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Thapsigargin Analogues for Targeting Programmed Death of Androgen-Independent Prostate Cancer Cells

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Abstract—A number of analogues of thapsigargin, a selective inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPases have been synthesized. In all of the prepared analogues the butanoyl residue at *O*-8 has been replaced with a residue containing an aromatic amine. The amine can be used as an anchoring point for attaching a peptide group sensitive to the proteolytic enzyme, prostate specific antigen, secreted by prostate cancer cells. Like thapsigargin, the analogues are capable of elevating the cytoplasmic Ca^{2+} concentration approximately sevenfold when tested at effective cytotoxic doses. The analogues in which the 8-*O*-butanoyl group has been replaced with 3-(4-aminophenyl)propanoyl or 4-aminocinnamoyl were found potently to induce programmed cell death of the prostate cancer cells.

Introduction

Currently there is no treatment that significantly prolongs survival in men with metastatic prostate cancer.[1] Androgen ablation therapy eventually fails because the metastatic prostate cancer within an individual patient is heterogenously composed of clones of both androgen dependent and independent cancer cells.[2] Thapsigargin (**1**, Tg, Fig. 1) is a sesquiterpene lactone[3] that selectively inhibits the sarcoplasmic and the endoplasmic reticulum Ca^{2+}-ATPases (SERCA).[4,5] Inhibition of the SERCA causes depletion of intracellular Ca^{2+} stores resulting in an initial rise in cytoplasmic Ca^{2+} concentration, which by capacitance influx of extracellular Ca^{2+} affords a secondary sustained elevation of cytoplasmic Ca^{2+}. This sustained elevation in the cytosolic Ca^{2+} concentration activates the proliferation independent programmed death of susceptible cells including androgen independent prostate cancer cells.[6] Since the SERCA is present in almost all cells Tg will also induce apoptosis in many normal host cells. Consequently a method of targeting the proliferation independent cytotoxicity of Tg selectively to prostate cancer cells is needed. In order to target Tg, advantage can be taken of the secretion of prostate-specific antigen (PSA) by prostate cancer cells.[7] PSA is a serine protease with an unusual specificity. Thus, PSA efficiently liberates 7-amino-4-methylcoumarin from a substrate in which the amino group of this coumarin is coupled to the six-amino acid peptide His-Ser-Ser-Lys-Leu-Gln. In contrast, this conjugate is a very poor substrate for other purified proteases and proteases present in sera.[8] The specificity has further been illustrated by incubating cancer cells with a prodrug in which the amino group of doxorubicin is coupled to this six-amino acid. Only PSA producing cells are killed, indicating that doxorubicin is only liberated in the vicinity of such cells.[9] A fast inactivation of PSA outside the prostate ductal system means that the hydrolytic activity only will be of significance in the close vicinity of prostate cells and prostate cancer cells. The aim of the present project is to develop a prodrug consisting of a primary amine containing Tg analogue, which via the amine group is coupled to a PSA cleavable peptide.[3] This paper describes design, synthesis and SERCA inhibitory potencies of analogues of Tg that can be coupled to promoiety groups.

Key words: Prostate cancer; prodrug; thapsigargin; prostate-specific antigen; apoptosis.
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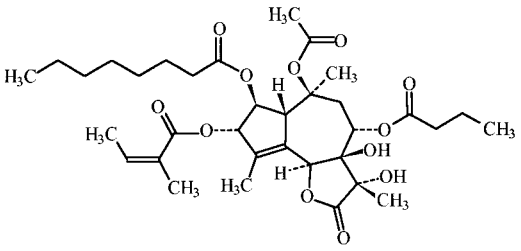


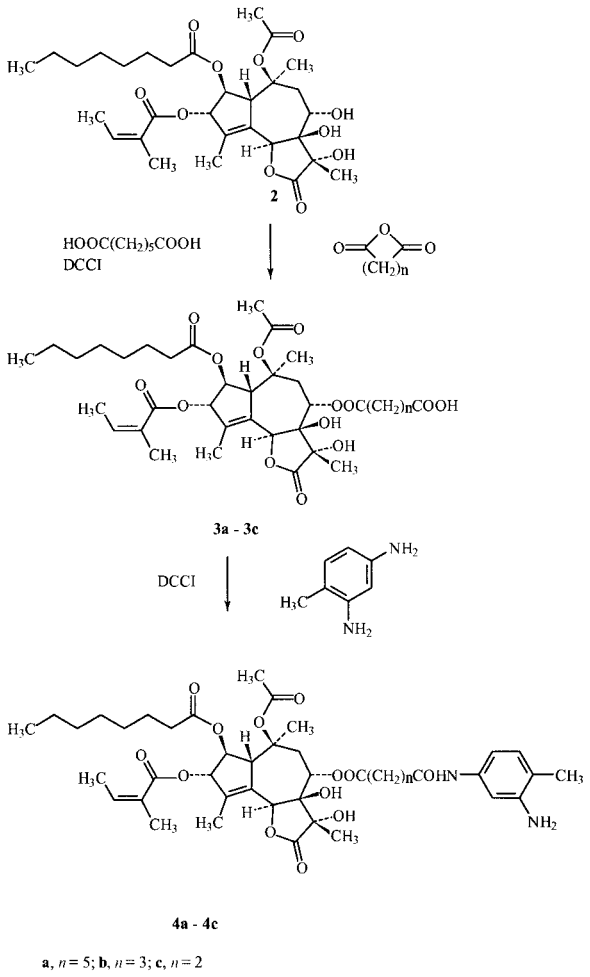
1 Tg

Figure 1. Structure of thapsigargin (**1**, Tg).

Synthesis

All the 13 analogues have been prepared from 8-*O*-debutanoylthapsigargin (**2**). For the preparation of **4a** pimelic acid was reacted with **2** in the presence of *N,N*'-dicyclohexylcarbodiimide (DCCI) to give 8-*O*-(6-carboxyhexanoyl)-8-*O*-debutanoylthapsigargin (**3a**), which in the presence of DCCI was coupled with 2,4-diaminotoluene. The carboxylic acids (**3b**) and (**3c**) were prepared using glutaric and succinic anhydride, respectively, as starting materials. The analogues (**4b**) and (**4c**) were obtained through dicyclohexylcarbodiimide (DCCI) promoted coupling of **3b** and **3c** with 2,4-diaminotoluene (Scheme 1).

