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Design, synthesis and anticancer activity of constrained sphingolipid-phenoxazine/phenothiazine hybrid constructs targeting protein phosphatase 2A

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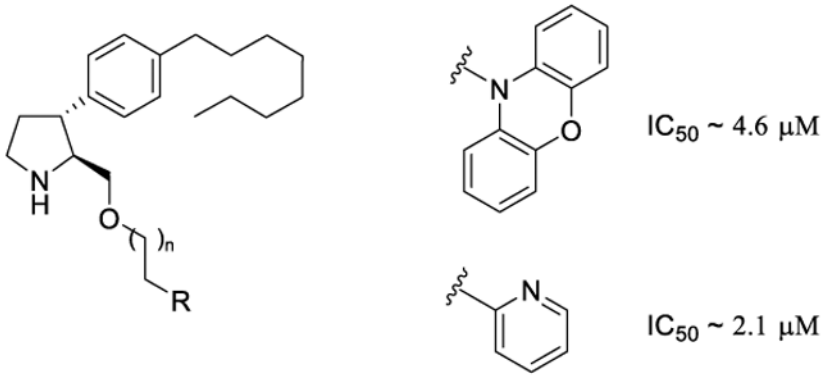
Abstract

Inspired by the cytotoxicity of perphenazine toward cancer cells and its ability to activate the serine/threonine protein phosphatase 2A (PP2A), we prepared series of ether-carbon linked analogs of a constrained synthetic sphingolipid analog **3,** known for its cytotoxicity, nutrient transporter down-regulation and vacuolation properties, incorporating the tricyclic neuroleptics phenoxazine and phenothiazine to represent hybrid structures with possible synergistic cytotoxic activity. While the original activity of the lead compound **3** was diminished by fusion with the phenoxazine or phenothiazine tethered moieties, the corresponding 3-pyridyltetryl ether analog **10** showed cytotoxicity and nutrient transporter down-regulation similar to the lead compound although it separated these PP2A-dependent phenotypes from that of vacuolation.

Graphical Abstract



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Keywords

sphingolipid; nutrient transport down-regulation; cytotoxicity FL5.12; hybrid structures; vacuolation

Endogenous sphingolipids including ceramides and sphingosine-1-phosphate are natural components of mammalian cells that have both pro-growth and pro-apoptotic functions.[1] Sphingolipids modulate a number of signaling processes that regulate the life cycle of cells.[1-3] Among the effects of sphingolipids is their ability to regulate the activity of the serine/threonine protein phosphatase 2A (PP2A).[1] The potential of small molecules to restore the tumor suppressor ability of functionally altered PP2A in cancer cells is of great interest.[4-6]

Sphingolipids such as ceramide (**1**) contribute to cell death in part by removing nutrient transporters from the cell surface downstream of PP2A activation, leading to death by starvation (Figure 5).[5, 7-10] In normal cells, nutrient stress triggers adaptive quiescence, prolonging their survival, while cancer cells that are locked in a rapidly proliferating state by oncogenic mutations experience bioenergetic crisis when they fail to undergo growth arrest.

While biologically active and tumor suppressive, ceramide is difficult to apply clinically due to its insolubility in water and ready metabolism.[1] The synthetic sphingolipid analogue FTY720 (**2**), originally conceived as an immunosuppressant and currently marketed for the treatment of multiple sclerosis under the trade name Gilenya, was found to possess anticancer activity *in vitro* and *in vivo*.[8, 11-15] However, FTY720 induces severe bradycardia at the anticancer dose, which has been attributed to activation of $S1P_1$ and $S1P_3$ receptors.[9, 16-18]

