Supporting Information

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SI Materials and Methods

Synthesis and Structural Details of Compounds Ia, Ib, IIa, IIb, IIIa, IIIb, and IXa. Compounds were synthesized as described in refs. 1–3. Briefly, a mixture of 6-amino-1,3-dimethyluracil (0.32 mmol, 1.0 eq), 1,3-indandione (**Ia, Ib, IIa, IIb, IIIa, IIIb**) or 1,3-cyclopentadione (**IXa**) (0.38 mmol, 1.16 eq), and 3-bromobenzaldehyde (**IIa, IIb, IXa**) or benzaldehyde (**Ia, Ib**) or 3-fluorobenzaldehyde (**IIIa, IIIb**) (0.38 mmol, 1.16 eq) in 7.0 ml of acetic acid was heated at 110°C for 40 min in a Discover microwave (CEM) at 250 W. Reactions were monitored by LC/MS and TLC. The mixture was then poured into a beaker and water was added until no more precipitate was formed. The orange precipitate was filtered, washed with cold water, dissolved in chloroform, and adsorbed on silica gel before purification by automated flash chromatography (Biotage). In the case of synthesis of compounds **IIa** and **IIb**, both compounds were isolated as an orange solid (**IIa**) and as a yellow solid (**IIb**) in 43% and 29% yields, respectively. Compound **IXb** was isolated in a similar manner as a white precipitate in 45% yield and washed with cold water; no flash chromatography was required to isolate it.

5-(3-Bromophenyl)-1,3-dimethyl-5,11-dihydro-1*H*-indeno-[2′,1′:5,6]pyrido[2,3-*d*]pyrimidine-2,4,6-trione (IIa, BPIPP). ^{1}H NMR (300 MHz, acetone-d_6): δ 9.05 (bs, 1H), 7.64 (ddd, 1H, J = 7.2, 0.8, 0.8 Hz), 7.54 (t, 1H, J = 1.7 Hz), 7.44–7.25 (m, 5H), 7.17–7.12 (m, 1H), 4.93 (s, 1H), 3.69 (s, 3H), 3.17 (s, 3H).

5-(3-Bromophenyl)-1,3-dimethyl-1*H*-indeno[2′,1′:5,6]-pyrido[2,3-*d*]pyrimidine-2,4,6-trione (IIb). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.97–7.94 (m, 1H), 7.69–7.59 (m, 3H), 7.56–7.51 (m, 1H), 7.38–7.33 (m, 2H), 7.17 (ddd, 1H, J = 7.7, 1.65, 1.1 Hz), 3.90 (s, 3H), 3.36 (s, 3H). 7.9, 2H), 7.17 (ddd, 1H, J = 7.7, 1.65, 1.1 Hz), 3.90 (s, 3H), 3.36 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 188.1, 168.5, 159.8, 155.4, 150.8, 150.7, 140.5, 136.4, 136.3, 134.9, 132.8, 131.2, 129.7, 129.2, 125.5, 123.9, 121.9, 121.8, 121.1, 106.9, 30.8, 28.7.

9-(3-Bromophenyl)-5,7-dimethyl-2,3,4,9-tetrahydro-4*H*-4,5,7-triazacyclopentanaphthalene-1,6,8-trione (IXa). ^{1}H NMR (300 MHz, DMSO): δ 10.00 (s, 1H), 7.37 (t, 1H, J = 1.7 Hz), 7.28 (dt, 1H, J = 7.4, 1.7 Hz), 7.23–7.12 (m, 2H), 4.65 (s, 1H), 3.46 (s, 3H), 3.08 (s, 3H), 2.73–2.68 (m, 2H), 2.30 (t, 2H, 4.7 Hz). ^{13}C NMR (75 MHz, DMSO): 201.1, 164.3, 160.8, 150.5, 148.1, 145.4, 130.4, 130.1, 129.0, 127.0, 121.3, 116.6, 89.2, 35.1, 33.8, 30.6, 27.9, 24.8.

