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Tanmoy Saha, Munshi Sahid Hossain, Debasis Saha, Mayurika Lahiri, and Pinaki Talukdar J. Am. Chem. Soc., Just Accepted Manuscript • DOI: 10.1021/jacs.6b01723 • Publication Date (Web): 25 May 2016 Downloaded from http://pubs.acs.org on May 26, 2016

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Chloride-Mediated Apoptosis-Inducing Activity of bis(Sulfonamide) Anionophores

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ABSTRACT: Transmembrane anion transport modality is enjoying a renewal interest because of recent advances towards anticancer therapy. Here we show bis(sulfonamides) as efficient receptors for selective Cl⁻ ion binding and transport across lipid bilayer membranes. Anion binding studies by ¹H NMR indicate a logical correlation between the acidity of sulfonamide N–H proton and binding strength. Such recognition is influenced further by the lipophilicity of a receptor during the ion transport process. Anion binding and transport activity of a bis(sulfonamide) system are far superior compared to corresponding bis(carboxylic amide) derivative. Fluorescent-based assays confirm the Cl⁻/anion antiport as the operational mechanism of the ion transport by bis(sulfonamides). The disruption of the ionic homeostasis by transported Cl⁻ ion, via bis(sulfonamide), is found to impose cell death. Induction of caspase depended intrinsic pathway of apoptosis is confirmed by monitoring the change in mitrochondrial membrane potential, cytochrome *c* leakage, activation of family of caspases and nuclear fragmentation studies.

1. INTRODUCTION

Ion transport process across the cell membrane controls the distribution of collective information stored in the building constituents of cells and perform specific functions.¹⁻⁵ Certain class of membrane proteins such as channels, carriers, etc. facilitates the ion transport process, overcoming the hydrophobic barrier of the phospholipid bilayer membranes.⁶⁻⁸ Regulation of ion movement across the bilayer membranes is essential for neuronal proliferation, cellular signalling, pH control of osmotic pressure, etc.⁹⁻¹¹ Chloride, bicarbonate, and phosphate are the most abundant anions in the physiological system, and the typical extracellular (110 mM) versus intracellular (5 - 15 mM) concentration gradient of Cl⁻ ion is maintained by the transmembrane proteins.12 Selective transport of Cl^{-} ion is indispensable for diverse biological processes *e.g.* transepithelial salt transport, acidification of internal and extracellular compartments, cell volume regulation, cell cycle and apoptosis.^{13,14} On the other hand, misregulated transport of Cl⁻ ion, occasionally resulted from mutation of the transmembrane proteins such as in CFTR-Cl channel, etc., can cause the life shorting disease like cystic fibrosis.¹⁵ Furthermore, defects in anion transport proteins can lead to various diseases e.g. Bartter Syndrome, Gitelman syndrome, Dent's diseases, renal tubular acidosis, deafness, etc.^{7,16}

Recently, the introduction of artificial ion channels as the "channel replacement therapy", has instigated as a novel combination treatment modality for targeting diseases associated with ion channel dysfunction.^{17,18} Therefore synthetic ion transport systems have immense potential in integrating a palliative care approach into several life-threatening diseases, including cystic fibrosis, cancer, etc. Prodigiosin¹⁹ is a natural Cl⁻ carrier that can function as an anion exchanger via either H⁺/Cl⁻ symport or Cl⁻/anion antiport mechanism.²⁰ Ion transport properties of prodigiosin and its synthetic analogs are also linked to their anticancer activities.^{21,22} Among diverse

synthetic anionophores,²³⁻⁴⁷ classes of urea/thiourea,^{30,32,36} calix[4]pyrrole²⁷ derivatives and tambjamine analogs²⁴ were also reported for their anticancer activities. These molecules either change the intracellular pH or disrupt the cellular ionic homeostasis which triggers the apoptosis-inducing pathway. Here we demonstrate bis(sulfonamides) as a new class of low molecular weight Cl⁻ transporters. The study provides thoughtful insight into the correlation between structure, lipophilicity, anion recognition, and anion transport activity. Transport of Cl⁻ into the cells by these anionophores facilitates the activation of caspase-dependent apoptotic pathways.

Sulfonamide was envisaged as a better scaffold for anion recognition compared to the carboxylic amide,^{48,49} due to the higher acidity of the N-H proton.^{50,51} The *m*-xylenediamine was selected as the rigid core, and the arylsulfonyl moieties were varied to tune the N-H proton acidity as well as lipophilicity of bis(sulfonamide) derivatives. According to Lipinski's rule,⁵² the structural modulation, based on lipophilicity, was expected to influence the membrane permeability of designed molecules and thereby, a variation in the ion transport activity was envisaged.^{36,58} The logP values of all designed molecules were calculated (*i.e.* logP = 2.77, 2.96, 4.11, 3.69, 3.62, 4.84 and 5.06 for **1a-1g**, respectively) using the calculator plugins of MarvinSketch program (Table 1).⁵ The program was also used for predicting pKa_1 value of sulfonamide N-H protons (Table 1). A bis(carboxylic amide) derivative 2 was designed to evaluate the importance of the sulfonamide groups in the anion recognition process.

Table 1. Structures, logP values and pKa values of N-H protonsof designed anionophores 1a-1g and 2.

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^[a] Calculator plugins of MarvinSketch program were used for structure property prediction and calculation.