In previous reports from our laboratory we described constrained analogs of FTY720 with remarkable selectivity toward sphingosine-1-phosphate receptors ($S1P_{1-5}$).[19] More recently, we have reported structurally related 2-hydroxymethyl-3-aryloctylpyrrolidines, represented by a pharmacologically viable analog (**3**), which possesses highly favorable properties as an anticancer agent *in vitro* and *in vivo*.[5, 9] This water soluble, orally bioavailable compound efficiently down-regulates nutrient transporters, kills cells at single-digit μM doses, and even when phosphorylated, does not activate $S1P_1$ or induce bradycardia in mice, avoiding the dose-limiting toxicity that precludes re-purposing of FTY720 for cancer.[5, 9] An added feature is the ability of the lead compound **3** to cause a PP2A-dependent disruption in lysosomal fusion, visualized as extensive cytosolic vacuolation (Figure 2). Preventing degradation of endosomally delivered macromolecules in the lysosome restricts cellular access to nutrients gained by processes such as macropinocytosis and autophagy and contributes to the anticancer activity of **3** *in vitro* and *in vivo*.[5, 20] Furthermore, both nutrient transporter loss and vacuolation phenotypes are reversed by known inhibitors of PP2A such as Calyculin A, okadaic acid, LB100, and the protein inhibitor SV40 small t (Calyculin A data shown in Figures 2 and 5).[5, 9, 20, 21] As would be expected for a key phosphatase, PP2A inhibition kills cells within several hours, precluding direct assessment of PP2A's role in cytotoxicity. However, it is clear that the metabolic effects of PP2A-dependent vacuolation and nutrient transporter loss are an important part of the mechanism of action of **3**, as cells can be protected from death by the addition of cell-permeant nutrients[5].

Activation of PP2A has also been reported with a series of tricyclic neuroleptics such perphenazine (**4)** [22] and by a synthetic compound incorporating a phenoxazine moiety lacking a basic nitrogen (**5**) (SMAP)[23] (Figure 3). We were intrigued that a functionally important cellular enzyme such as PP2A was activated by compounds with such diverse chemical structures as **3** and the tricyclic neuroleptic **4** in which a piperazine unit was linked to a phenothiazine via a three carbon chain. With the likely premise that the lead compound **3** and perphenazine **4** were interacting at different sites within PP2A, we considered the synthesis of hybrid molecules in which the C-2 hydroxymethyl group in **3** was extended with 3-5 carbon alkyl ether chain tethers containing terminally situated phenoxazine and phenothiazine cores (Figure 4).

If these compounds bind PP2A at different sites, linking these two activators could potentially increase avidity above either parent compound alone, enhancing potency. We have previously shown that the positively charged nitrogen is critical for the activity of **3**.[9, 24] A simple overlay of the structures of perphenazine on the intended hybrid shown as in Figure 4A indicated a spatially viable possible juxtaposition of the basic nitrogen atoms. It remained to be determined if the hydrophobic aryloctyl appendage, which is an important component of the lead compound **3**, would also be beneficial for the intended hybrid structures. To this end we prepared compounds **6-9** containing phenoxazine and phenothiazine appended units with varying chain lengths (Figure 4).

We were curious to see if the presence of the phenoxazine or phenothiazine moiety in such hybrids would enhance the activity of **3** in a synergistic manner. As potential markers for the anticancer activity of these novel hybrid compounds, we would determine their cytotoxicity and monitor the PP2A-dependent phenotypes of nutrient transporter down-regulation and cytoplasmic vacuolation[5, 21].

Results

Given that our lead compound **3**, perphenazine (**4**), and SMAP (**5)** have all been reported to activate PP2A,[5, 9, 22, 23] we first sought to compare their ability to kill murine hematopoietic FL5.12 cells, which exhibit a rapid, cancer-like metabolism when supplied with high levels of the cytokine IL-3. While all of the compounds tested were less cytotoxic than **3**, the SMAP compound **5** was at least 8-fold less active, with an IC_{50} value of 15 μM (Figure 5 and Table 1).

We next determined the degree to which these compounds could induce surface nutrient transporter loss and vacuolation. Transporter loss was evaluated by measuring surface levels of the amino acid transporter-associated protein CD98 in cells treated with 10 μM compound, a concentration at which **3** achieves maximum down-regulation. Despite its slightly higher IC_{50}, perphenazine (**4**) matched the activity of **3** in CD98 down-regulation assays, while SMAP (**5**) did not reduce surface CD98 at 10 μM, consistent with its lower potency (Figure 5A, Table 1). However, all three compounds down-regulated CD98 similarly at concentrations equal to twice the IC_{50}, suggesting an overlap in mechanism of action. Furthermore, this phenotype was PP2A-dependent, as the PP2A inhibitor Calyculin A protected from CD98 loss in all three cases (Figure 5B). Interestingly, neither perphanazine

nor SMAP vacuolated this cell line at 10 μM (Figure 5A, Table 1). We have previously reported that the endogenous PP2A activator ceramide also fails to produce vacuolation and in fact protects from **3**-induced vacuoles[21].

