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Authors

Chen, Bin
Roy, Saurabh G
McMonigle, Ryan J
et al.

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Azacyclic FTY720 Analogues that Limit Nutrient Transporter Expression but Lack S1P Receptor Activity and Negative Chronotropic Effects Offer a Novel and Effective Strategy to Kill Cancer Cells in vivo

Bin Chen[a,‡], **Saurabh G. Roy**[b,‡], **Ryan J. McMonigle**[b], **Andrew Keebaugh**[c], **Alison N. McCracken**[b], **Elizabeth Selwan**[b], **Rebecca Fransson**[a], **Daniel Fallegger**[d], **Andrea Huwiler**[d], **Michael T. Kleinman**[c], **Aimee L. Edinger**[b,*], and **Stephen Hanessian**[a,*]

[a]Department of Chemistry, Université de Montréal, PO Box 6128, Station Centre-Ville, Montréal, QC, H3C 3J7, Canada [b]Department of Developmental and Cell Biology, University of California, Irvine, 2128 Natural Sciences 1, CA 92697-2300, USA [c]Community & Environmental Medicine, University of California, Irvine, FRF 100, CA 92697-1825, USA [d]Institute of Pharmacology, University of Bern, Inselspital INO-F, CH-3010 Bern, Switzerland

Abstract

FTY720 sequesters lymphocytes in secondary lymphoid organs through effects on sphingosine-1-phosphate (S1P) receptors. However, at higher doses than are required for immunosuppression, FTY720 also functions as an anti-cancer agent in multiple animal models. Our published work indicates that the anti-cancer effects of FTY720 do not depend on actions at S1P receptors, but instead stem from FTY720's ability to restrict access to extracellular nutrients by down-regulating nutrient transporter proteins. This result was significant because S1P receptor activation is responsible for FTY720's dose-limiting toxicity, bradycardia, that prevents its use in cancer patients. Here we describe diastereomeric and enantiomeric 3- and 4-*C*-aryl 2-hydroxymethyl pyrrolidines that are more active than the previously known analogues. Of importance is that these compounds fail to activate S1P1 or S1P3 receptors in vivo but retain inhibitory effects on nutrient transporter proteins and anticancer activity in solid tumor xenograft models. Our studies reaffirm that the anticancer activity of FTY720 does not depend upon S1P receptor activation and uphold the promise of using S1P receptor-inactive azacyclic FTY720 analogues in human cancer patients.

Graphical abstract

Corresponding Authors: Stephen Hanessian, Department of Chemistry -Université de Montréal, Pavillon R. Gaudry, C.P. 6128, Succursale Centre-Ville, Montréal, Québec, H3C 3J7, CANADA. Aimee Edinger, Department of Developmental and Cell Biology, 2128 Natural Sciences 1, University of California Irvine, Irvine, CA 92697-2300, USA.

†Present Addresses: Rebecca Fransson, AstraZeneca, Molndal, SWEDEN

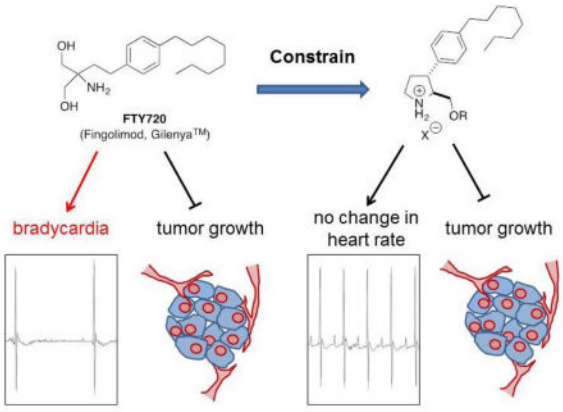
‡These authors contributed equally.

Author Contributions

The manuscript was written by A.L.E and S.H.. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information.



FTY720 (Fingolimod, Gilenya) is the first FDA-approved oral drug for use in multiple sclerosis patients.[1–3] The immunosuppressive activity of FTY720 depends on its ability to activate and persistently down-regulate sphingosine-1-phosphate receptor 1 (S1P1) once it has been phosphorylated by sphingosine kinase 2. As such, FTY720 is a pro-drug that also activates S1P receptor isoforms 3, 4, and 5.[4–6] The immunoregulatory activity of FTY720 results from the sequestration of lymphocytes in secondary lymphoid organs thereby reducing inflammation.[1–3] FTY720 is also a potent anti-proliferative agent, but only at doses above those required for S1P receptor activation and immunosuppression.[7–11] Although effective in animal models, FTY720 cannot be used in human cancer patients. The active, phosphorylated form of FTY720 triggers profound bradycardia through actions on S1P1 in humans and S1P3 in mice at the elevated, anti-cancer dose.[12–14] This known toxicity has marginalized studies assessing FTY720 as an anticancer drug since it was not clear that its antineoplastic activity was independent of its effects on S1P receptors.

Recent studies from our group conclusively demonstrated that FTY720 has anti-cancer activity independent of phosphorylation and S1P receptor activation.[15] Similar to structurally related sphingolipids, FTY720 mimics starvation by down-regulating plasma membrane transporters for amino acids and glucose by activating the serine/threonine phosphatase, protein phosphatase 2A (PP2A).[15–17] These findings suggested that if FTY720 analogues that lack S1P1/3 activity retain the ability to reduce nutrient transporter expression, they might be safe and effective anti-cancer agents with a novel mechanism of action namely, starving cancer cells to death. Here we demonstrate that constraining the aminodiol portion of FTY720 and removing a hydroxymethyl group can separate these two activities, eliminating S1P receptor activity without impeding nutrient transporter loss secondary to PP2A activation.

