

Development of Artificial Anion Transport Systems and Evaluation of Their Biological Activity

A Thesis

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of the Degree of**

Doctor of Philosophy

By

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ID: 20162022

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Indian Institute of Science Education and Research Pune

2023

Dedicated to...

Maa Baba



भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान, पुणे

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CERTIFICATE

Certified that the work incorporated in the thesis entitled “*Development of Artificial Anion Transport Systems and Evaluation of Their Biological Activity*”, submitted by **Mr. Abhishek Mondal**, was carried out by the candidate under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.

Date: 10 May 2023

Place: Pune

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DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will cause disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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A

α	Alpha
Å	Angstrom
A	Absorbance
Ar	Aromatic
Ac	Acetyl
ATP	Adenosine Tri-Phosphate

B

β	Beta
br	Broad singlet
BLM	Black lipid membrane
Boc	<i>tert</i> -Butoxycarbonyl
BF ₃ ·OEt ₂	Boron trifluoride diethyl etherate

C

<i>c</i>	Concentration
cm	Centimeter
CF	Carboxyfluorescein
Calc	Calculated
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CLC	Chloride Channel
CLCA	Calcium-activated Chloride Channel
CLIC	Chloride intracellular channel protein
CLNS1A	Chloride Nucleotide-Sensitive Channel 1A
CHCl ₃	Chloroform
CDCl ₃	Deuterated chloroform
CH ₃ CN	Acetonitrile
CsCl	Cesium chloride
CCDC	Cambridge Crystallographic Data Centre
Cbz	Carboxybenzyl

D

δ	Delta (Chemical shift)
$^{\circ}\text{C}$	Degree Celsius
d	Doublet
dd	Doublet of doublet
dt	Doublet of triplet
DPhPC	2-diphytanoyl- <i>sn</i> -glycero-3-phosphocholine
DMSO	Dimethylsulfoxide
DMF	Dimethylformamide
DCM	Dichloromethane
D ₂ O	Deuterated water
DMEM	Dulbecco's Modified Eagle's Medium
DFT	Density Functional Theory
DTT	Dithiothreitol

E

EYPC	L- α -phosphatidylcholine from egg-yolk
EC_{50}	Effective concentration at half maximal activity
EtOAc	Ethylacetate
EtOH	Ethanol
Et ₃ N	Triethylamine
ESI	Electrospray ionization
EDC·HCl	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

F

F_t	Fluorescence intensity at time t
FCCP	Carbonyl cyanide- <i>p</i> -trifluoromethoxyphenylhydrazone

G

G_{hyd}	Free energy of hydration
------------------	--------------------------

H

Hz	Hertz
h	Hour
HPTS	8-Hydroxypyrene-1,3,6-trisulfonate trisodium salt
HOBt	Hydroxybenzotriazole
HPF ₆	Hexafluorophosphoric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRMS	High Resolution Mass Spectrometry

I

I_F	Normalized Fluorescence Intensity
IR	Infrared spectroscopy
ISE	Ion selective electrode
ITC	Isothermal titration calorimetry

J

J	Coupling constant
J	Joule

K

k	Rate constant
K	Equilibrium constant
K_a	Association constant
K ₂ CO ₃	Potassium carbonate
KCl	Potassium chloride
KBr	Potassium bromide
KOH	Potassium Hydroxide

L

Abbreviations and Symbols

λ	Lambda
LUV	Large unilamellar vesicle
logP	Partition Coefficient
LiCl	Lithium chloride
M	
m	Multiplet
M	Molar
μ M	Micromolar
μ L	Microliter
M.P.	Melting Point
MHz	Mega hertz
min	Minute(s)
max	Maximum
mg	Milligram(s)
mol	Mole
mmol	Millimole(s)
mM	Millimolar
mL	Milliliter
M. Wt.	Molecular weight
MALDI	Matrix Assisted Laser Desorption Ionization
MeOH	Methanol
Mon	Monensin
Me	Methyl
N	
n	Hill coefficient
nm	Nanometer
nM	nanomolar
NMR	Nuclear magnetic resonance
Na ₂ SO ₄	Sodium sulfate
NaF	Sodium fluoride

NaCl	Sodium chloride
NaBr	Sodium bromide
NaI	Sodium iodide
NaNO ₃	Sodium nitrate
NaClO ₄	Sodium perchlorate
NaSCN	Sodium thiocyanate
NaOAc	Sodium acetate
NaOH	Sodium hydroxide

O

obs	Observed
ORTEP	Oak Ridge Thermal Ellipsoid plot

P

Ph	Phenyl
pM	Picomolar
ppm	Parts per million

R

R	Ideal gas constant
ρ	Resistivity
rt	Room temperature
RbCl	Rubidium chloride

S

s	Second(s)
SCXRD	Single crystal X-ray diffraction

T

t	Triplet
td	Triplet of doublets
t	Time
Tx	Triton X-100

Abbreviations and Symbols

TLC	Thin Layer Chromatography
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid
TBA	Tetrabutyl ammonium
TOF	Time of flight

V

V_m	Membrane Potential
V or Vln	Valinomycin
VDAC	Voltage activated anion channel
Val	Valine

Y

Y	Fractional fluorescence intensity
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The thesis entitled “**Development of Artificial Anion Transport Systems and Evaluation of Their Biological Activity**” comprises five chapters.

The main objective of this thesis is to develop artificial ion transport systems and explore their potential applications in the field of biomedicine. During my doctoral research, I focused on designing, synthesizing, and characterizing biomimetic artificial ion channels and ion carriers that have shown promising biological applications for future therapeutics. Specifically, this thesis primarily focuses on the supramolecular architecture of artificial ion channels that selectively transport chloride ions. The thesis addresses the ability to fine-tune ion transport by controlling pore diameter and enhancing ion selectivity by incorporating multiple selectivity filters. Additionally, the thesis demonstrates the correlation between ion transport activity and lipophilicity in anion transport systems. The potential use of ion carriers and ion channels for inducing apoptosis via disruption of autophagy in live cells through chloride ion transport was evaluated, indicating that it may be a promising therapeutic tool for combating cancer in due course.

Chapter 1: Introduction to membrane transport

The translocation of solute molecules across cellular membranes is vital to fundamental biological processes such as cell-to-cell communication, energy production, biosynthesis, and metabolism. However, due to the hydrophobic nature of the membrane's domain, the transmembrane transport of solutes is limited by a thermodynamic barrier. Specific classes of membrane proteins overcome this thermodynamic barrier to facilitate the exchange of meticulous solutes, particularly ions. Among anions in the physiological system, chloride is the most abundant. The selective transport of chloride ions, typically facilitated by chloride-selective ion transporters, has significant implications in various biological processes, including transepithelial salt transport, acidification of internal and extracellular compartments, cell volume regulation, cell cycle, and apoptosis. Dysfunction of ion transport proteins can result in severe and life-threatening diseases such as myotonia congenita, cystic fibrosis, Bartter syndrome, Gitelman syndrome, Dent's disease, renal tubular acidosis, deafness, and more. A novel treatment approach referred to as "channel replacement therapy" has been recently developed to repair impaired ion transport using engineered protein-based ion channels. Additionally, it has been

discovered that disrupted chloride transport can trigger programmed cell death. Manipulating healthy chloride transport with external transporting molecules has created a new avenue for cancer treatment drug development.

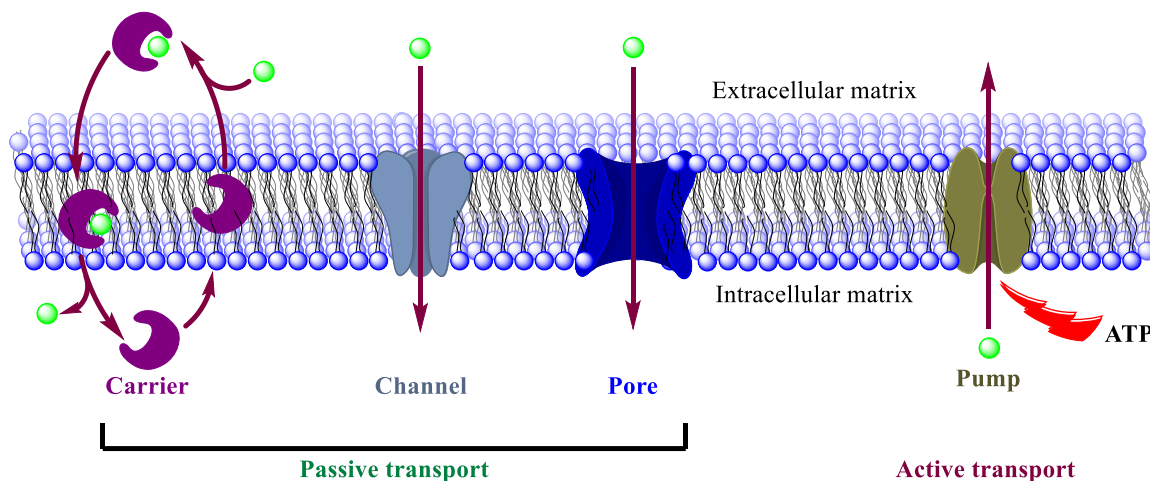


Figure 1 Schematic description of ion transport process through the plasma membrane.

Chapter 2: Nontoxic Artificial Chloride Channel Formation in Epithelial Cells by Isophthalic Acid-Based Small Molecules

Artificial channels facilitating Cl^- ion transport across cell membranes while remaining nontoxic to cells are a rarity. They can potentially replace defective ion channels and treat channelopathies by mimicking the functions of membrane transport proteins. In this study, we developed a series of isophthalic acid-based small molecules, which self-assemble into supramolecular nanochannels that enable the selective transport of Cl^- ions. Our findings revealed that intermolecular hydrogen bonding and π - π stacking interactions govern the self-assembly, as confirmed by single-crystal X-ray diffraction analysis. Electrophysiological studies, in addition to Molecular dynamics simulation, demonstrated the formation of stable Cl^- channel assembly in the lipid membrane and efficient Cl^- transport through them. The efficacy of the compounds in delivering Cl^- ions into cells was demonstrated through the MQAE assay, while the MTT assays confirmed that the compounds are nontoxic to cells, even at a concentration of 100 μM .