2. RESULTS AND DISCUSSIONS

2.1. Synthesis:

Compounds 1a-1g were synthesized from commercially available *m*-xylenediamine **3** by treating with corresponding sulfonyl chloride derivatives (Scheme 1). Compound **2**, on the other hand, was synthesized from 4-(trifluoromethyl)benzoic acid **4** by treating with SOCl₂ followed by addition of **3**. All compounds were purified by column chromatography, and



Scheme 1. Synthesis of bis(sulfonamide) derivatives 1a–1g (A) and bis(carboxylic amide) derivative 2 (B).

characterized by ¹H NMR, ¹³C NMR, HRMS, IR and melting point (see ESI for detailed experiments and characterization).

2.2. Anion Binding Studies:

At first, Cl⁻ ion binding properties of **1a-1g** and **2** were investigated by ¹H NMR titrations in either CD₃CN (for **1a**, **1b**, **1c**, 1f, 1g and 2) or CDCl₃ (for 1d and 1e), selected based on compatibility. Upon stepwise addition of tetrabutylammonium chloride (TBACl) to compounds 1a-1g, downfield shifts of H_a, H_b and H_c signals and upfield shifts of H_d protons were observed (Figure 1, Figure S1-S7). These data indicate the presence of C-H_a...Cl⁻, N-H_b...Cl⁻ and C-H_c...Cl⁻ interactions with the anion. For calculating stoichiometry and binding constant, the change in chemical shift ($\Delta\delta$) of H_a proton was monitored rather than the $\Delta\delta$ of H_b proton due to the line broadening of the H_b proton upon addition of excess TBACl. When the receptor 1f and TBACl were mixed in CD₃CN by varying mole fraction (\varkappa) of the receptor (Table S1), and the $\Delta\delta$ of H_a proton was monitored (Figure S9A), the maximum interaction was observed at $\varkappa = 0.5$ indicating 1:1 complexation between **1f** and Cl⁻ (Figure S9B). The δ of the H_a proton from figure S1-S7 was plotted against the increasing concentration of TBACl and the association constant for the complexation of 1f with Cl⁻ was calculated by fitting the doseresponse curve to the 1:1 binding model of WinEQNMR2 program.⁵⁴ The Cl⁻ ion recognition studies for 1a, 1b, 1c, 1f and 1g provided the dissociation constants, $K_d = 21.28 \pm 3.42$, 2.56 ± 0.34 , 19.50 ± 2.24 , 2.39 ± 0.21 and 14.15 ± 0.81 mM, respectively. Similarly, Cl⁻ ion binding studies with 1d and 1e in CDCl₃ provided $K_d = 4.08 \pm 0.44$ and 2.48 ± 0.20 mM, respectively. Therefore, an electron withdrawing group on Ar/Ar' moieties increases the acidity of sulfonamide N-H proton, and this is responsible for better Cl⁻ ion binding. Halide ion binding studies by **1f** provided the sequence: $Cl^{-}(K_d =$ $2.39 \pm 0.21 \text{ mM}$ > Br⁻ ($K_d = 19.47 \pm 0.94 \text{ mM}$) > I⁻ ~ F⁻ ($K_d =$ not determined) confirming better Cl⁻ ion recognition by the anionophore (Figure S10). It is noteworthy that the ¹H NMR



Figure 1. Partial ¹H NMR (400 MHz, 298 K) spectral change of **1f** in CD₃CN upon stepwise addition of TBACl (0 - 10 equiv).

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59 60 titration of compound **2** with TBACl did not provide any significant change of proton chemical shift (Figure S8) suggesting poor Cl⁻ ion binding by the bis(carboxylic amide) derivative. This observation was corroborated to the higher acidities of sulfonamide N–H protons ($pKa_1 = 10.10$) of **1f** compared to the carboxylic amide N–H protons ($pKa_1 = 13.23$) of **2**.

Table 2. Experimentally determined K_d and EC_{50} values for **1a-1g** and **2**.

Compound	logP	<i>K</i> _d (mM)	<i>EC</i> ₅₀ (μM)
1 a	2.77	$21.28 \pm 3.42^{[a]}$	26.56 ± 1.13
1b	2.96	$2.56\pm0.34^{\left[a\right]}$	17.18 ± 0.77
1c	4.11	$19.50\pm2.24^{[a]}$	15.84 ± 0.23
1d	3.69	$4.08 \pm 0.44^{[b]}$	10.26 ± 0.42
1e	3.62	$2.48\pm0.20^{[b]}$	8.19 ± 0.11
1f	4.84	$2.39\pm0.21^{[a]}$	5.89 ± 0.15
1g	5.06	$14.15 \pm 0.81^{[a]}$	ND ^[c]
2	5.33	ND ^{[a],[c]}	ND ^[c]

^[a] K_d values were determined in CD₃CN. ^[b] K_d values were determined in CDCl₃. ^[c] ND = could not be determined.