The analogues **6a** through **6e** all have been prepared by DCCI promoted esterification of the BOC protected aminocarboxylic acids **7a**–**7e** with **2** (Scheme 2).



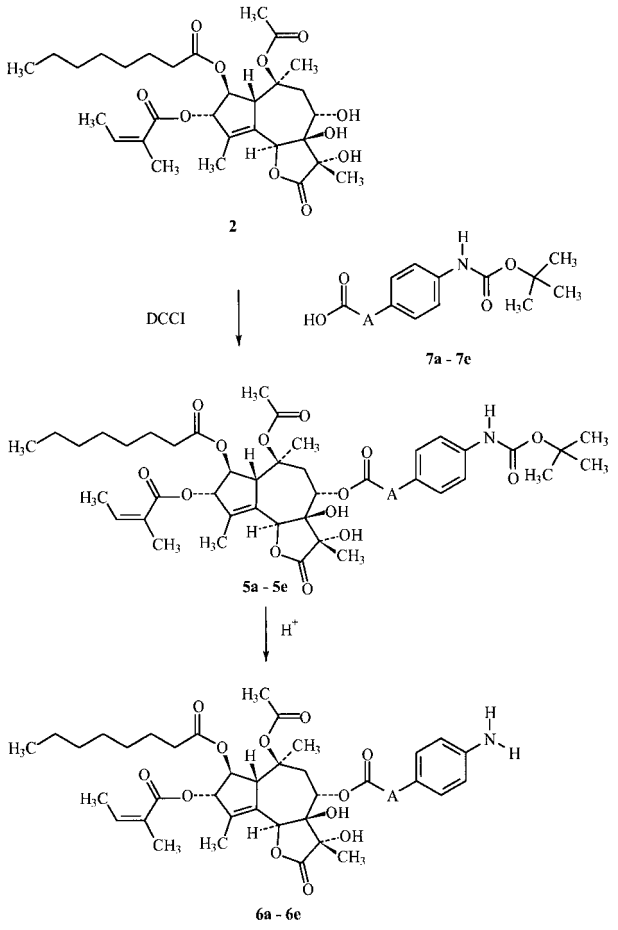
Scheme 1. Synthesis of the analogues containing a 2,4-diaminotoluene residue (**4a–4c**).

Results and Discussion

Previously, it has been shown that Tg (**1**) can induce programmed cell death in androgen independent prostate cancer cell lines with a LC_{50} value of approximately 100 nM.[6] In order to irreversibly commit cells to undergo apoptosis chronic exposure for greater than 24 h is needed; exposure to 500 nM for up to 12 h did not result in loss of clonogenic ability or viability.[6] In the present study TSU-Pr1 human prostate cancer cells were exposed for 72 h to Tg or a series of Tg analogues, in which the butanoyl group of the 8-*O*-butanoyl group has been replaced with an acyl group containing an amino group. The amino group might be used as an anchoring point for attachment of a promoiety peptide. It was decided to replace the butanoyl group because a model of the binding site indicates that when Tg is bound to the SERCA this group is situated in a cave.[10] Consequently, replacement of the butanoyl group with a more voluminous group should preserve the affinity. After exposure, cells were assayed for the loss of clonogenic survival (i.e. cytotoxic response) and LC_{50} were determined for the various analogues, Table 1. Table 1 also gives the IC_{50} values of the analogues for inhibition of skeletal muscle Ca^{2+}-ATPase (SERCA1). The affinity of SERCA1 towards Tg equals that of SERCA2b, a housekeeping SERCA isoform expressed in all cell types.[4] The SERCA1 activity was assayed as the ATP-dependent $^{45}Ca^{2+}$ uptake in a preparation of sarcoplasmic reticulum (SR) membranes isolated from rabbit skeletal muscle (see the Experimental). At least 50% of the protein in this preparation consisted of Ca^{2+}-ATPase, as determined by densitometric scanning of SDS-polyacrylamide gels. The data in Table 1 were obtained with either 2 or 5 μg/mL of the SR membrane protein in the assay. Two different preparations have been used to give SERCA1 enzymes, in which the concentration of SERCA differs. The low IC_{50} value of Tg[5] causes Tg to bind to the binding site in reality in an irreversible way. Consequently different concentrations of SERCA in the assay will appear to give different IC_{50} values. To overcome this problem activities relative to that of Tg are given. Notice, that in the case of **4a** the IC_{50} value for inhibition of the SERCA is only approximately 4.5 times larger than that of Tg, whereas the ability to kill TSU cells is approximately 100 times smaller.

For analogues **4a** and **4b** whereas the IC_{50} value for inhibition of the SERCA is only approximately 4–5 times larger than that of Tg the concentration needed to kill TSU cells is approximately 100–300 times higher. More encouraging results were obtained with the aminobenzenecarboxylic acid esters **6a**–**6e**. The analogues **6c**–**6e** possess submicromolar LC_{50} values. The corresponding IC_{50} values are of the same order of magnitude

as that of Tg. Surprisingly the LC_{50} value of **6a** was 100 times less active than Tg and **6b** only showed minor activity in concentrations up to 10 μM.



a, A = -$(CH_2)_0$-; **b**, A = -(CH_2)-, **c**; A = -$(CH_2)_2$; **d**, A = -CH=CH-; **e**, A = -$(CH_2)_3$-

Scheme 2. Synthesis of the analogues containing a 4-aminobenzene carboxylic acid (**5a–5e** and **6a–6e**).