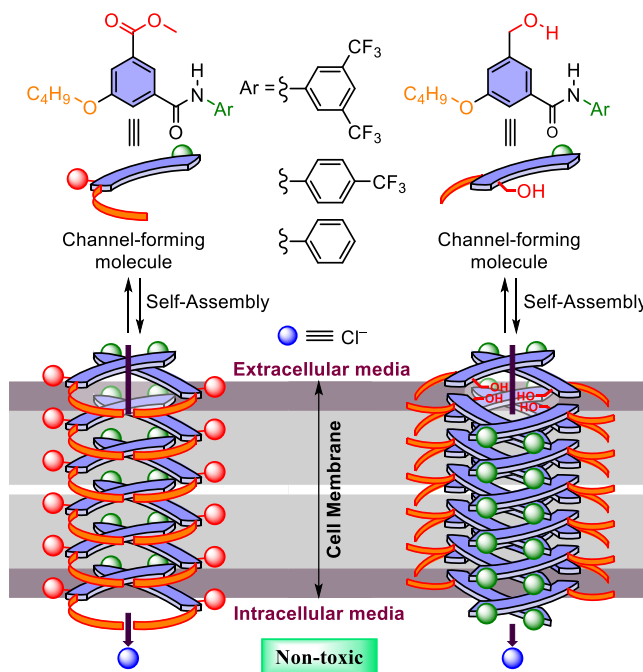


Figure 2 Structures of isophthalic acid derivatives and schematic representation of their self-assembly in crystalline form and lipid membranes.

Chapter 3: A Benzohydrazide-based Artificial Ion Channel that Modulates Chloride Ion Concentration in Cancer Cells and Induces Apoptosis by Disruption of Autophagy

Previous research has shown that changes in the concentration of chloride ions within cells, induced by synthetic ion transporters, can cause cell death by disrupting the balance of ions within the cell. However, the effects of these transporters on autophagy, the process by which cells break down and recycle their own components, have not been extensively studied. This chapter introduced a benzoylbenzohydrazide molecule that can assemble into a supramolecular nanochannel within cell membranes. This nanochannel selectively transports chloride ions, disrupting the balance of ions within cancer cells and causing them to undergo programmed cell death (apoptosis). Importantly, this transporter did not cause significant harm to noncancerous cells. We also found that the transporter disrupts autophagy in cancer cells by causing lysosomes (organelles involved in autophagy) to become less acidic. These results demonstrate a novel approach for targeting cancer cells by disrupting autophagy and inducing apoptosis using an artificial ion channel.

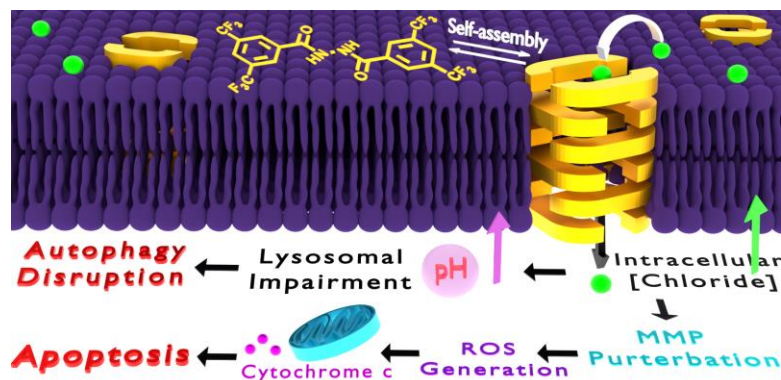


Figure 3 Structure of the benzohydrazide transporter and schematic representation of its self-assembly across membranes.

Chapter 4: A Pyridyl-Linked Benzimidazolyl Tautomer Facilitates Prodigious H^+/Cl^- Symport through a Cooperative Protonation and Chloride Ion Recognition

In this chapter, we introduced two pyridyl-linked benzimidazolyl hydrazones that exhibit superior HCl cotransporting ability compared to prodigiosin, a natural product whose synthetic counterparts have never achieved such high transport efficiency. These hydrazones possess a cooperative protonation and chloride ion recognition mechanism that facilitates the binding of HCl. The transport of HCl by the most potent compound results in the deacidification of lysosomes. Viability assays indicated that these compounds induce cytotoxicity in human breast cancer MCF-7 cells while exhibiting relatively low toxicity towards noncancerous HEK293T cells. These findings suggest that these hydrazone-based HCl transporters are promising therapeutic agents for cancer treatment.

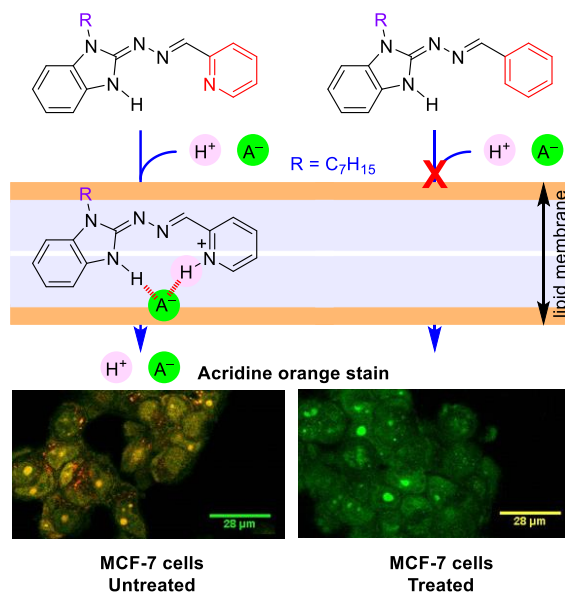


Figure 4 Mode of HCl transport by the hydrazone-based transporter. HCl transport by the transporter deacidifies lysosomal pH, as demonstrated by the acridine orange assay.

Chapter 5: A Pyrrole-linked Benzimidazolyl Hydrazone Self-Assembles to Form HCl Channel and Induces Apoptosis in Cancer Cells

Modulation of intracellular chloride ion concentration by artificially developed ion transporters has been known to cause apoptosis in cancer cells. However, most of these transporters cause harm to healthy cells as well, limiting their applicability in real systems. In this chapter, we report a pyrrole-linked benzimidazolyl hydrazone that self-assembles to form a nanochannel assembly across the membrane for the selective translocation of HCl – a system which is rarely reported in the literature. We also demonstrate solid-state evidence that the compound self-assembles in the presence of HCl to produce organized arrays that include chloride-filled and water-filled channels. When tested on cellular systems, the compound showed extremely high toxicity toward cancerous cell lines while remaining relatively nontoxic to cell lines of noncancerous origin. The compound also showed better efficacy when compared with the commercially available anticancer drug, doxorubicin, in inhibiting the growth of MCF-7 3D spheroid cultures.

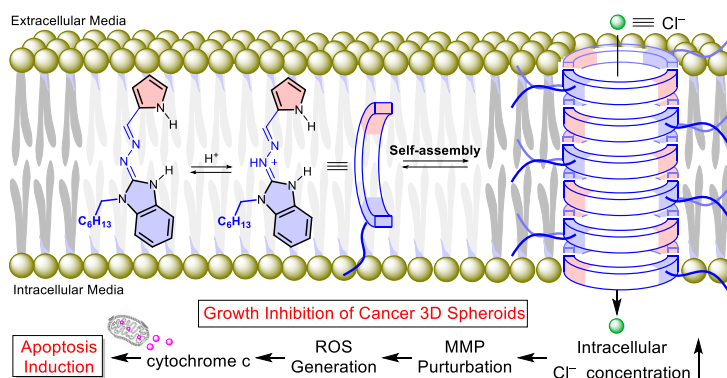
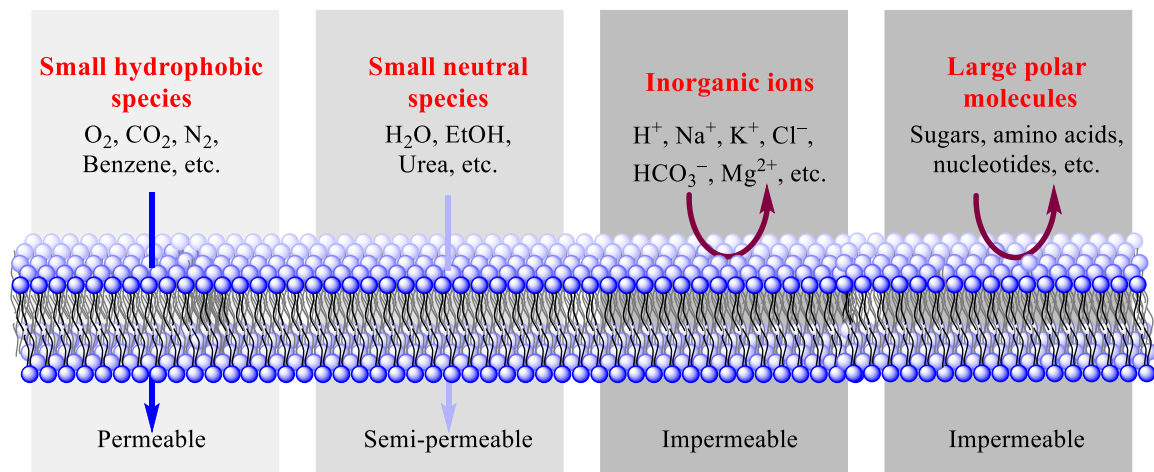


Figure 5 Structure of the pyrrole-linked hydrazone transporter and schematic representation of self-assembly of these molecules. The transporter increases the intracellular chloride concentration, leading to cancer cell apoptosis.

Chapter 1

Introduction to Membrane Transport



Compartmentalization, according to Daniel Koshland's 2002 proposal, is one of the fundamental principles of life.¹ It pertains to the ability of living organisms to isolate distinct chemical reactions and metabolic pathways within separate compartments. These compartments can take the form of specialized structures in prokaryotic cells or organelles in eukaryotic cells. This isolation allows for the regulation of chemical concentrations and reactions, as well as the development of unique environments that facilitate specialized functions.

For instance, the genetic material is protected in eukaryotic cells, and gene expression is regulated by the nuclear envelope separating the nucleus from the cytoplasm. Additionally, specialized metabolic processes are facilitated by the presence of distinct membranes in mitochondria and chloroplasts, which create controlled environments for ATP generation and other metabolic functions.