2.3. Anion Binding Model:

To obtain the theoretical insight about the geometry of [1f+Cl⁻] complex, initial structures were generated by using CONFLEX 7 program.^{55,56} Initial conformations for density functional theory (DFT) calculations were predicted by using MMFF94s force field of the software. First seven conformations, obtained according to their decreasing Boltzmann population percentages (Figure S11), were selected for further geometry optimization studies (see ESI for details). The geometry optimization by Gaussian 09 program⁵⁷ using B3LYP functional and 6-311G++(d,p) basis set⁵⁸ provided the most stable conformation of the [1f+Cl⁻] complex (Figure 2A). This optimized structure also correlates to the noncovalent interactions (i.e. two N-H····Cl⁻, and three C-H····Cl⁻ interactions) those observed in NMR titration studies. On the other hand, the direct experimental evidence of anion recognition of 1f was obtained from the electrospray ionization-mass spectrometric (ESI-MS) study. A sample was prepared in CH₃CN by mixing 1f and TBACl in 1:1 molar ratio, and electrosprayed under as mild as possible ionization conditions. The mass



Figure 2. Geometry optimized structure of $[1f+Cl^-]$ (**A**). Expanded region of the ESI-MS spectrum obtained by injecting the solution containing 1:1 mixture of **1f** and TBACl in CH₃CN (**B**).

spectrometric data provided peaks at m/z = 587.0313 and 589.0287, which correspond to the [1f+Cl⁻] complex in the solution state (Figure 2B and S13).

2.4. Ion Transport Activity of 1a-1g and 2:

Anion recognition properties of bis(sulfonamide) compounds 1a-1g encouraged us to evaluate their ion transport activity across large unilamellar vesicles (LUVs). The vesicles were prepared from egg-volk phosphatidylcholine (EYPC) lipid with entrapped 8-hydroxypyrene-1,3,6-trisulfonate (HPTS, pKa = 7.2) dye to get EYPC-LUVs \supset HPTS (Figure S14A).⁵⁹⁻⁶¹ Subsequently, a pH gradient, $\Delta pH = 0.6$ (pH_{in} = 7.0 and $pH_{out} = 7.6$) was applied across the EYPC bilayer by adding NaOH in the extravesicular solution. The destruction of pH gradient by either H⁺ efflux or OH⁻ influx, upon addition of each compound, was monitored by measuring the fluorescence intensity of HPTS at $\lambda_{em} = 510$ nm ($\lambda_{ex} = 450$ nm). Finally, vesicles were collapsed by the addition of Titron X-100 to get the maximum fluorescence intensity (Figure S14B). All synthetic receptors **1a-1g** exhibited significant fluorescence intensity enhancement indicating transport of ions across the LUVs. Comparison of ion transport activity at identical concentration $c = 10 \,\mu\text{M}$ provided the activity sequence: 1f > 1e >1g > 1d > 1c > 1b > 1a (Figure 3A). The dose-response plots of bis(sulfonamides) **1a–1f** (Figure S15-S20) provided $EC_{50} =$ 26.56 \pm 1.13, 17.18 \pm 0.77, 15.84 \pm 0.23, 10.26 \pm 0.42, 8.19 \pm 0.11 and 5.89 \pm 0.15 μ M, respectively (Table 2). *EC*₅₀ calculation could not be performed on 1g due to the precipitation observed at higher concentrations of the compound upon its addition in the buffer. The lowest activity of 1a was primarily correlated to its poor anion binding ability ($K_d = 21.28 \pm 3.42$ mM). In addition to this, low log P = 2.77 of **1a** also contribute to its preferential distribution in the aqueous media over permeation through the hydrophobic lipid bilayer membranes. Compound 1c with better Cl⁻ binding ability ($K_d = 19.50 \pm$ 2.24 mM) and permeability (logP = 4.11) compared to 1a displayed higher transport activity. Although, the binding of 1b with Cl⁻ is much higher than 1c, the lower logP value of the nitro-substituted derivative restricts its membrane permeation which results in the poor ion transport property. Similarly, higher ion



Figure 3. Comparison of ion transport activities of bis(sulfonamide) derivatives 1a-1g (10 μ M each) across EYPC-LUVs \supset HPTS (A). Comparison of ion transport activities of 1f (10 μ M) and 2 (10 μ M) across EYPC-LUVs \supset HPTS (B).

transport activity of the compound 1e was corroborated to its better anion binding ability than 1d, even if having close logP values. The effect of permeability (logP = 4.84) and anion binding ($K_d = 2.39 \pm 0.21$ mM) was most significant for 1f which provided the lowest value of $EC_{50} = 5.89 \ \mu\text{M}$. Despite of having low anion binding property ($K_d = 14.15 \pm 0.81$ mM), appreciably high ion transport activity was observed for compound 1g. This result can be justified from the highest logP value (logP = 5.06) among all bis(sulfonamide) compounds. However, the solubility of the compound 1g in the aqueous solution was compromised because of its hydrophobic nature. On the other hand, ion transport activity of the bis(carboxylic amide) 2 was negligible compared to 1f (Figure 3B). In spite of identical aromatic moieties and comparable logP values $(\log P = 4.84 \text{ and } 5.33 \text{ for 1f and 2}, respectively}), higher acidi$ ty of sulfonamide N-H proton was crucial for better anion binding by 1f.

