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Facilitated phospholipid translocation in vesicles and nucleated cells using synthetic small molecule scramblases

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ABSTRACT

A series of 16 synthetic scramblase candidates were prepared from a tris(aminoethyl)amine (TREN) scaffold and evaluated for ability to facilitate translocation of fluorescent phospholipid probes across vesicle membranes and endogenous phosphatidylserine across the plasma membrane of nucleated cells. More than half of the compounds were found to greatly accelerate phospholipid translocation in vesicles. However, they were generally unable to induce large increases in the amount of phosphatidylserine on the surface of nucleated mammalian cells, which contrasts with previous results using erythrocytes. Fluorescence microscopy showed that the synthetic scramblases are rapidly trafficked out of the cell plasma membrane and into the membranes of internal organelles. Future molecular designs of synthetic scramblases should focus on structures that are more amphiphilic, a structural feature that is expected to increase plasma membrane residence time.

1. Introduction

The translocation of phospholipids across synthetic bilayer membranes is known to be a very slow process with half-lives on the order of hours to days; in comparison, lateral diffusion within a phospholipid monolayer is much more rapid (Fig. 1).[1,2] If the membrane is not highly curved then the distribution is simply a scrambled equilibrium. In contrast, most biological membranes have a highly asymmetric distribution of phospholipids.[3] In the case of mammalian plasma membranes, the outer monolayer is enriched in zwitterionic choline-based phospholipids, such as phosphatidylcholine (PC), while the inner monolayer is enriched in aminophospholipids, such as anionic phosphatidylserine (PS) (Scheme 1).[4] This asymmetric phospholipid distribution is maintained by the cumulative action of a family of endogenous membrane transporters called scramblases, flippases and floppases.[5] The asymmetric distribution is crucial for normal cellular activity, but it becomes scrambled during certain pathological disorders and also the early stages of cell death.[6] The consequent exposure of PS on the cell surface is signal for cell clearance by the innate immune system.[7]

For several years, our group has investigated the use of small synthetic molecules to facilitate translocation of phospholipids across bilayer membranes.[8,9] The practical goal is to produce synthetic scramblases for use as research tools to remodel cell membrane structure and perhaps eventually as pharmaceutical leads. We have reported previously that synthetic scramblases based on a TREN (tris(2-aminoethyl)amine) scaffold can scramble the phospholipid distribution in vesicles and erythrocytes.[8] Mechanistic studies indicate that these TREN scramblases can associate with the phospholipid head group and form lipophilic, hydrogen-bonded phospholipid:scramblase complexes that diffuse through the membrane (Scheme 1). Initially, we examined simple tris(sulfonamide) and tris(amide) derivatives, but a screening study found that the urea-based scramblases **1** and **2** (Scheme 2) cannot only promote translocation of fluorescently-labeled phospholipid probes across vesicle membranes, but also induce surface exposure of endogenous PS in erythrocytes.[8f]

These promising results motivated us to pursue an expanded study of TREN scramblases. We report here the preparation of 16

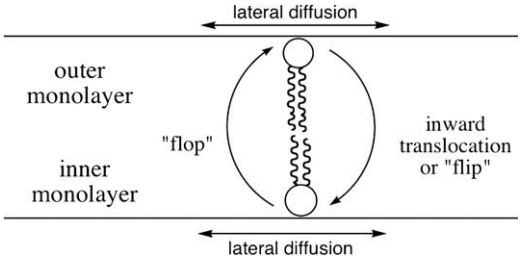

Figure 1.

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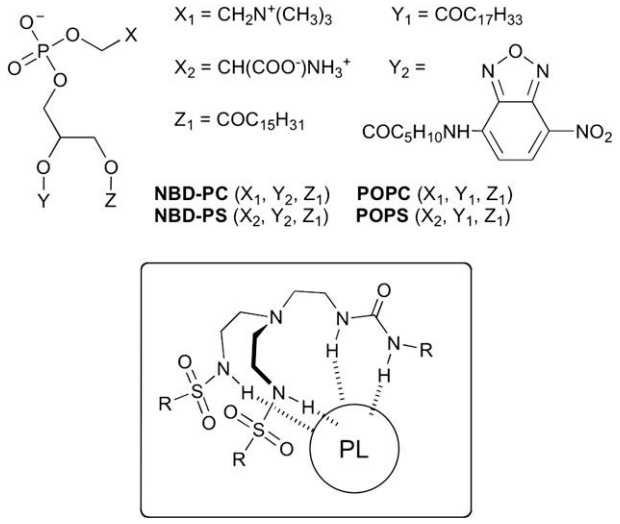