Incorporation of the phenoxazine or phenothiazine moiety as in compounds **6**-**9** decreased the activity of the parent **3**. The degree to which the phenoxazine moiety reduced activity depended on the length of the alkyl chain. Compounds with a 3-4 carbon linker such as **6** and **7** had IC_{50} values approximately 2-3 times higher than **3** and induced modest nutrient transporter down-regulation at 10 μM. Increasing the chain length as in **8** and **9** was even more detrimental to CD98 loss and cytotoxicity (Figure 6, Table 1).

It was evident that appending tricyclic heterocycles such as phenoxazine and phenothiazine as hybrid structures was not beneficial to the intrinsic activity of the lead compound **3**. In a previous study involving modifications of the octyl aryl appendage,[25] we realized the importance of maintaining its hydrophobic character, since the introduction of polar groups resulted in substantial loss to cytotoxicity. However, it now appears that appending ether-linked heterocyclic units at the 2-hydroxymethyl group as in compounds **6**-**7** in the current series, resulted in only a 2-4 fold loss of cytoxicity and modest down-regulation at 10 μM compared to the lead compound **3**. This result encouraged us to probe the tolerance of side-chain appendages with less rigid and spatially more favorable monocyclic heteroaromatics containing a basic nitrogen atom. To test the hypothesis, we chose to attach 3-pyridyltetryl and 5-pyrimidinyl tetryl units to the extremity of the 2-hydroxymethyl group as first generation probes. Compounds **10**-**12** were easily prepared from a 4-carbon chain aldehyde derived from N-Boc **3** and Grignard reagents prepared *in situ* from the corresponding heteroaromatic iodides (see SI). Interestingly, compounds **10**-**12** maintained low micromolar cytotoxicity and good down-regulation, but lacked the ability to vacuolate, indicating that this phenotype was sensitive to functional variations compared to the lead compound **3** (Figure 7-8, Table 1). Of further relevance, removal of the benzylic hydroxyl group as in the 3-pyridyltetryl ether analog **10** in particular, maintained good cytotoxicity and nutrient transporter down-regulation compared to **3**. As with **3** and the other reported PP2A activators, Calyculin A successfully protected from nutrient transporter loss (Supplementary Figure 1).

Conclusion

Inspired by the anticancer activity of perphenazine and its activation of PP2A, we synthesized series of analogs of the synthetic constrained cytotoxic sphingolipid **3** in which tricyclic neuroleptic heterocycles phenoxazine and phenothiazine were appended to the hydroxymethyl group as ether linked moieties. Cytotoxicity was at best maintained and often dramatically decreased compared to **3**, suggesting that the incorporation of flat tricyclic heteroaromatic moieties as hybrid constructs with the aim of achieving synergistic cytotoxicity by combining structural elements of multiple PP2A activators was not a viable strategy. However, appending monocyclic heteroaromatics such as a 3-pyridyl unit attached by a 4-carbon chain as an ether linkage exemplified by compound **10,** maintained excellent levels of cytotoxicity and nutrient down-regulation at lower doses than **11** or **12**. This augurs well for diversification of the lead compound **3** with other heterocyclic entities attached as

alkyl ethers and the prospects of improved potency and nutrient transport down-regulation as anticancer agents. Moreover, the selective loss of vacuolation capability may render compound **10** a useful tool to study the cellular factors involved in producing this phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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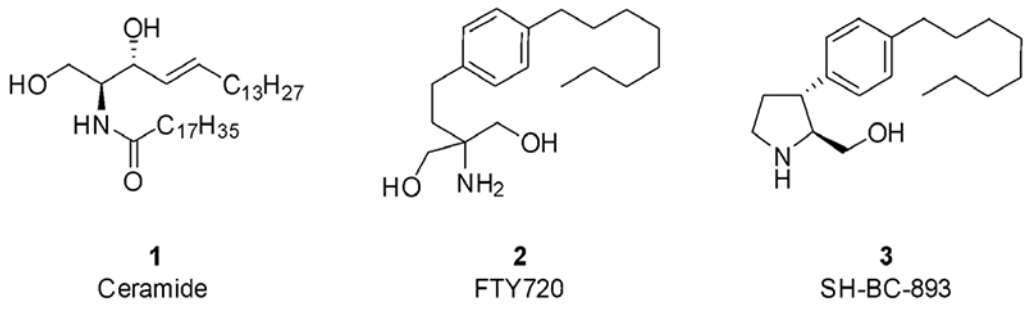


