 97.29, 96.55, 81.81, 78.56, 66.67, 55.90, 55.74, 55.00, 31.91, 30.70, 29.64, 29.53, 29.48, 29.34, 24.94, 22.67, 14.09; m/z (ESI) 180.1 (100 %), 317.0 (5 %).

tert-butyl ((2S,3S)-1-(diisopropylamino)-3-(methoxymethoxy)octan-2-yl)carbamate (36b)

Solid; yield 69 %; mp 54 °C; R_f (30% EtOAc/Hexane) 0.16; ^{1}H NMR (400 MHz, $CDCl_3$) 6.36 (1H, s, -NH), 4.75-4.67 (2H, m, $-CH_2$), 4.43-4.41 (2H, m, $-(CH)_2$), 4.13-4.12 (1H, m, $-CH_2$), 3.91-3.88 (1H, m, $-CH_2$), 3.46-3.38 (5H, m, $-CH_2$, $-CH_3$), 1.42-1.25 (47H, m, ^{t}Bu-H, aliphatic H), 0.90 (3H, J= 8Hz, t, $-CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) 159.68, 97.19, 81.39, 78.54, 66.71, 55.88, 55.66, 31.90, 30.63, 29.66, 29.63, 29.60, 29.53, 29.48, 29.33, 24.97, 22.66, 14.08; m/z (ESI) 437.2 (100 %), 515.4 (22 %. MH^+); HRMS (ESI): MH^+ found 515.4793 $C_{30}H_{62}N_2O_4$ requires 515.4788.

tert-butyl ((2S,3S)-1-(dibutylamino)-3-(methoxymethoxy)octan-2-yl)carbamate(36c)

Solid; yield 71 %; mp 50 °C; R_f (30% EtOAc/Hexane) 0.17; ^{1}H NMR (400 MHz, $CDCl_3$) 6.27 (1H, s, -NH), 4.69-4.66 (3H, m, $-CH_2$), 4.43-4.39 (1H, m, -CH), 4.12-4.09 (1H, m, -CH), 3.89-3.87 (2H, m, $-CH_2$), 3.45-3.38 (6H, m, $-CH_3$, $-CH_2$, -CH), 1.41-1.25 (49H, m, aliphatic H), 0.87 (9H, J= 6.5 Hz, t, $-(CH_3)_3$); ^{13}C NMR (100 MHz, $CDCl_3$) 159.50, 97.23, 96.57, 81.56, 78.55, 66.69, 55.88, 55.68, 31.90, 30.65, 29.67, 29.66, 29.63, 29.60, 29.53, 29.48, 29.33, 24.97, 22.67, 14.09; m/z (ESI) 543.3 (100 %), 544.3 (30 %, MH^+); HRMS (ESI): MH^+ found 543.5114 $C_{32}H_{66}N_2O_4$ requires 543.5101.

tert-butyl ((2S,3S)-3-(methoxymethoxy)-1-thiomorpholinooctan-2-yl)carbamate(36d)

Solid; yield 67 %; mp 53 °C; R_f (30% EtOAc/Hexane) 0.14; ^{1}H NMR (400 MHz, $CDCl_3$) 6.25 (1H, s, -NH), 4.69-4.67 (3H, m, -CH_2, -CH), 4.43-4.39 (2H, m, -CH_2), 4.12-4.08 (1H, m, -CH), 3.90-3.85 (1H, m, -CH), 3.52-3.50 (1H, m, -CH), 3.45-3.38 (6H, m, -CH_3, -CH_2, -CH), 2.78-2.72 (1H, m, -CH), 2.05-2.00 (1H, m, -CH), 1.41 (35H, m, aliphatic H), 0.87 (3H, J= 6.5 Hz, t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 159.49, 97.23, 81.55, 66.68, 55.88, 55.68, 46.75, 31.90, 30.65, 29.63, 29.53, 29.48, 29.33, 24.96, 22.66, 14.09; m/z (ESI) 357.9 (100 %), 517.2 (75 %), 518.2 (23 %, MH^+); HRMS (ESI): MH^+ found 517.4045 $C_{28}H_{56}N_2O_4S$requires 517.4039

tert-butyl ((2S,3S)-3-(methoxymethoxy)-1-(piperidin-1-yl)octan-2-yl)carbamate (36e)