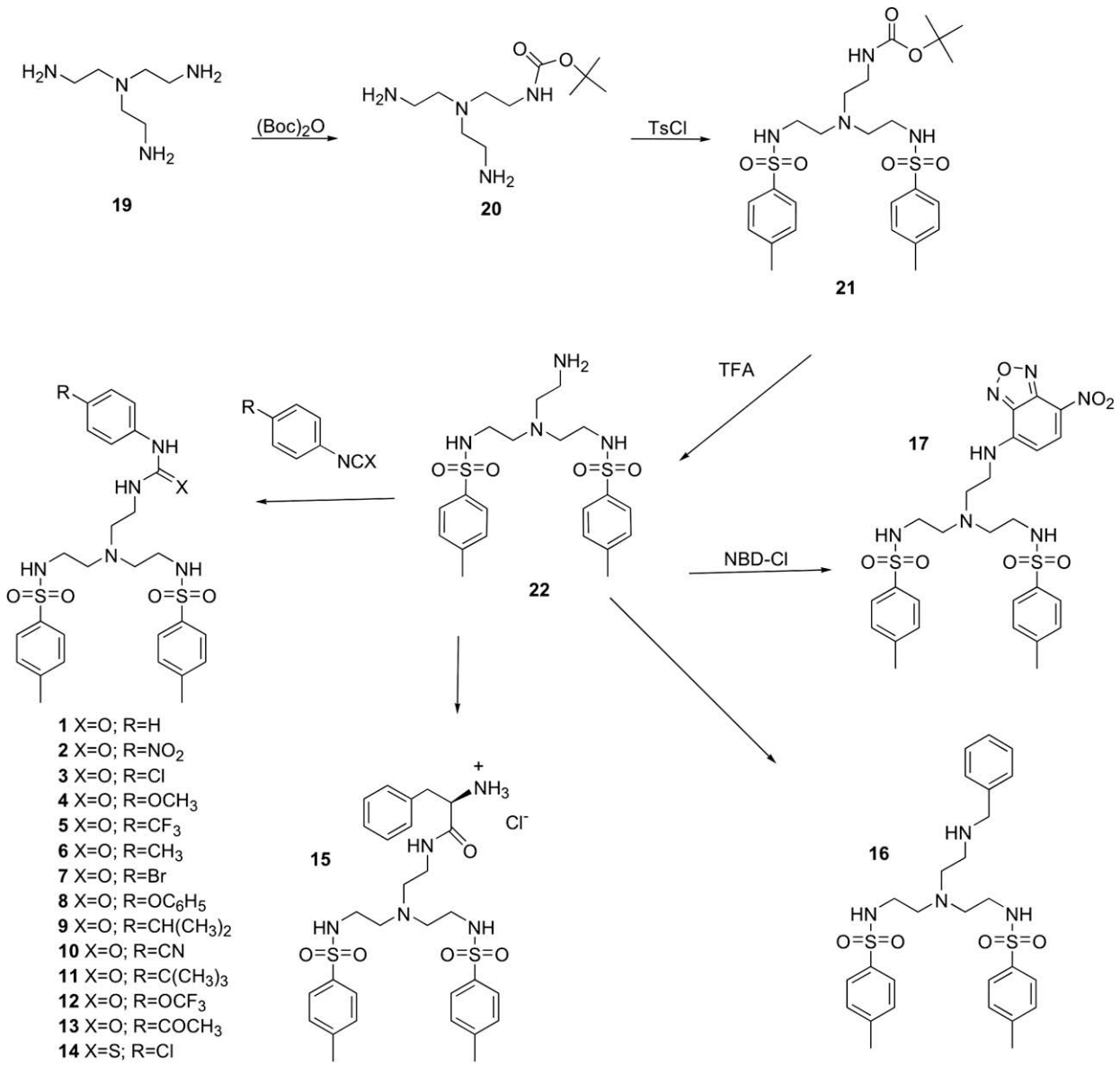
Scheme 1. (Top) Phospholipid structures, (Bottom) Scramblase hydrogen-bonded to anionic residues within the phospholipid (PL) head group.

candidate compounds and their evaluation for facilitated translocation activity in vesicles. The most active compounds were subsequently tested in live mammalian cells, to see if they increased the amount of PS exposed on the cell surface. In contrast to our previous success with erythrocytes we find that the TREN scramblases are less effective with nucleated cells. Microscopic imaging of a fluorescent analog suggests that the scramblases are rapidly trafficked away from the plasma membrane and distributed throughout the organelle membranes inside the cell. It appears that residence time in the plasma membrane is a key factor controlling synthetic scramblase activity.

2. Results

2.1. Synthetic chemistry

As shown in Scheme 2, the central intermediate, TREN disulfonamide (**22**) was synthesized in three steps. It is worth noting that improved reaction conditions were found to produce the mono-*N*-Boc-protected TREN (**20**) in 92% yield. From **22** the urea



Scheme 2. Structures and syntheses of TREN scramblases.

Table 1
Translocation half-lives for NBD–PC and NBD–PS in vesicles

Scramblase (12.5 μM)	$t_{1/2}$ (min)[a]	
	NBD–PC (2 mol%)	NBD–PS (2 mol%)
1	60	60
2	8	12
3	0.5	2
4	90	>90
5	0.5	
6	20	
7	0.5	0.25
8	0.5	
9	0.5	
10	20	60
11	0.5	
12	0.5	
13	>30	
14	0.5	
15	>60	
16	>60	

[a] Translocation half-life in 7:3 POPC/cholesterol vesicles, TES buffer (5 mM TES, 100 mM NaCl, pH 7.4; 25 °C); error ± 33%.

derivatives **1**–**13** and thiourea **14** were prepared in one step processes. Coupling of **22** with L-phenylalanine gave the amide **15**, and reductive benzylation of **22** gave **16**. The fluorescent derivative **17** with an NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) group was prepared by aromatic substitution with commercially available NBD–Cl.

2.2. Phospholipid translocation in vesicles

The well-established NBD/dithionite quenching assay was used to measure the translocation half-lives for fluorescent phospholipid probes containing an NBD chromophore in one of its acyl chains.[8a,10,11] Exo-labeled vesicles were prepared by addition of a small aliquot of NBD–PC or PBD–PS (2 mol.%) to a dispersion of unilamellar 7:3 POPC/cholesterol vesicles (25 μM). The NBD–lipid rapidly inserts into the vesicles. Bilayer membranes are effectively impermeable to sodium dithionite; therefore, only NBD–phospholipid located in the membrane outer leaflet is chemically quenched upon dithionite addition. The background rate of phospholipid translocation across vesicle membranes is many hours to days and at final equilibrium there is 60% of the probe in the outer leaflet, reflecting the difference in surface area of inner and outer leaflets. Thus, the translocation half-life is defined operationally as the time required for 20% of the exo-NBD–phospholipid to be translocated to the inner leaflet. Listed in Table 1 are the translocation half-lives for NBD–PC observed upon treatment of the vesicles with TREN scramblases **1**–**16**. In general, the scramblases with withdrawing groups on the 4-aryl urea ring produced shorter translocation half-lives. This agrees with earlier studies that withdrawing groups enhance association with phospholipid head group due to increased hydrogen bonding.[8] Six of the scramblases were also evaluated in a NBD–PS translocation assay and some highly effective PS scramblases were identified (Table 1). In most cases, scramblase induced acceleration of NBD–PC was slightly greater than NBD–PS.

Table 2
Toxicity of TREN scramblases[a]

Scramblase	LD_{50} (μM)		
	Jurkat cells	PC3 cells	LNCaP cells
14	15	25	15
15	25	15	40
16	25	25	40

[a] As determined by the MTT assay; ±10 μM. The IC_{50} for compounds **1**–**16** was >100 μM.

