Letter

Synthetic sphingolipids with 1,2-pyridazine appendages improve anti-proliferative activity in human cancer cell lines

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ACS Med. Chem. Lett., **Just Accepted Manuscript** • DOI: 10.1021/acsmedchemlett.9b00553 • Publication Date (Web): 12 Feb 2020

Synthetic sphingolipids with 1,2-pyridazine appendages improve anti-proliferative activity in human cancer cell lines

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KEYWORDS Sphingolipid, anti-cancer, KRAS mutant cancer, nutrient transporter, vacuolation.

ABSTRACT. A synthetic sphingolipid related to a ring-constrained hydroxymethyl pyrrolidine analog of FTY720 that was known to starve cancer cells to death was chemically modified to include a series of alkoxy tethered 3,6- substituted 1,2-pyridazines. These derivatives exhibited excellent anti-proliferative activity against 8 human cancer cell lines from 4 different cancer types. A 2.5-9 fold reduction in IC_{50} in these cell lines was observed relative to the lead compound which lacked the appended heterocycle.

Sphingolipids are a family of evolutionarily-conserved natural compounds that play essential roles in the life cycle of cells.[1–3] A subset of sphingolipids, such as phytosphingosine, slow cell growth, induce differentiation, and trigger cell death (Figure 1, compound **1**). As part of the adaptive response to stress, compound **1** down-regulates nutrient transporters in yeast, reducing access to amino acids and uracil and inducing a stress-resistant quiescent state.[4] Although mammalian cells do not produce phytosphingosine, the sphingolipid ceramide, a 4,5-unsaturated congener, has a similar effect on mammalian nutrient transporters for amino acids and glucose.[5,6] While limiting nutrient access produces adaptive quiescence in normal cells, the oncogenic mutations in cancer cells constitutively drive growth, rendering transformed cells hypersensitive to interruptions in the nutrient supply.[7,8] Indeed, many standard of care chemotherapies target biosynthetic pathways and cancer therapies that disrupt anabolism are under development.[9]

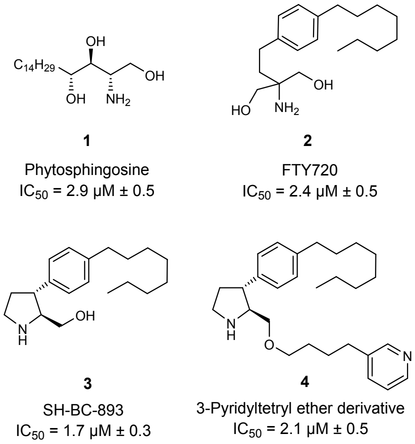


Figure 1. Structures of anti-proliferative synthetic compounds related to sphingosine (**1** – **3**); 3-pyridyl appended analog of **3** (**4**). IC_{50} in FL5.12 cells shown.

Our group has developed synthetic sphingolipid analogs that recapitulate the ability of natural sphingolipids to starve cancer cells to death.[8,10,11] Being cognizant of the challenges associated with using a natural molecule like ceramide as a drug (e.g. poor solubility and rapid metabolism), we turned to the synthetic sphingolipid FTY720 (Fingolimod). At higher doses than are required to affect sphingosine-1-phosphate receptors, FTY720 limits nutrient access and starves cancer cells to death.[10,11] In addition to reducing the levels of glucose and amino acid transporters on the cell surface, FTY720 also disrupts lysosomal fusion reactions.[6,11] This disruption, visualized as cellular vacuolation, additionally limits access to nutrients acquired from the digestion of LDL particles, autophagosomes, and macropinosomes.[8,11,12] Despite its activity in multiple pre-clinical cancer models, FTY720 cannot be repurposed as a cancer therapy because the phosphorylated form triggers severe bradycardia at the doses required to kill neoplastic cells.[10,13–16]

We have reported that the synthetic sphingolipid compound **3** (SH-BC-893), a constrained variant of FTY720, exhibits favorable activity as an anti-cancer agent without the negative cardiovascular effects associated with FTY720.[11,16,17] Like FTY720, compound **3** triggers the internalization of nutrient transporters and blocks lysosome-dependent nutrient generation pathways.[6,11] Compound **3** significantly inhibits the growth of colon cancer xenografts and autochthonous prostate tumors in a genetically modified mouse model.[11] Importantly, compound **3** affects both normal and transformed cells, but is only toxic to tumor cells. Compound **3** did not disrupt normal organ function, cause bone marrow suppression, or negatively affect the rapidly proliferating cells of the gut even upon chronic administration of the anti-cancer dose.[11] Thus, compound **3** is both efficacious and well-tolerated, at least in mice.