The cytotoxicity of Tg on prostate cancer cells is due to its ability to sustain an increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) more than 10 h leading to the subsequent activation of the programmed death pathway in these cells.[6] Several of the analogues were tested for their ability to elevate $[Ca^{2+}]_i$ in TSU cells using appropriate drug levels as based upon their LC_{50} values. The 4 analogues **4a** and **6c–6e** produced the same initial rise in $[Ca^{2+}]_i$ as Tg and also like Tg sustained an approximately sevenfold elevation in $[Ca^{2+}]_i$ for more than 4 h of treatment in TSU cells, Table 2. If EGTA was added to lower the extracellular Ca^{2+} concentration to below 100 nM in the media, then the $[Ca^{2+}]_i$ levels rapidly (i.e. minutes) return to below 50 nM. After 4 h of exposure to Tg or the analogue, further addition of 1 μM Tg to the media did not result in any further increase in $[Ca^{2+}]_i$. This indicates that theses analogues like Tg itself [6] deplete the ER calcium stores leading to the capacitive entrance of extracellular Ca^{2+} into the cells to elevate $[Ca^{2+}]_i$.[11] The analogue **6b** that did not induce programmed cell death was also unable to increase the $[Ca^{2+}]_i$ up to a concentration of 10 μM, Table 2.

In conclusion, coupling of the analogues **6c–6e** to peptides, which are selectively cleaved by PSA will allow for targeted delivery of these cytotoxic agents to sites of PSA producing metastatic prostate cancer. Currently we are coupling the analogues to the peptide carrier for further in vitro and in vivo testing for activity against PSA producing prostate cancers.

Experimental

Chemistry

The spectra have been recorded on a AF200X Bruker spectrometer in deuterated chloroform solutions using tetramethylsilane as an internal standard. In all the spectra the signals originating in the acetyl, angeloyl,

Table 1. IC_{50} values for inhibition of the skeletal muscle Ca^{2+}-ATPase and LC_{50} values for loss of clonogenic survival for Tg (**1**) and some analogues against TSU-Pr1 human prostate cancer cells

Compound	To inhibit Ca^{2+}-ATPase 50%[a] (IC_{50} in nM)	To inhibit Ca^{2+}-ATPase 50%[b] (IC_{50} in nM)	Activity relative to Tg (**1**)[c]	To kill 50% of clonogenic cells (LC_{50} in μM)
Tg (**1**)	3.9 ± 0.2 (6)	11.0 ± 1.1 (4)	1	0.030 ± 0.004 (5)
dBTg (**2**)	21 ± 1 (3)		0.19	10.0 ± 0.5 (5)
4a sch1	17.2 ± 2.4 (3)		0.22	3.1 ± 0.2 (5)
4b sch1	316 ± 22 (3)		0.012	16.6 ± 0.4 (5)
4c sch1	1278 ± 91 (3)		0.0030	> 25
6a sch2		18.4 ± 0.3 (3)	0.59	4.0 ± 0.3 (5)
6b sch2		n.d.		> 10
5c sch2		21.2 ± 0.8 (3)	0.52	0.87 ± 0.02 (5)
6c sch2		16.2 ± 0.9 (3)	0.68	0.28 ± 0.06 (5)
5d sch2		20.3 ± 0.2 (3)	0.54	1.9 ± 0.1 (5)
6d sch2		18.7 ± 0.2 (3)	0.59	0.11 ± 0.03 (5)
5e sch2		14.4 ± 0.8 (3)	0.76	n.d.
6e sch2		18.4 ± 1.4	0.60	0.23 ± 0.02 (5)

Results expressed as mean ± standard error (number of experiments).
[a] The concentration of SR protein in the test solution was 2 μg/mL.
[b] The concentration of SR protein in the test solution was 5 μg/mL.
[c] The ratio of the IC_{50} value of Tg/IC_{50} value of the analogue.

Table 2. Increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in TSU-Pr1 cells induced by Tg and the Tg analogues at effective cytotoxic concentrations[a]

Compound	Concentration in μM	$[Ca^{2+}]_i$ in nM
Control	—	51 ± 20
Tg (**1**)	0.10	283 ± 19
4a	10	275 ± 32
6b	10	54 ± 6
6c	0.80	253 ± 26
6d	0.30	295 ± 56
6e	0.70	233 ± 11

[a] The cells were treated wi th Tg or the analogue for 1 h prior to the determinations of $[Ca^{2+}]_i$. All experiments were performed in triplicate. Results expressed as mean ± standard error.

butanoyl, and octanoyl residues have been found as previously reported[12] and are not listed. The ^{1}H NMR spectra were recorded at 200 MHz. The signals of H-9′ have in many cases been overlapped by the signals from the α protons in the octanoyl residue. The ^{13}C NMR spectra were recorded at 50 MHz. In the ^{13}C NMR spectra the assignments of signals with similar chemical shift values might be interchanged. The signals originating in C-2 and C-6 are hidden by the signals of chloroform, but have been visualised in a few cases by recording the DEPT spectra. The small amounts of compounds available have in some cases precluded observation of signals of poor intensities. Unless otherwise stated column chromatography has been performed over silica gel 60, 0.040–0.063 mm, Merck. The purity of all target compounds has been verified by HPLC to be more than 95%.

8-*O*-{6-(*N*-[3-Amino-4-methylphenyl]-carboxamido)hexanoyl}-8-*O*-debutanoylthapsigargin (4a). A solution of **2**[13] (103 mg, 0.177 mmol), pimelic acid (185 mg, 1.16 mmol), DCCI (233 mg, 1.15 mmol) and dimethylaminopyridine (100 mg, 0.71 mmol) in dichloromethane is left for 3 h. The reaction mixture was filtered and the filtrate washed twice with hydrochloric acid (0.5 M, 10 mL). The organic phase was concentrated and 8-*O*-(6-carboxyhexanoyl)-8-*O*-debutanoylthapsigargin (**3a**, 63 mg, 49%) isolated by repeated chromatography using an eluent consisting of toluene/ethyl acetate (6/1) added 1% of acetic acid, to which increasing amounts of ethyl acetate were added. A solution of 8-*O*-{6-carboxyhexanoyl}-8-*O*-debutanoylthapsigargin (52 mg, 72 μmol), 2,4-diaminotoluene (75 mg, 635 μmol), and DCCI (27.3 mg, 132 μmol) in methylene chloride (6 mL) was left for 1 h at room temperature. The mixture was filtered, and the filtrate concentrated in vacuo to give a residue from which 26 mg (43%) of **4a** was isolated as as a colourless amorphous powder by column chromatography using an eluent consisting of toluene/ethyl acetate (9/1). ^{1}H NMR ($(CD_3)_2CO$) δ guaianolide 4.38 (br s, H-1), 5.52 (dd, J = 4.0 and 3.2 Hz, H-2), 5.80 (br q, J = 3 Hz, H-3), 5.67 (m, H-6), 5.70 (t, J = 3.6 Hz, H-8), 3.02 (dd, J = 15 and 3 Hz), 1.86 (br s H-15), 1.43 (s, H-13), 1.42 (s, H-14); diaminotoluene 2.00 (br s, CH_3), (7.12, d J = 2.0 Hz, H-2), 7.08 (d, J = 8.2 Hz, H-5), 7.18 (dd, J = 8.2 and 2.0 Hz, H-6), pimeloyl 2.25–2.35 (m, H-2 and H-6), 1.55–1.65 (m, H-3 and H-5), 1.35–1.40 (m, H-4). MS FAB$^-$ m/z 825 [M−H$^+$].