2.3. Effect of TREN scramblases on nucleated cells

A major goal of the study was to evaluate if the TREN scramblases could alter the PS distribution across the plasma membrane of nucleated mammalian cells. Since PS exposure is a signature of most if not all cell death processes, it was important to first determine if the scramblases induced any cell toxicity. Thus, they were screened for effects on the viability of Jurkat, PC3 and LNCaP cells using an MTT assay (Table 2). Briefly, 10^6 cells were plated out in 48-well plates and left to adhere and multiply for 48 h. The media were exchanged with fresh media that were supplemented with MTT. In healthy cells, the MTT is converted by mitochondrial reductase enzymes into the purple formazan product. None of the urea-based TREN scramblases **1**–**13** had any measurable effect on mitochondrial reductase activity (LD_{50} > 100 μM); however, the three scramblases **14**, **15** and **16** exhibited modest toxicity (LD_{50} > 15 μM). Overall the TREN scramblases are not inherently toxic to cells.

The next step was to determine if TREN scramblases can alter the endogenous transmembrane distribution of PS in nucleated cells. The exposed PS was detected by staining with the dye-labeled protein Annexin V-FITC.[8f] Briefly, separate samples of PC3 cells (1×10^5 cells/mL) in TES buffer were treated with TREN scramblase (100 μM) and incubated at 37 °C for 2 h. Flow cytometry was used to identify cells that were stained by Annexin-FITC and simultaneous 7AAD co-staining of the nucleus was used to eliminate necrotic cells that had lost plasma membrane integrity. Previously, we observed that treatment of erythrocytes with scramblase **2** raised the fraction of Annexin-stained cells from 1% to 34%. In comparison, the fraction of PC3 cells that were stained by Annexin-FITC after scramblase treatment was low. The best result was obtained with scramblase **5** (100 μM) which increased the fraction of Annexin-stained cells from a background value of 2–10% (Fig. 2). Three other TREN scramblases were tested (**1**, **2** and **9**) and in each case the fraction of Annexin-stained cells was <5% of the cell population (data not shown). The same outcome was observed with PC3 cells that were pretreated with vanadate, a well-known inhibitor of the ATP-dependent flippases.[12]

2.4. Partitioning of fluorescent TREN probe into vesicles and cells

To help understand why the TREN scramblases are unable to greatly alter endogenous PS surface levels, a fluorescent NBD-version, **17**, was prepared and used for partitioning studies. A dithionite protection study was employed to measure partitioning into

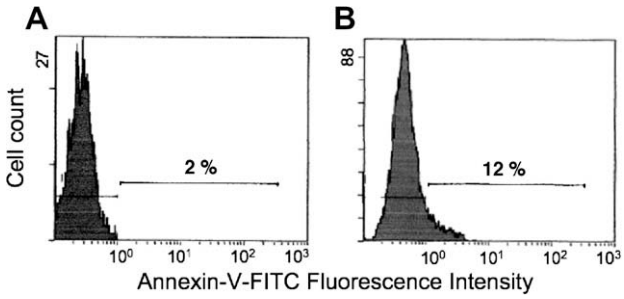


Figure 2. Flow cytometry analysis of PC3 cells stained by Annexin V-FITC. (A) Untreated cells. (B) Cells treated with scramblase **5** (100 μM). The data shown is a representative histogram from three independent experiments.

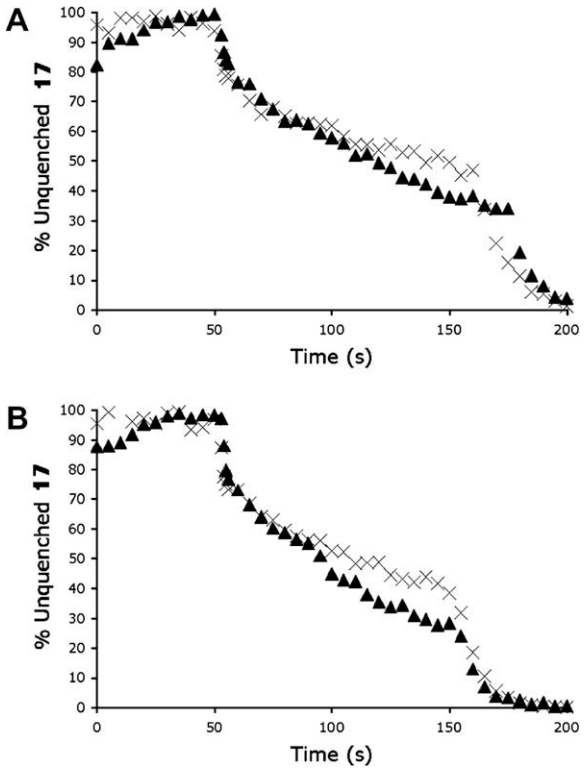


Figure 3. Chemical quenching of **17**, protection by vesicles. At $t = 0$, vesicles composed of 7:3 POPC/cholesterol (A) or 3.5:3.5:3 POPC/POPS/cholesterol (B) vesicles (25 μM) in TES buffer (5 mM TES, 100 mM NaCl, pH 7.4) were treated with **17** (12.5 μM). After 0.5 min (⑩) and 20 min (▭), samples (3 mL) were withdrawn and assayed by adding sodium dithionite and Triton X-100 at times of 50 and 150 s, respectively. The data shown are a representative plot from three independent experiments. The error for each point is ±5%.

vesicles composed of 7:3 POPC/cholesterol (mimic of zwitterionic plasma membrane outer leaflet) and 3.5:3.5:3 POPC/POPS/cholesterol (mimic of anionic plasma membrane inner leaflet). Briefly, probe **17** was added to a solution of vesicles and the amount of probe that had moved to the membrane interior surface (and thus was protected from chemical quenching by the subsequent addition of sodium dithionite) was measured after 0.5 and 20 min (Fig. 3). The chemical quenching involved addition of sodium dithionite at 50 s to quench the fluorescence of surface exposed **17** and subsequent vesicle lysis at 150 s with Triton X-100 allowed complete quenching. With both vesicle compositions, there was substantial protection of the probe from dithionite quenching even 0.5 min after the probe was added, indicating that the weakly lipophilic probe **17** equilibrates very rapidly across the vesicle membrane.