Overall, compartmentalization plays a crucial role in living systems, enabling the coordination and regulation of chemical reactions and metabolic pathways. This regulation allows for the maintenance of homeostasis and the capacity to adapt to changing environments.²

1.1 Phospholipid Bilayer Membrane

Phospholipids are the main components of cellular membranes, and their amphiphilic nature enables them to form a bilayer structure. The polar head-groups of the phospholipids are hydrophilic and interact with water molecules, while the hydrophobic tails are nonpolar and interact with each other, creating a hydrophobic region in the center of the bilayer (Figure 1.1). This hydrophobic region acts as a barrier to the free movement of polar or charged molecules, such as ions and sugars, between the intracellular and extracellular environments. Therefore, compartmentalization is achieved by the formation of these phospholipid bilayers, which enable the cell to maintain a distinct internal environment that is separate from the external environment.^{2, 3}

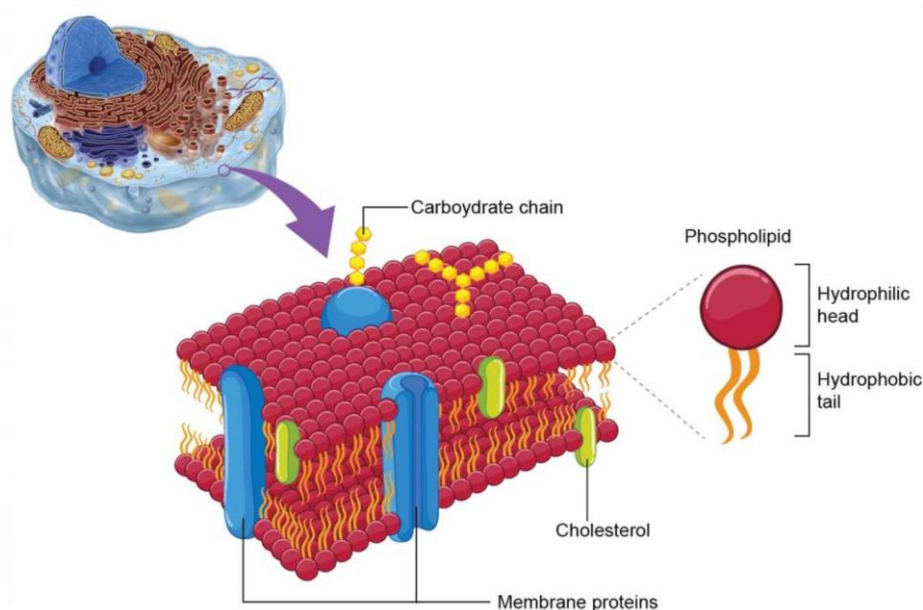


Figure 1.1 The representative structure of a eukaryotic cell and the plasma membrane. The image of the cell is obtained from the link <https://rsscience.com/cell-membrane>.

1.2 Membrane Permeability

The cell's interior is separated from the external environment by the hydrophobic plasma membrane, which selectively allows certain solute particles to pass while restricting others. The membrane's bilayer structure enables controlled exchange of various solutes, including small polar molecules, inorganic ions, large polar molecules, and nonpolar solutes. Figure 1.2 demonstrates the relative permeability of small molecules and charged ions across lipid bilayer membranes. This exchange is critical for the smooth functioning of intracellular processes, such as regulating pH and cell volume, maintaining osmotic balance, and signal transduction. While nonpolar species can easily pass through the membrane via diffusion, small polar molecules have a slower diffusion rate. Large polar molecules and ions cannot permeate the membrane due to the high thermodynamic barrier of the hydrophobic interior. To transport these essential substances, specific transmembrane proteins form ion channels or pores, creating a hydrophilic path to facilitate their transport.²

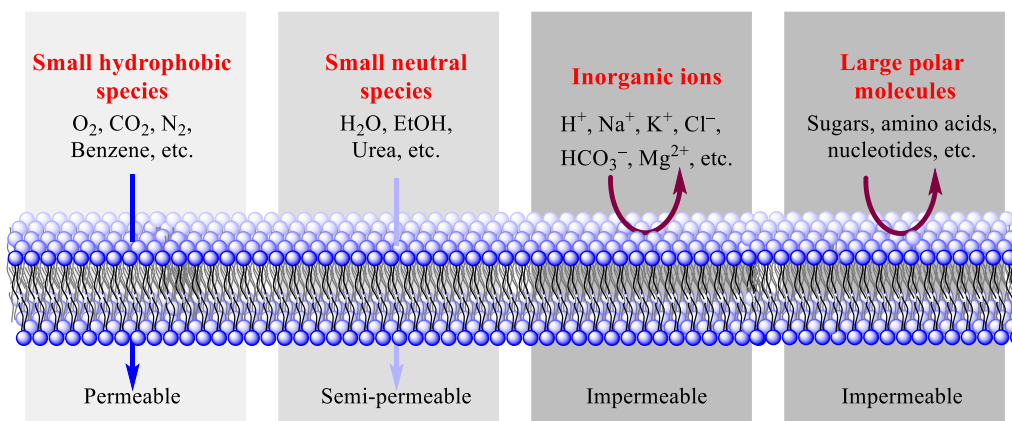


Figure 1.2 The relative permeability of small molecules and charged ions.

1.3 Classifications of Ion Transport

The movement of ions through biological membranes and the maintenance of ion gradients are crucial for many essential cellular processes. The majority of ion transport processes are driven by the electrochemical gradient of ions passively. Active membrane transporters (ion pumps) create the electrochemical gradient by using ATP hydrolysis as an energy source. These processes are responsible for regulating various tasks, such as sensory transduction, pH regulation, osmotic stress, cell volume, nutrient transport, electrical signal production, cell proliferation, and secretion across epithelial layers.⁴

The transport of ions across the membrane mainly occurs through ion channels or carriers.⁵ While natural channels are membrane-bound proteins that provide a static path for ions to move across the membrane, carriers can be mobile molecules that shuttle along with an encased ion through the hydrophobic interior of the bilayer membrane.

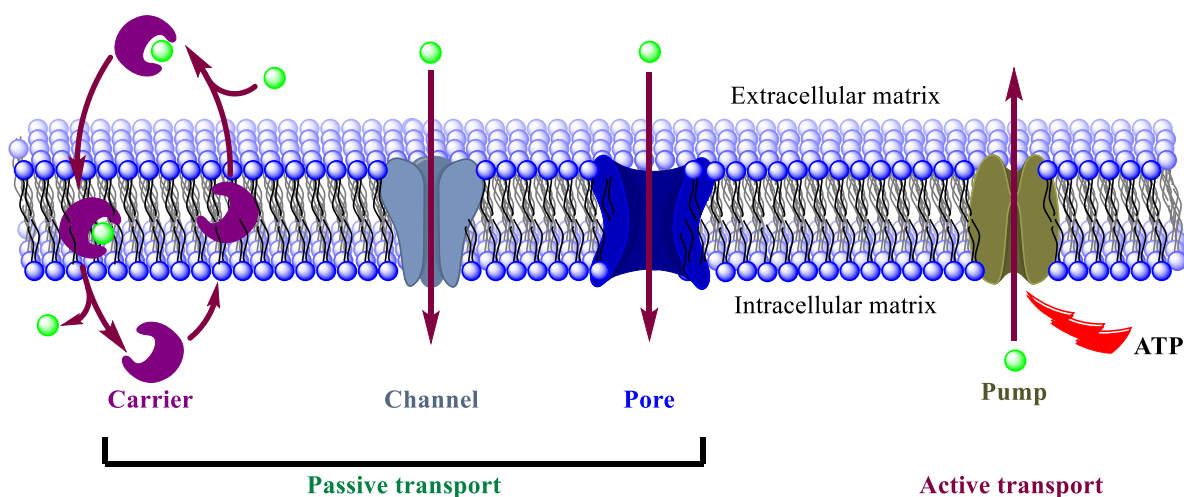


Figure 1.3 The process of transmembrane ion transports via a pump, channels, and carriers.

Transporters are categorized based on their transport stoichiometry (number of solutes transported) and direction of transport. Uniporters transport a single species in one direction, while cotransporters carry two or more species across the membrane. Cotransporters are further classified as symporters and antiporters. In symport, two species are transported in the same direction, whereas in antiport, two species are carried in opposite directions. Natural ion proteins selectively impose the required electrochemical gradient by uniporting ions.

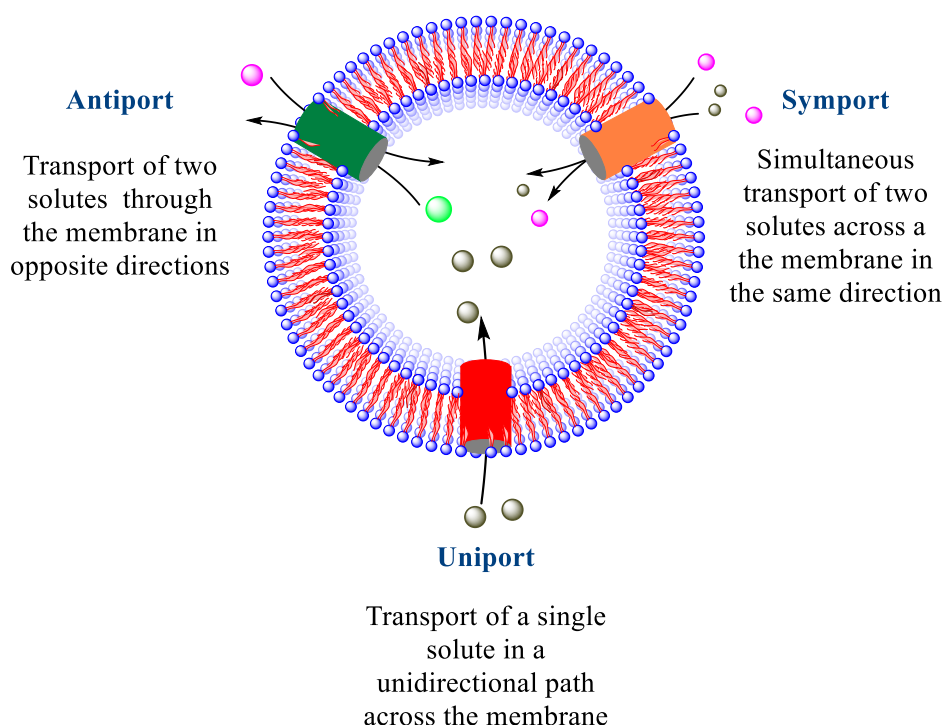


Figure 1.4 Modes of ion transport processes based on the directions of ion transport.