2.5. Ion selectivity Studies:

The top three active compounds 1d–1f was used further to investigate the selectivity and the mechanism of ion transport. Ion transport activity across EYPC-LUVs DHPTS with intravesicular NaCl and isoosmolar extravesicular NaA (where, $A^{-} = F^{-}, Cl^{-}, Br^{-}, I^{-}, NO_{3}^{-}, SCN^{-}, OAc^{-} and ClO_{4}^{-})$ was monitored to determine anion selectivity (Figure S22). Similarly, an isoosmolar extravesicular MCl (where, $M^+ = Li^+$, Na^+ , K^+ , Rb^+ and Cs⁺) was used to determine the selectivity among cations. The difference in ion transport activity upon changing extravesicular anions implies the involvement of anions in the transport process. For compound 1f ($c = 6 \mu M$), an activity sequence: $Cl^- >> SCN^- > OAc^- > F^- > ClO_4^- \ge I^- > NO_3^- >$ Br⁻ was observed (Figure 4A and S23C). The observed Cl⁻ ion selectivity of **1f** rationalized by the strong binding of the ion in the cavity of the bis(sulfonamide) molecule. Lower transport activities for other anions indicate poor binding of these anions due to the mismatch of their sizes with the binding pocket of the bis(sulfonamide). The little-enhanced activity observed for Γ compared to Br⁻ may be because of the higher membrane permeation of more hydrophobic Γ anion.⁶² Variation of monovalent cations in the extravesicular buffer provided marginally enhanced ion transport activities for K⁺, Rb⁺ and Cs⁺ compared to Li⁺ and Na⁺ (Figure 4B and S24C). In the absence of any of ditopic binding motif in 1f, the observations suggest the possibility of cation recognition as an alternate binding



Figure 4. Anion (A) and cation (B) selectivity of **1f** (6 μ M) determined from the ion transport studies across EYPC-LUVs \supset HPTS. The fluorescence intensity values at 100th s, after addition of **1f**, were normalized with respect to the intensity value obtained with extravesicular NaCl.

motif in the molecular cavity.⁶³ Anion selectivity studies for **1d** and **1e** also inferred excellent transport of Cl^- ion (Figure S23A and B).

2.6. Chloride Leakage Studies:

In the next stage, Cl⁻ ion transport activities of 1d-1f across LUVs were investigated in details by monitoring the fluorescence intensity of intravesicular lucigenin dye at $\lambda_{em} =$ 535 nm (λ_{ex} = 450 nm).⁶⁴ EYPC/cholesterol-LUVs⊃lucigenin were prepared with 7:3 EYPC/cholesterol by entrapping lucigenin dye and NaNO₃ (200 mM). Subsequently, a Cl⁻/NO₃⁻ gradient was applied by addition of concentrated solution of NaCl in the extravesicular buffer (Figure S25A). Quenching of lucigenin fluorescence due to the influx of Cl- ion by bis(sulfonamides) was monitored with time. Finally, Triton X-100 was added to get the complete quenching of lucigenin fluorescence (Figure S25B). A sharp quenching in fluorescence, observed upon addition of 1f, confirmed the Cltransport activity of the compound (Figure 5A). Compounds 1e and 1d also displayed quenching of lucigenin fluorescence in the presence of extravesicular NaCl (Figure S26A and S26B), and the observed trend of Cl^{-} transport activity 1f > 1e> 1d (Figure 5B) resembles that obtained from HPTS assays (Figure 3A).



Figure 5. Cl⁻ ion influx across EYPC/cholesterol-LUVs \supset lucigenin upon addition of **1f** (0-80 μ M) (**A**). Comparison of Cl⁻ influx across EYPC/cholesterol-LUVs \supset lucigenin for **1d**, **1e** and **1f** (60 μ M each) (**B**).

Numerical analysis of the activity profiles (Figure 5B) were done to get the quantitative data of Cl⁻ ion transport across EYPC/cholesterol-LUVs⊃lucigenin. At first, the normalized fluorescence quenching curves were fitted to the single exponential decay function (Equation S5) and then the half-life ($t_{1/2}$) was calculated by using Equation S6. Similarly, the initial rate (r_i) was estimated by fitting the dose-response curve to a double exponential decay function (Equation S7 and S9). An increase in initial rate (r_i) with a concomitant decrease in half-life ($t_{1/2}$) was encountered while moving from compound 1d, 1e to 1f (Table 3).

Table 3: Determination of half-lifes $(t_{1/2})$ and initial rates (r_i) of Cl⁻ ion influx across EYPC/cholesterol-LUVs \supset lucigenin by **1d–1f** (60 μ M each).

Compound	<i>t</i> _{1/2} (s)	$r_{\rm i} ({\rm s}^{-1})$
1d	32.36 ± 0.10	0.0165 ± 0.0014
1e	26.04 ± 0.09	0.0232 ± 0.0026
1 f	17.86 ± 0.05	0.0335 ± 0.0087

2.7. Ion Transport Mechanism:

In the aforestated lucigenin experiments, either Cl⁻/NO₃⁻ antiport or Cl⁻/Na⁺ symport was considered as the probable mechanism as the ion transport pathway. Therefore, experiments were carried out by varying either the cations or the anions in the extravesicular media to identify the correct mechanism. To evaluate the presence of a symport process, vesicles containing lucigenin in NaNO3 were suspended in extravesicular MCl $(M^+ = Li^+, Na^+, K^+, Rb^+ and Cs^+)$ salt solution (Figure S27). The addition of **1f** displayed small differences in the transport rates which rule out the possibility of Cl⁻/Na⁺ symport mechanism (Figure 6A). On the other hand, the variation of extravesicular of Na_mA (A⁻ = F⁻, ClO₄⁻, NO₃⁻, SO₄⁻²⁻, Cl⁻; m = valency of an anion) with isoosmolar intravesicular NaCl (Figure S28) provided remarkable differences in Cl⁻ efflux rates upon addition of 1f (Figure 6B). These experiments suggest that the Cl⁻/A⁻ antiport is the effective ion transport process.