This picture of rapid membrane transport was confirmed with a fluorescence microscopy study of **17** partitioning into mammalian cells. Briefly, separate samples of Jurkat cells were incubated for 30 min with **17** (12.5 μM) and either of the targeted fluorescent probes, ER Tracker™ or Mito Tracker™. Cells were then visualized using epi-fluorescence microscopy. The large amount of co-localized staining clearly indicates that probe **17** is localized primarily in the ER and the mitochondria and that relatively little is in the plasma membrane (Fig. 4). These membrane partitioning results indicate that the TREN scramblases are trafficked rapidly away from the plasma membrane of mammalian cells and they are sequestered in the relatively large volume of intracellular organelle membranes.[13]

3. Discussion

As expected from previous work,[8] most of the urea-based TREN scramblases with electron withdrawing groups on the urea aryl ring are able to greatly accelerate the translocation of fluorescent PC–NBD and PS–NBD probes across vesicle membranes. Most likely, the electron withdrawing substituents enhance association with phospholipid head group due to increased hydrogen bonding. Although the TREN scramblases can greatly enhance the fraction of erythrocytes that expose PS on their exterior surface, they are substantially less efficient with nucleated mammalian cells. In both cases, the scramblases have to compete with the endogenous flippase enzymes in the cell plasma membranes, and it may be that flippase activity is inherently higher in nucleated cells. However, evidence against this explanation is the fact that pretreatment of the PC3 cells with vanadate, a well-known inhibitor of the ATP-dependent flippases,[12] does not help a TREN scramblase to increase PS exposure on the cell surface. A more likely explanation for the lack of scramblase activity in nucleated cells comes from the microscopy results, which indicate that fluorescent TREN scramblases are trafficked rapidly away from the plasma membrane and

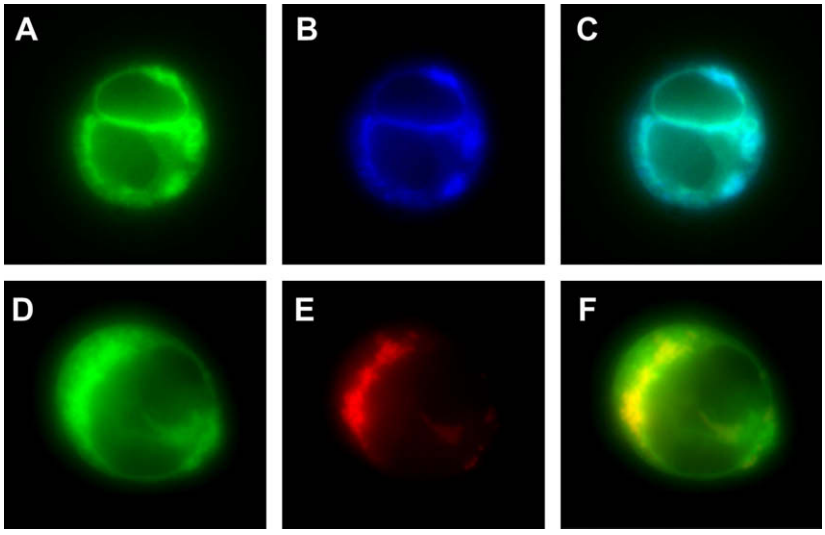


Figure 4. Fluorescence microscopy images of two separate Jurkat cells incubated with **17** (12.5 μM) and the following co-stains. Top row: (A) **17**, (B) ER Tracker™, (C) color overlay. Bottom row: (D) **17**, (E) Mito Tracker™, (F) color overlay.

into the membranes of internal organelles such as the ER and mitochondria. Erythrocytes, of course, do not have these internal membranes. A shortened residence time in the plasma membrane would diminish the inherent ability of the TREN scramblases to promote PS flip-flop.

4. Conclusion

Although many of the TREN scramblases reported in this study can accelerate the translocation of phospholipids in model membrane systems, they are unable to significantly increase the exposure of PS on the surface of nucleated cells because they are rapidly trafficked out of the plasma membrane and into the membranes of internal organelles. Future designs should focus on scramblase structures that are more amphiphilic, a structural feature that is expected to increase plasma membrane residence time.[14] This structural strategy should also be considered when designing other synthetic membrane transport systems that require the transporter to remain in the plasma membrane of nucleated cells for extended periods (e.g., synthetic chloride transporters for treatment of cystic fibrosis).[15]

5. Experimental

5.1. Synthetic chemistry

5.1.1. General procedure for 1–14

A solution of the *p*-substituted phenylisocyanate (1.1 equiv) in dichloromethane (60 mL per mmol **22**) was added drop wise to a stirred solution of TREN disulfonamide (**22**) in dichloromethane (120 mL per mmol **22**) during 20 min at 0 °C. The reaction mixture was allowed to stir for 16 h at room temperature. After removal of the solvent *in vacuo* the crude product was purified by column chromatography on silica gel using ethyl acetate/hexanes gradient elution (60–100 vol% EtOAc).

*Urea (**1**)*. According to the general procedure product **1** was obtained as a colorless solid (34.5 mg, 62%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.34 (s, 6H), 2.43 (br m, 6H), 2.90 (br m, 4H), 3.21 (br m, 2H), 6.00 (unresolved t, 1H), 6.29 (unresolved t, 2H), 6.96 (t, J = 7.4 Hz, 1H), 7.20 (d, J = 8.0 Hz, 4H), 7.23 (m, 2H), 7.40 (d, J = 7.6 Hz, 2H), 7.57 (br s, 1H), 7.74 (d, J = 8.3 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.5, 37.5, 41.1, 53.7, 119.2, 122.4, 127.0, 128.9, 129.8, 136.5, 139.6, 143.5, 156.3; TLC (9:1 EtOAc/acetone) R_f = 0.47.

*Urea (**2**)*. According to the general procedure product **2** was obtained as yellow solid (38.4 mg, 34%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.36 (s, 6H), 2.56 (br m, 6H), 3.01 (br m, 4H), 3.26 (br m, 2H), 6.19 (unresolved t, 2H), 6.26 (unresolved t, 1H), 7.22 (d, J = 8.2 Hz, 4H), 7.57 (d, J = 9.2 Hz, 2H), 7.72 (d, J = 8.3 Hz, 4H), 8.06 (d, J = 9.2 Hz, 2H), 8.18 (s, 1H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.4, 37.5, 40.9, 53.3, 117.4, 125.0, 126.8, 129.8, 136.2, 141.6, 143.8, 146.2, 155.1; TLC (EtOAc) R_f = 0.57.