Results and discussion

In a previous study, we prepared a series of 2,3,5-trisubstituted pyrrolidines as constrained azacyclic analogues of FTY720 represented by a generic pyrrolidine core scaffold **A** (Figure 1). These phosphorylated versions of (2*R*,3*R*,5*R*)-2,5-bis-hydroxymethyl-3-(4-octyl)phenyl pyrrolidine **1** and the corresponding enantiomer **2** exhibited a remarkable selectivity for S1P4 and S1P5 over S1P1 and S1P3 compared to FTY720 phosphate.[18] This observation

affirmed that chemical modification of the conformationally flexible aminodiol portion of FTY720 could lead to selective affinities towards S1P receptors.[19–21]

Recently, we reported on synthetically more accessible constrained analogues of FTY720 in a series of stereo-chemically distinct 2-hydroxymethyl 4-O-arylmethyl pyrrolidines which exhibited remarkable anti-leukemic activity in BCR-Abl-expressing cell lines as exemplified by the (2*R*,4*S*)-analogue **B** (Figure 1).[23] We also demonstrated a stereochemical dependence, since the enantiomeric (2*S*,4*R*)-enantiomer was six times less active. However, this series of constrained FTY720 analogues exhibited much lower potency against other types of tumor cell lines (vide infra). We therefore returned to the original *C*-aryl constrained pyrrolidine analogue series[18] since it displayed S1P isoform selectivity. We prepared compounds **1–4** which represent four out of the eight possible diastereomers and enantiomers [24] as initial probes to evaluate their anticancer activity and to assess possible stereo-chemical preferences as in the *O*-arylmethyl pyrrolidine series [23] relative to FTY720 in PC3 and DU145 cancer cell lines. Although not as potent as FTY720, compounds **1** and **2** limited cell proliferation in a Cell Titer Glo assay that measures ATP content per well as an indicator of live cell mass (Table 1). Once phosphorylated, FTY720 does not induce nutrient transporter loss or kill cells.[1,2] Consistent with this, analogue **2** which is not phosphorylated by either sphingosine kinase 1 or 2 in vitro,[18] was slightly more active than analogue **1** which can be phosphorylated by these enzymes. Two additional new enantiomers **3** and **4** in the bis-hydroxymethyl series (Figure 1), were synthesized and tested for effects on cell growth. Both compounds were similarly active to **1** and **2** in PC3 and DU145 cells in growth and viability assays (Table 1). Thus, the set of four enantiomeric conformationally constrained *C*-aryl pyrrolidine analogues of FTY720 represented by compounds **1–4** retain its anti-cancer activity.

Our goal was to design analogues that maintained anti-cancer activity against a broader panel of cancer cell lines but did not activate S1P1/3 receptors, which are responsible for the dose-limiting toxicity shown by FTY720. Cognizant that the phosphate esters of analogues **1**and **2** were not active at S1P1/3 receptors,[18] we chose to remove one of the 5-hydroxymethyl groups altogether from the generic structure **A** (Figure 1) and to prepare two sets of enantiomeric 2-hydroxymethyl 3-aryl pyrrolidine analogues **5**–**8** of FTY20 as deshydroxymethyl variants of analogues **1**–**4** (Figure 2).

This structural modification did not diminish the activity of the original analogues **1**–**4,** since **5** and **6** were as active as FTY720 in prostate cancer cell lines (Figure 3 and Table 1). To determine whether the charge on the nitrogen in the pyrrolidine ring was important for activity, we also prepared lactams **9**–**11** corresponding to analogues **6**–**8** (Figure 2). This caused a dramatic loss of activity, suggesting that electrostatic interactions with the target may be critical for compound activity as lactams should be equally stable (Figure 3 and Table 1). These findings also suggest that, in contrast to our earlier report of the *O*-arylmethyl pyrrolidine analogues[23] of FTY720, stereochemistry in this series of *C*-aryl analogues is *not* important for interaction with the anticancer target. We therefore did not prepare the remaining diastereoisomers and enantiomers in this series.

Given that analogue **5** reduced prostate cancer cell growth and viability with similar potency to FTY720 (Figure 3), we next tested whether it was phosphorylated by sphingosine kinases and competent to activate S1P receptors. As an immunosuppressant, FTY720 is a pro-drug that is converted to its active form in vivo upon phosphorylation by sphingosine kinase 2.[4–6] Analogue **5** was phosphorylated to similar degree as FTY720 in PC3 and SW620 cells and exported into the medium[24] (Supplemental information).