1.4 Natural Ion Transporters

The formation of a phospholipid bilayer provides a suitable barrier for cells, but it is also necessary for cells to have a mechanism for selectively transporting ions and molecules in and out of the cell or organelle. While the transport of larger polar species through endocytosis and exocytosis has been extensively studied, the following discussion focuses on the transport of ions across membranes.

Ion transport across phospholipid membranes may have been one of the earliest processes in the evolution of life. In the early stages, a small amphiphilic molecule would have bound with the ion in the aqueous media and transported it through the membrane. Over time, these simple

molecules became more complex, and ion channels were developed. Ion channels are molecules or aggregates that span the lipid bilayer and create a water-filled cavity through which ions can pass. Natural ion channels are amphiphilic protein molecules that insert into the membrane bilayer and span the distance between the separated aqueous domains. These channels present an outer hydrophobic surface to the non-polar domain of the bilayer and an ion-conducting pore lined with polar, hydrophilic residues. The polar residues effectively lower the energetic barrier in the microenvironment of the pore for the passage of ions between the aqueous domains.

Through the process of evolution, ion channels have been refined to form pores of optimal geometry with selectivity filters and gating mechanisms that can determine what is transported and what conditions are required for transport. Some channels have evolved into pumps that are able to actively transport ions or molecules against the concentration gradient. With these structures, cells are able to maintain ion homeostasis and concentration gradients.

Several natural products composed of one or more protein subunits have been discovered to facilitate the transport of cations across cell membranes. These products, such as gramicidin A^{6,7} and valinomycin⁸, have been extensively employed to explore the transport mechanism of synthetic ion transporters. Gramicidin A is a monovalent cation channel comprised of two identical alpha-helical peptide units that form a dimeric structure within biological membranes. Valinomycin, on the other hand, is a cyclic dodecadepsipeptide that occurs naturally. It selectively transports K^+ ions and is frequently used as a biochemical research tool. Valinomycin is capable of transporting approximately 104 ions per second and forms a six-fold coordination complex with K^+ ions. The stability of the potassium-valinomycin complex is roughly 100,000 times greater than that of the sodium-valinomycin complex.⁹

Anion transporters facilitate the movement of anions across the bilayer membrane by following the concentration gradient. There are two categories of naturally occurring anion transporters based on their structural unit: non-protein and protein-based anion channels. One example of a non-protein-based anion transporter is Prodigiosin, which is found in Gram-negative bacteria such as *Serratia marcescens*.¹⁰ Prodigiosin is lipophilic and has an optimal anion-binding site that can transport Cl^- and HCO_3^- via H^+/Cl^- symport and Cl^-/HCO_3^- exchange¹¹⁻¹³, with high permeability across the membrane.¹⁴⁻¹⁶ Its high ion transport activity has been linked to its cytotoxic effects against cancer cells^{15, 17, 18} and its potential in various biological applications,

such as antimalarial, immunosuppressive, and antifungal activities.¹⁸⁻²⁰ Tambjamines²¹⁻²³, which are structurally similar to Prodigiosin, have also shown anticancer and antimicrobial activities. Ceramides, a group of sphingolipids, are another type of naturally occurring anion transporter that participates in various signaling processes in eukaryotic cells via the anion-exchange mechanism.^{24, 25} At lower concentrations, they transport anions via the carrier mechanism, while at higher concentrations, they act as a channel.²⁶ Various types of Cl^- transporters exist naturally^{4, 27-29}, such as cystic fibrosis transmembrane conductance regulator (CFTR), Chloride-conducting channels (CLC), chloride intracellular channel (CLIC), Chloride Nucleotide-Sensitive Channel 1A (CLNS1A), and Calcium-Dependent Chloride Channel (CLCA), light gated chloride channel GtACR1, voltage dependent anion channel mitochondrial VDAC etc.

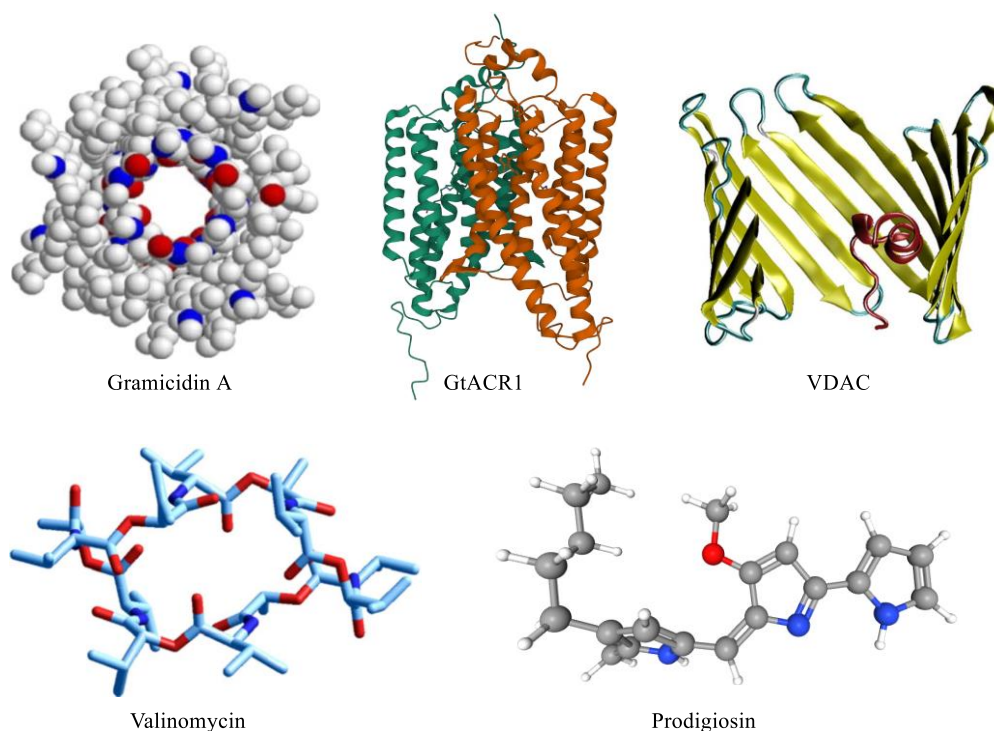


Figure 1.5 Some representative examples of naturally occurring ion transporters.

1.5 Need for Synthetic Anion Transporters

Anions are crucial for various cellular functions, including pH regulation, osmotic balance, and cellular signaling. Dysregulation of natural anion transporters can lead to medical complications known as "channelopathies."^{30, 31} Chloride (Cl^-) is the most abundant extracellular anion in the human body and is essential for physiology.³⁰ However, a defective

Cl^- ion channel has been linked to various diseases, including Bartter's syndrome, osteopetrosis, Dent's disease, and cystic fibrosis (CF).^{31, 32} The transport of CO_2 in the form of HCO_3^- through the blood from respiring tissue to lungs plays a crucial role in acid/base secretion and pH homeostasis. Although bicarbonate transport across the membrane is low in abundance, defects in HCO_3^- transport proteins have been associated with diseases affecting most organ systems, including the brain, heart, bones, and CF.³³

There is growing evidence that aberrant channel activity is associated with the development of cancer, metastasis, and drug resistance. The process of cell proliferation requires an increase in cell volume to produce a daughter cell, whereas cell shrinkage is a characteristic of cell death. The maintenance of constant cell volume is crucial, and volume-regulated anion channels (VRAC) play a significant role in this process.³⁴⁻³⁶ In many cancer cells, VRAC is encoded by CLC-3 as a major component,³⁷ which is a voltage-gated chloride channel belonging to the CLC superfamily. Abnormal proliferation of various types of cells in the body characterizes cancer cells, and this is linked to significant changes in cell volume.³⁸ Unlike healthy cells, cancer cells have an elevated intracellular pH ($\text{pH}_i \geq 7.4$) compared to the extracellular pH ($\text{pH}_e = 6.7-7.1$), which limits apoptosis and facilitates cell proliferation.³⁹ CLC-3, CLC-4, and CLC-5 act as a Cl^-/H^+ antiporter in intracellular membranes, contributing to the acidification of late endosomes and lysosomes. Proton flux regulators such as V-ATPase and Na^+/H^+ exchangers (NHE) regulate the unusual acidic pH in the microenvironment of tumor cells, protecting them from weak basic drugs.⁴⁰ The acidic microenvironment induces drug resistance by promoting the protonation of weak base drug molecules, resulting in reduced effective concentrations of these drugs. Chemical agents that can modify tumor cell acidity could be utilized to restore the efficiency of such drug candidates.

In recent decades, the field of supramolecular chemistry has seen significant developments in synthetic ion transport systems. These systems have been utilized for various purposes such as sensing, catalysis, and organic porous materials.⁴¹ However, the concept of "ion channel replacement therapy" has rekindled interest in the development of new ion transport systems to treat diseases related to channelopathies, which involve the dysfunction of natural ion channels. Yang et al. created a harmless ion channel system⁴² and demonstrated that CF epithelial cells could recover their Cl^- permeability through this Cl^- channel.⁴³ In a separate study, Davis et al. introduced bis-(p-nitrophenyl)ureidodecalin as effective and non-toxic ion carriers, and suggested that synthetic anionophores could be a promising therapy option for CF

either alone or in combination with CFTR modulators.⁴⁴ Burke et al. used Amphotericin B, which can impair respiratory host defences in people with cystic fibrosis, as it forms non-selective ion channels.⁴⁵ Talukdar and coworkers has recently developed 3-(1,2,3-triazol-1-yl)benzamide derivatives as non-toxic transmembrane anion carriers.⁴⁶ Very recently, they have also developed isophthalic acid-based artificial ion channels that were found to be non-toxic to cells even at concentrations up to 100 μM .⁴⁷

On the contrary, it has been discovered through recent research that various natural substances, including prodigiosin, tambjamines, and sphingolipids derived ionophores, have the potential to inhibit the growth of numerous types of cancer cells^{20, 21, 48-53}. This has led to the investigation of numerous artificial ion transport systems for their possible use in anticancer therapy. These systems comprise a range of compounds, such as Indole, benzimidazole/imidazole-based anionophores⁵⁴⁻⁵⁷, urea/thiourea-based anionophores⁵⁸⁻⁶⁹, squaramides^{70, 71}, and other anion transporters⁷²⁻⁸⁰. Additionally, apart from their ability to trigger apoptosis in cancer cells, some of these synthetic anion transporters were also observed to regulate autophagy in cancer cells, which could prove to be a powerful combination in the fight against cancer.^{70, 76, 81, 82}

1.6 Design Principles of Synthetic Anion Transporters

Nature synthesizes the ion channels by genetically engineered biosynthesis. Due to the extremely complex structures and large sizes, synthetically creating and mimicking the natural peptide-based ion channels is very difficult. In natural channels, the induced mutations and sequencing of protein residues have been identified by crystallizing the ion channels. These discoveries provided the sufficient knowledge about conserved protein residues and their contributions to the channel gate and selectivity filter. This information is useful in the new designs of protein and non-protein based channels. However, this task of identifying the mutations and sequencing is eminently resource-intensive and challenging to execute. The small natural products or small synthetic molecules can offer a feasible, resource-superficial and easily adaptable alternative natural ion channels.