These results showed the benefits and feasibility of concurrently blocking parallel nutrient access pathways that are essential for proliferating cancer cells. Because compound **3** simultaneously reduces access to glucose, amino acids, and cholesterol,[6,11] it is expected to be effective even against heterogeneous tumors and may be less susceptible to the rapid development of resistance observed with many targeted therapies. While it has many activities desirable in an anti-cancer agent, the low micromolar potency exhibited by compound **3** might be improved by additional derivatization.

In previous studies, we delineated the structural and functional features in compound **3** that contributed to cytotoxicity.[18] The phenyloctyl appendage was found to be susceptible to changes in the length of the octyl chain and the presence of polar groups, except at the benzylic position. Extension of the hydroxymethyl group to include alkyl ethers containing heterocyclic moieties such as a 3-pyridyl appended variant (Figure 1, compound **4)** was tolerated.[19] We were intrigued that the inclusion of a 3-pyridyl unit as an appendage did not negatively affect the cytotoxicity of the original compound **3**.

In an effort to further explore the effects of heteroaromatic appendages, we turned our attention to 1,2-pyridazine as a heterocycle that has found many applications as a versatile biological probe.[20–26]

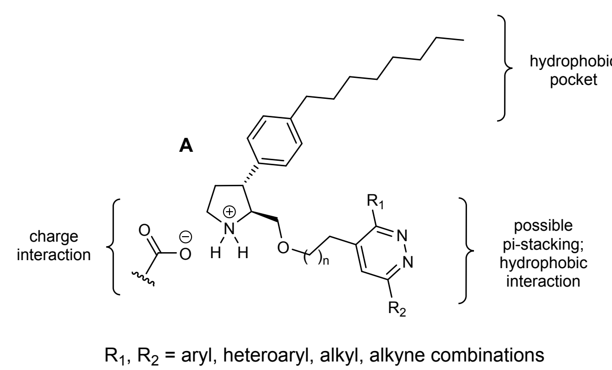
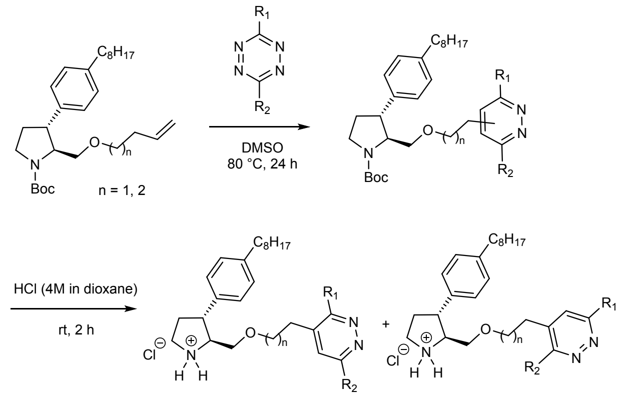


Figure 2. Hypothetical interactions of compound **3**/pyridazine ether-appended constructs (**A**). Only one regioisomer is shown in the 1,2-pyridazine ring.

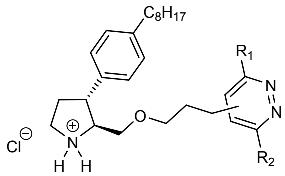
In view of the presence of an extended (or folded) hydrophobic chain, and an appended aromatic heterocycle anchored on a basic pyrrolidine core, it is possible to envisage certain hypothetical interactions with a biological target as exemplified by structure **A** in Figure 2. The synthetic protocol we chose allowed for the generation of a series of 3,6-disubstituted 1,2-pyridazines appended to the lead compound **3** via a 2-3 carbon chain ether linker. We further surmised that the relatively flexible single bonds of the 3,6-substituents would adopt conformationally favorable spatial orientations. Although we have previously established that compound **3** activates PP2A[6,11,27], we do not as yet know the mechanism by which this occurs.