Figure 6. Influx of Cl⁻ ion across EYPC/cholesterol-LUVs \supset lucigenin upon addition of **1f** (60 μ M) with intravesicular NaNO₃ and extravesicular MCl (M⁺ = Li⁺, Na⁺, K⁺, Rb⁺ and Cs⁺) (**A**). Exflux of Cl⁻ ion across EYPC/cholesterol-LUVs \supset lucigenin upon addition of **1f** (40 μ M) with intravesicular NaCl and extravesicular Na_mA salt (A⁻ = F⁻, Cl⁻, NO₃⁻, SO₄²⁻ and ClO₄⁻; m = valency of an anion) (**B**).

Finally, in pursuit to obtain direct experimental insight of preferential transporting mechanism for **1f**, valinomycin (V), a K⁺ selective transporter, was used. EYPC/cholesterol-LUVs⊃lucigenin with intravesicular NaNO₃ were suspended in the KCl solution (Figure S29), and ion transport rate of **1f** was monitored in absence and in the presence of valinomycin. A significant enhancement in the rate of transport was observed in the presence of valinomycin (Figure 7A). Numerical analysis of the normalized fluorescence quenching curves provided $r_i = 0.0120 \pm 0.0004 \text{ s}^{-1}$ and $t_{1/2} = 47.04 \pm 0.14 \text{ s}$ for only **1f**, whereas the combination of **1f** and valinomycin gave $r_i = 0.1333 \pm 0.0007 \text{ s}^{-1}$ and $t_{1/2} = 5.83 \pm 0.02 \text{ s}$ indicating more than 11-fold enhancement in the Cl⁻ transport rate. The remarkable improvement of transport rate in the presence of

valinomycin clearly indicates the Cl^{-}/NO_{3}^{-} antiport mechanism of **1f** which gets accelerated because of the synergistic effect of **1f** and valinomycin.



Figure 7. Comparison of Cl⁻ transport activity of **1f** (40 μ M) in the presence and the absence of valinomycin (2.5 μ M) (**A**). Calculated half-life ($t_{1/2}$) and initial rate (r_i) of Cl⁻ influx for **1f** in the presence and the absence of valinomycin (**B**).

2.8. Evidence of Carrier Mechanism:

The required evidence of ion transport through carrier mechanism was obtained through the classic U-tube experiment.^{33,36,40,65} In this experiment the mimic of lipid membrane was established by an organic phase, separating two aqueous phases. The large dimension of organic phase restricts the movement of ions from one aqueous layer to the other whereas, ion carriers can easily act as a vehicle by overcoming the organic phase barrier. In each experiment, equimolar NaCl and NaNO₃ were added to the aqueous phase of the donor and receiver phase respectively. Remarkable enhancement in Cl[−] level was observed in the receiver aqueous phase only when **1f** was taken in the organic phase (Figure 8 and S30). This result confirm the Cl[−] transport of **1f** by carrier mechanism.



Figure 8. Concentration of Cl^- in the aqueous phase of receiver end of U-tube as the function of time. Data were recorded in presence and absence of 1f in the organic phase.

2.9. Effect of Ion Transport in Biological Systems:

The excellent Cl⁻ transport activity of designed bis(sulfonamide) derivatives **1a–1f** prompted us to explore their bio-applicability. Recent studies have demonstrated that

dysregulation of ionic homeostasis, via the influx of Cl- ion into cells, can induce apoptosis.^{27,66,67} At first, the viability of various cancer (e.g. human breast cancer MCF-7, human bone osteosarcoma U2OS, human cervical cancer HeLa, and lung adenocarcinoma epithelial A549) and normal (e.g. mouse fibroblast NIH3T3) cell lines were screened for compounds 1a-1f and 2. A single point screening was done by MTT assay with these compounds (20 µM each). The maximum cell death was observed for the most efficient anionophore 1f while the least active bis(carboxylic amide) derivative 2 caused minimum cell death (Figure 9). Overall, the differences in cell viability were inversely proportional to the ion transport abilities of these molecules. The dose-dependent cell viability studies for compounds 1e and 1f provided $IC_{50} = 56.9-14.6 \ \mu M$ (Figure S31) and $IC_{50} = 12.2-7.5 \ \mu M$ (Figure S32 and Table S3), respectively depending on cell lines used. The compound 1f was selected to perform the further studies because of its least IC_{50} values in the cell viability experiments.



Figure 9. Cell viability obtained from single point screening of compounds 1a-1f and 2 (20 μ M each) by MTT assay. Cell viability was checked after 24 h treatment of each compound in various cell lines, from left to right, MCF7, NIH3T3, U2OS, HeLa and A549. Each bar diagram represents mean cell viability, calculated from three independent experiments. Compound 1g was excluded because of the precipitation in higher concentration.