1 Ceramide **2** FTY720 **3** SH-BC-893

Figure 1.
Structures of natural and synthetic anti-proliferative compounds.

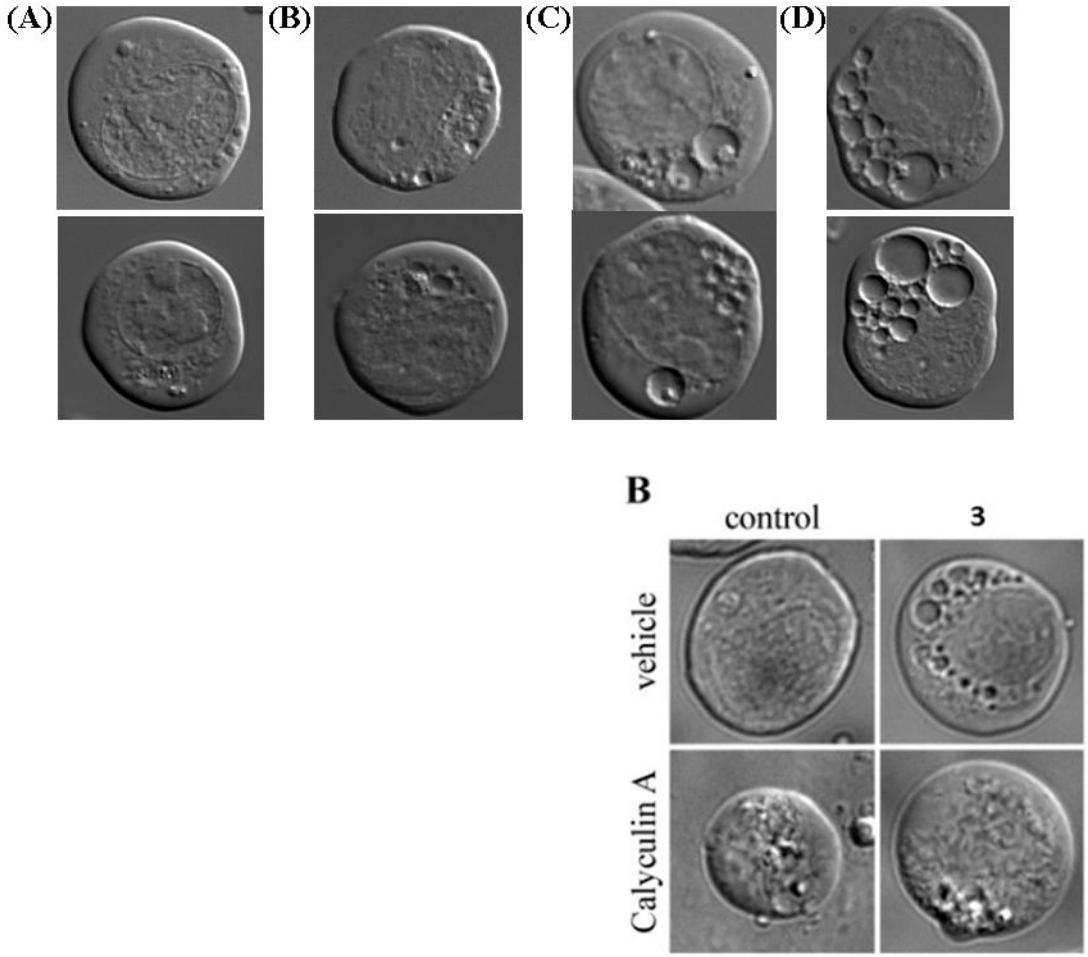
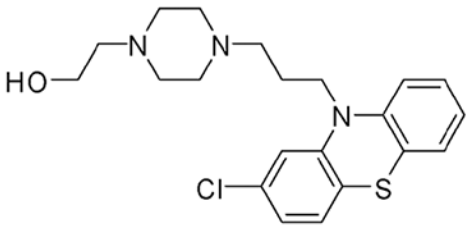
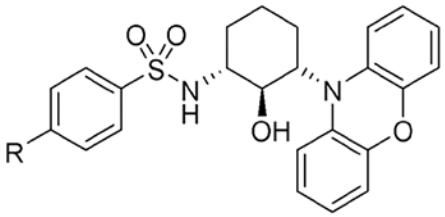