1,3-Dimethyl-5-phenyl-5,11-dihydro-1*H*-indeno[2′,1′:5,6]-pyrido[2,3-*d*]pyrimidine-2,4,6-trione (Ia). Yield 39%. ^{1}H NMR (300 MHz, acetone-d_6): δ 9.18 (bs, 1H), 7.67 (ddd, 1H, J = 7.2, 1.2, 1.2 Hz), 7.42–7.28 (m, 5H), 7.20–7.14 (m, 2H), 7.07 (dddd, 1H, J = 7.4, 7.4, 1.4, 1.4 Hz), 4.94 (s, 1H), 3.70 (s, 3H), 3.16 (s, 3H).

1,3-Dimethyl-5-phenyl-1*H*-indeno[2′,1′:5,6]pyrido[2,3-*d*]pyrimidine-2,4,6-trione (Ib). Yield 14%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.96–7.93 (m, 1H), 7.67–7.62 (m, 2H), 7.54–7.48 (m, 4H), 7.24–7.20 (m, 2H), 3.90 (s, 3H), 3.34 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 188.4, 168.4, 159.9, 155.4, 152.8, 150.9, 140.6, 136.5, 134.8, 134.4, 132.7, 128.4, 128.3, 128.1, 127.7, 126.6, 123.8, 121.8, 121.2, 107.1, 30.8, 28.6.

5-(3-Fluorophenyl)-1,3-dimethyl-5,11-dihydro-1*H*-indeno-[2′,1′:5,6]pyrido[2,3-*d*]pyrimidine-2,4,6-trione (IIIa). Yield 59%. ^{1}H NMR (300 MHz, acetone-d_6): δ 9.03 (bs, 1H), 7.62 (d, 1H, J = 6.9 Hz), 7.43–7.29 (m, 3H), 7.23–7.10 (m, 3H), 6.88–6.81 (m, 1H), 4.96 (s, 1H), 3.69 (s, 3H), 3.17 (s, 3H).

5-(3-Fluorophenyl)-1,3-dimethyl-1*H*-indeno[2′,1′:5,6]-pyrido[2,3-*d*]pyrimidine-2,4,6-trione (IIIb). Yield 22%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.96–7.93 (m, 1H), 7.68–7.63 (m, 2H), 7.55–7.50 (m, 1H), 7.44 (ddd, 1H, J = 8.0, 8.0, 5.8 Hz), 7.17 (dddd, 1H, J = 8.5, 8.5, 2.5, 1.1 Hz), 6.99 (ddd, 1H, J = 7.7, 1.4, 0.8 Hz), 6.93 (ddd, 1H, J = 9.0, 2.2, 1.4 Hz), 3.90 (s, 3H), 3.35 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 188.1, 168.5, 162.2 (d, J = 245.7 Hz), 159.8, 155.4, 150.9 (d, J = 1.7 Hz), 150.8, 140.6, 136.5, 136.4, 134.9, 132.8, 129.4 (d, J = 8.3 Hz), 123.9, 122.5 (d, J = 3.2 Hz), 121.9, 121.1, 115.2 (d, J = 20.9 Hz), 114.2 (d, J = 23.2 Hz), 106.9, 30.8, 28.7.

BPIPP Binding. Binding of BPIPP to T84 cells was assayed by spectrophotometry using its characteristic absorption maximum at 525 nm in 0.1 M NaOH and extinction coefficient of 2.22 $mM^{-1}{\cdot}cm^{-1}$ determined in a separate experiment. Cells were treated with 50 μM BPIPP for 10 min in DPBS and then washed to remove the excess of unbound BPIPP three times with DBPS or DPBS containing 1% BSA. Cells were then solubilized with 0.1 M NaOH containing 2% SDS, and optical density was measured to quantify bound BPIPP in nmol/mg of protein. Binding of BPIPP to BSA was assessed in DBPS containing 5–10 μM BSA and 5–50 μM BPIPP incubated for 20 min. Unbound BPIPP was removed by ultrafiltration through a YM-10 membrane (Microcon, Millipore). The amount of free and bound BPIPP was plotted to calculate the stoichiometry of binding and the apparent equilibrium binding constant by a Scatchard plot.