8-*O*-(4-Carboxybutanoyl)-8-*O*-debutanoylthapsigargin (3b). A solution of 8-*O*-debutanoylthapsigargin (**2**,[13] 100 mg, 0.17 mmol), glutaric anhydride (400 mg, 3.5 mmol) and 4-dimethylaminopyridine (100 mg, 0.82 mmol) in methylene chloride (10 mL) was left for 21 h at room temperature and filtered. The filtrate was added 4 M hydrochloric acid (10 mL) and extracted twice with ethyl acetate (10 mL). The organic phase was concentrated in vacuo to give 320 mg of a residue from which 68 mg (58%) of **3b** was isolated by column chromatography using an eluent consisting of toluene/ethyl acetate/acetic acid (5/1/0.01), to which increasing amounts of ethyl acetate were added. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.22 (br s, H-1), 5.46 (br t, J = 2 Hz, H-2), 5.7–5.5 (br, H-3, H-8, and H-6), 2.95 (dd, J = 15 and 3 Hz, H-9), 1.78 (br s H-15), 1.43 (s, H-13), 1.38 (s, H-14); glutaryl 2.2–2.4 (m, H-2 and H-4), 1.5–1.7 (m, H-3); ^{13}C NMR ($CDCl_3$) δ. The guaianolide nucleus 57.6 (C-1), 83.9 (C-3), 141.2 (C-4), 130.1 (C-5), 78.3 (C-7/C-11), 66.2 (C-8), 37.9 (C-9), 84.2 (C-10), 78.5 (C-11/C-7), 176.3 (C-12), 15.6 (C-13), 21.8 (C-14), 12.5 (C-15); glutaroyl 171.2 (C-1), 33.4 (C-2), 20.5 (C-3), 31.6 (C-4). MS FAB$^-$ m/z 693 [M−H$^+$].

8-*O*-{4-(*N*-[3-Amino-4-methylphenyl]-carboxamido) butanoyl}-8-*O*-debutanoylthapsigargin (4b). A solution of **3b** (14 mg, 20 μmol), 2,4-diaminotoluene (10 mg, 80 μmol), and DCCI (7.9 mg, 40 μmol) in methylene chloride (2 mL) was left for 2 h at room temperature. The mixture was filtered, and the filtrate concentrated in vacuo to give a residue from which 8 mg (50%) of **4b** was isolated as a colourless amorphous powder by column chromatography using an eluent consisting of toluene/ethyl acetate (9/1), to which increasing amounts of ethyl acetate were added. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.17 (br s, H-1), 5.46 (br t, J = 2 Hz, H-2), 5.6–5.4 (br, H-3, H-8, and H-6), 2.80 (dd, J = 15 and 3 Hz, H-9), 1.78 (br s H-15), 1.43 (s, H-13), 1.38 (s, H-14); diaminotoluyl 2.00 (br s, CH_3), (7.03, d J = 2.0 Hz, H-2), 6.90 (d, J = 8.2 Hz, H-5), 6.72 (dd, J = 8.2 and 2.0 Hz, H-6), glutaroyl 2.2–2.4 (m, H-2 and H-4), 1.5–1.7 (m, H-3); ^{13}C NMR ($CDCl_3$) δ the guaianolide nucleus 57.6 (C-1), 83.4 (C-3), 141.4 (C-4), 130.3 (C-5), 78.2 (C-7), 66.2 (C-8), 37.9 (C-9), 84.2 (C-10), 78.2 (C-11), 175.9 (C-12), 15.5 (C-13), 22.0 (C-14), 12.8 (C-15); glutaroyl 170.6 (C-1), 33.2 (C-2), 22.2 (C-3), 31.3 (C-4), 161.9 (C-5); diaminotoluyl 138.4 (C-1), 106.5 (C-2), 136.9 (C-3), 118.4 (C-4), 129.6 (C-5), 110.0 (C-6). MS FAB$^-$ m/z 797 [M−H$^+$].

8-*O*-{3-(*N*-[3-Amino-4-methylphenyl]-carboxamido) propanoyl}-8-*O*-debutanoylthapsigargin (4c). The intermediate 8-*O*-{6-carboxypropanoyl}-8-*O*-debutanoylthapsigargin (**3c**) was prepared as described above for **3b** using **2** and succinic anhydride as starting materials. A solution of **3c** (25 mg, 33 μmol), 2,4-diaminotoluene (14 mg, 120 μmol), and DCCI (6 mg, 30 μmol) in methylene chloride (3 mL) was left for 4.5 h at room temperature. The mixture was filtered and the filtrate concentrated in vacuo to give a residue from which 8 mg (33%) of **4c** was isolated as a colourless amorphous powder by column chromatography using an eluent consisting of toluene/ethyl acetate (9/1), to which

increasing amounts of ethyl acetate were added. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.20 (br s, H-1), 5.46 (br t, J = 2 Hz, H-2), 5.59 (br t, H-8), 5.62 (br, H-3, and H-6), 2.92 (dd, J = 15 and 3 Hz, H-9), 2.42 (dd, J = 15 and 3 Hz, H-9'), 1.85 (br s H-15), 1.40 (s, H-13), 1.38 (s, H-14); diaminotoluyl 1.95 (br s, CH3), (7.00, d J = 2.0 Hz, H-2), 6.90 (d, J = 8 Hz, H-5), 6.72 (dd, J = 8 and 2.0 Hz, H-6), succinyl 2.68 (m, H-2 and H-3); ^{13}C NMR ($CDCl_3$) δ the guaianolide nucleus 57.6 (C-1), 83.4 (C-3), 141.4 (C-4), 130.1 (C-5), 78.6 (C-7), 67.1 (C-8), 38.1 (C-9), 84.6 (C-10), 176.0 (C-12), 15.9 (C-13), 22.6 (C-14), 12.9 (C-15); succinyl 172.6 (C-1), 29.1 (C-2), 29.0 (C-3), 167.1 (C-5); diaminotoluyl 138.8 (C-1), 136.4 (C-3), 119.7 (C-4), 127.4 (C-5). (C-2 and C-6 not seen). MS FAB$^-$ m/z 783 [M−H$^+$].