When it comes to ion transport, the ion and transporter must interact in some way. Synthetic ion transporters are often based on supramolecular chemistry and ion receptors. The ion receptor field typically focuses on solid-state binding geometries and maximizing ion-binding strength. However, in the field of transmembrane ion transport, stronger binding is not always better, and binding in the solid state may not translate to binding in solution. In solution, both

direct transporter-ion interactions and indirect interactions via organized water molecules can occur. Direct interaction is not always preferred, as shedding a solvation shell can be energetically expensive. Additionally, an active transporter requires a dynamic interaction with the transported ion, and excessively strong binding could impede ion passage through the channel or further uptake by a carrier. Therefore, a compound that interacts with the solvation sphere rather than the ion itself may be a more effective transporter. While ion binding and transport may be based on similar principles, the goals for each field are distinct in terms of designing an optimal set of compounds.

Non-covalent interactions that are reversible play a crucial role in both ion binding and transmembrane ion transport. Electrostatic interactions are considered to be among the strongest non-covalent bonds, but their strength is influenced by the charge density across the surface of the ionic species. Diffuse charges, such as those found in iodide anions, or sterically hindered charges, result in weaker electrostatic attractions. When compared to a tetrabutylammonium cation that lacks a directly accessible charge, a chloride anion is more attracted to a charge-dense sodium cation.

Host-guest interactions with these charged species can occur in either polar or non-polar solvents if the proper host or transporter binding geometries and strengths are present. These binding interactions must, however, be energetically and kinetically competitive with the counter ion and/or solvation shell if either is displaced. In non-polar solvents, binding competition mainly comes from the counter-ion, while in polar solvents, contact energies with the counter-ion are almost negated, with competition from the ion's solvation sphere dominating.

While most interactions in the cytosol or aqueous cell exterior rely on charge-charge, charge-dipole, or dipole-dipole contacts, the non-polar interior of the membrane bilayer is characterized by moieties that aim to minimize contacts with polar and charged species. This region can also accommodate molecules with hydrophobic substructures like alkyl chains or arenes with minimal dipole moments. Amphiphilic compounds must interact with the membrane as the surrounding phospholipids do, with polar or charged moieties directed outward and lipophilic residues directed towards the membrane interior.

Unlike cations attracted to anions, hydrophobic residues of a membrane monomer or other amphiphiles are not inherently attracted to each other. Water, on the other hand, is essentially

repelled by these residues, making membrane insertion an entropy-driven process. No favorable hydrogen-bonding interactions exist between polar water and non-polar substructures. As a result, proximate water molecules lose the energetic advantage of full hydrogen bonding capacity. These water molecules, therefore, must form more rigid, "ice-like" contacts with surrounding water molecules, resulting in a high entropic penalty. When the quantity of rigid water contacts is minimized, the entropic energy penalty is proportionately reduced.

The forces between lipophilic residues themselves are extremely weak, allowing for the free movement and lateral diffusion of amphiphilic and hydrophobic species throughout the bilayer, similar to the diffusion of charged and polar species in polar solvents. Under the right conditions, amphiphiles that insert into one leaflet of the bilayer can flip to the other leaflet. Gramicidin, a natural cation transporter, takes advantage of these characteristics in biological membranes. Channel functionality is elicited by two monomers in each leaflet of the bilayer, diffusing across the face of its respective leaflet before aligning to form a transient pore.

To operate as an ion transporter, molecules must possess a polar cavity that can interact with multiple ion-binding sites. Additionally, the receptor's outer surface must be nonpolar to interact with the lipid's hydrophobic tail in the membrane. According to Lipinski's rule^{83, 84}, the $\log P$ values of receptors should be around 5 to enhance transport activity and better membrane permeability. Ion carriers are typically designed in various structural forms, such as bipodal, tripodal, foldameric, macrocyclic, and cage receptors.⁸⁵⁻⁸⁷ These designs offer different structural diversities.

Compared to anion carriers, the creation and development of anion channels that emulate the structure and functionality of membrane-embedded protein molecules present a greater challenge for synthetic chemists. Successful design of synthetic channels necessitates the consideration of various noncovalent supramolecular interactions in order to construct a channel assembly that can properly bind a specific type of ion. Significant strides have been made in the development of synthetic channel systems, with each design adhering to specific principles. For instance, the length of the channel in the membrane must either be equal to or greater than the thickness of the lipid membrane, achieved by single or multiple self-assembled units of channel-forming molecules. Additionally, the outer surface of the channel should be hydrophobic to facilitate insertion into the lipophilic membrane, while the inner pore surface

must be hydrophilic to allow for interaction with the polar ion and permit its passage through the channel lumen.

1.7 Strategies for Identifying and Measuring Anion Transport

The accurate detection and measurement of ion transporter activity is crucial, as these transporters change the concentration of solutes across the membrane. Two primary techniques are used to measure transporter activity: conductance measurement across planar bilayer membranes, known as black lipid membrane (BLM), and liposome-based assays. The BLM assay directly measures voltage and distinguishes between ion channel and carrier mechanisms. Liposome-based assays are used by chemists to study ion carriers. Unilamellar phospholipid vesicles are reliable and reproducible models that can mimic and study ion transport across cell membranes. Liposomes offer great versatility in controlling intra- and extra-vesicular solutions. Concentration gradients can be created across vesicular membranes by replacing extravesicular ions using size exclusion chromatography or dialysis techniques. Fluorescence, ion-selective electrodes, and NMR can quantify ion transport enabled by transporter compounds.

1.7.1 Fluorimetric Assays

Fluorescence-based assays are utilized to quantify transport activity by measuring changes in fluorescence emission of a specific encapsulated dye sensitive to changes in pH, anion concentration, or electric potential over a period. Common fluorescent dyes employed to measure ion transport in liposomal assays include HPTS, Lucigenin, and Safranin O. HPTS is a highly water-soluble pH indicator with a pKa of approximately 7.3 in aqueous buffers.⁸⁸ The two forms of HPTS, protonated and deprotonated, have distinct excitation wavelengths (403 nm and 460 nm, respectively) but give emission at the same wavelength (510 nm). In these assays, a pH gradient is created across the HPTS-entrapped vesicles, and the transport is characterized by monitoring the HPTS emission intensity after adding the transporter molecule.⁸⁸⁻⁹⁰ The intravesicular pH change following the addition of anion transporter indicates whether transport occurs through H^+/A^- efflux (symport) or OH^-/A^- exchange process (Figure 1.9A). This assay permits measurement of intravesicular pH by determining the ratio of protonated and deprotonated dye. Lucigenin assays directly measure anion transport. Lucigenin fluorescence is quenched selectively in the presence of halide anions but is insensitive to oxoanions, such as nitrate or sulfate. In the standard assay, vesicles entrapped with NO_3^- and

lucigenin dye are used, and a chloride gradient is created by adding Cl^- to the extravesicular matrix. The transport process is characterized by monitoring the quenching of fluorescence after adding transporter molecules. The $\text{NO}_3^-/\text{Cl}^-$ exchange is followed during transport⁹¹ (Figure 1.9B). Similarly, Safranin O, which is sensitive to membrane potential, is employed to detect unidirectional ion transport. This cationic dye is associated with the membrane, and the addition of transporter leads to an increase in Safranin O fluorescence.⁹²

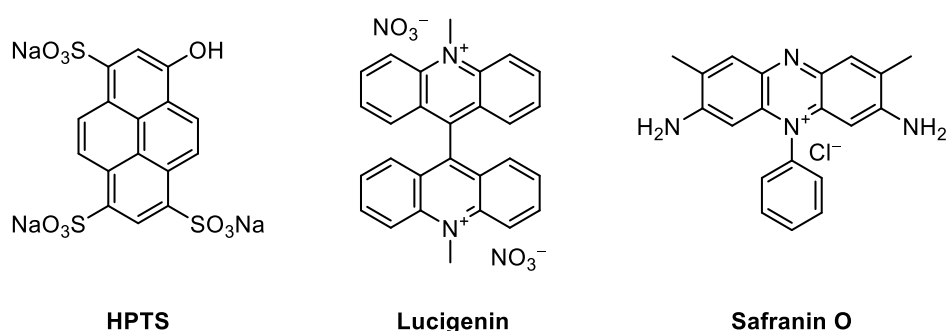


Figure 1.8 Commonly used fluorescent dyes in characterization of ion transport by vesicle-based assays.