Assay of Adenylyl and Guanylyl Cyclase Activity. Membranes from T84 cells were prepared by scraping intact cells from the dish with a rubber policeman in calcium- and magnesium-free DPBS containing 1 mM EDTA. Cells were then lysed with ice-cold 50 mM Tris·HCl buffer (pH 7.4) containing 2.5 mM EGTA and protease inhibitor mixture (Roche). In additional experiments, membranes were prepared in the presence of protein phosphatase inhibitor cocktails 1 and 2 (Sigma; catalog nos. P2850 and P5726, respectively). After sonication and centrifugation at 100,000 × *g* for 1 h, the membrane pellet was resuspended in 50 mM Tris·HCl (pH 7.4) containing 2 mM $MgCl_2$, 1 mM EDTA, and protease inhibitors and membranes (5–10 mg of protein per ml) were stored at −80°C. Guanylyl cyclase activity in the membranes was assayed in a mixture containing 50 mM Tris·HCl (pH 7.4), 1 mg/ml ovalbumin, 50 μg/ml creatine kinase, 5 mM phosphocreatine, 1 mM IBMX, 5 mM $MgCl_2$, 1 mM GTP, and 20–50 μg of membrane protein in a final volume of 50 μl. Samples were incubated for 10 min at 37°C, reaction was stopped by boiling, and the amount of cGMP formed was determined by RIA. Adenylyl cyclase in T84 cell membranes was assayed in a similar buffer substituting ATP for GTP, and cAMP was then measured with a cAMP assay kit (Cayman Chemical).

Transport of Chloride Ion in T84 Cells. Cells were grown in black, clear bottom 96-well plates (Costar; catalog no. 3603) loaded with the corresponding dye (10 mM in 100 μl of growth medium) overnight and washed three times with equilibration buffer 1 (pH 7.4) containing 3 mM phosphate, 10 mM Hepes, 10 mM glucose, 1 mM $MgSO_4$, 5.4 mM K^+, 130 mM Na^+, 1 mM Ca^{2+}, 130 mM Cl^-, and 2 mM SO_4^{2-} at ambient temperature. The cells were then pretreated with BPIPP or other agents or corresponding vehicle in buffer 1 for 10 min. Efflux assay was started by aspirating buffer 1 and replacing it with buffer 2 (130 mM NO_3^- instead of 130 mM Cl^- compared with buffer 1) and contained same additions as used for pretreatment plus other agents as indicated in figure legends. Fluorescence was recorded with a SpectraMAX M2 plate reader (Molecular Devices) controlled

by SoftMax Pro software (version 4.7) at 30-s intervals (355-nm excitation and 460-nm emission for MQAE and 345-nm excitation and 445-nm emission for SPQ) for up to 15 min. Difference between maximal fluorescence (F_t, achieved in 4–5 min after aspiration of buffer 1 and addition of buffer 2) and baseline fluorescence (F_0 in buffer 1) was used for calculations. Fluorescence of control samples ($F_c - F_0$) containing vehicle (0.1% DMSO) was subtracted from the measured values, and because none of the agents tested in the assay influenced F_0 values, increase in fluorescence corresponding to increase in Cl^- efflux from the cells was calculated as $F_t - F_c$. Addition of 50 μM BPIPP had no significant influence on the Stern-Volmer quenching constant of MQAE or SPQ fluorescence by chloride ions as determined in a separate set of experiments in the presence of 10 μM tributyltin chloride and 5 μM nigericin (4).