Because **5** could be phosphorylated, the activity of **5** and its phosphate **5-P** (Figure 1) on S1P receptors was evaluated[24] (Supplemental information). Of significance is that analogues **5** and **6** failed to activate S1P receptors 2, 3, 4, or 5 in cell-based assays and only weakly activated S1P1 at 1000-fold higher doses than S1P. Bradycardia, the dose-limiting toxicity that prevents the use of FTY720 in cancer patients, stems from FTY720-P's actions on S1P1/3.[12–14] Preliminary studies evaluating the phosphates of **5-P** and **6-P** in vitro indicated a loss of activity at S1P3, but possible activity at S1P1 receptors. To evaluate whether these compounds activate S1P1/3 receptors in vivo, we determined the effect of **5** and **5-P** on heart rate (S1P3 dependent effect) and lymphocyte sequestration (S1P1 dependent effect) in mice. While FTY720 reduced heart rate by 50% as expected, neither **5** nor its phosphate **5-P** altered heart rate relative to the vehicle control (Figure 4 and Supplemental information).[24] Circulating numbers of B and T lymphocytes were evaluated 12 h after intraperitoneal injection of FTY720, **5**, or **5-P**. While FTY720 and FTY720-P reduced the number of circulating lymphocytes by more than 90%, neither **5** nor **5-P** caused lymphocyte sequestration[24] (Supplemental information). Together, this in vivo data demonstrates that **5** lacks the dose-limiting S1P1 and S1P3 activities that preclude the use of FTY720 in cancer patients. It is also of interest that phosphorylation of active compounds such as **5** has no detrimental effect on heart rate in mice, unlike the parent FTY720.

Because **5** was phosphorylated in cells, we synthesized the two pairs of enantiomeric pyrrolidine analogues **12**–**15** [24] to eliminate any possibility of in vivo phosphorylation (Figure 5). Interestingly, analogues **12**–**15** were nearly as potent as the parent 2-hydroxymethyl compounds and limited cell growth similar to FTY720 (Table 1). Moreover, **12** and **14** did not activate S1P receptors[24] (Supplemental information). This result further demonstrates that the presence of the hydroxymethyl group and S1P receptor activation is not a critical determinant of anti-cancer activity for this series.

To further evaluate the impact on anti-cancer activity of this series, we synthesized positional isomers with stereochemical variations of the 3-*C*-aryl pyrrolidines **12–15** [24] and evaluated them in cell growth and viability assays. 4-*C*-Aryl pyrrolidines **16** and **17** were as active as 3-*C*-aryl pyrrolidines **12**–**15** and FTY720, while **18** and **19** were active but somewhat less potent (Table 1). These results suggest that the relative positions and stereochemistry of substituents on the pyrrolidine core scaffold in this series do not have a negative effect on anti-cancer activity. In summary, analogues **5**, **6**, and **12**–**15** are good candidates for further investigation as potentially therapeutic anti-cancer compounds in solid tumor models because they lack the toxic S1P receptor activity of FTY720 but retain its anti-cancer effects.

The Cell Titer Glo assay used for compound screening is high throughput but does not discriminate well between compounds that are cytostatic and compounds that are cytotoxic. To determine whether these analogues are cytotoxic, we analyzed cell viability using vital dye exclusion and flow cytometry. The most active compounds (**3**–**6** and **14**) were indeed cytotoxic, triggering cell death in PC3 prostate cancer cells with IC_{50}'s similar to that observed in the Cell Titer Glo assays (Table 1 and Supplemental information). To determine whether the activity of these compounds was restricted to prostate cancer cells, we tested them against cell lines derived from other cancer classes. Compounds that were active in prostate cancer cell lines also killed BCR-Abl-positive leukemia cells with similar potency to FTY720 (Table 2). However, compound **21** [23] (Figure 5), which is a more potent analogue than an FTY720 in SupB15 cells, is also more potent than compounds in the *C*-aryl series in this cell line. It is of interest that compound **5** was more active than compound **21** or **22** in prostate cancer cell lines (Table 1) suggesting that the *C*-aryl series is better suited to use against solid tumors while the *O*-benzyl **21** is the most potent against leukemia. Moreover, unlike the enantiomeric 4-*O*-arylmethyl ether analogues **20** and **21,** 4-*C*-aryl-2-hydroxymethyl pyrrolidines **16**–**19** did not show distinct stereochemical differences in their activities toward BCR-Abl positive leukemia cells.[24]

To further test the potential activity of the series of 3- and 4-C-aryl constrained analogues of FTY720, we extended the panel to colon, lung, pancreas, and breast cancer cell lines. Compounds **5** and **6** were active in these adherent cancer cell lines (Table 2), although like FTY720, they were slightly less active against a lung cancer cell line. The broad activity of the new analogues described herein is consistent with our proposed bioenergetic mechanism of action against cancer cells that includes nutrient transporter down-regulation secondary to PP2A activation. Thus, we evaluated surface levels of the amino acid transporter associated protein 4F2hc following treatment with several analogues. As predicted, compounds **4**, **5**, and **6** reduced 4F2hc surface expression with similar efficacy. Transporter loss was prevented by the selective PP2A inhibitor Calyculin A consistent with a conserved, PP2A-dependent mechanism for cancer cell killing among these analogues (Figure 7)[24].

As a preliminary test of the anticancer activity of these compounds, we compared their effects on colon cancer xenograft growth. Nude mice bearing subcutaneous SW620 xenograft tumors were treated daily with 10 mg/kg FTY720 [15] or 20 mg/kg of the detoxified analogues **5** or **15** by intraperitoneal injection using normal saline as a vehicle. All three compounds inhibited tumor growth by about 50% (Supplemental information)[24]. A minor decrease in body weight was observed in treated animals as expected, given that amino acid and glucose uptake would be compromised in all cells. In tumor samples taken at sacrifice 24 h after the last dose, **5** was present at approximately 20 μM, well above the IC_{50} (Supplemental information)[24]. The phosphorylated form of **5** was present at concentrations below the limit of quantification in vivo and therefore very unlikely to contribute to the anti-tumor effects of **5** (Supplemental information). These results suggest that further in vivo evaluation of compounds **5** and **15** to treat solid tumors is warranted.