Solid; yield 68 %; mp 50 °C; R_f (30% EtOAc/Hexane) 0.15; ^{1}H NMR (400 MHz, $CDCl_3$) 6.16 (1H, s, -NH), 4.69(3H, m, -CH_2, -CH), 4.43-4.39 (1H, m, -CH), 4.11-4.08 (1H, m, -CH), 3.90-3.84 (1H, m, -CH), 3.45-3.38 (6H, m, -CH_3, -CH_2, -CH), 3.19-3.17 (1H, m, -CH), 1.41-1.25 (41H, m, ^{t}Bu-H, aliphatic H), 0.87 (3H, J= 6.5 Hz, m, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 159.38, 97.27, 81.71, 66.67, 55.89, 55.72, 46.75, 31.91, 30.68, 29.67, 29.66, 29.64, 29.62, 29.60, 29.53, 29.48, 29.34, 25.36, 24.96, 22.67, 14.09; ; m/z (ESI) 288.26 (18 %), 316.29 (8 %, MH^+).

tert-butyl((2S,3S)-3-(methoxymethoxy)-1-(4-methylpiperazin-1-yl)octan-2-yl)carbamate (36f)

Solid; yield 64 %; mp 41°C; R_f (30% EtOAc/Hexane) 0.12; ^{1}H NMR (400 MHz, $CDCl_3$) 6.19 (1H, s, -NH), 4.70-4.66 (5H, m, -CH), 4.43-4.39 (2H, m, -CH_2), 4.12-4.08 (2H, m, -CH_2), 3.90-3.85 (2H, m, -CH_2), 3.45-3.38 (9H, m, -$(CH_3)_2$, -CH_2, -CH), 1.29-1.25 (35H, m, aliphatic H), 0.88 (3H, J= 5.4 Hz, t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 159.38, 97.26, 81.68, 66.67, 55.89, 55.71, 31.91, 30.67, 29.64, 29.53, 29.48, 29.34, 24.96, 22.67, 14.09; m/z (ESI) 356.0 (100 %), 431.9 (15 %).

General procedure for the synthesis of compounds (37a-d)

To an ice cooled solution of **36a-d** (0.21 mmol) in dry DCM (2 ml), $BF_3.OEt_2$ (0.25 mmol) was added slowly and stirred for 5 min. After 5 min, ethane dithiol (0.25 mmol) was added and stirred at rt for 2 h. After completion, water was added to the reaction mixture, the aqueous layer was extracted with CH_2Cl_2 (3x30 ml). The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Purification of the crude product by silica gel column chromatographaffordeddiastereomerically pure compounds **37a-d** in good yields (69-72 %).

tert-butyl ((2S,3S)-1-(diethylamino)-3-hydroxyoctan-2-yl)carbamate(37a)

Solid; yield 71 %; mp 80 °C; R_f (50% EtOAc/Hexane) 0.2; ^{1}H NMR (400 MHz, $CDCl_3$) 6.37 (1H, s, -NH), 4.46-4.38 (2H, m, -CH_2), 4.20-4.09 (2H, m, -CH_2), 3.83-3.67 (2H, m, -CH_2), 3.48 (1H, s, -CH), 3.05-2.96 (1H, m, -CH), 1.76 (1H, s, -OH), 1.50-1.25 (43 H, s, aliphatic H), 0.87

(3H, J= 6.36 Hz, t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 160.60, 73.02, 67.36, 57.35, 33.25, 31.92, 29.69, 29.66, 29.61, 29.56, 29.50, 29.35, 25.71, 25.48, 22.68, 14.10; m/z (ESI) 258.2 (60 %), 288.3 (55 %), 385.0 (45 %), 414.4 (42 %), 474.2 (100 %, MCH_3OH^+).

tert-butyl ((2S,3S)-1-(diisopropylamino)-3-hydroxyoctan-2-yl)carbamate(37b)