Figure 2.

Vacuolation in FL5.12 cells. (A) Two representative vacuolation images of FL5.12 cells with vacuolation scores of: (i) 0 (no vacuoles); (ii) 1 (a few small vacuoles; (iii); 2 (multiple vacuoles); (iv) 3 (multiple large vacuoles). (B) Calyculin A protects from vacuolation caused by **3**.

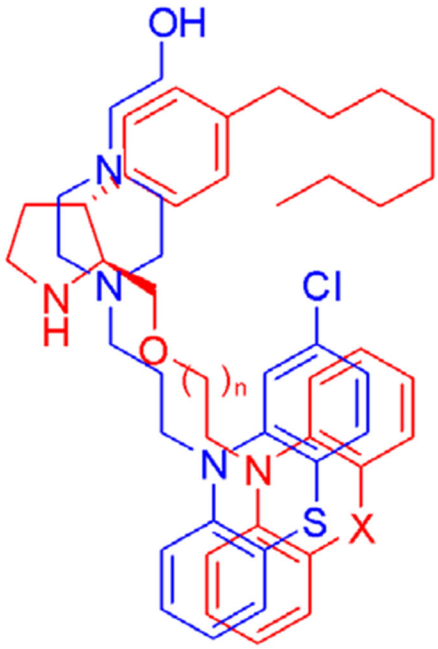


4
Perphenazine



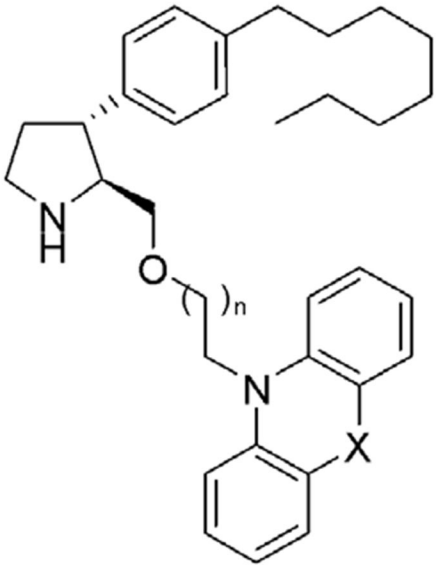
5
SMAP

Figure 3.
Structures of known PP2A activators.



A

Expression A is the overlay of the intended hybrid (red) and perphenazine (blue).



6: n = 2, X = O
7: n = 3, X = O
8: n = 4, X = O
9: n = 4, X = S

Figure 4.
Structures of synthetic hybrid compounds **6-9**.