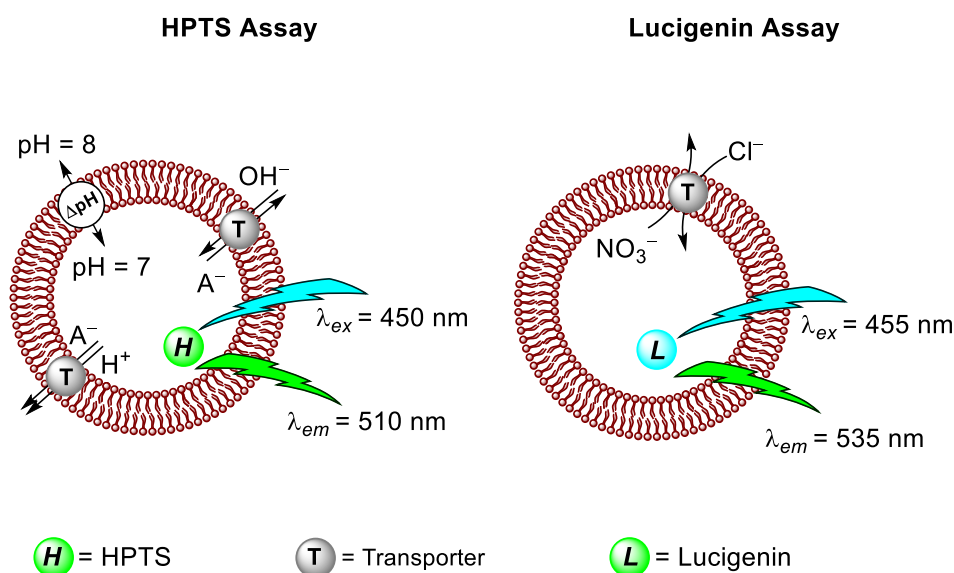


Figure 1.9 Representative fluorescent-based assay in liposomes entrapped with the pH-sensitive HPTS (A), and lucigenin captured vesicles (B).

1.7.2 Ion Selective Electrode (ISE)-based Assay

A direct and simple method for detecting anion transport is through the use of ion selective electrodes, similar to the lucigenin-based assay. The process involves measuring the conductivity of the extravesicular solution to determine the efflux of specific ions from vesicles. In a typical chloride efflux assay, high concentrations of MCl are enclosed within liposomes and added to an iso-osmolar solution of MNO_3 or M_2SO_4 (where M represents monovalent alkali cations). The chloride efflux is then measured using a chloride selective electrode by monitoring the conductivity of the extravesicular solution in the absence and presence of the transporter (as shown in Figure 1.10A).

1.7.3 NMR-based Assay

It is necessary for the transported species to be NMR active (e.g., ^{35}Cl , $\text{H}^{13}\text{CO}_3^-$, or $^{33}\text{SO}_4^{2-}$). In a typical experiment, NaA is used to prepare unilamellar vesicles with A^- being a NMR inactive anion. Paramagnetic shift reagents like Co^{2+} and Mn^{2+} are added to the extravesicular buffer. The presence of paramagnetic shift reagents outside the liposome causes a significant difference in either the NMR chemical shift or line-broadening of the peak for intra- and extravesicular anions (Figure 1.10B). Therefore, the line broadening or chemical shift change of the peak for extravesicular NMR active anion can be monitored to check anion transport across the membrane when an ion transporting agent is present.^{93, 94}

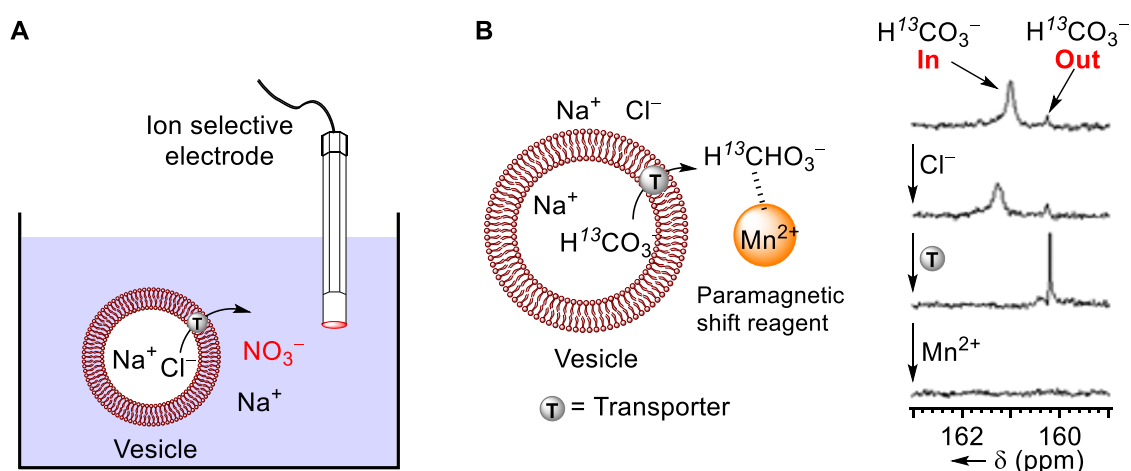


Figure 1.10 Schematic representation of ion transport study using ion selective electrode (A), and representative bicarbonate transport by ^{13}C NMR technique (B).

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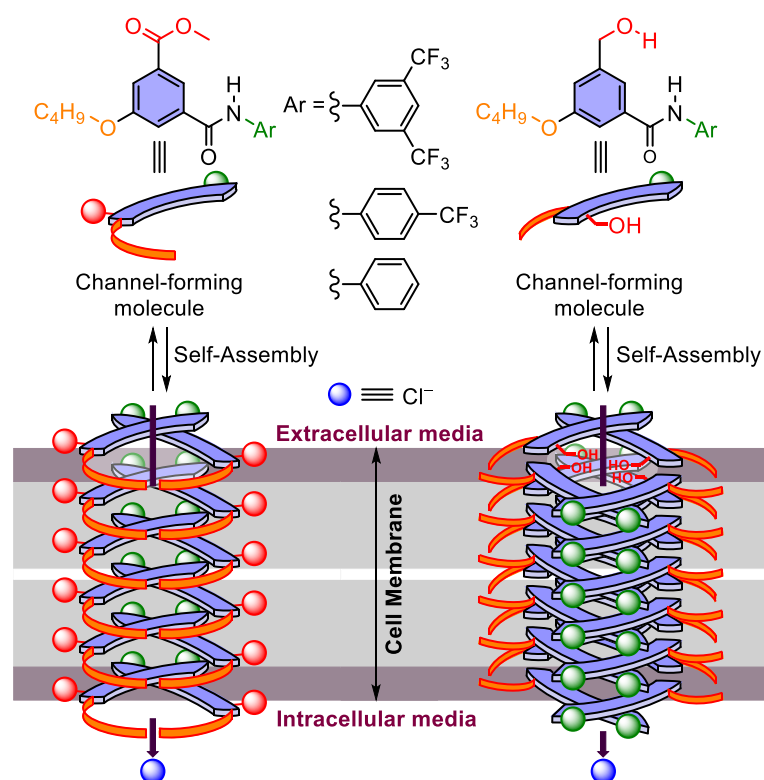
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Chapter 2

Nontoxic Artificial Chloride Channel Formation in Epithelial Cells by Isophthalic Acid-Based Small Molecules



2.1 Introduction

Ion transport across cell membranes is essential in the cellular domain to maintain ionic homeostasis, regulate cell volume, electrical excitability, cell-to-cell communication, produce energy, etc.¹⁻⁴ The structural incompatibility of the hydrophobic interior of cell membranes and hydrophilic ions prevents the ions from diffusing through the cell membranes. Thus, nature has created cell membrane-embedded ion transport proteins classified into channels, carriers and pumps.^{5, 6} Any defect or mutation in such transport proteins can lead to detrimental diseases like Bartters syndrome, Dent's disease, Best disease, Startle illness, and most notably, cystic fibrosis (CF), etc.; collectively called "channelopathies".^{7, 8} The prevalence of CF is one in 3500 in the United States and one in 2500 in the European Union, making it one of the most common lethal autosomal-recessive diseases.⁹ It is caused by ~2000 mutations in the gene that encodes cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel located at the top of the epithelial cells.⁹ Recent clinical trials have approved some drugs that target defective CFTR channels, but their efficacy may vary between mutations in the same class, potentially limiting their usefulness.⁹⁻¹² Conversely, the development of synthetic Cl^- channels, which can mimic the functions of the membrane transport proteins, may bring up a brand new opportunity for treating CF. They can also open a window of combination therapy with the drugs which target defective CFTR.¹³

Structurally simple low molecular weight molecules carry several intrinsic advantages due to their facile synthesis, structural tunability, long shelf-life, high permeability, flexible modes of action, etc.,¹⁴ and have greatly influenced the progress of biomedical science. To date, most synthetic Cl^- channel structures are relatively complicated, and their high molecular weights impede their applications in drug discovery.^{2, 15-17} Moreover, most compounds showing anion transport in large unilamellar vesicles (LUVs) are toxic to the cells (potentially useful as anticancer agents), therefore, incompatible with the treatment of CF.^{3, 18-25} As a whole, synthetic Cl^- channels, composed of small organic molecules with nontoxic behavior, are rarely reported in the literature. For instance, recent work by Yang and colleagues has shown that a synthetic Cl^- channel can restore Cl^- permeability in CF

epithelial cells, thereby accounting for its potential use in channel replacement therapy.²⁶ Burke and coworkers recently used a non-selective ion channel-forming molecule, amphotericin B, which restores the host defenses in CF airway epithelia with a genotype-independent mechanism.²⁷ Davis and colleagues reported an anion carrier based on decalin bis-urea, which was not toxic to the cells up to a concentration of 30 μM .²⁸ Further, they demonstrated that synthetic anionophores alone or in conjugation with CFTR modulators might represent a potentially useful treatment strategy for CF.²⁹ Recently, our group reported 3-(1,2,3-triazol-1-yl)benzamide as a nontoxic transmembrane anion carrier.³⁰ Herein, we report our newly designed isophthalic acid-based small molecule channels capable of transporting Cl^- ions across cell membranes and exhibiting nontoxic behavior to cells even at a loading concentration of 100 μM .

In 2001, Fyles and coworkers reported alkoxy-substituted isophthalic acid derivatives, which form well-defined cation-selective ion channels in planar and vesicle bilayer membranes.³¹ They came up with the lowest molecular weight compound that can render ion channels. Their study suggests that alkoxy isophthalates are capable of forming self-assembled oligomeric structures that are large enough to span membranes. To regulate the selectivity of the transport, we anticipated that the core structure, i.e., the alkoxy isophthalic acid, can be used to develop a supramolecular channel selective for anion by converting one of the carboxylic acid groups to the corresponding ester/alcohol. At the same time, an amide was introduced to ensure efficient self-assembly as well as anion recognition through hydrogen bond interactions (Figure 2.1). We envisaged that the intermolecular $\text{C}=\text{O}\cdots\text{H}-\text{N}$ interactions, in the case of the ester molecule, would provide a preorganized geometry, and the $\pi-\pi$ stacking interactions among the aromatic rings would lead to aggregation, which would result in the formation of an active channel in the membrane. The alcohol molecules, on the other hand, should gain additional structural rigidity from the intermolecular $\text{O}-\text{H}\cdots\text{O}-\text{H}$ hydrogen bonding interactions. In order to fine-tune the lipophilicity of the compounds, as predicted from the *clogP* values obtained from the ChemDraw program³² (Figure 3H), we chose butyl chain as the alkyl group and incorporated different aromatic moieties to the amide linkage.