In conclusion, we have synthesized a series of stereo-chemically related 4-*C*-aryl-2-hydroxymethyl pyrrolidines as constrained azacyclic analogues of FTY720, and found them to be potent inhibitors of proliferation and survival using human prostate, leukemia, colon,

breast and pancreatic cancer cell lines independent of phosphorylation. Several of the active compounds did not activate S1P receptors 1 and 3 in vivo even when chemically phosphorylated and showed promise as inhibitors of tumor growth. Compound **5** in this series can be considered as a potential broadly active anti-cancer agent against solid tumors while lacking the undesirable negative chronotropic effect known for the parent compound FTY720 and warrants further development. Efforts toward this goal, including more in-depth pharmacokinetics/pharmacodynamics evaluation, optimization of vehicle and dosing schedule, and the identification of possible cellular targets are under way and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

IC_{50}	concentration that inhibited growth or survival by 50%
S1P	sphingosine-1-phosphate
S1P1/3	S1P receptor isoform 1 or 3
4F2hc	4F2 heavy chain amino acid transporter associated protein

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24.

See Supporting Information

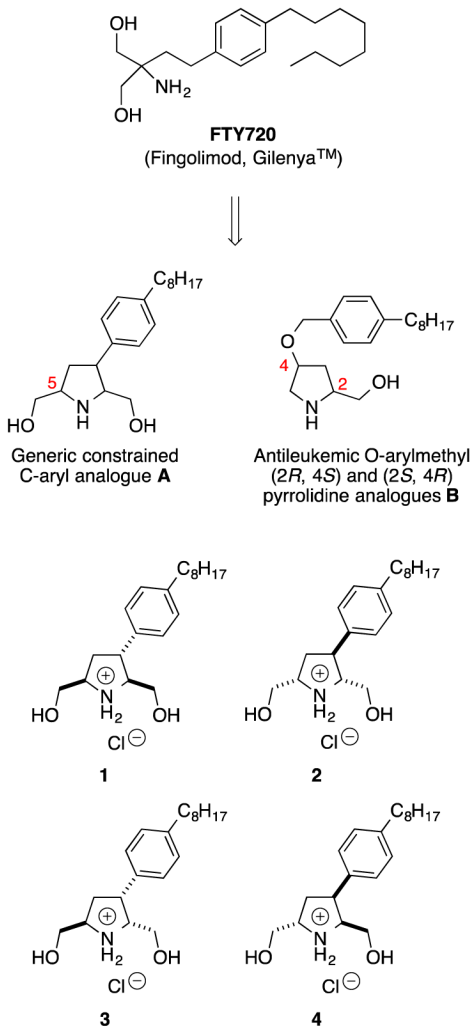

Figure 1. Structure of FTY 720, and constrained *C*-aryl and *O*-arylmethyl pyrrolidine analogues