Assay of Tyrosine-Specific Phosphorylation in T84 Cell Extracts. Cells were treated with vehicle or 50 μM BPIPP for 20 min and then extracted with PhosphoSafe extraction reagent (Novagen) mixed with EDTA-free protease inhibitor mixture (set V, Calbiochem). The extract was diluted to yield 5–7 μg of protein per sample (final volume 50 μl) with reaction buffer containing 20 mM Hepes (pH 7.4), 30 mM $MgCl_2$, 2 mM $MnCl_2$, 0.05% Triton X-100, 0.15% Brij 35,1% glycerol, 1 mM DTT, 0.2% 2-mercaptoethanol, 0.5 mM activated sodium orthovanadate, 0.2 mM ATP, and protease inhibitors and incubated in wells of a 96-well plate (K-LISA PTK screening kit, Calbiochem) coated with tyrosine kinase substrate polypeptides composed of glutamate (E), tyrosine (Y), and alanine (A) at various ratios as indicated in Fig. 4*A*. Plates were incubated for 30 min at 30°C, and reaction was terminated with 50 μl of 500 mM EDTA. Plates were then washed and developed with peroxidase-conjugated anti-phosphotyrosine antibody PY-20 as instructed by the supplier. Blank values (wells coated with BSA only) were subtracted and data were expressed in absorption units (a.u.) per mg of protein.

To asses subcellular distribution of tyrosine kinase activity, the cells were homogenized in a hypotonic buffer containing 50 mM Tris·HCl (pH 7.6), 1 mM DTT, 1 mM EDTA, protease inhibitors, and a mixture of phosphatase inhibitor cocktails 1 and 2 (Sigma; catalog no. P2850 and P5726, respectively) with a glass–glass homogenizer on ice. The homogenate was then centrifuged at 100,000 $\times$ *g* for 1 h to separate crude membrane and cytosolic fractions. The fractions were then mixed with PhoshoSafe extraction reagent and assayed as described above in K-LISA PTK EAY reaction plates (Calbiochem) because previous experiments demonstrated that activity of tyrosine kinase with EAY as a substrate was the highest.

Assay of Inositol Phosphate Generation in T84 Cells. For assay of inositol phosphate (IP_x) generation, cells in 24-well plates were loaded overnight with 37 MBq/ml *myo*-[^{3}H]inositol in inositol-free RPMI medium 1640 in the absence of serum. On a day of experiment, cells were treated with 10 mM LiCl in serum-free DMEM with corresponding additions in a final volume of 150 μl per well for 20 min and reaction was stopped with 0.3 ml of 20% (wt/vol) ice-cold trichloroacetic acid. Samples were diluted to 5 ml with water and loaded onto 1-ml Dowex 50 columns. Inositol phosphates were eluted sequentially with 15-ml portions of 0.1 M formic acid containing 0.2 M ammonium formate (IP_1), 0.4 M ammonium formate (IP_2), 1 M ammonium formate (IP_3), and 1.5 M ammonium formate (IP_4). The first 3 ml of the eluate were collected and quantified by liquid scintillation counting. IP_3 generation was measured with a radioreceptor assay kit according to the supplier's protocol (Perkin–Elmer). An activator of phosphatidylinositol-specific phospholipase C, 2,4,6-trimethyl-*N*-(*m*-3-trifluoromethylphenyl)benzenesulfonamide (M3-M) and the control compound 2,4,6-trimethyl-*N*-(*o*-3-trifluoromethylphenyl)benzenesulfonamide (O-3M) (5) were purchased from Sigmol.

Assay of Phosphocholine and Choline Generation in T84 Cells. Cells were labeled with [^{3}H]choline (Amersham) at 74 MBq/ml for 48 h in DMEM containing 2% FBS. Cells were washed with DMEM containing 20 mM Hepes and 1% BSA (DHB) three times and then three more times with DHB without BSA. Cells were incubated for 40 min in 150 μl of DH per well with vehicle or various additions as indicated. At this stage medium was removed and extracellular production of phosphocholine and choline was assayed. Samples were extracted with chloroform/methanol and the aqueous phase was removed and diluted with water to a final volume of 5 ml. Phosphocholine was separated from choline by chromatography on Dowex 50 resin (1 ml) eluted with water and 1 M KCl, respectively.