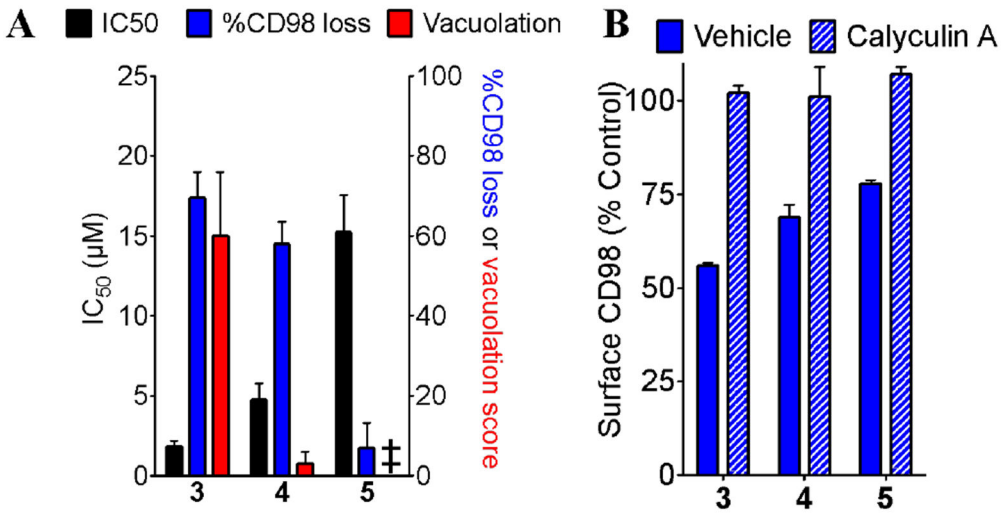


Figure 5.

Perphenazine (**4**) and SMAP (**5**) are less potent than **3** but share PP2A dependence. (A) IC_{50}, nutrient transporter loss, and vacuolation of PP2A activators **3-5**. ‡Denotes a score of 0. (B) Nutrient transporter loss at 2x IC_{50} of PP2A activators shown in (A) ± pretreatment with 5 nM Calyculin A. Data in (A) are means ± S.D. and data in (B) are means ± range.

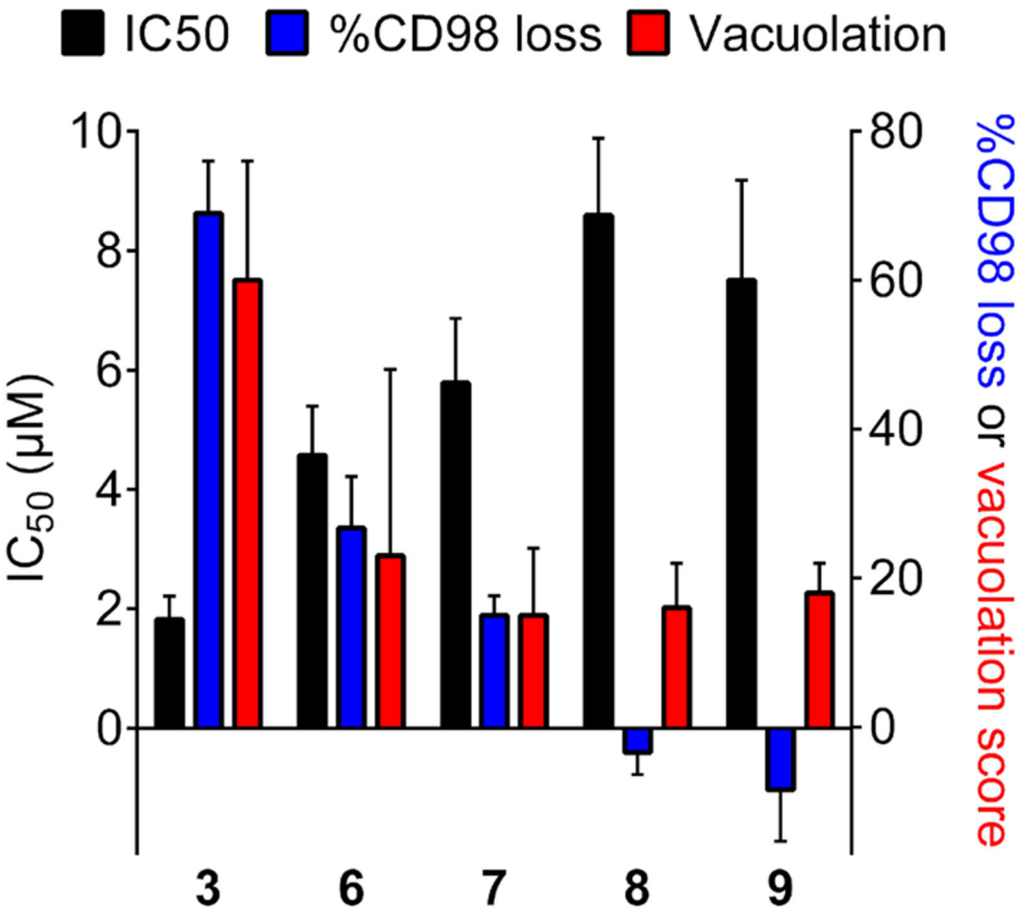


Figure 6.