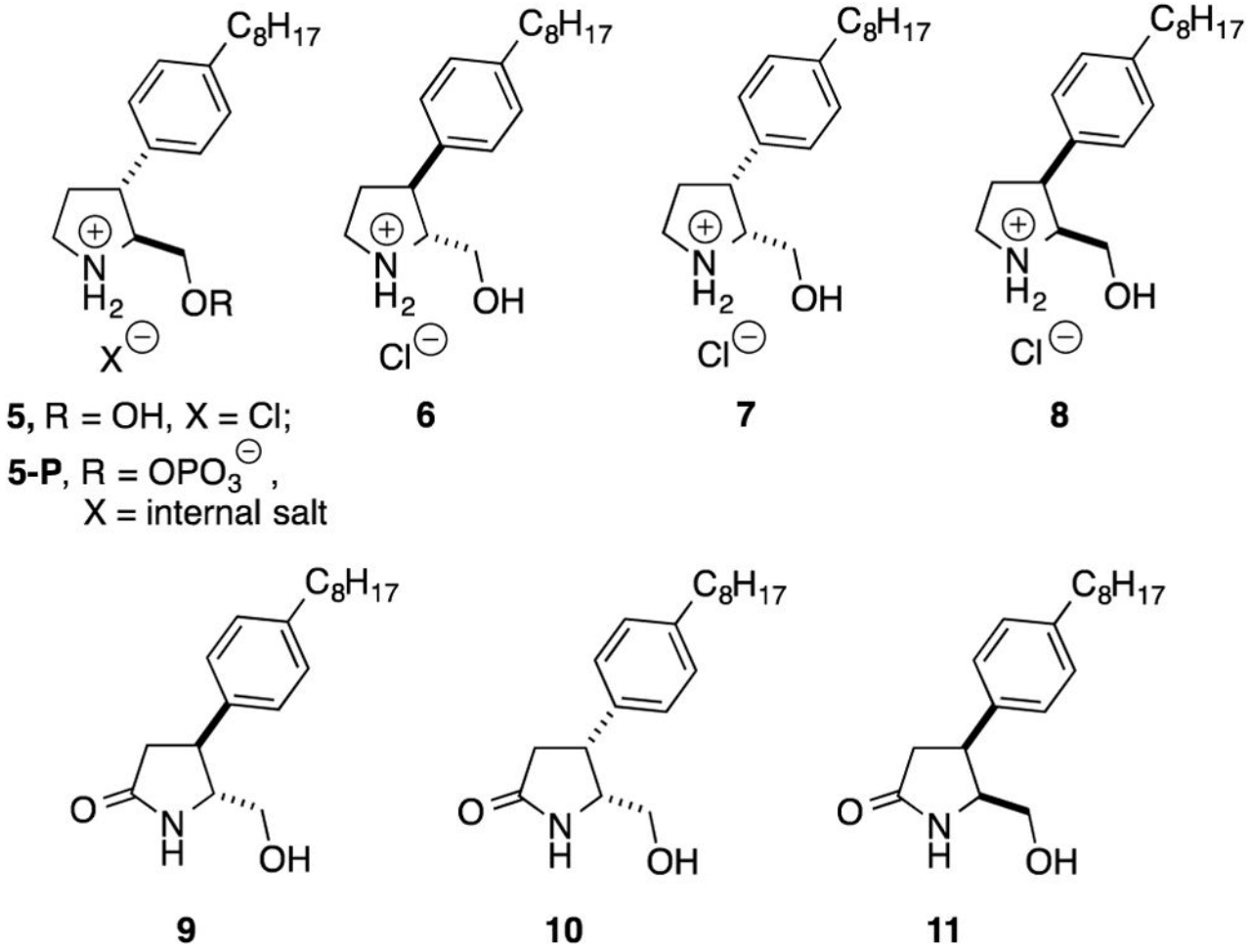

Figure 2. Enantiomeric 2-hydroxymethyl-3-aryl pyrrolidines and selected lactams

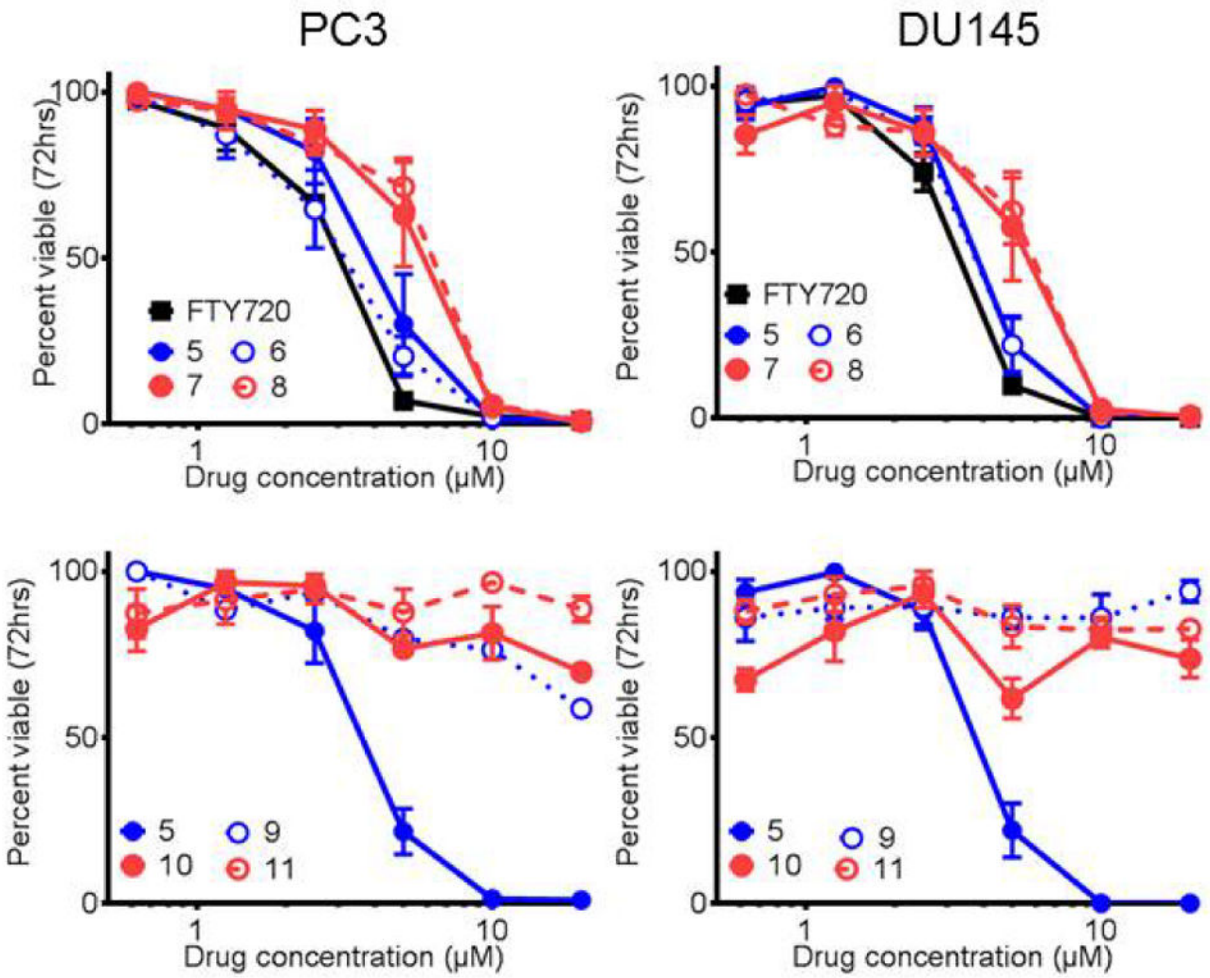

Figure 3. IC_{50} curves in Cell Titer Glo assays with prostate cancer cell lines