With this premise in mind we developed a strategy that engaged the terminal olefinic ether appendages of **3** with 3,6-disubstituted 1,2,4,5-tetrazines in an inverse electron demand Diels–Alder reaction to give the corresponding 3,6 disubstituted 1,2-pyridazines[28] as 1:1 mixtures of 4,5-regioisomers (Scheme 1). Cytotoxicity, nutrient transporter down-regulation, and vacuolation induced by the products were then measured in FL5.12 murine hematopoietic cells, a cell line that grows in suspension and is therefore well-suited to flow cytometry-based assays for transporter loss. Cytotoxicity was also evaluated in a panel of human cancer cell lines for compounds of interest (see Figure 3 and Supplementary Table 3)**.**



Scheme 1. General scheme for the synthesis of the 3,6-disubstituted 1,2-pyridazine derivatives as 1:1 mixtures of 4,5-regioisomers.

Table 1 lists the activity of a variety of analogs. We were pleased that a 1:1 mixture of regioisomers of compound **5** having methyl and 1,3-pyrimidine substituents on the 1,2-pyridazine core surpassed the activity of the parent **3**, reaching the high nanomolar threshold for the first time in this series. In fact, the two regioisomers **5a** and **5b** could be separated by chromatography and exhibited IC_{50} values of 0.5 and 0.7 μM respectively. Despite its increased potency in cytotoxicity assays, compound **5** did not down-regulate amino acid transporters or vacuolate FL5.12 cells at the concentrations that killed cells; 10 μM was required to observe these effects (See Supplementary Table 1). However, it is of particular interest that compound **5** efficiently triggered transporter loss and vacuolation in MDA-MB-468 breast cancer cells (See Supplementary Figure 1). These discrepancies may arise from differences in the subunit composition of PP2A heterotrimers, post-translational modification of PP2A subunits, and/or the subcellular localization of PP2A in these various cell lines. In sum, given its ability to fully phenocopy the PP2A-dependent effects of compound **3** in cancer cells, it is likely that compound **5** shares the PP2A-related target of compound **3**. On the other hand, the ability of compound **5** to efficiently kill FL5.12 cells without inducing transporter loss or vacuolation suggests for the first time that there may be another target of this chemical series that makes an important, and partially redundant, contribution to its anti-cancer effects. On-going target deconvolution studies are expected to provide insight on these points.



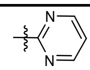
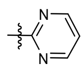
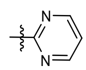
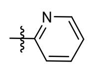
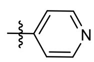
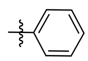
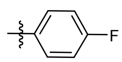
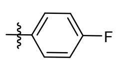
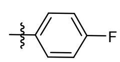
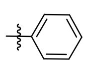
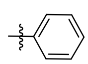
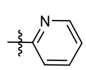
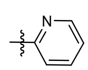
Comp.	n	R_1	R_2	IC_{50}
5a	2	CH_3		0.5 µM ± 0.2
5b	2		CH_3	0.7 µM ± 0.4
6	1	CH_3		1.7 µM ± 0.4
7	2	CH_3		1.6 µM ± 0.5
8	2	CH_3		1.3 µM ± 0.4
9	2	CH_3		2.8 µM ± 0.4
10	2	CH_3		3.3 µM ± 0.3
11	2	CH_3	CH_3	1.3 µM ± 0.3
12	2			1.8 µM ± 0.6
13	2			1.4 µM ± 0.3
14	2			0.9 µM ± 0.1

Table 1. Cytotoxicity of the synthetic pyridazine derivatives in FL5.12 cells. Values represent mean ± SD. Compounds **6-10** are 1:1 mixtures of 4- and 5- substituted isomers. The IC_{50} value for a 1:1 mixture of **5a** and **5b** was 0.9 µM ± 0.1

Although there was a clear preference for a methyl and a pyrimidine group attached to the 3- and 6- positions of the pyridazine in analogs **5a** and **5b** compared to all the others in Table 1, the differences in IC_{50} values only varied by a factor of 5 for the least active compound (**10**). Decreasing the number of carbons in the linker from 3 to 2 as in compound **6** resulted in a 3-fold increase in IC_{50} compared to **5a** or **5b** without affecting its ability to down-regulate nutrient transporters or to vacuolate (Supplemental Table 1). The relatively non-remarkable SAR within this limited series of derivatives suggests that the central pyridazine core with a 3,6-substitution pattern of small or large groups such as methyl and aryl or heteroaryl respectively is well-tolerated. Importantly, compounds consisting of only the pyridazine moiety (**15**), and compound **6** devoid of the phenyloctyl chain (**16**), were inactive (Figure 3, Table 2, Supplementary Table 2). However, compound **17** with the ether linker but without the pyridazine minimally affected the cytotoxicity of **3**. Clearly, adding the pyridazine moiety to the linker in compound **17**, particularly exemplified by compounds **5a** and **5b** (or the 1:1 mixture of regioisomers), improves the activity of the current lead compound **3**.