IC_{50}, nutrient transporter loss and vacuolation of synthetic hybrid compounds with increasing linker length. Data shown are means ± S.D.

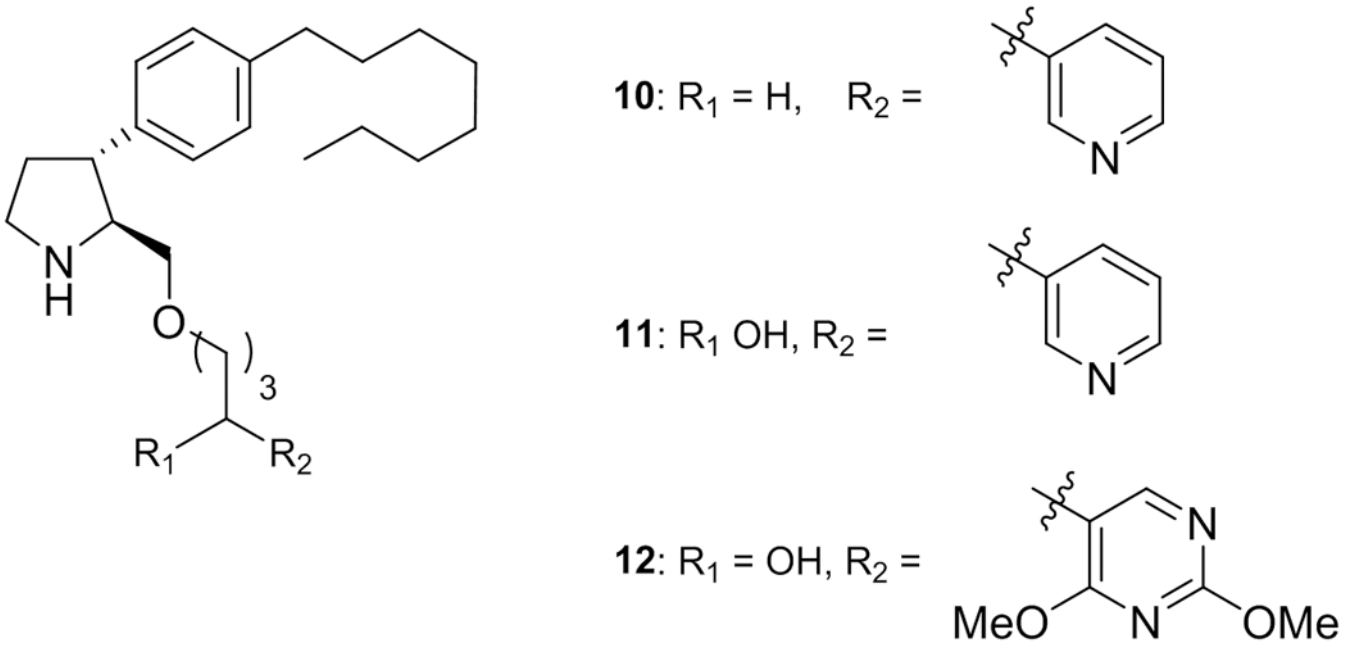


Figure 7.
Structures of heteroaromatic linked analogs of **3**.