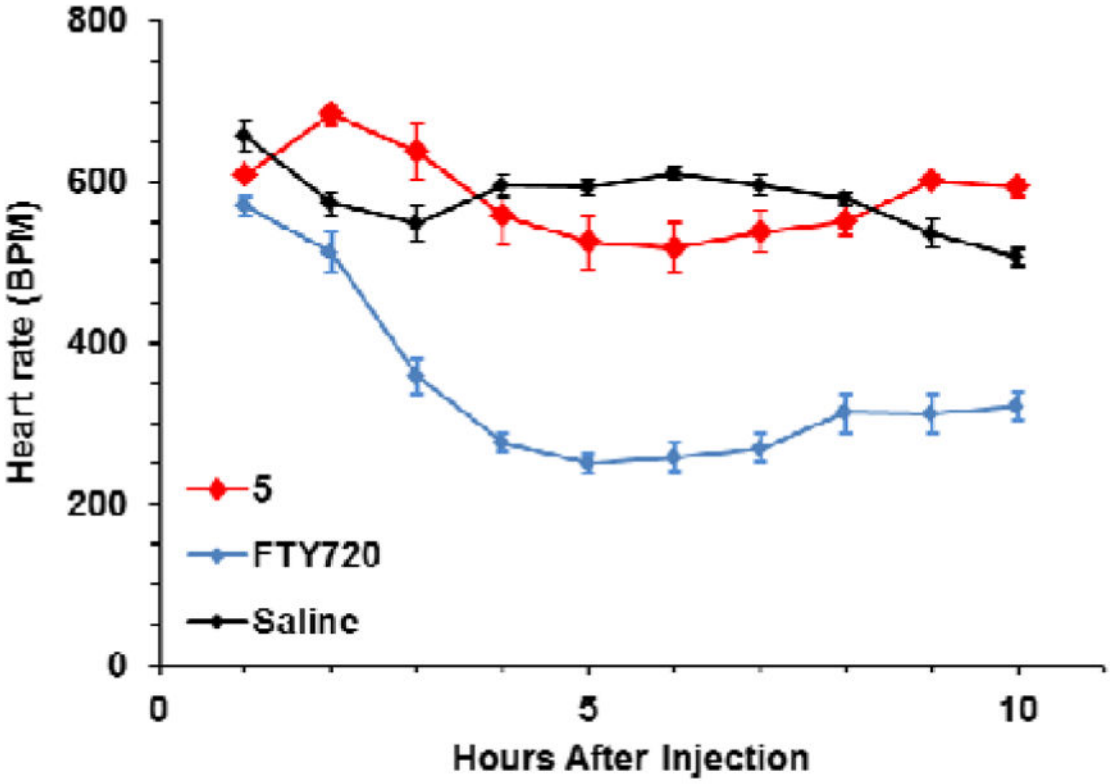

Figure 4. Effect of 5 and FTY720 (10 mg/kg IP) on heart rate in mice

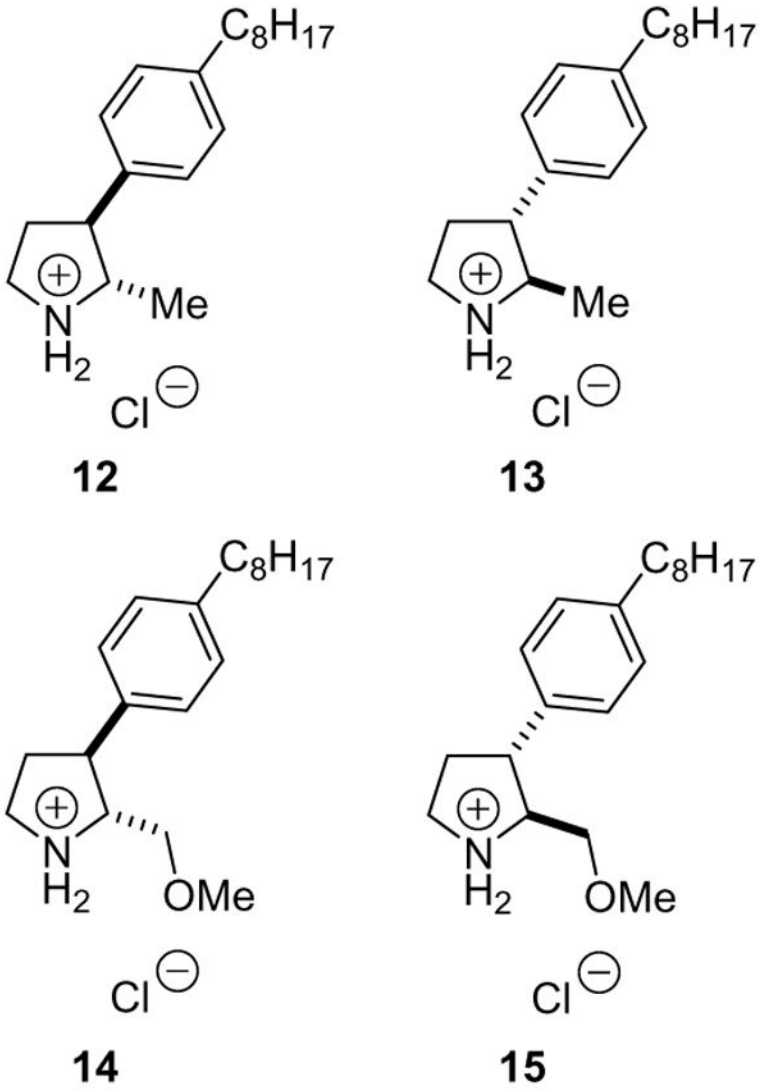

Figure 5. Enantiomeric 2-methyl and 