1. Agarwal A, Chauhan PMS (2004) *Synth Commun* 34:4447–4461.
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3. Agarwal A, *et al*. (2005) *Bioorg Med Chem* 13:6678–6684.
4. West MR, Molloy CR (1996) *Anal Biochem* 241:51–58.
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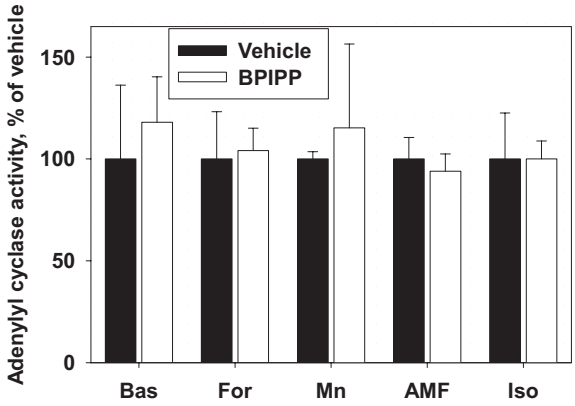

Fig. S1. Effect of BPIPP on adenylyl cyclase activity in membranes. T84 cells were pretreated with 50 μM BPIPP or vehicle for 10 min and membranes were isolated in the presence of phosphatase and protease inhibitors and assayed for adenylyl cyclase activity; basal (Bas) (84.5 $\pm$ 30.1 pmol of cAMP per min per mg of protein assumed as 100%); 50 μM forskolin (For) (4.66 $\pm$ 1.01 nmol of cAMP per min per mg of protein assumed as 100%); 5 mM $MnCl_2$ (Mn) (288 $\pm$ 10 pmol of cAMP per min per mg of protein assumed as 100%); 50 μM $AlCl_3$ and 6 mM NaF (AMF) (8.50 $\pm$ 0.90 nmol of cAMP per min per mg of protein assumed as 100%); 100 μM isoproterenol (Iso) (151 $\pm$ 34 pmol of cAMP per min per mg of protein assumed as 100%). $n = 3$.