*Urea (**3**)*. According to the general procedure product **3** was obtained as a colorless solid (66.0 mg, 49%); ^{1}H NMR ($CDCl_3$, TMS, 600 MHz) δ 2.36 (s, 6H), 2.45 (m, 6H), 2.93 (m, 4H), 3.20 (m, 2H), 5.99 (t, J = 5.0 Hz), 6.22 (unresolved t, 4H), 7.15 (d, J = 8.8 Hz, 2H), 7.20 (d, J = 7.9 Hz, 4H), 7.35 (d, J = 8.8 Hz, 2H), 7.54 (s, 1H), 7.72 (d, J = 8.3 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 150 MHz) δ 21.5, 37.6, 41.2, 53.6, 53.6, 120.1, 126.9, 126.9, 128.7, 129.8, 136.4, 138.3, 143.6, 155.9; TLC (9:1 EtOAc/acetone) R_f = 0.51.

*Urea (**4**)*. According to the general procedure product **4** was obtained as a colorless solid (85.6 mg, 66%); ^{1}H NMR ($CDCl_3$, TMS, 600 MHz) δ 2.34 (s, 6H), 2.39 (m, 6H), 2.87 (m, 4H), 3.17 (m, 2H), 3.74 (s, 3H), 5.91 (unresolved t, 1H), 6.34 (unresolved t, 2H), 6.78 (d, J = 8.9 Hz, 2H), 7.20 (d, J = 8.1 Hz, 4H), 7.28 (d, J = 9.0 Hz, 2H); 7.39 (s, 1H); 7.74 (d, J = 8.2 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 150 MHz) δ 21.5, 37.7, 41.1, 53.7, 54.1, 55.5, 114.1, 121.7, 127.0, 129.8, 132.3, 136.6, 143.4, 155.5, 156.9; TLC (9:1 EtOAc/acetone) R_f = 0.49.

*Urea (**5**)*. According to the general procedure product **5** was obtained as a colorless oil (84.4 mg, 58%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.35 (s, 6H), 2.50 (m, 6H), 3.24 (m, 2H), 6.07 (t, J = 4.8 Hz, 1H), 6.14 (unresolved t, 2H), 7.19 (d, J = 8.2 Hz), 7.44 (d, J = 8.8 Hz, 2H), 7.53 (d, J = 8.7 Hz, 2H), 7.72 (d, J = 8.2 Hz, 4H), 7.77 (s, 1H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.4, 37.7, 41.1, 53.5, 118.0, 123.54 (q, $^2J_{CF}$ = 32.6 Hz), 124.4 (q, $^1J_{CF}$ = 271.2 Hz), 126.0 (q, $^3J_{CF}$ = 3.51), 126.9, 129.8, 136.4, 143.0, 143.7, 155.6; TLC (EtOAc) R_f = 0.46.

*Urea (**6**)*. According to the general procedure product **6** was obtained as a colorless oil (43.7 mg, 66%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.27 (s, 3H), 2.34 (s, 6H), 2.43 (br m, 6H), 2.90 (br m, 4H), 3.19 (br m, 2H), 5.96 (unresolved t, 1H), 6.33 (unresolved t, 2H), 7.02 (d, J = 8.3 Hz, 2H), 7.19 (d, J = 8.3 Hz, 4H), 7.27 (d, J = 8.3 Hz, 2H), 7.48 (s, 1H), 7.74 (d, J = 8.2 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 20.7, 21.5, 37.6, 41.1, 53.8, 54.1, 119.6, 127.0, 129.4, 129.8, 131.9, 136.5, 136.8, 143.4, 156.6; TLC (EtOAc) R_f = 0.50.

*Urea (**7**)*. According to the general procedure product **7** was obtained as a colorless oil (39.7 mg, 55%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.36 (s, 6H), 2.49 (br m, 6H), 2.95 (br m, 4H), 3.21 (br m, 2H), 6.07 (unresolved t, 1H), 6.28 (unresolved t, 2H), 7.19 (d, J = 8.0 Hz, 4H), 7.30 (m, 4H), 7.67 (br s, 1H), 7.72 (d, J = 8.3 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.5, 37.5, 41.0, 53.6, 53.7, 114.4, 120.4, 126.9, 129.8, 131.6, 136.4, 138.8, 143.6, 156.0; TLC (EtOAc) R_f = 0.57.

*Urea (**8**)*. According to the general procedure product **8** was obtained as a colorless oil (30.4 mg, 51%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.35 (s, 6H), 2.49 (br m, 6H), 2.95 (br m, 4H), 3.22 (br m, 2H), 6.02 (unresolved t, 1H), 6.33 (unresolved t, 2H), 6.90 (d, J = 9.0 Hz, 2H), 6.94 (m, 2H), 7.04 (t, J = 7.4 Hz, 1H), 7.22 (d, J = 8.1 Hz, 4H), 7.28 (m, 2H), 7.38 (d, J = 9.0 Hz, 2H), 7.57 (br s, 1H), 7.75 (d, J = 8.3 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.5, 37.6, 41.0, 53.7, 54.1, 117.9, 119.9, 120.9, 122.6, 127.0, 129.6, 129.8, 135.3, 136.5, 143.5, 151.8, 156.5, 158.0; TLC (EtOAc) R_f = 0.43.

*Urea (**9**)*. According to the general procedure product **9** was obtained as a white solid (58%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 1.21 (d, J = 6.86 Hz, 6H), 2.34 (s, 6H), 2.51 (br m, 6H), 2.84 (sep, J = 6.9 Hz, 1H), 2.95 (br m, 4H), 3.21 (br m, 2H), 6.11 (unresolved t, 1H), 6.48 (unresolved t, 2H), 7.08 (d, J = 8.5 Hz, 2H), 7.19 (d, J = 8.1 Hz, 4H), 7.33 (d, J = 8.5 Hz, 2H), 7.67 (s, 1H), 7.76 (d, J = 8.3 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.5, 24.1, 33.5, 37.4, 40.7, 40.8, 54.0, 119.6, 126.7, 127.0, 129.8, 136.5, 137.0, 143.1, 143.5, 156.7; TLC (EtOAc) R_f = 0.62; HPLC/MS RT = 9.55 min, exact mass calcd for $C_{30}H_{41}N_5O_5S_2$ $[M+H]^+$ 616.26: found: 616.4.