8-*O*-(4-*t*-Butoxycarbonylaminobenzoyl)-8-*O*-debutanoylthapsigargin (5a). A solution of **2**[13] (52.9 mg, 91 μmol) 4-*t*-butoxycarbonylaminobenzoic acid (**7a**, 40.0 mg, 169 μmol), DCCI (24.5 mg, 122 μmol) and DMAP (7.3 mg, 60 μmol) in dichloromethane (5 mL) was left for 7 h at room temperature. The solution was filtered and the filtrate was concentrated in vacuo. The residue was dissolved in a few milliliters of cold ethyl acetate and the solution filtered and concentrated in vacuo. Compound **5a** (40.1 mg, 55%) was isolated from the residue as a colourless amorphous powder by chromatography using toluene/ethyl acetate (5/1) to which increasing amounts of ethyl acetate were added as an eluent followed by chromatography over silanized silica gel 60 RP 2 (0.070–0.230 mm) using methanol/water (9/1) as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.20 (br s, H-1), 5.50 (br t, J = 2 Hz, H-2), 5.8–5.85 (H-3 and H-6), 5.72 (H-8), 3.14 (dd, J = 15 and 3 Hz, H-9), 2.45 (dd, J = 15 and 3 Hz, H-9'), 1.87 (br s H-15), 1.48 (s, H-13), 1.42 (s, H-14); 4-aminobenzoyl 7.85 (H-2 and H-6), 7.42 (H-3 and H-5); boc 1.52 (CH_3); ^{13}C NMR ($CDCl_3$) δ the guaianolide nucleus 57.2 (C-1), 84.0 (C-3), 141.2 (C-4), 130.2 (C-5), 78.6 (C-7), 67.1 (C-8), 38.1 (C-9), 84.5 (C-10), 78.5 (C-11), 175.6 (C-12), 15.9 (C-13), 22.5 (C-14), 12.9 (C-15); 4-aminobenzoyl 165.4 (C=O), 123.6 (C-1), 130.9 (C-2 and C-6), 117.6 (C-3 and C-5), 138.6 (C-4); boc 28.1 (CH_3), 81.3 (C-O), 152.2 (C=O). MS FAB$^-$ m/z 798 [M−H$^+$].

8-*O*-(4-Aminobenzoyl)-8-*O*-debutanoylthapsigargin (6a). A solution of **5a** (18.1 mg, 23 μmol) and trifluoroacetic acid (100 μL) in dichloromethane (2 mL) was left for 3.5 h at room temperature and concentrated in vacuo. Compound **6a** (14.0 mg, 88%) was isolated from the residue as a yellow amophous powder by column chromatography using an eluent consisting of toluene/ethyl acetate/acetic acid (4/1/0.01) to which increasing amounts of ethyl acetate were added. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.32 (br s, H-1), 5.51 (br t, J = 2 Hz, H-2), 5.8–5.85 (H-3, H-6, and H-8), 3.10 (dd, J = 15 and 3 Hz, H-9), 2.48 (dd, J = 15 and 3 Hz, H-9′), 1.88 (br s H-15), 1.48 (s, H-13), 1.45 (s, H-14); 4-aminobenzoyl 7.72 (H-2 and H-6), 6.60 (H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ the guaianolide nucleus 57.2 (C-1), 83.9 (C-3), 141.4 (C-4), 130.1 (C-5), 79.1 (C-7), 66.8 (C-8), 38.3 (C-9), 84.5 (C-10), 79.0 (C-11), 175.2 (C-12), 16.3 (C-13), 23.3 (C-14), 12.9 (C-15); 4-aminobenzoyl 163.6 (C=O), 118.3 (C-1), 131.8 (C-2 and C-6), 113.8 (C-3 and C-5), 151.3 (C-4). MS FAB$^-$ m/z 698 [M−H$^+$].

8-*O*-([2-*t*-Butoxycarbonylaminophenyl]acetyl)-8-*O*-debutanoylthapsigargin (5b). A solution of **2**[13] (60.0 mg, 100 mol), 4-*t*-butoxycarbonylaminophenyla-cetic acid (**7b**, 100.0 mg, 0.40 mmol), DCCI (39.1 mg, 0.19 mmol) and DMAP (23.0 mg, 0.19 mmol) in dichloromethane (5 mL) was left for 90 min at room temperature. The solution was filtered and the filtrate was evaporated in vacuo. The residue was dissolved in a few milliliters of cold ethyl acetate and the solution was filtered. The filtrate was concentrated in vacuo to give a residue from which **5b** (54.0 mg, 66%) was isolated as a colourless amorphous powder by column chromatography using toluene/ethyl acetate/acetic acid (4/1/0.01) to which increasing amounts of ethyl acetate were added as an eluent. ^{1}H NMR ($CDCl_3$) guaianolide 4.27 (br.s, H-1), 5.42 (t, J = 2 Hz, H-2), 5.48 (br.s, H-3), 5.63 (br.s, H-6), 5.55 (br.s, H-8), 2.94 (dd, J = 15 and 3 Hz, H-9), 2.20 (dd, J = 15 and 3 Hz, H-9′), 1.80 (br.s H-15), 1.38 (s, H-13), 1.18 (s, H-14); 4-aminophenylacetyl 7.30 (d, J = 7 Hz, H-2 and H-6), 7.11 (d, J = 7 Hz, H-3 and H-5), 3.55 (s, CH_2); boc 1.50 (s, CH_3); ^{13}C NMR ($CDCl_3$) guaianolide 57.4 (C-1), 76.7 (C-2), 84.1 (C-3), 141.4 (C-4), 130.3 (C-5), 77.6 (C-6), 78.4 (C-7), 66.7 (C-8), 37.9 (C-9), 84.7 (C-10), 78.4 (C-11), 176.1 (C-12), 15.9 (C-13), 22.5 (C-14), 12.8 (C-15); 2-aminophenylacetyl 171.1 (C=O), 130.7 (C-1), 130.2 (C-2 and C-6), 118.6 (C-3 and C-5), 137.5 (C-4), 40.9 (CH_2); boc 28.4 (CH_3), 80.7 (C−O), 153.1 (C−O). MS FAB$^-$ Found 812.3879. Calc. for $C_{43}H_{58}NO_{14}$ 812.3857.