2-methoxymethyl-3-aryl pyrrolidines

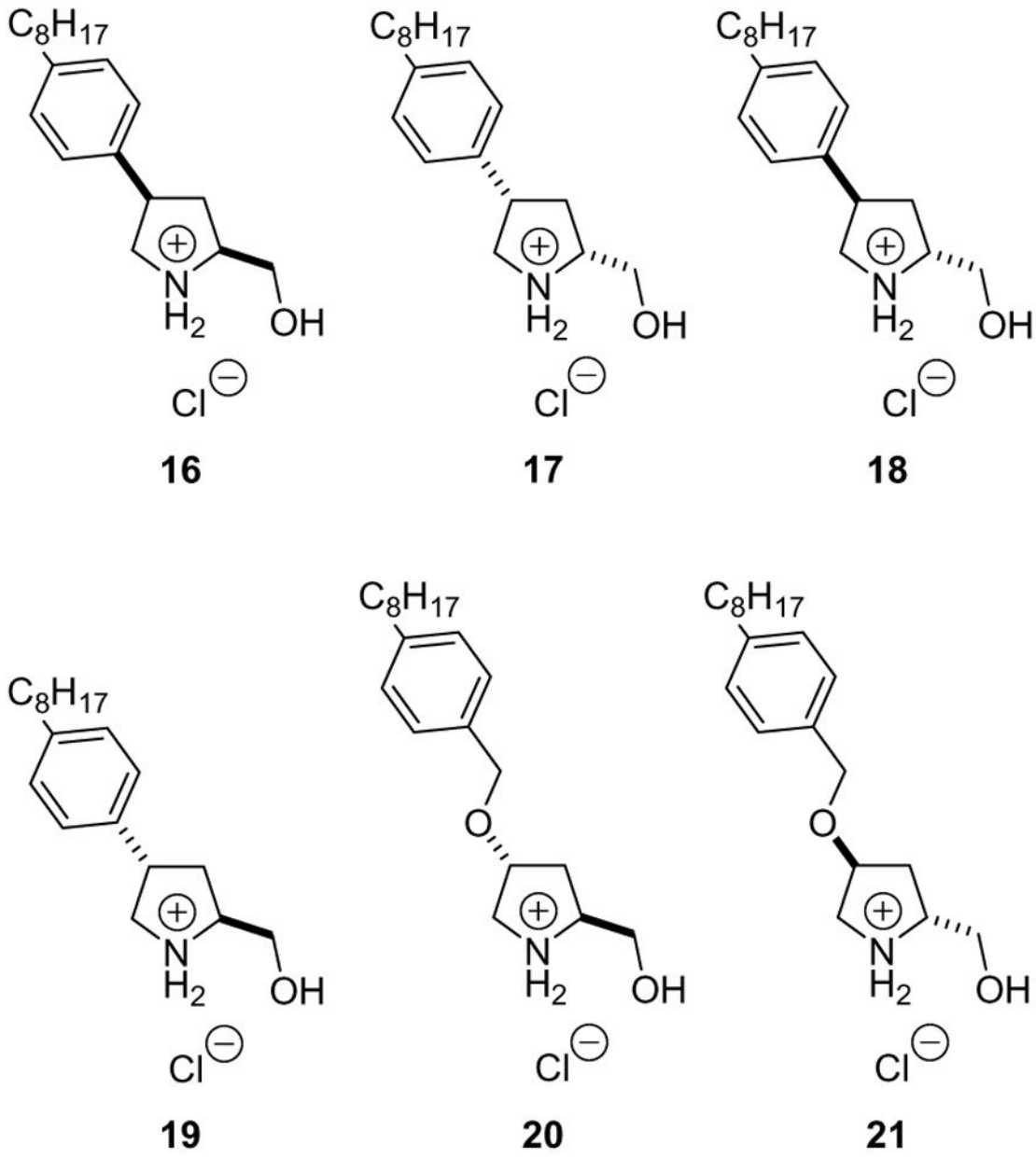

Figure 6. Enantiomeric 2-hydroxymethyl-4-*C*-aryl pyrroli-dines and 2-hydroxymethyl-4-*O*-arylmethyl pyrrolidines