Solid; yield 69 %; mp 83 °C; R_f (50% EtOAc/Hexane) 0.21; ^{1}H NMR (400 MHz, $CDCl_3$) 6.71 (1H, s, -NH), 4.45-4.41 (1H, m, -CH), 4.19-4.16 (1H, m, -CH), 3.83-3.68 (2H, m, -CH_2), 3.41 (2H, s, -$(CH)_2$), 2.13-2.07 (1H, m, -OH), 1.50-1.11(47H, m, aliphatic H), 0.87 (3H, J= 6.11 Hz, t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 160.60, 73.02, 67.36, 57.35, 33.25, 31.92, 29.69, 29.66, 29.61, 29.56, 29.50, 29.35, 25.71, 25.48, 22.68, 14.10; m/z (ESI) 300.6 (58 %), 318.0 (100 %), 371.4 (55 %), 502.2 (45 %, MCH_3OH^+).

tert-butyl ((2S,3S)-1-(dibutylamino)-3-hydroxyoctan-2-yl)carbamate(37c)

Solid; yield 71 %; mp 81°C; R_f (50% EtOAc/Hexane) 0.22; ^{1}H NMR (400 MHz, $CDCl_3$+ CD_3OD) 4.46-4.39 (2H, m, -CH_2), 4.19-4.17 (2H, m, -CH_2), 3.88-3.76 (2H, m, -CH_2), 3.56-3.45 (2H, m, -$(CH)_2$), 1.53-1.35 (46H, m, ^{t}Bu-H, aliphatic H), 0.88 (6H, m, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$+ CD_3OD) 164.63, 76.57, 71.12, 61.23, 36.78, 35.77, 33.52, 33.49, 33.45, 33.41, 33.39, 33.19, 31.15, 29.34, 26.51, 17.85; m/z (ESI) 358.3 (15 %), 386.5 (28 %), 398.5 (30 %), 414.5 (15 %), 485.0 (40 %), 530.2 (40 %, MCH_3OH^+).

tert-butyl ((2S,3S)-3-hydroxy-1-thiomorpholinooctan-2-yl)carbamate(37d)

Solid; yield 72 %; mp 94 °C; R_f (50% EtOAc/Hexane) 0.21; ^{1}H NMR (400 MHz, $CDCl_3$) 6.56 (1H, s, -NH), 4.45-4.38 (2H, m, -CH_2), 4.20-4.17 (2H, m, -$(CH)_2$), 3.84-3.68 (2H, m, -CH_2), 3.48 (2H, s, -CH_2), 3.29-3.03 (2H, m, -CH_2), 2.17-188 (2H, m, -CH_2), 1.50-1.48 (1H, m, -OH), 1.27 (35H, m, aliphatic H) 0.88 (3H, J= 6.2 Hz, t, -CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) 160.47, 73.03, 67.32, 57.29, 33.28, 31.92, 29.69, 29.65, 29.60, 29.54, 29.49, 29.35, 25.67, 25.46, 22.68, 14.10; m/z (ESI) 386.1 (30 %), 394.2 (100 %), 398.5 (30 %).

Biological Assays:

Cell Culture

Various human cancer cell line including DLD-1, HT-29 (colorectal adenocarcinoma) A-549, NCI-H358 (lung carcinoma) MCF-7, MDA-MB-231(Breast cancer cell line), were obtained from ATCC, USA. We used early passage cells and cultured according to manufacturer's instructions. Cells were plated at 40% confluency, after overnight incubation different drug concentrations used for cell treatment as indicated in results section.

Evaluation of in vitro anticancer activity of spisulosine derivative

In-vitro cytotoxic efficacy of compounds against various cancer cell lines were assayed using standard sulforhodamine B (SRB) cytotoxicity assay[53,54]. Absorbance was measured on a plate reader (epoch microplate reader, biotek) at 510 nm to calculate the percent inhibition in cell growth by using the formula: [100(absorbance of compound treated cells/absorbance of untreated cells)] 100.