8-*O*-(2-[4-Aminophenyl]acetyl)-8-*O*-debutanoylthapsigargin (6b). A solution of **5b** (63.4 mg, 78 mol) and trifluoroacetic acid (500 L) in dichloromethane (5 mL) was left for 3 h at room temperature and concentrated in vacuo. Compound **6b** (46.3, 83%) was isolated as an amorphous powder by chromatography using toluene/ethyl acetate/acetic acid (4/1/0.01) to which increasing amounts of ethyl acetate were added as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.26 (br.s, H-1), 5.43 (br.t, J = 2 Hz, H-2), 5.52 (s, H-3 and H-8) 5.64 (s, H-6), 2.97 (dd, J = 15 and 3 Hz, H-9), 2.20 (dd, J = 15 and 3 Hz, H-9′), 1.82 (br.s H-15), 1.34 (s, H-13), 1.21 (s, H-14); aminophenylacetyl 6.98 (d, J = 8 Hz, H-2 and H-6), 6.62 (d, J = 8 Hz, H-3 and H-5), 3.47 (s, CH_2); ^{13}C NMR ($CDCl_3$) guaianolide 57.3 (C-1), 76.7 (C-2), 84.1 (C-3), 141.4 (C-4), 130.5 (C-5), 77.7 (C-6), 78.4 (C-7), 66.6 (C-8), 37.1 (C-9), 84.8 (C-10), 78.4 (C-11), 176.2 (C-12), 15.7 (C-3), 22.5 (C-14), 12.6 (C-15); 2-aminophenylacetyl 171.3 (C=O), 40.7 (CH_2), 131.0 (C-1), 130.5 (C-2 and C-6), 115.6 (C-3 and C-5), 141.4 (C-4). FABMS: Found 712.3347. Calc. for $C_{38}H_{51}NO_{12}$ 712.3333.

8-*O*-(3-[4-*t*-Butoxycarbonylaminophenylpropionyl)-8-*O*-debutanoylthapsigargin (5c). A solution of **2**[13] (29.3 mg, 51 μmol), 3-(4-*t*-butoxycarbonylaminophenyl) propionic acid (**7c**, 30.2 mg, 114 μmol), DCCI (14.2 mg, 71 μmol) and DMAP (7.3 mg, 60 μmol) in dichloromethane (2 mL) was left for 3 h at room temperature. The solution was filtered and the filtrate was evaporated in vacuo. The residue was dissolved in a few milliliters of cold ethyl acetate and the solution was filtered. The filtrate was concentrated in vacuo to give a residue from which **5c** (30 mg, 72%) was isolated as a colourless

amorphous powder by column chromatography using toluene/ethyl acetate (5/1) to which increasing amounts of ethyl acetate were added as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.20 (br s, H-1), 5.39 (br t, J = 2 Hz, H-2), 5.5–5.6 (H-3, H-6, and H-8), 2.92 (dd, J = 15 and 3 Hz, H-9), 1.75 (br s H-15), 1.33 (s, H-13), 1.28 (s, H-14); aminophenylpropionyl 2.81 (br.t, J = 7.5 Hz, H-α), 2.51 (br.t, J = 7.5 Hz, H-β), 7.19 (H-2 and H-6), 7.02 (H-3 and H-5), boc 1.42 (CH_3); ^{13}C NMR ($CDCl_3$) δ the guaianolide nucleus 57.4 (C-1), 76.8 (H-2), 84.0 (C-3), 141.2 (C-4), 130.3 (C-5), 77.7 (C-6), 78.4 (C-7), 66.2 (C-8), 38.0 (C-9), 84.6 (C-10), 78.3 (C-11), 175.7 (C-12), 15.7 (C-13), 22.5 (C-14), 12.8 (C-15); 4-aminophenylpropionyl 171.9 (C=O), 36.1 (C-α), 29.6 (C-β), 134.8 (C-1), 128.6 (C-2 and C-6), 119.2 (C-3 and C-5), 136.3 (C-4), boc 28.2 (CH_3), 80.5 (C-O), 153.0 (C=O). MS FAB$^-$ m/z 826 [M−H$^+$].

8-*O*-(3-[4-Aminophenyl]propionyl)-8-*O*-debutanoylthapsigargin (6c). A solution of **5c** (40.5 mg, 49 μmol) and trifluoroacetic acid (400 μL) in dichloromethane (4 mL) was left for 45 min at room temperature and concentrated in vacuo. Compound **6c** (33.7 mg, 96%) was isolated as an amorphous powder by chromatography using toluene/ethyl acetate/acetic acid (4/1/0.01) to which increasing amounts of ethyl acetate were added as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.25 (br.s, H-1), 5.48 (br.t, J = 2 Hz, H-2), 5.55–5.70 (H-3, H-6, and H-8), 2.85 (dd, J = 15 and 3 Hz, H-9), 1.75 (br.s H-15), 1.28 (s, H-13), 1.23 (s, H-14); aminophenylpropionyl 2.71 (br.t, J = 7 Hz, H-α), 2.45 (br.t, J = 7 Hz, H-β), 6.95 (d, J = 7 Hz, H-2 and H-6), 6.65 (d, J = 7 Hz, H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ guaianolide 57.6 (C-1), 76.2 (C-2), 84.0 (C-3), 141.4 (C-4), 130.1 (C-5), 77.7 (C-6), 78.4 (C-7), 66.2 (C-8), 38.3 (C-9), 84.5 (C-10), 78.4 (C-11), 175.6 (C-12), 15.7 (C-13), 22.5 (C-14), 12.9 (C-15); 4-aminophenylpropionyl 172.0 (C=O), 36.3 (C-α), 29.8 (C-β), 131.0 (C-1), 129.1 (C-2 and C-6), 116.1 (C-3 and C-5), 143.4 (C-4). MS FAB$^-$ m/z 726 [M−H$^+$].