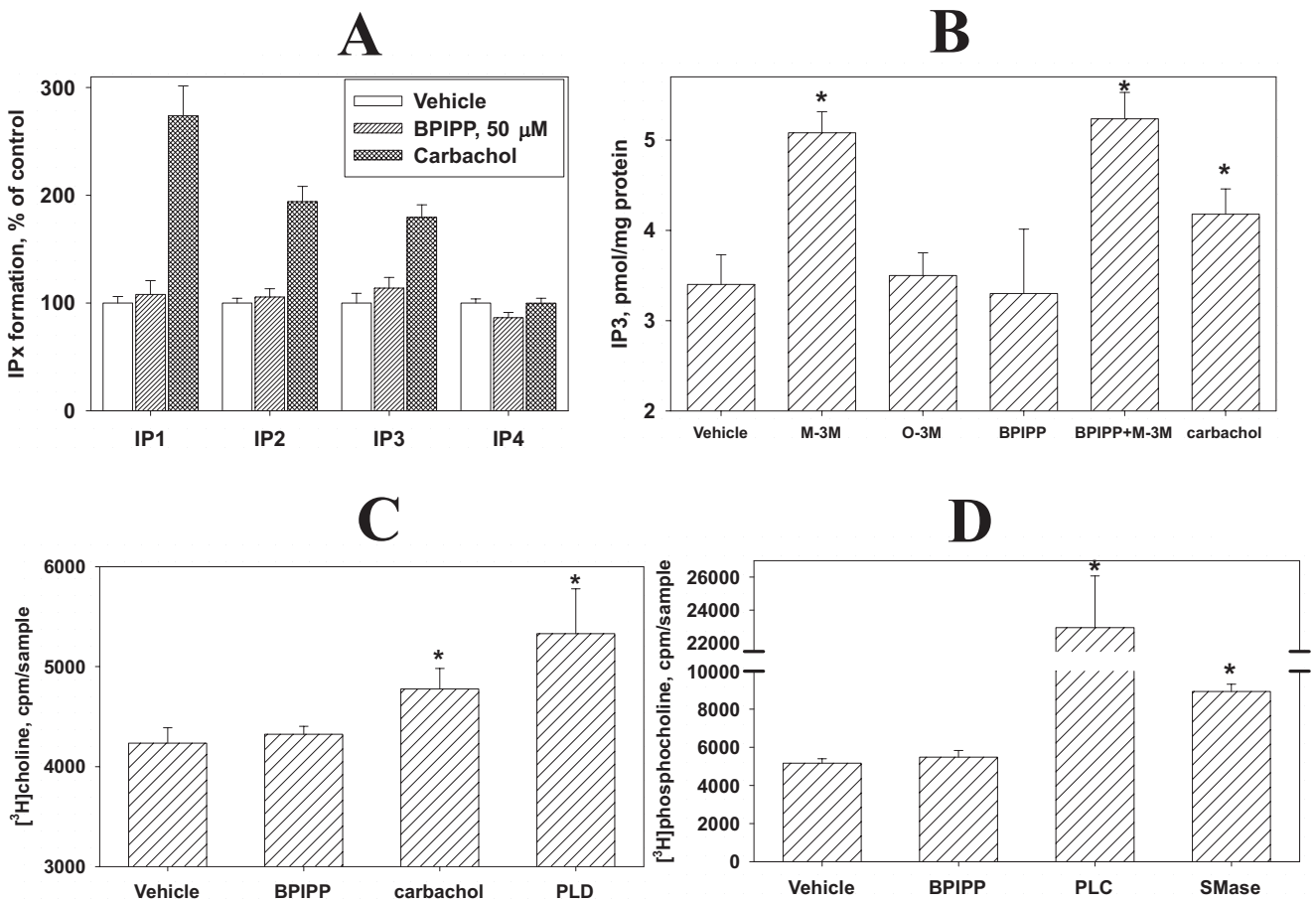

Fig. S2. Effect of BPIPP on phospholipase C and D activities in T84 cells. (*A*) Cells were treated for 10 min in the presence of 10 mM LiCl and 50 μM BPIPP or 200 μM carbachol. Inositol phosphates (IP_x) were extracted and measured as described in *SI Materials and Methods*. $n = 6$. (*B*) Cells were treated in the presence of vehicle (0.2% DMSO), 50 μM M-3M (2,4,6-trimethyl-*N*-(*m*-3-trifluoromethylphenyl)benzenesulfonamide; activator of phospholipase C), O-3M (2,4,6-trimethyl-*N*-(*o*-3-trifluoromethylphenyl)benzenesulfonamide; control compound for M-3M), or BPIPP, a combination of BPIPP and M-3M, and 200 μM carbachol for 20 min, and accumulation of IP_3 was assayed with a radioreceptor kit; asterisks correspond to significant enhancement of IP_3 formation; $P < 0.05$; $n = 6$. (*C*) Cells were loaded with [^{3}H]choline and treated for 20 min in the presence of vehicle (0.1% vol/vol DMSO), 50 μM BPIPP, 200 μM carbachol, or 20 units/ml phospholipase D from peanut (PLD); release of [^{3}H]choline was measured as described in *SI Materials and Methods*; asterisks denote significant difference versus vehicle at $P < 0.05$; $n = 4$. (*D*) Cells were loaded with [^{3}H]choline and treated for 20 min in the presence of vehicle (0.1% DMSO), 50 μM BPIPP, 10 units/ml phospholipase C (PLC), or 2.5 units/ml of sphingomyelinase from *Bacillus cereus* (SMase). Release of phospho[^{3}H]choline was measured as described in *SI Materials and Methods*; asterisks denote significant difference versus vehicle at $P < 0.05$; $n = 4$.