The direct evidence of Cl⁻ ion transport into the live cells upon treatment with **1f** was obtained by using MQAE, a Cl⁻ selective dye ($\lambda_{ex} = 350$ nm and $\lambda_{em} = 460$ nm), that gives quenching of fluorescence in the presence of the ion.^{68,69} A remarkable stepwise quenching of fluorescence was observed upon post incubation of either HeLa (Figure 10A) or MCF7 (Figure 10B) cells with **1f** (0 – 50 µM). The gradual quenching of MQAE fluorescence indicates a concentration dependent influx of Cl⁻ ion in the intracellular matrix facilitated by the transporter molecule.

The correlation between the enhanced Cl⁻ ion level in the intracellular matrix, and the death of either HeLa or MCF7 cells was evaluated by altering the Cl⁻ ion concentration in the extracellular matrix. HBSS (Hank's balanced salt solution) buffers of two different salt compositions (with and without Cl⁻ ions) were prepared, and cells were suspended separately in these buffers. Then, both sets of cells were treated separately with **1f** (0 – 20 μ M) for 24 h and the cell viability between the sets was compared. Remarkably higher viability was observed for cells suspended in Cl⁻ ion free HBSS than those dispersed in the HBSS buffer containing the ion (Figure 11). These results confirm that the enhanced level of intracellular Cl⁻ ion, transported by **1f**, is potentially related to the amount

of cell death. Such Cl^- ion mediated cell death is known to trigger the apoptotic pathways in live cells.^{24,27,66,70}



Figure 10. Normalized fluorescence intensity of HeLa (**A**) and MCF7 (**B**) cells incubated with MQAE (5 mM) for 3 h followed by treatment of **1f** (0 – 50 μ M) for 24 h. Fluorescence intensities were recorded by the plate reader at $\lambda_{em} = 460$ nm ($\lambda_{ex} = 350$ nm), and normalized with respect to the fluorescence intensity of untreated cells. Each bar represents the mean intensity of three independent experiments, and the differences in mean intensity are statistically significant (P < 0.01 for HeLa and P < 0.001 for MCF7) according to one-way analysis of variance (ANOVA).



Figure 11. Comparison of HeLa cell viability in Cl⁻ containing and Cl⁻ free HBSS buffer upon dose dependent treatment of **1f** (0 – 20 μ M) for 24 h (**A**). Similar studies were done with MCF7 cells (**B**). Each bar represents the mean intensity of three independent experiments, and the differences in mean intensities are statistically significant (P < 0.05 for HeLa and P < 0.01 for MCF7) according to one-way analysis of variance (ANOVA).

A number of experiments have been carried out to demonstrate the appropriate apoptotic pathway. In the intrinsic pathway of apoptosis, disruption of mitochondrial membrane potential (MMP) induces the release of cytochrome c from mitochondria to cytosol.^{71,72} Released cytochrome c subsequently binds to the Apaf-1 to form apoptosome. Then the cytochrome c/Apaf-1 complex activates caspase-9, which further activates multiple pathways, including activation of caspase-3, to induce apoptosis.⁷³⁻⁷⁶ Therefore, the process of apoptosis can be monitored at several stages, e.g. (a) by monitoring the mitochondrial membrane potential (MMP) and cytochrome c release as a pre-apoptotic symptom, (b) by monitoring proteins of the caspase family involving in the caspase cascade mechanism, and (c) by observing the nuclear fragmentation of the cells. The collapse of MMP, observed during the early stages of apoptosis, can be monitored by using JC-1, a membrane potential sensitive fluorescent probe.77,78 This dye exhibits intense red fluorescence when present in the mitochondrial membrane of healthy cells due to the formation of J-aggregate. The disruption of MMP does not allow the dye to stay in the membrane resulting in its dispersion in the cytosol. In the cy-

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toplasm, the JC-1 dye exhibits green fluorescence due to the loss of aggregation. When HeLa cells were treated with **1f** $(0-20 \ \mu\text{M})$ for 24 h followed by staining with JC-1, a stepwise decrease in the red fluorescence with concomitant enhancement of the green fluorescence was observed (Figure 12A–C). Subsequently, the ratio of pixel intensities (*i.e.* red/green) was calculated from the live cell images of the stained cells (Figure 12D). The decrease in the ratio of pixel intensities can be correlated to the disruption of MMP upon treatment of **1f**.



Figure 12. Live cell imaging of HeLa cells upon treating with 0 μ M (**A**), 10 μ M (**B**) and 20 μ M (**C**) of **1f** for 24 h followed by staining with JC-1 dye. Each image was generated by merging the images obtained from red and green channels. The bar graph (**D**) represents the ratio of pixel intensities (red/green) for each set of cells. One way ANOVA analysis of seven replicate images show the statistically significant (*P* < 0.001) mean difference in the ratio of pixel intensity.

Cytochrome *c* is a well conserved electron-transport protein and confined between mitochondrial intermembrane spaces. Apoptotic stimulation triggers the release of cytochrome *c* from mitochondria which subsequently instigate the caspase dependant apoptotic pathway.⁷⁹⁻⁸¹ HeLa cells were incubated with increasing concentration of **1f** (0, 10 and 20 μ M) for 8 h and immunostaining analysis was done with cytochrome *c* antibody. The release of cytochrome *c* was monitored by enhancement in emission intensity which further diffused upon treatment of higher dose. The release of cytochrome *c* upon ion transport endorses the induction of mitochondrial dependent apoptosis by **1f** (Figure 13).