8-*O*-(4-*t*-Butoxycarbonylaminocinnamoyl)-8-*O*-debutanoylthapsigargin (5d). A solution of **2**[13] (62.1 mg, 107 μmol), 4-*t*-butoxycarbonlyaminocinnamic acid (**7d**, 69.7 mg, 265 μmol), DCCI (21.3 mg, 106 μmol) and DMAP (12.8 mg, 106 μmol) in dichloromethane (5 mL) was left for 3 h at room temperature. The solution was filtered and the filtrate was evaporated in vacuo. The residue was redissolved in a few milliliters of cold ethyl acetate and the solution was filtered and evaporated in vacuo to give yellowish foam. Compound **5d** (31.5 mg, 36%) was isolated as a white amorphous powder by chromatography using toluene/ethyl acetate (5/1) to which increasing amounts of ethyl acetate were added followed by chromatography over silanized silica gel 60 RP-2 (0.070–0.230 mm, Merck) using methanol/water (9/1) as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.31 (br s, H-1), 5.51 (br t, J = 2 Hz, H-2), 5.65–5.8 (H-3, H-6, and H-8), 3.08 (dd, J = 15 and 3 Hz, H-9), 2.40 (dd, J = 15 and 3 Hz, H-9′), 1.87 (br s H-15), 1.48 (s, H-13), 1.28 (s, H-14); 4-aminocinnamoyl 6.28 (d, J = 16 Hz, H-α), 7.59 (d, J = 16 Hz, H-β), 7.42 (H-2 and H-6), 7.28 (H-3 and H-5), boc 1.50 (CH_3); ^{13}C NMR ($CDCl_3$) δ the guaianolide nucleus 57.4 (C-1), 84.0 (C-3), 141.4 (C-4), 130.2 (C-5), 78.6 (C-7), 66.7 (C-8), 38.1 (C-9), 84.5 (C-10), 78.6 (C-11), 175.4 (C-12), 15.7 (C-13), 22.5 (C-14), 12.9 (C-15); 4-aminocinnamoyl 166.4 (C=O), 117.7 (C-α), 140.7 (C-β), 131.1 (C-1), 127.4 (C-2 and C-6), 118.2 (C-3 and C-5), 139.6 (C-4), boc 28.2 (CH_3), 81.9 (C-O), 152.3 (C=O). MS FAB$^-$ m/z 824 [M−H$^+$].

8-*O*-(4-aminocinnamoyl)-8-*O*-debutanoylthapsigargin (6d). A solution of **5d** (65.5 mg, 79 μmol) and trifluoroacetic acid (0.7 mL) in dichloromethane (7 mL) was left for 15 min at room temperature and concentrated in vacuo. Compound **6d** (45.1 mg, 78%) was isolated as a amorphous powder by chromatography using toluene/ethyl acetate/acetic acid (4/1/0.01) as an eluent to which increasing amounts of ethyl acetate were added as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.32 (br s, H-1), 5.48 (br t, J = 2 Hz, H-2), 5.65–5.8 (H-3, H-6, and H-8), 3.04 (dd, J = 15 and 3 Hz, H-9), 2.40 (dd, J = 15 and 3 Hz, H-9′), 1.89 (br s H-15), 1.50 (s, H-13), 1.48 (s, H-14); 4-aminocinnamoyl 6.18 (d, J = 16 Hz, H-α), 7.58 (d, J = 16 Hz, H-β), 7.32 (H-2 and H-6), 6.63 (H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ the guaianolide nucleus 57.3 (C-1), 84.1 (C-3), 141.1 (C-4), 130.2 (C-5), 78.6 (C-7), 66.8 (C-8), 38.2 (C-9), 84.5 (C-10), 78.5 (C-11), 175.6 (C-12), 15.9 (C-13), 22.5 (C-14), 12.5 (C-15); 4-aminocinnamoyl 167.0 (C=O), 112.4 (C-α), 149.0 (C-β), 124.2 (C-1), 130.0 (C-2 and C-6), 115.0 (C-3 and C-5), 146.0 (C-4). MS FAB$^-$ m/z 724 [M−H$^+$].

8-*O*-(4-[4-*t*-Butoxycarbonylaminophenyl]butan-oyl)-8-*O*-debutanoylthapsigargin (5e). A solution of **2**[13] (57.7 mg, 100 μmol), 4-(4-*t*-butoxycarbonylaminophenyl)butanoic acid (**7e**, 63.3 mg, 230 μmol), DCCI (25.8 mg, 120 μmol) and DMAP (20.1 mg, 170 μmol) in dichloromethane (3 mL) was left for 100 min at room temperature. The solution was filtered and the filtrate was evaporated in vacuo. The residue was dissolved in a few milliliters of cold ethyl acetate and the solution was filtered. The filtrate was concentrated in vacuo to give a residue from which **5e** (81.5 mg, 97%) was isolated as a colourless amorphous powder by column chromatography using toluene/ethyl acetate (2/1) to which increasing amounts of ethyl acetate were added as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.25 (br s, H-1), 5.42 (br t, J = 2 Hz, H-2), 5.5–5.6 (H-3, H-6, and H-8), 2.93 (dd, J = 14 and 3 Hz, H-9), 1.80 (br s H-15), 1.38 (s, H-13), 1.35 (s, H-14); aminophenylbytanoyl 2.53 (br.t, J = 7 Hz, H-α), 1.80 (m, H-β), 2.25 (t, J = 7 Hz, H-γ), 7.18 (H-2 and H-6), 7.05 (H-3 and H-5), boc 1.50 (CH_3); ^{13}C NMR ($CDCl_3$) δ the guaianolide nucleus 58.1 (C-1), 76.7 (H-2), 84.0 (C-3), 141.2 (C-4), 130.3 (C-5), 77.5 (C-6), 78.4 (C-7), 66.7 (C-8), 38.0 (C-9), 84.6 (C-10), 78.3 (C-11), 175.6 (C-12), 15.7 (C-13), 22.5 (C-14), 12.8 (C-15); 4-aminopheylbutanoyl 172.4 (C=O), 33.3 (C-α and C-γ), 26.0 (C-β), 135.8 (C-1), 128.8 (C-2 and C-6), 119.0 (C-3 and C-5), 136.1 (C-4), boc 28.2 (CH_3), 80.5 (C−O), 152.8 (C=O). MS FAB$^-$ Found 840.4093. Calc. for $C_{45}H_{63}NO_{14}$ 840.4170.