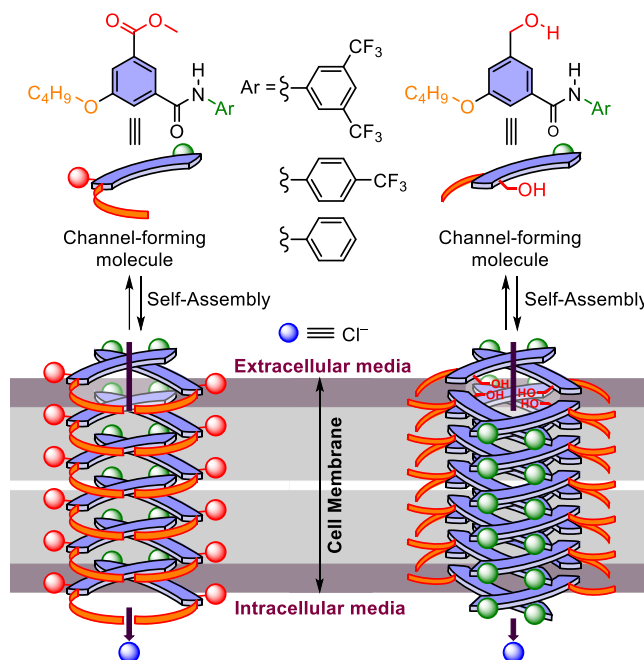
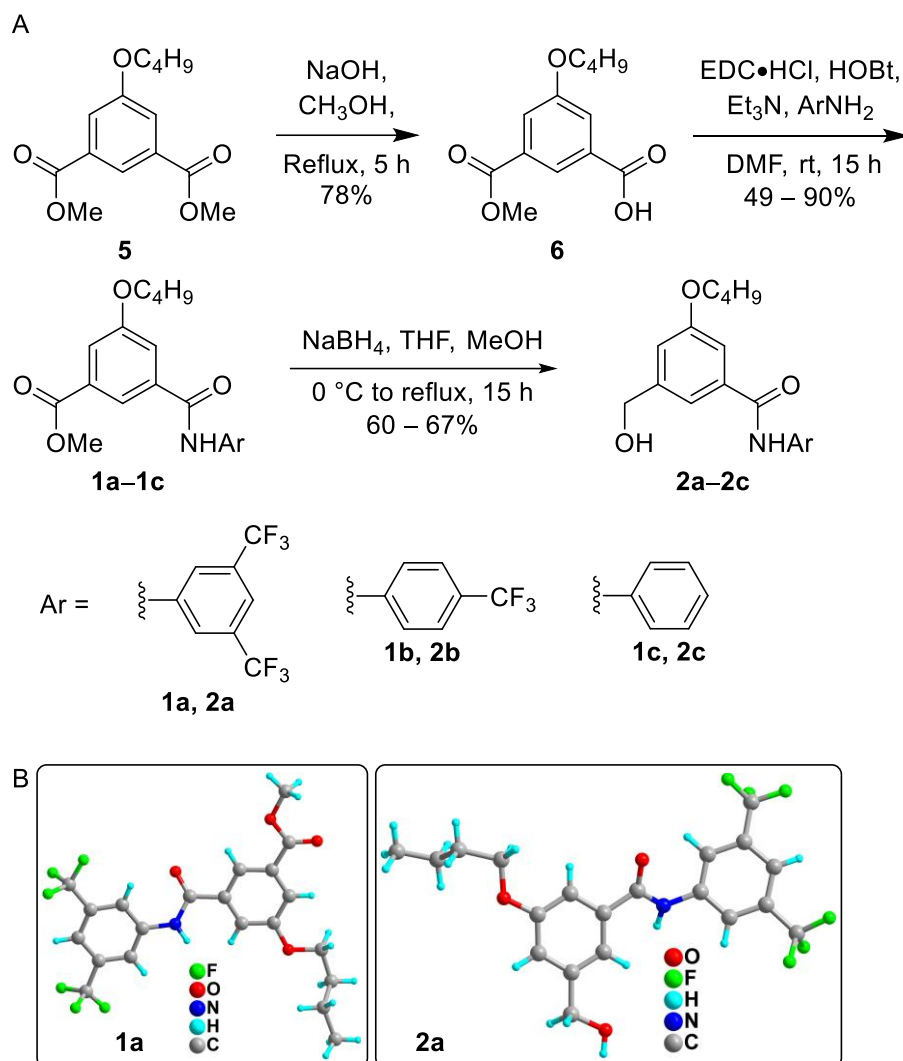


Figure 2.1 Structures of isophthalic acid derivatives and schematic representation of their self-assembly in crystalline form and lipid membranes.

2.2 Results and Discussions

2.2.1 Synthesis

Compounds **1a–1c** and **2a–2c** were synthesized from dimethyl 5-butoxyisophthalate **5**, which itself was synthesized following a literature protocol.^{33 34} Partial hydrolysis of compound **5** provided 3-butoxy-5-(methoxycarbonyl)benzoic acid **6**, which upon coupling with different aromatic amines, furnished the compounds **1a–1c**. Compounds **1a–1c** were finally subjected to ester reduction to obtain compounds **2a–2c**. (Scheme 2.1).



Scheme 2.1 The synthetic scheme of the compounds **1a–1c**, and **2a–2c** (A). Single-crystal X-ray diffraction structures of **1a** and **2a** (B). Very congested

2.2.2 Self-assembly Behavior

After the synthesis of the compounds, initially, the self-assembly behavior of compounds **1a** and **2a** was analyzed by single crystal x-ray crystallographic and field emission scanning electron microscopy (FESEM) studies. Suitable crystals of the compounds were obtained by slow evaporation of the acetonitrile/methanol (1:1) solutions of ester-based compound **1a** and hydroxyl-based compound **2a**. Multiple monomer units of either **1a** or **2a** self-aggregate via intermolecular hydrogen bonding and π - π interactions to form stacked anti-parallel architectures (Figure 2.2A, B). In the case of compound **2a**, the antiparallely aggregated structure further ensembles through intermolecular hydrogen bonding interactions between the hydroxyl groups (Figure 2.2B). In the assemblies of **1a** and **2a**, the hydrophilic

polar groups are facing inwardly, while the hydrophobic phenyl and alkyl groups are outwardly directed, making them ideal candidates to be inserted into the lipid bilayer membrane. We also recorded field emission scanning electron microscopy (FESEM) images for **1a** and **2a** to gain more insights into the aggregation pattern of these compounds in the solid-state. The samples were prepared by dissolving the compounds **1a** and **2a** in methanol. Interestingly, distinct self-aggregated morphology was observed for both compounds **1a** and **2a** (Figure 2.9). Single crystal X-ray diffraction analysis and the FESEM images confirm that compounds **1a** and **2a** possess strong self-assembly properties in solid state.

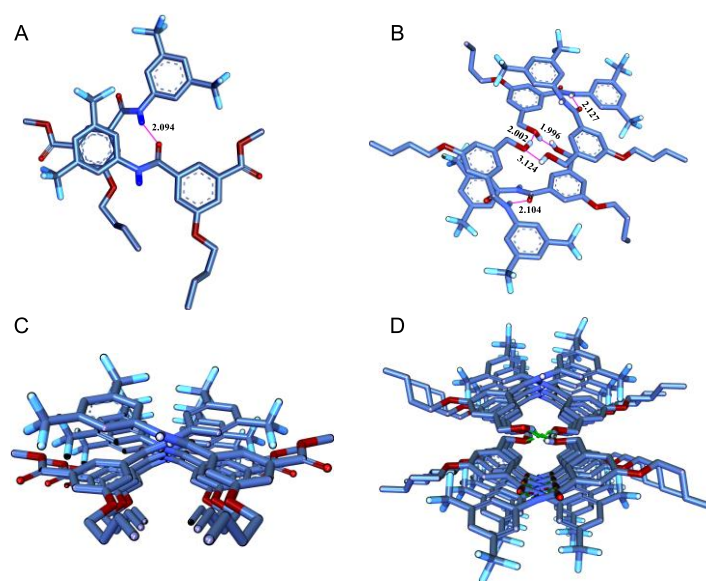


Figure 2.2 Intermolecular hydrogen bonding and π - π stacking interactions of **1a** (A) and **2a** (B). The top view of self-assembled structures of **1a** (C) and **2a** (D) as obtained from the X-ray single crystal analysis.

To gain insight into the solution-phase assembly, we recorded ^1H -NMR spectra of compounds **1a** and **2a** in CDCl_3 at different concentrations (i.e., 0.5 mM, 1.0 mM, 5.0 mM, 10 mM, 20 mM, and 30 mM). For both compounds, the $-\text{NH}$ proton shifted significantly downfield ($\Delta\delta = 0.31$ ppm for **1a** and 0.17 ppm for **2a**) with increasing concentration. (Figure 2.10, 2.11). In the case of **2a**, an $-\text{OH}$ proton shift was also found toward the downfield direction ($\Delta\delta = 0.28$ ppm) with increasing concentration (Figure 2.11). In addition to these observations, a significant broadening in the line width (full width at half maximum) of the peaks was found with increasing the concentrations for both **1a** and **2a**. Thus, it is evident that compounds **1a** and **2a** strongly aggregate and experience concentration-dependent strong intermolecular interactions confirming their strong self-assembly property in solution.