*Urea (**10**)*. According to the general procedure product **10** was obtained as a colorless solid (38.5 mg, 76%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.37 (s, 6H), 2.55 (br m, 6H), 3.00 (br m, 4H), 3.25 (br m, 2H), 6.19 (br m, 3H), 7.22 (d, J = 8.0 Hz, 4H), 7.46 (d, J = 8.9 Hz, 2H), 7.55 (d, J = 8.9 Hz, 2H), 7.72 (d, J = 8.3 Hz, 4H), 8.00 (br s, 1H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.5, 37.5, 41.0, 53.4, 104.2, 118.2, 119.5, 126.9, 129.9, 133.0, 136.3, 143.8, 144.2, 155.3; TLC (EtOAc) R_f = 0.49.

*Urea (**11**)*. According to the general procedure product **11** was obtained as a colorless oil (44.4 mg, 71%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 1.27 (s, 9H), 2.33 (s, 6H), 2.41 (br m, 6H), 2.89 (br m, 4H), 3.19 (br m, 2H), 5.98 (unresolved t, 1H), 6.36 (unresolved t, 2H), 7.18 (d, J = 8.0 Hz, 4H), 7.23 (d, J = 8.9 Hz, 2H), 7.31 (d, J = 8.8 Hz, 2H), 7.50 (s, 1H), 7.75 (d, J = 8.3 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.5, 31.4, 34.2, 37.6, 41.1, 53.9, 54.2, 119.2, 125.6,

127.0, 129.8, 136.5, 136.8, 143.4, 145.3, 156.5; TLC (EtOAc) R_f = 0.57.

*Urea (**12**)*. According to the general procedure product **12** was obtained as a colorless oil (35.9 mg, 57%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.34 (s, 6H), 2.52 (br m, 6H), 2.96 (br m, 4H), 3.22 (br m, 2H), 6.17 (unresolved t, 1H), 6.37 (unresolved t, 2H), 7.04 (d, J = 8.4 Hz, 2H), 7.19 (d, J = 8.1 Hz, 4H), 7.43 (d, J = 9.0 Hz, 2H), 7.73 (d, J = 8.3 Hz, 4H), 7.83 (s, 1H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.4, 37.4, 40.9, 53.7, 54.0, 120.5 (q, $^1J_{CF}$ = 256.1 Hz), 119.7, 121.6, 127.0, 129.8, 136.4, 138.5, 143.7, 156.1; TLC (EtOAc) R_f = 0.62; HPLC/MS RT = 9.42 min, exact mass calcd. for $C_{28}H_{34}F_3N_5O_6S_2$ $[M+H]^+$ 658.20: found: 658.3.

*Urea (**13**)*. According to the general procedure product **13** was obtained as a beige solid (42.4 mg, 62%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.33 (s, 6H), 2.44 (br m, 6H), 2.91 (br m, 4H), 3.18 (br m, 2H), 4.99 (s, 2H), 6.00 (unresolved t, 1H), 6.44 (unresolved t, 2H), 6.85 (d, J = 8.9 Hz, 2H), 7.20 (d, J = 8.1 Hz, 4H), 7.35 (m, 7H), 7.51 (s, 1H), 7.74 (d, J = 8.2 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.5, 29.7, 37.2, 40.7, 54.0, 70.2, 115.2, 121.5, 127.0, 127.5, 127.9, 128.5, 129.8, 132.7, 136.5, 137.1, 143.4, 154.6, 157.1; TLC (EtOAc) R_f = 0.54.

*Thiourea (**14**)*. According to the general procedure product **14** was obtained as a white solid (23.2 mg, 67%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.39 (s, 6H), 2.60 (br m, 6H), 2.98 (br m, 4H), 3.62 (br m, 2H), 6.09 (unresolved t, 2H), 7.14 (unresolved t, 1H), 7.25 (m, 6H), 7.44 (d, J = 8.7 Hz, 2H), 7.74 (d, J = 8.3 Hz, 4H), 8.42 (br s, 1H); ^{13} NMR ($CDCl_3$, TMS, 75 MHz) δ 21.6, 41.0, 42.3, 52.9, 53.7, 125.6, 127.0, 128.9, 129.9, 130.6, 136.4, 137.1, 143.8, 181.1; TLC (7:3 EtOAc/acetone) R_f = 0.46.

*Phenylalanine disulfonamide (**15**)*. EDC (27.8 mg, 1.5 equiv), HOBt (29.6 mg, 2.0 equiv), triethylamine (19.6 mg, 2.0 equiv) and *N*-Boc-protected phenylalanine (30.8 mg, 1.2 equiv) were dissolved in DMF (5 mL) and stirred at room temperature for 1 h. Next, compound **22** (44.0 mg, 0.977 mmol) was dissolved in DMF (2 mL) and added drop wise to the solution. The mixture was stirred for 16 h after which the solvent was removed in vacuo. Purification of the crude product took place by column chromatography on SiO_2 using ethyl acetate as the eluent and yielding a colorless oil which was dissolved in dichloromethane (10 mL). Dry HCl gas was slowly bubbled through the stirred solution for 30 min. The reaction mixture became cloudy and was stirred for additional 30 min and the solvent was removed in vacuo. The product **15** was obtained as a colorless oil (19.2 mg, 90%); ^{1}H NMR (CD_3OD, 300 MHz) δ 2.41 (s, 6H), 3.08 (dd, J = 8.3 Hz, J = 13.9 Hz, 1H), 3.25 (m, 5H), 3.47 (br m, 7H), 3.71 (br m, 1H), 4.18 (m, 1H), 7.31 (m, 5H), 7.40 (d, J = 8.0 Hz, 4H), 7.78 (d, J = 8.3 Hz, 4H); ^{13}C NMR (CD_3OD, 75 MHz) δ 21.5, 36.0, 38.4, 38.8, 54.4, 55.2, 56.0, 128.4, 129.0, 130.3, 130.6, 131.1, 135.6, 137.4, 145.6, 171.7.