8-*O*-(4-[4-Aminophenyl]butanoyl)-8-*O*-debutanoylthapsigargin (6e). A solution of **5e** (33.1 mg, 39 μmol) and trifluoroacetic acid (400 μL) in dichloromethane (4 mL) was left for 45 min at room temperature and concentrated in vacuo. Compound **6e** (27.7 mg, 96%) was isolated as an amorphous powder by chromatography using toluene/ethyl acetate/acetic acid (2/1/0.01) to

which increasing amounts of ethyl acetate were added as an eluent. ^{1}H NMR ($CDCl_3$) δ guaianolide 4.25 (br.s, H-1), 5.68 (br.s, H-6), 5.63 (br.s. H-8), 5.59 (t, $J=3$ Hz, H-3), 5.47 (br.t, $J=2$ Hz, H-2), 2.97 (dd, $J=20$ and 3 Hz, H-9), 1.84 (br.s H-15), 1.40 (s, H-13), 1.37 (s, H-14); aminophenylbutanoyl 2.52 (t, $J=9$ Hz, H-α), 2.24 (t, $J=9$ Hz, H-γ), 1.80 (m, H-β), 6.94 (d, $J=7$ Hz, H-2 and H-6), 6.64 (H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ guaianolide 57.5 (C-1), 76.7 (C-2), 84.1 (C-3), 141.6 (C-4), 130.5 (C-5), 77.8 (C-6), 78.5 (C-7), 66.1 (C-8), 38.1 (C-9), 84.6 (C-10), 78.5 (C-11), 178.5 (C-12), 15.7 (C-13), 22.5 (C-14), 12.8 (C-15); 4-aminophenylbutanoyl 172.7 (C=O), 34.0 (C-α), 33.6 (C-γ), 26.9 (C-β), 129.3 (C-1), 129.3 (C-2 and C-6), 115.7 (C-3 and C-5), 143.9 (C-4). MS FAB$^-$ Found 740.3672. Calc. for $C_{40}H_{55}NO_{12}$ 740.3646.

Pharmacology

SERCA assay. Sarcoplasmic reticulum (SR) vesicles from rabbit skeletal muscle were prepared according to De Meis and Hasselbach[14] and stored in *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) 20 mM (pH 7.0), sucrose 300 mM, at −80 °C.

ATPase activity was measured as $^{45}Ca^{2+}$ uptake to SR vesicles at 25 °C, pH 7.0, using 2–5 μg/mL of protein. Triplicate samples of SR membranes were preincubated for 10 min in the uptake medium minus ATP, in the presence of the desired inhibitor concentrations (added as 100- or 200-fold concentrated stock solutions in DMSO; corresponding amount of DMSO was present in control incubations). The medium composition was then completed by adding ATP (or TES buffer for the blank determinations) to start the uptake reaction. The complete medium contained (concentrations in mM): TES 20; sucrose, 60; KCl 110; $MgCl_2$ 5; K^+-oxalate 6; EGTA 1; $^{45}CaCl_2$ 0.2 (about 2 μCi/mL); ATP 2.5 mM. (The concentration of free Ca^{2+} ions was 0.1 μM, calculated according to Foehr et al. 1993[15]). The uptake was stopped after 4 min by filtration through Whatman GF/F filters (presoaked in 0.1% polyethyleneimine to reduce the background radioactivity) on a Brandel Harvester M24 apparatus (SEMAT Technical Ltd., UK). The filters were washed immediately in an ice-cold buffer containing (concentrations in mM): tris/HCl 20 (pH 7); NaCl 140; $MgCl_2$ 10; and counted using Opti-Fluor scintillation liquid (Packard Instruments, DK) at 100% efficiency. The time course of the uptake was linear at least up to 4 min.

The reported IC_{50} values are means of the numbers obtained from 4-parameter logistic equation fitting (GRAFIT program, Erithacus Software Ltd.) of the doses–response data, with the number of individual experiments indicated for each compound. For thapsigargin derivatives, these values are reported relative to that of thapsigargin when a corresponding amount of SR protein was used. This normalization of the thapsigargin analogue IC_{50} values to those of thapsigargin was necessary because of the stoichiometric nature of thapsigargin binding to Ca^{2+}-ATPase, resulting in shifts of the apparent inhibitory potency in parallel with the concentration of the enzyme.[16]

Determination of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). TSU cells (10^6) were treated with the indicated analogues for 1 h at 37 °C. Cells were then loaded with Fura 2-acetoxymethyl ester (5 μM) (0.1% DMSO/0.001% Plutonic) (Molecular Probes, Eugene OR) for 30 min at 33 °C in phenol red free RPMI media. Cells were then pelleted and resuspended in buffer of (4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES)-Krebb's medium [108 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 11.5 mM glucose, and 10 mM HEPES-KOH (pH 7.4)]) for an additional 20 min. Cells were then repelleted and washed just prior to assay then suspended in a quartz cuvette and fluorescence emission at 510 nm was measured after excitation at 340 nm and 380 nm using a photomultiplier from PTI (New Brunswick, NJ) at 25 °C. $[Ca^{2+}]_i$ were calculated from ratios from fluorescence intensities obtained every 1 second using the calculations of Grynkiewicz et al. and a K_d of 150 nM.[17] Dye was considered saturated on addition of 10 μM ionomycin while the minimum fluorescence ratio was determined in the presence of 5 mM EGTA.

Cytotoxicity assays. Percentage clonogenic survival of TSU-Pr1 (2×10^5 cells) following 2 h exposure to varying concentrations of Tg analogues was performed as described previously.[18]

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