Table S1. Pharmacological analysis of inhibition of STa-stimulated accumulation of cGMP in T84 cells pretreated with 50 μM BPIPP

Treatment	Target	Change in BPIPP inhibition, Δ%
Calcium-related pathways		
Ca-free DPBS containing 0.1 mM EGTA[a]	Ca^{2+}	7.1 ± 2.6
A23187, 1 μM[a]	Ca^{2+}	2.2 ± 1.5
BAPTA-AM, 50 μM[a]	Ca^{2+}	11.8 ± 2.8
ALLM, 100 μM[b]	Calpain	−2.9 ± 3.9
ALLN, 100 μM[b]	Calpain	6.0 ± 2.5
Calmidazolium, 10 μM[b]	Calmodulin	−19.7 ± 4.4
Calpeptin, 100 μM[b]	Calpain	22.8 ± 1.6
Nifedipine, 100 μM[b]	Ca^{2+} channels	10.9 ± 2.5
Protein kinase and phosphatase regulators		
AAG, 10 μM[c]	Hsp90	−16.0 ± 0.6
BIM I, 100 μM[b]	Protein kinase C	20.1 ± 2.2
Genistein, 50 μM[b]	Tyrosine kinases	1.5 ± 4.4
Go6976, 5 μM[b]	Protein kinase C	−13.5 ± 12.0
H7, 50 μM[b]	Protein kinases	−13.5 ± 6.8
H8, 50 μM[b]	Protein kinases	6.5 ± 3.2
KN-62, 10 μM[b]	Ca^{2+}/calmodulin kinase	5.0 ± 6.5
Orthovanadate[b]	Tyrosine-specific protein phosphatases	
0.5 mM		−11.4 ± 2.5
1 mM		−19.5 ± 3.6
2 mM		−30.8 ± 2.6*
PMA, 100 nM[b]	Protein kinase C	16.9 ± 4.0
Staurosporine[b]	Protein kinases	
10 nM		−13.7 ± 0.3
100 nM		6.6 ± 3.6
Phospholipase-related agents		
AACOCF3, 50 μM[b]	Phospholipase A2	−17.0 ± 5.2
Carbachol[b]	Muscarinic receptors	
0.1 mM		5.0 ± 4.6
1 mM		−1.8 ± 1.4
D609, 50 μM[b]	Phospholipase C	−52.6 ± 5.7*
Et-18OCH3, 50 μM[b]	Phospholipase C	−4.2 ± 1.0
U73122[b]	Phospholipase C	
10 μM		−11.7 ± 1.5
20 μM		−54.0 ± 15.8*
50 μM		−60.5 ± 12.8*
Wortmannin[c]	PI3-kinase	
50 nM		−3.1 ± 2.1
250 nM		−28.6 ± 3.9
Reactive oxygen species		
Catalase, 100 units/ml[b]	H_2O_2	−21.8 ± 13.9
SOD, 100 units/ml[b]	Superoxide	−20.8 ± 7.2

Cells were grown in 12-well plates as described in *SI Materials and Methods* and then washed with DPBS containing 1 mM IBMX and treated for 10 min with vehicle (0.1% DMSO) or BPIPP in the absence or presence of indicated additions and stimulated with STa for another 10 min in the same medium. The accumulation of cGMP was then measured with ELISA. Percentage of inhibition with BPIPP was then calculated and change in inhibitory activity of BPIPP was estimated. Positive values indicate that inhibition with BPIPP was enhanced and negative values indicate that inhibition with BPIPP was suppressed. Significance of differences was evaluated by Student's *t* test comparing directly nonnormalized values of percentage of inhibition. The data are mean ± SEM of three to six independent experiments assayed in duplicate. *, $P < 0.05$, suppression of BPIPP inhibition was statistically significant. AACOCF3, arachidonyl trifluoromethyl ketone; AAG, 17-allylamino-17-demethoxygeldanamycin; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester; BIM I, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide; D609, tricyclodecan-9-yl-xanthogenate; Et-18OCH3, edelfosine; Go6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole; KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; U73122, 1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione.

[a]Pretreatment for 1 h in Ca-free Hanks balanced salt solution (HBSS) containing 1 mM EGTA; incubation with BPIPP and STa was also preformed in the same medium.

[b]Pretreatment for 10 min in DPBS during incubation with BPIPP and STa.

[c]Pretreatment for 1 h in growth medium and also for 10 min in DPBS during incubation with BPIPP and STa.