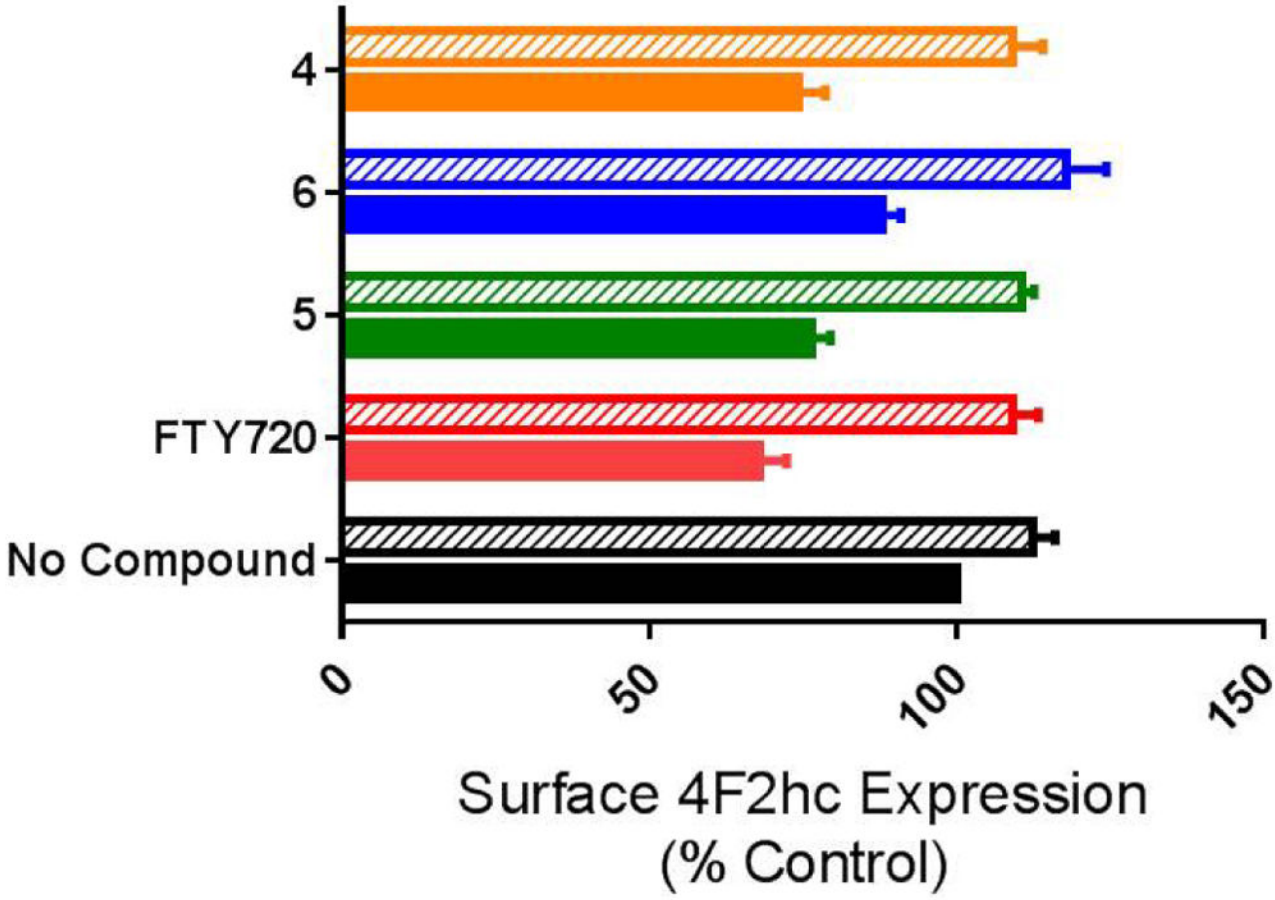

Figure 7. Nutrient transporter down-regulation in CCRF-CEM cells treated with 5 μM compound with (hatched bars) or without (solid bars) 10 nM Calyculin A. Means +/− SEM are shown, n =3

Table 1

IC_{50} values (μM) in prostate cancer cell lines.

	PC3	DU145
	PC3	DU145
FTY720	2.6 ± 0.2	3.1 ± 0.2
1	7.0 ± 0.3	7.3 ± 0.8
2	6.1 ± 1.0	5.7 ± 0.4
3	4.9 ± 0.6	5.3 ± 0.2
4	3.4 ± 0.6	4.0 ± 0.6
5	4.0 ± 0.7	3.8 ± 0.4
6	3.0 ± 0.4	3.7 ± 0.4
7	5.5 ± 0.7	5.3 ± 0.7
8	6.5 ± 0.3	5.4 ± 0.3
9	>20	>20
10	>20	>20
11	>20	>20
12	4.1 ± 0.4	5.0 ± 0.1
13	4.4 ± 0.6	5.1 ± 0.6
14	1.9 ± 0.3	5.6 ± 0.1
15	6.3 ± 0.3	5.9 ± 0.3
16	6.3 ± 0.1	4.9 ± 0.1
17	6.6 ± 0.1	5.8 ± 0.2
18	14.4 ± 0.9	17.6 ± 2.3
19	9.8 ± 1.3	10.6 ± 1.0
20	8.0 ± 0.9	6.1 ± 0.5
21	7.9 ± 0.3	7.6 ± 0.6
22	5.5 ± 0.1	5.0 ± 0.1
23	5.7 ± 0.1	5.1 ± 0.1

Mean +/− SEM is shown, n ≥ 3.

Table 2

IC_{50} values in cancer cell lines in μM.

	SW-620	A-549	PANC-1	MDA-MB-231	SupB-15
	Colon	**Lung**	**Pancreas**	**Breast**	**Leukemia**
FTY720	2.8 ± 0.1	6.0 ± 0.4	4.6 ± 0.5	4.0 ± 0.1	6.8 ± 0.7
4	2.6 ± 0.0	4.7 ± 0.6	3.5 ± 0.3	4.6 ± 0.5	ND
5	2.5 ± 0.1	8.9 ± 1.4	3.3 ± 0.5	2.1 ± 0.2	5.1 ± 0.9
6	2.1 ± 0.2	8.4 ± 1.2	5.0 ± 0.9	4.9 ± 0.6	5.9 ± 0.1
14	2.7 ± 0.2	7.8 ± 1.8	4.8 ± 0.6	4.0 ± 0.0	7.5 ± 0.4
20	7.0 ± 1.2	10.7 ± 0.2	8.0 ± 1.5	9.1 ± 0.3	7.7 ± 0.8
21	4.9 ± 0.9	9.5 ± 1.1	8.8 ± 0.6	6.4 ± 0.4	2.0 ± 0.2

Mean +/− SEM is shown, n≥3. SupB-15 viability was determined by flow cytometry, Cell Titer Glo assays were performed with the other cell lines. ND, not determined.