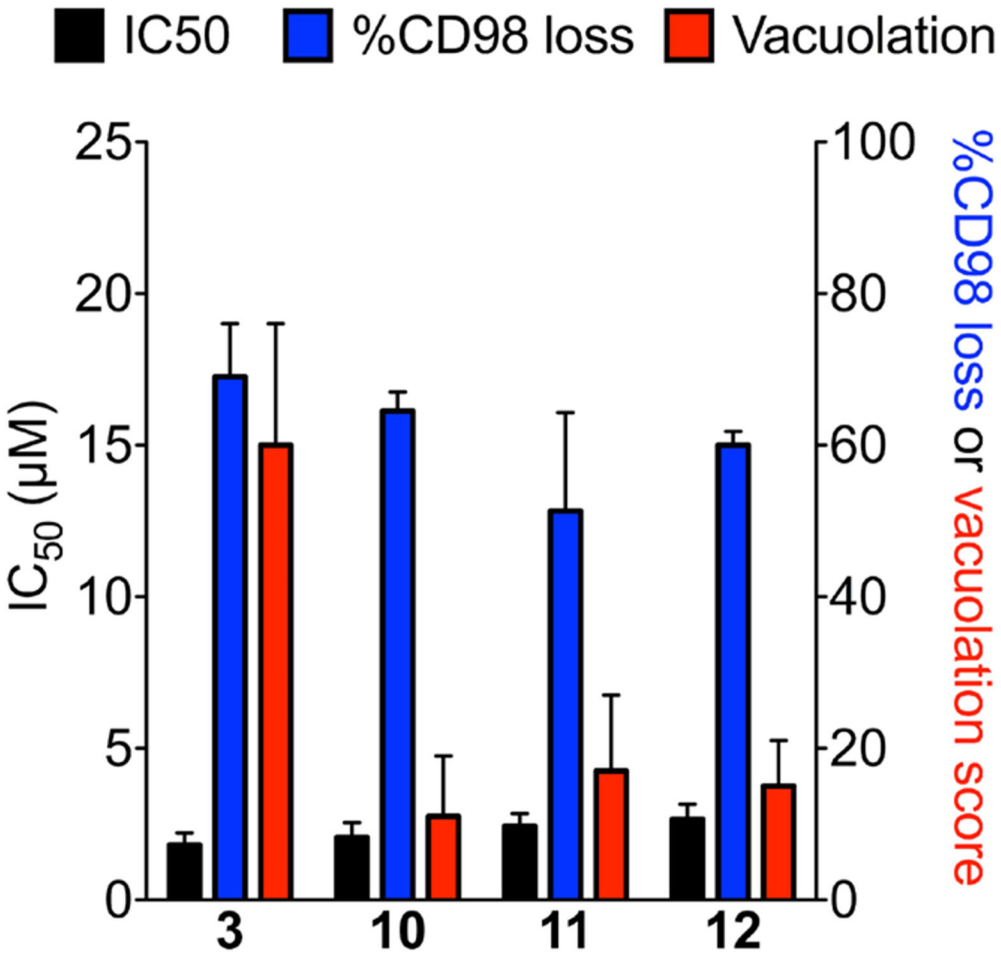


Figure 8.

IC_{50}, nutrient transporter loss, and vacuolation of heteroaromatic linker analogs of **3**. Data shown are means ± S.D.

Table 1.

IC_{50}, nutrient transporter down-regulation, and vacuolation scores in FL5.12 cells.

Comp	IC50(μM) ± SD	%CD98 down-regulation ± SD			Vacuolation score ± SD		
		2.5 μM	10 μM	40 μM	2.5 μM	10 μM	40 μM
3	1.8 ± 0.4	32 ± 6	69 ± 7	n.d.	46 ± 12	60 ± 16	n.d.
4	4.8 ± 1.1	19 ± 5	58 ± 6	n.d.	1 ± 2	3 ± 3	n.d.
5	15.2 ± 2.3	n.d.	7 ± 6	31 ± 8	n.d.	0 ± 0	11 ± 1
6	4.6 ± 0.8	4 ± 5	27 ± 7	n.d.	2 ± 2	23 ± 25	n.d.
7	5.8 ± 1.1	5 ± 3	15 ± 3	n.d.	7 ± 11	15 ± 9	n.d.
8	8.6 ± 1.3	n.d.	−3 ± 3	38 ± 4	n.d.	16 ± 6	66 ± 8
9	7.5 ± 1.7	n.d.	−8 ± 7	29 ± 6	n.d.	18 ± 4	42 ± 20
10	2.1 ± 0.5	40 ± 1	64 ± 3	n.d.	12 ± 10	11 ± 8	n.d.
11	2.4 ± 0.5	24 ± 5	51 ± 13	n.d.	8 ± 5	17 ± 10	n.d.
12	2.7 ± 0.5	10 ± 1	60 ± 2	n.d.	8 ± 4	15 ± 6	n.d.