Figure 13. HeLa cells, treated first with 0 μ M (**A**), 10 μ M (**B**) and 20 μ M (**C**) of **1f** for 8 h, then fixed and analyzed for cytochrome *c* release by immunostaing with cytochrome *c* antibody. DMSO was used as negative control and the nuclei were stained with Hoechst 33342.

It is already established that mitochondria dependent apoptotic pathway can proceed via activation of family of caspases.^{75,82-84} In the next step, for better understanding of ion transporter induced caspase cascade, the activation of initiator

caspase-9 and the executioner caspase-3 were investigated. Immunoblot assay was performed on HeLa cells upon treatment with 1f $(0 - 40 \,\mu\text{M})$ for 24 h by using appropriate primary antibodies. A dose dependent enhancement of the amount of activated caspase-9 and caspase-3 with simultaneous degradation of procaspase-3 indicates the caspase dependent intrinsic pathway of the apoptosis program (Figure 14 and S33). On the other hand apoptosis can also be initiated via extrinsic pathway involving caspase-8.75,85 No such activation of caspase-8, and no degradation of procaspase-8 ruled out the possibility of extrinsic pathway of apoptosis program (Figure 14 and S33B). Similarly, apoptosis can also proceed via activation of p53 mediated pathway.⁸⁶ Posphorilation at Ser-15 position of the p53 protein is a common phenomenon in p53 mediated apoptosis pathway.⁸⁷ Absence of phosphorilation at Ser-15 position of p53 protein, monitored by immunoblot assay, eliminate the possibility of p53 mediated apoptosis program (Figure S34).



Figure 14. Immunoblot assay for active caspase-9, caspase-3 and caspase-8 in HeLa cells, after 24 h incubation with various concentrations (0, 10, 20 and 40 μ M) of **1f**. Data were quantified with respect to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels.

Moreover, to elucidate the caspase dependent intrinsic pathway of apoptosis, cell viability was screened in presence of caspase inhibitors. The small polypeptide derivatives, Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-

fluoromethylketone (z-DEVD-fmk), and Benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (z-LEHDfmk) are known cell permeable, irreversible inhibitors for caspase-3 and caspase-9, respectively, which can inhibit the caspase dependent apoptotic pathway of cell.^{83,88,89} Almost negligible amount of caspase-3 and caspase-9 activation was observed in immunoblot assay, upon preincubation of HeLa cells with z-DEVD-fmk and z-LEHD-fmk (50 μ M each) followed by treatment of **1f** (20 μ M) for 24 h (Figure 15A and S35). Similarly, in MTT assay, significant increase in cell viability was encountered upon treatment of **1f** when cells were preincubated with caspase inhibitors (Figure 15B). This enhanced cell viability can be correlated to the protection process of cells form ion transporters by inhibition of caspase dependent intrinsic pathway of apoptosis program.



Figure 15. Immunoblot analysis of HeLa cells preincubated with z-DEVD-fmk and/or z-LEHD-fmk (50 μ M each) for 3 h followed by incubation with **1f** (20 μ M) for 24 h (**A**). Difference in cell viability upon treatment with **1f** and caspase inhibitors measured by MTT assay. HeLa cells were preincubated with z-DEVD-fmk and/or z-LEHD-fmk (50 μ M each) for 3 h followed by incubation with **1f** (10 μ M) for 24 h (**B**). Each bar represents the mean intensity of three independent experiments, and the differences in mean intensity are statistically significant (P < 0.05), in each case, according to one-way analysis of variance (ANOVA).

Apoptosis can also be characterized by distinct changes in cellular morphology, including membrane blebbing, chromatin condensation, the appearance of membrane-associated apoptotic bodies, internucleosomal DNA fragmentation, as well as by cleavage of poly(ADP-ribose) polymerase (PARP).^{36,90,91} Nuclear condensation, fragmentation and formation of apoptotic bodies can be identified by Hoechst 33342, a fluorescent nuclear staining dye. HeLa cells were treated with **1f** (20 μ M) for 24 h and then, stained with the dye. A significant change in nuclear morphology was observed in treated HeLa cells compared to untreated ones (Figure 16). The appearance of nuclear condensation and fragmentation indicates apoptotic cell death.



Figure 16. Live cell imaging of HeLa cells stained with Hoechst 33342. Typical oval nuclei in control (untreated) cells (**A** and **B**), fragmented nuclei of cells after 24 h treatment with $1f(20 \ \mu M)$ (**C** and **D**).

It is also well-known that PARP cleavage, by endogenous caspases, serves to prevent futile repair of DNA strand breaks during the apoptotic program.^{90,92} An appearance of cleaved PARP-1 (86 kD) with concomitant degradation of full-length PARP-1 (116 kD) was observed upon treatment of HeLa cells with **1f** (40 μ M) for 24 h (Figure 17). The activation of cleaved PARP-1 by endogenous caspase substrate can be considered as an additional validation of caspase mediated apoptosis.



Figure 17. The expression of cleaved PARP-1 and degradation of full length PARP-1 in HeLa cells, after 24 h incubation with **1f** (40 μ M), determined by immunoblot analysis (**A**). Statistically significant difference in cleaved PARP-1 expression was observed upon applying unpaid *t*-test on three replicate data (**B**).