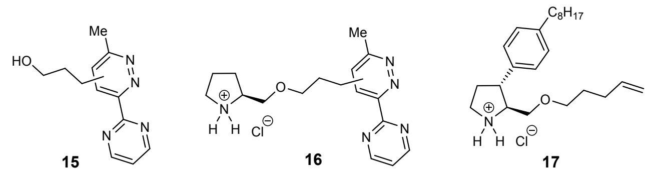


Figure 3. 1,2-Pyridazine derivatives and a 1-butenyl ether of **3** for comparisons of biological activities.

Comp.	IC_{50}
3	1.7 ± 0.3
15	> 40
16	> 40
17	2.9 ± 0.2

Table 2. Cytotoxicity of pyridazine control compounds in FL5.12 cells. Values represent mean ± SD.

Encouraged by the improved cytotoxicity of compound **5** in FL5.12 cells, we conducted additional tests to evaluate its anti-neoplastic potential compared to the parent compound **3** in 8 human cancer cell lines from 4 different cancer types. We were pleased to find that the superior activity of compound **5** was indeed reproduced. In fact, as a 1:1 mixture of isomers, compound **5** was consistently more potent than the parent compound **3**, with IC_{50} values from ~2.5 to 9-fold lower than **3** (Figure 4), substantiating the role of the pyridazine core unit as a preferred heterocyclic appendage. We further confirmed that, as in FL5.12, the presence of a pyridine (compound **4**) is not sufficient for increased potency, and that the nature of the substituents on the pyridazine plays a role in activity (compound **5** vs **9)** in HCT116 colon cancer cells (see Supplementary Table 3). Finally, it is worth noting that in 3 KRAS mutant cancer cell lines, compound **5** is between 7 and >20 times more potent than the published small molecule activator of PP2A (SMAP), which is reported to inhibit proliferation of KRAS mutant lung cancers[29] (see Supplementary Figure 2). Even the parent compound **3** is at least 2-fold more potent than SMAP in these cell lines.

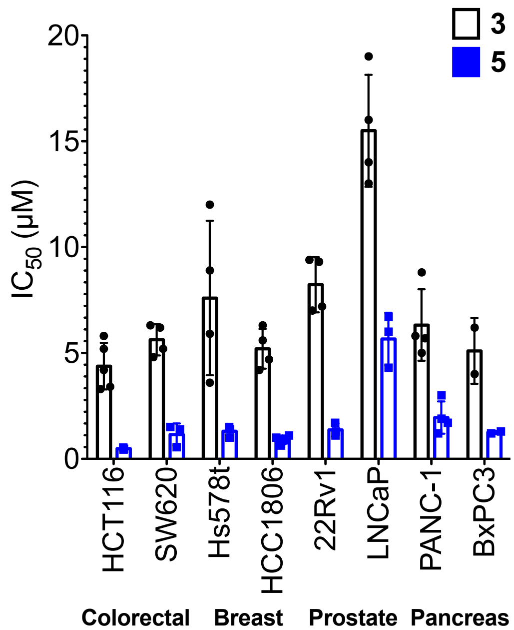


Figure 4. Compound **5** has a lower IC_{50} than compound **3** across human cancer cell lines.

In conclusion, we have established a novel series of synthetic sphingolipids related to a lead compound **3**, with appended 1,2-pyridazine units bearing substituents at the 3,6-positions via a 3-carbon ether linker. While a combination of substituents were tolerated in maintaining cytotoxicity against FL5.12 cells, inclusion of a methyl and a pyrimidinyl group proved to be particularly beneficial in rendering high nanomolar cytotoxicity for the first time in this series. Extension of these activities to selected human cancer cell lines at the nanomolar level augurs well for the development of well-tolerated cancer therapies that target multiple nutrient import pathways to starve cancer cells to death. Studies are in progress to identify the specific cellular targets of these structurally related natural and synthetic sphingolipids. The 3,6-disubstituted 1,2-pyridazines developed here will be invaluable in these efforts to pinpoint the protein targets of the anti-proliferative compound **3** and its analogs.

ASSOCIATED CONTENT

The Supporting Information contains the biological and chemical methods and chemical experimental procedures and is available free of charge via the internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

The Montreal group thanks NSERC for financial assistance. Funding for A.L.E. was provided through a grant from the Chao Family Comprehensive Cancer Center Anti-Cancer Challenge.

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