*Benzylated disulfonamide (**16**)*. Under argon atmosphere, benzaldehyde (8.6 mg, 0.081 mmol) and **22** (40.5 mg, 1.1 equiv) were dissolved in 3 mL dichloroethane at room temperature. Sodium triacetoxyborohydride (34.3 mg, 2.0 equiv) was added to the stirred solution, which then was allowed to stir for 24 h. The reaction mixture was quenched by addition of saturated sodium hydro carbonate solution. The product was extracted with ethyl acetate (5 × 5 mL). The combined organic phases were washed with brine (15 mL) and dried over $MgSO_4$. After removal of the solvent in vacuo the crude product was purified by column chromatography using silica gel and ethyl acetate/methanol (8:2) elution. The product was obtained as a colorless oil (22.2 g, 46%);[7] ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.39 (s, 6H), 2.56 (br m, 6H), 2.77 (br m, 2H), 2.89 (br m, 4H), 4.06 (s, 2H), 5.00 (br s, 3H), 7.24 (d, J = 8.3 Hz, 4H), 7.34 (m, 3H), 7.45 (d, J = 7.5 Hz, 2H), 7.75 (d, J = 8.3 Hz, 4H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.5, 41.2, 45.5, 52.2, 52.4, 54.9, 127.1, 128.3, 128.9, 129.5, 129.6, 134.8, 137.3, 143.1; TLC (7:3 EtOAc/MeOH) R_f = 0.40.

*NBD disulfonamide (**17**)*. TREN disulfonamide **22** (35.1 mg, 0.0772 mmol) was dissolved in MeOH (5 mL) under argon at 0 °C. K_2HCO_3 (32.0 mg, 3.0 equiv) was added. The resulting suspension was allowed to stir for 5 min at 0 °C and for additional 10 min at room temperature. After that a solution of NBD–Cl (15.4 mg, 1.0 equiv) in MeOH (5 mL) was added drop wise over 5 min. The reaction was stirred at rt for 24 h after which time the solvent was removed in vacuo. The residue was extracted with ethyl acetate. After that the solvent was removed in vacuo and the crude product was purified by column chromatography on Al_2O_3 using 0.5–1.0% MeOH:ethyl acetate gradient elution. The product **17** was obtained as a orange-green oil (19.6 mg, 41%); ^{1}H NMR ($CDCl_3$, TMS, 300 MHz) δ 2.36 (s, 6H), 2.52 (t, J = 6.1 Hz, 4H), 2.69 (t, J = 6.3 Hz, 2H), 2.84 (q, J = 6.1 Hz, 4H), 3.43 (br s, 2H), 5.59 (unresolved t, 2H), 6.25 (br m, 1H), 7.30 (d, J = 8.0 Hz, 5H), 7.67 (d, J = 8.3 Hz, 4H), 8.46 (d, J = 8.8 Hz, 1H); ^{13}C NMR ($CDCl_3$, TMS, 75 MHz) δ 21.4, 41.9, 42.3, 52.6, 54.2, 99.9, 123.5, 127.7, 130.6, 138.2, 144.4, 145.5; TLC (EtOAc, SiO_2) R_f = 0.72.

*Mono-N-Boc TREN (**20**)*. To a stirred solution of tris-(2-aminoethyl)amine (**19**) (5.1 mL, 35 mmol) in dioxane (30 mL) under nitrogen a solution of di-*tert*-butyl-dicarbonate (1.2 mL, 5.5 mmol) in dioxane (30 mL) was added over 1 h at rt. The reaction mixture was stirred for 17 h. The solvent was removed in vacuo and the residue was dissolved in water (10 mL). The aqueous solution was extracted with dichloromethane (6× 15 mL). The organic phases were combined. The removal of the solvent in vacuo gave the product **20** (1.247 g, 92%) as a viscous oil; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.45 (s, 9H), 1.69 (s, 4H), 2.54 (t, J = 6.0 Hz, 4H), 2.57 (t, J = 5.8 Hz, 2H), 2.78 (t, J = 6.0 Hz, 4H), 3.20 (m, 2H), 5.32 (br s, 1H); TLC (10:2:1 $CH_3CN/H_2O/NH_4OH$) R_f = 0.20.

*Mono-N-Boc TREN disulfonamide (**21**)*. A solution of **20** (1.247 g, 5.062 mmol) and triethylamine (2.8 mL, 4.0 equiv) in acetonitrile (80 mL) was prepared at room temperature under nitrogen. While stirring, a solution of toluenesulfonylchloride in acetonitrile (40 mL) was added drop wise during 5 min. The reaction mixture was allowed to stir as rt for 17 h after which time the solvent was removed in vacuo. The residue was dissolved in dichloromethane (40 mL), washed with water (2× 10 mL) and with brine (10 mL). After removal of the solvent the crude product was purified by column chromatography using silica gel and ethyl acetate/hexanes gradient elution (30–60 vol% ethyl acetate). The product **21** (2.091 g, 74%) was obtained as a viscous oil; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.43 (s, 9H), 2.38 (t, J = 5.8 Hz, 2H), 2.41 (s, 6H), 2.47 (t, J = 5.2 Hz, 4H), 2.91 (m, 4H), 3.03 (m, 2 H), 5.07 (t, J = 5.5 Hz, 1H), 5.80 (t, J = 5.0 Hz, 2H), 7.30 (d, J = 8.0 Hz, 4H), 7.78 (d, J = 8.0 Hz, 4H); TLC (6:4 EtOAc/cyclohexane) R_f = 0.31.

*TREN disulfonamide (**22**)*. Over 15 min TFA (36 mL) was added drop wise to a stirred solution of **21** (700 mg, 1.26 mmol) in dichloromethane (36 mL) at 0 °C under nitrogen. The reaction mixture was allowed to stir at 0 °C for 60 min after which the solvents were removed in vacuo. The residue was dissolved in 60 mL ethyl acetate and extracted with 1 M HCl (5× 10 mL). To the combined aqueous phases saturated aqueous potassium carbonate solution was added until the solution became basic. The basic solution was extracted with ethyl acetate (4× 20 mL). The combined organic phases were washed with brine (20 mL) and dried with magnesium sulfate. Removal of the solvent in vacuo gave the product **22** (324 mg, 57%) as a viscous oil; ^{1}H NMR (CD_3OD, 300 MHz) δ 2.38 (s, 6H), 2.48 (m, 6H), 2.71 (t, J = 5.3 Hz, 2H), 2.87 (t, J = 5.8 Hz, 4H), 7.34 (d, J = 8.2 Hz, 4H), 7.76 (d, J = 8.2, 4H).

5.2. Biochemical assays

5.2.1. Vesicle inward translocation assay

NBD–PC, NBD–PS and POPC were purchased from Avanti Polar Lipids. Cholesterol was purchased from Sigma–Aldrich. All

phospholipid stocks were stored in $CHCl_3$ in a −20 °C freezer. An appropriate mixture of cholesterol and POPC was dried in vacuo for 1 h. A stock solution of 10 mM vesicles was made by rehydration at room temperature with TES buffer (5 mM TES, 100 mM NaCl, pH 7.4). Multilamellar vesicles were extruded to form unilamellar vesicles with a Basic LiposoFast device purchased from Avestin, Incorporated. The vesicles were extruded 29 times through a 19-mm polycarbonate Nucleopore filter with 100-nm diameter pores.