Finally, to establish the relation between the change in ionic homeostasis and induction of apoptosis, reactive oxygen species (ROS) level of cells were measured. It is well known in the literature that ROS level of cell can be effected by the change in ionic homeostasis via interruption of respiratory chain in mitochondria.93,94 ROS level in HeLa cells were measured fluoremetrically by using a ROS sensitive probe, 3methyl-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-2Hchromen-2-one (ROS probe).95,96 An enhancement of ROS level was observed when HeLa cells were incubated with increasing concentration of 1f (0 - 100 μ M) for 8 h (Figure 18A). Furthermore, a significantly higher enhancement in ROS level was observed when media with Cl⁻ was used as external buffer (HBSS buffer with Cl⁻) than Cl⁻ free media (Cl⁻ free HBSS buffer) (Figure 18B). Thus, the Cl⁻ dependent enhancement of ROS level signifies the ion transport mediated ROS production in HeLa cells upon incubation with 1f. The enhanced level of ROS instigates the opening of mitochondrial permeability transition pore which results in the leakage of cytochrome c from mitochondria *i.e.* the induction of intrinsic apoptotic pathway.⁹⁷⁻¹⁰⁰



Figure 18. Measurement of ROS production in HeLa cells upon incubation with varied concentration of **1f** (0 – 100 μ M) for 7 h, followed by treatment with the **ROS probe** (50 μ M) for 1 h in the same solution (**A**). Comparison of ROS production in HeLa cells in absence and presence of Cl⁻ ion (HBSS buffer with and without Cl⁻ ion) in extracellular media (**B**). Fluorescence intensities were recorded from the plate reader at $\lambda_{em} = 460$ nm ($\lambda_{ex} =$ 315 nm), and normalized with respect to the fluorescence intensity of untreated cells. Each bar represents the mean intensity of three independent experiments, and the differences in mean intensity are statistically significant (P < 0.001), in each case, according to one-way analysis of variance (ANOVA).

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3. CONCLUSION

In this study, we have described bis(sulfonamides) as efficient artificial anionophores for selective Cl⁻ ion transport. These anion transporters were synthesized by reacting the 1,3phenylenedimethanamine core with arylsulfonyl chlorides to create a molecular library of varied sulfonamide N-H proton acidity, and lipophilicity. ¹H-NMR titrations confirmed strong anion binding when an electron withdrawing group is connected to the arylsulfonyl group. The bis(sulfonamide) system displayed strongest binding with Cl⁻ ion compared to other halides, and a 1:1 binding model was established with Cl⁻ ion. Anion recognition of the system was also much stronger compared to the corresponding bis(carboxylic amide) derivative. Transmembrane ion transport studies confirmed the compound with appropriate lipophiliciity and strong anion binding ability is the most efficient transporter. Selective transport of Cl⁻ ion and Cl7/anion antiport mechanism across large unilamellar vesicles were also confirmed by several fluorescent based assays.

MTT assay indicated an inverse correlation between cell viability and ion transport activity of bis(sulfonamides) derivatives. The elevated intracellular Cl⁻ ion level and the Cl⁻ mediated cell death were also confirmed. The disruption of ionic homeostasis of cells led to the change in the mitrochondrial membrane potential which subsequently initiate the release of cytochrome c from mitochondrial intermembrane spaces to cytosol. The mitochondria dependent intrinsic apoptotic pathway of cell death was confirmed by expression of a family of caspases and cleaved PARP-1. The change in the nuclear morphology was also observed as a postapoptotic symptom. The caspase-9 and caspase-3 dependent intrinsic pathway of apoptosis was further confirmed by monitoring transporter mediated cell death in presence of caspase inhibitors. Finally, the induction of apoptosis mechanism by ion transport involving excessive production of ROS was demonstrated. Hence, artificial Cl⁻ transporters as apoptotic inducing agents, via disrupting ionic homeostasis of cell, could be a potential therapeutic tool for cancer treatment in next generation.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI: http://pubs.acs.org.

Experimental procedures, compound characterization data, theoretical calculations and biological evaluation and data (Pdf)

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Funding Sources

This work was supported in part by collaborative grants from SERB, DST, Govt. of India (Grant No. SR/S1/OC-65/2012 and EMR/2014/000873).

Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENT

TS thanks UGC (University Grant Commission), India for research fellowships. We also thank Dr. Aloke Das, Dr. Arnab Mukherjee, Ms. Libi Anandi, Ms. Vaishali Chakaborty, Mr. Satish Bodakuntla, Mr. Ashiq K. A., Mr. Avishek Karmakar and Mr. Abhik Mallick of IISER Pune for their valuable discussions.

ABBREVIATIONS

DPPC, 1,2-Dipalmitoyl-*sn*-phosphocholine; ESI-MS, Electrospray ionization-mass spectrometry; EYPC, Egg yolk phosphatidylcholine; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HPTS, 8-Hydroxypyrene-1,3,6-trisulfonate; JC-1, 5,5',6,6'-Tetrachloro-1,1',3,3'-

tetraethylbenzimidazolylcarbocyanine iodide; LUV, Large unilammelar vesicle; MD, Molecular dynamics; MQAE, *N*-(Ethoxycarbonylmethyl)-6-methoxy-quinolinium bromide; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, Reactive oxygen species; Tx, Triton X-100; z-DEVD-fmk, Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; z-LEHD-fmk, Benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone.

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