Visualization of MDC vacuoles

DLD-1 colon cancer cells were incubated in the presence of drug for 20 hours and labeled with 50mM concentration of autofluorescent marker monodensylcadaverine MDC (Sigma Chemical[55] Co.) in PBS at 37°c for 20 min in dark, after washing with PBS cells were immediately analyzed by a laser scanning confocal microscopy, (excitation 390nm; emission 460nm)

Western blot analysis

After treatment cells were lysed in NP-40 buffer supplemented with phosphatase and protease inhibitors cocktail. Lysates were quantitated using pierce BCA protein assay kit. Equal amount of protein in SDS-PAGE sample buffer were loaded in 15% SDS polyacrylamide gel and tranfered to PVDF membranes. Immunoreactivity was detected by enhanced chemiluminescence solution (immobilon western ECL, Millipore) after incubation of membrane with recommended amount of primary and secondary. Beclin-1 (#3495), LC3A/B (#12741), Atg5 (#12994), Atg3 (#3415), Atg7 (#8558), cleaved PARP (#5625) were purchased from cell signaling technology and GAPDH (#25778) antibody were purchased from Santa Cruz biotechnology.

Apoptosis analysis by Flowcytometry

Different stages of apoptosis were distinguished using an Annexin-V-FITC/Propidium Iodide apoptosis kit (BD Biosciences). After overnight incubation DLD1- cells were treated for 24h in presence of compound 26b and spisulosine, For Annexin-V staining cells were washed by PBS and resuspended in 1x binding buffer provided by Sigma then treated and vehicle control cells were stained with FITC conjugated Annexin-V and propidium iodide and incubated for 20 min in dark followed by washing with 1x binding buffer. The stained cells were acquired in a FACS caliber (Becton Dickinson) and analyzed by FlowJo software.

Confocal Microscopy

DLD-1 colon cancer cells were grown on coverslips for overnight and the next day after treatment cells were fixed with 4% paraformaldehyde in PBS and permeabilized by 0.1%

TritonX-100 for 10 minutes followed by blocking with 2% BSA for 45 minutes at RT. After overnight primary antibody incubation at 4°C washed cells were then incubated with fluorescent-conjugated secondary antibodies at RT for 2 hours, followed by DAPI staining for 5 min at RT. After washing, cells were mounted with anti-fade mounting medium on glass slides and viewed under an inverted confocal laser scanning microscope (Zeiss Meta 510LSM; Carl Zeiss, Jena, Germany). Plan Apochromat 63X/1.4 NA Oil DIC objective lens was used for imaging and data collection. Appropriate excitation lines, excitation and emission filters were used for imaging.

Transmission Electron Microscopy (TEM)

Cells were fixed in 2.5% Glutaraldehyde in phosphate buffer, pH 7.4. Post-fixation was performed in 1% OsO4 and encapsulated in agarose. Cells were subsequently dehydrated in acending series of ethanol and embedded and polymerised in Spurr resin. Ultrathin sections were obtained using a Leica UC7 ultramicrotome and double stained with uranyl acetate and lead citrate. 150 mesh copper grids were used to pick up the sections. Grids were observed under a Jeol JEM-1400 Transmission Electron Microscope at 80 kV after proper alignment. Images were recorded using a Gatan Orius SC200 CC camera. At least 200 cells were analyzed from 4 grids for each set.

5. SUPPORTING INFORMATION

The characterization data of all new compounds are attached.

6. ACKNOWLEDGMENTS

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New Spisulosine Derivative Promotes Robust Autophagic Response to Cancer Cells

Asha Ganesher[⊥,a], Priyank Chaturvedi[⊥,b], Rohit Sahai[c], Sanjeev Meena[b], Kalyan Mitra[c], Dipak Datta*[,b], Gautam Panda*[,a]

[a] Medicinal and Process Chemistry Division, CSIR-Central Drug Research Institute, Sitapur Road, Jankipuram Extension, Lucknow 226031, UP, India
[b] Division of Cancer Biology, CSIR-Central Drug Research Institute, Sitapur Road, Jankipuram Extension, Lucknow 226031, UP, India
[c] Electron Microscopy Unit, Sophisticated Analytical Instrument Facility, CSIR-Central Drug Research Institute, Sector-10, Jankipuram Extension, Lucknow, 226 031, UP, India

Phone: 915222772450, ext 4659; Fax: 915222771941

- **Highlights**
  New Spisulosine Derivative as novel autophagy inducer.
- Robust autophagic cell death in diverse cancer cells
- sparing normal counterpart

Declaration of interests

√The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Asha Ganesher
Priyank Chaturvedi
Rohit Sahai
Sanjeev Meena
Kalyan Mitra
Dipak Datta
Gautam Panda