To a stock solution of vesicles (final concentration 25 μM) in TES buffer, 0.5 mol% NBD–PC (in 200 proof ethanol) was added. From this stock solution 3 mL samples were assayed using a Perkin-Elmer Luminescence Spectrometer LS50B (excitation was set at 460 nm while the emission was measured at 530 nm using a 515 nm cut off filter). During this 200 s assay time, at 50 s 180 μL of sodium hydrosulfate (sodium dithionite; 60 mM in 1 M Tris, pH ~10) was added and at 150 s 20 μL of 20% Triton X-100 (v/v) was added. The amount of total NBD–phospholipid which was translocated to the inside of the vesicles was calculated by the following equation:

$$\% \text{ Exo NBD-PX} = ((F_i - F_f)/F_i) * 100$$

where F_i and F_f are the fluorescence intensities just prior to the additions of sodium dithionite and Triton X-100. The values reported are the averages of 2 or 3 repeated experiments. All percent exo-probe values contain a 5% error and were normalized to the initial time point taken.

5.2.2. NBD protection assay

To a solution of vesicles (final concentration 25 μM) in TES buffer was added **17** from a DMSO stock solution (25 mM). From this vesicle solution, 3 mL samples were withdrawn and assayed using a Perkin-Elmer Luminescence Spectrometer LS50B (excitation was set at 460 nm while the emission was measured at 530 nm using a 515 nm cut off filter). An aliquot of sodium dithionite (180 μL of a 60 mM stock in Tris buffer) was added at 50 s, and a 20 μL aliquot of 20% Triton X-100 (v/v) was added at 150 s.

5.2.3. Cell culture

Jurkat and LNCap cells were cultured separately in 75 cm^2 tissue culture flasks using RPMI media with 10% FBS. PC3 cells were cultured in 75 cm^2 tissue culture flasks using Ham's F-12K media supplemented with 10% FBS. All cell lines were incubated at 37 °C in a 5% CO_2 atmosphere.

5.2.4. Cell uptake of fluorescent TREN

TREN compound **17** (12.5 μM, 25 mM DMSO stock) was added to 200 μL of Jurkat cells (1×10^6 cells/mL) in growth media with a subsequent incubation at 37 °C, 5% CO_2 for 30 min. ER Tracker™ and Mito Tracker™ were purchased from Molecular Probes and used as directed by manufacture protocols. The sample was centrifuged at 1500 rpm for 2 min and washed twice with TES buffer (5 mM TES, 100 mM NaCl, pH 7.4). After washing, the cells were visualized on an Axiovert S100 TV microscope (Carl Zeiss) equipped with filter sets DAPI/Hoechst/AMCA, FITC/RSGFP/Bodipy/Fluo3/DiO, Cy3 (Chroma). Images were acquired using a black and white digital camera and colored upon acquisition using Metamorph software version 6.2.

5.2.5. Endogenous PS exposure

At 90% confluency the PC3 cells were washed twice with PBS buffer then incubated at 37 °C, 5% CO_2 with 2 mL of 2.5× trypsin until the cells detached. Fresh media (8 mL) was added and the cell sample was centrifuged for 5 min at 4 °C and re-suspended in TES buffer to obtain a cell count of 1×10^5 cells/mL. The TREN scramblase (25 mM stock solution in DMSO) was added to 0.5 mL of the cell dispersion and incubated for 2 h at 37 °C, 5% CO_2. The samples were pelleted through centrifugation at 1500 rpm for 5 min and re-suspended in binding buffer. Annexin V-FITC (5 μL) and the nuclear stain 7AAD (500 ng/mL) were added to the samples and incubated for 15 min at 37 °C. Necrotic cells, as well as those cells in the advanced stages of apoptosis, have permeablized membranes and allow 7AAD to stain the cell nucleus. Immediately after staining, flow cytometry was performed on a Beckman Coulter Cytomics FC 500 MPL (Fullerton, CA) flow cytometer. Data analysis was performed using CXP Software (Fullerton, CA).

Another set of experiments used vanadate as a flippase inhibitor. At 60% confluencey, PC3 cells were added to fresh media and incubated with varying concentrations of sodium orthovanadate for 2 h at 37 °C. The concentrations of vanadate ranged from 0 to 800 μM. The TREN scramblase **2** (1 mM stock solution in DMSO) was added to the cells and incubated for 1 h at 37 °C. Cells were labeled with Annexin V-FITC (15 μL), incubated at room temperature for 15 min, washed with 1 mL of PBS, and then incubated at 37 °C with trypsin to detach them. Samples were re-suspended in 1 mL of Ham's F-12K media and flow cytometry was performed as described above.

5.2.6. MTT assay

The Vibrant® MTT cell proliferation assay kit was purchased from Molecular Probes (Invitrogen), Inc. At the time of assay 200 μL of cells (at least 5×10^4 cells/mL) in growth media were dispensed in each well of a clear 96-well plate. TREN scramblases (in a DMSO stock) were administered and incubated for a 24 h period. For the Jurkat cells the plate was centrifuged at 1500 rpm for 5 min and the growth media was exchanged for 100 μL of fresh RPMI media and 10 μL of a 12 mM MTT stock (5 mg MTT in 1 mL filtered PBS buffer). For the PC3 cells or the LNCap cells the media were exchanged for 100 μL of fresh growth media and 10 μL of a 12 mM MTT stock. After 4 h, 100 μL of a SDS stock (1 g SDS in 10 mL 0.01 M HCl) was added to each well and the plate was incubated for 18 h at 37 °C, 5% CO_2. The absorbance was measured using a Labsystems Multiskan Ascent plate reader at the λ_{max} is 570 nm. In order to determine the percent living cells in each sample the following equation was used:

$$\% \text{ Living cells} = (A - A_{Neg})/(A_{Con} - A_{Neg}) * 100$$

where A is the absorbance of the well tested, A_{Neg} is the absorbance of the negative control (no cells added) and A_{Con} is the absorbance of the positive control (10 μL DMSO added).

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Supporting information

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.11.011.

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