To study how temperature affects the aggregates, ^1H -NMR of compounds **1a** and **2a** (at 20 mM concentration each) in $\text{C}_2\text{D}_2\text{Cl}_4$ (boiling point 146 °C) were recorded at temperatures from -20 °C to 100 °C. A significant upfield shift of the $-\text{NH}$ proton of both **1a** and **2a** was observed ($\Delta\delta = 0.59$ ppm for **1a** and 0.71 ppm for **2a**) upon increasing the temperature from -20 °C to 100 °C (Figure 2.12, 2.13). In addition to these findings, a significant decrement in the line width (full width at half maximum) of the peaks was observed for both the channel-forming molecules. These results signify that the intermolecular interactions are interrupted and aggregate states are disrupted as a result of the increase in thermal energy.

2.2.3 Binding Ability

To assess the Cl^- ion binding ability of **1a** and **2a**, we carried out ^1H -NMR titration experiments for **1a** and **2a** in CDCl_3 (20 mM each) with increasing equivalents of tetrabutylammonium chloride (TBACl) (Figure 2.14, 2.16). From these experiments, the binding constant for **1a** was found to be $26.81 \text{ M}^{-1} \pm 2.22 \%$ with 1:1 stoichiometry (Figure 2.15) and that of **2a** was calculated to be $213.67 \text{ M}^{-1} \pm 17.03 \%$ with 1:1 stoichiometry (Figure 2.17). It suggests that **2a** is a stronger binder for Cl^- as compared to **1a**, which is expected given that only one strong binding site (i.e., $-\text{NH}$) is available with **1a** while **2a** possesses an additional strong binding site in the form of $-\text{OH}$. However, the transport efficiency of an ion channel relies on the additional factor of ion-releasing efficiency as well. It is because of this reason, the transport rate of chloride across the bilayer membrane through the channel lumen is faster for **1a** ($EC_{50} = 0.92 \text{ }\mu\text{M}$) as compared to **2a** ($EC_{50} = 19.37 \text{ }\mu\text{M}$), which was also validated by the MD simulation studies (discussed below).

2.2.4 Ion Transport Activity

The ion transport activities of **1a–1c** and **2a–2c** were assessed across large unilamellar vesicles comprised of egg yolk phosphatidylcholine (EYPC–LUVs)⊃lucigenin by a Cl^- ion-sensitive lucigenin dye-based fluorometric methods.¹⁸ A Cl^- gradient was created by adding NaCl in the extravesicular media (see ESI for details), and the anion influx was monitored by measuring the rate of quenching of lucigenin fluorescence intensity at $\lambda_{\text{em}} = 535 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$) upon addition of compounds **1a–1c** and **2a–2c**. The comparison of activities provided the activity sequence: **1a** > **1b** > **2a** > **2b** >> **1c** $\approx$ **2c**, inferring that compound **1a**, among the esters, and compound **2a**, among the alcohols, work as the most efficient ion transporters (Figure 2.3A). These observations indicate that the $-\text{CF}_3$ groups

contribute to the increase in clogP values of the molecules, thus enhancing the membrane permeability and hence efficient ion transport activity³⁵ (Figure 2.3H). Among the ester series, based on the dose-dependent activity profile of compounds **1a–1b**, the Hill analysis provided the half-maximal effective concentration, $EC_{50} = 0.92 \mu\text{M}$ for compound **1a** and $3.69 \mu\text{M}$ for compound **1b**. The Hill coefficient value $n \approx 2$ (Figure 3H) for the esters **1a–1b** corroborates that the nanochannel assembly is achieved through the formation of a supramolecular dimeric form of the esters. This dimeric channel structure is supported by the self-assembled solid-state crystal structure of compound **1a**. More interestingly, it can be seen that the chemistry of self-assembly plays a crucial role in making a compound with only one binding site (i.e., the amide $-NH$) an excellent transmembrane anion transporter. Among the alcohol series, the EC_{50} values were found to be $19.37 \mu\text{M}$ and $27.89 \mu\text{M}$ for compounds **2a** and **2b**, respectively (Figure 2.3H). The Hill coefficient value $n \approx 4$ indicates that a tetrameric self-assembled structure is responsible for the active channel formation, as supported by the crystal structure of compound **2a**. The most active compound from the ester series, i.e., **1a**, and the most active compound from the alcohol series, i.e., **2a**, were selected for further studies.

To determine whether cations are involved in the overall transport process, the extravesicular buffer of EYPC–LUVs $\supset$ lucigenin was treated with MCl ($M^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+$ and Cs^+) to create the ion gradient,³⁶ followed by the addition of compounds **1a** and **2a** (Figure 2.3B, C). No significant difference in the fluorescence quenching was observed, which directly excludes the participation of cations in the transport activity. On the other hand, the effect of different salts of anions was studied across EYPC–LUVs $\supset$ HPTS, to investigate the selectivity among the different anions.³⁶ The vesicles were prepared by entrapping 8-hydroxypyrene-1,3,6-trisulfonate (HPTS), a pH sensitive dye. During the experiment, a pH gradient ($\Delta\text{pH} = 0.8$) was created by adding NaOH in the extravesicular buffer and then the change

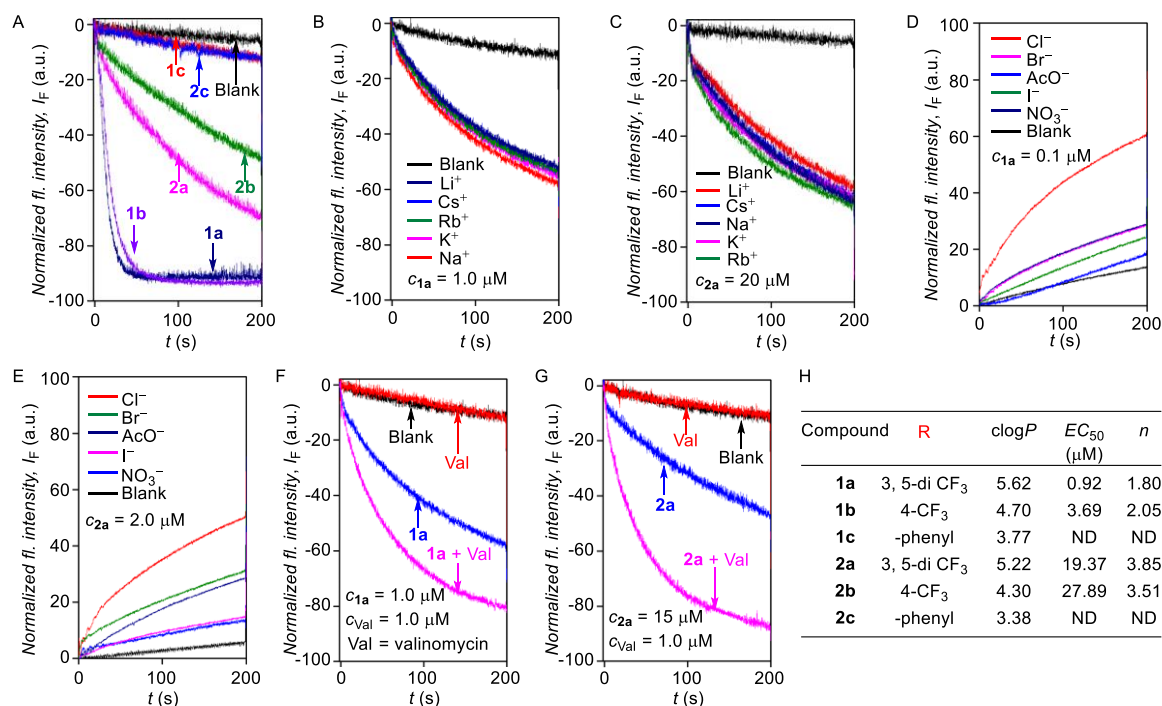


Figure 2.3 The comparison of ion transport activities of **1a–1c** and **2a–2c** in the lucigenin assay at 20 μM transporter concentration (A). The ion transport activity of **1a** ($c = 1.0 \mu\text{M}$) (B) and **2a** ($c = 20 \mu\text{M}$) (C) in the lucigenin assay by varying the extravesicular cations i.e. Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+ . The ion transport activity of **1a** ($c = 0.1 \mu\text{M}$) (D) and **2a** ($c = 2.0 \mu\text{M}$) (E) in the HPTS assay by varying the extravesicular anions i.e. Cl^- , Br^- , I^- , NO_3^- and OAc^- . The ion transport activity of **1a** ($c = 1.0 \mu\text{M}$) (F) and **2a** ($c = 15 \mu\text{M}$) (G) in the lucigenin assay in the absence and the presence of valinomycin ($1.0 \mu\text{M}$). The R groups, corresponding cLogP values, EC_{50} values and Hill coefficient values of the molecules **1a–1c** and **2a–2c** (H).

in the HPTS fluorescence intensity was recorded at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$) upon the addition of compounds **1a** and **2a** separately. (See ESI for details). Variation in anions (Cl^- , Br^- , I^- , OAc^- , and NO_3^-) in the extravesicular buffer gave the highest selectivity toward Cl^- for both **1a** and **2a** (Figure 2.3D, E).

To rationalize the origin of ion selectivity, the fractional activity Y with the reciprocal anionic radius or hydration energy of the anion (Figure 2.27) was plotted. During the transport process, ion selectivity can originate from the balance of two distinct pathways. First, a size-exclusion mechanism physically prevents bigger (partially solvated) ions from entering (higher barrier for larger ions). The second concern arises from the necessity of partial ion desolvation during bilayer crossing. When all other factors are equal, an ion with lower solvation energy will be transported more frequently and, as a result, will have higher

permeability. The activities of both **1a** and **2a** were found to be decreased with increasing halide radius. The halide transport sequence ($\text{Cl}^- > \text{Br}^- > \text{I}^-$) found from the selectivity profile of **1a** and **2a** is opposite to the common dehydration-dominated Hofmeister series.^{37, 38} The Eisenman hypothesis suggests that strong anion binding is what causes the selectivity order because it offsets the cost of dehydration by interacting with anion binding sites in **1a** and **2a**.^{39, 40} Again, the large yet hard-to-dehydrate weakly basic anion AcO^- is considered to be a standard probe.^{41, 42} Poor and moderate transport activity of AcO^- for **1a** and **2a**, respectively, suggests that the size exclusion effect also plays an important role in determining the selectivity. Moreover, when the abundance of water molecules associated with the Cl^- ion was examined during the passage of Cl^- along the channel lumen by MD simulation study (Figure 2.5J, K), no considerable amount of water molecules was observed inside both the channels as the Cl^- ion moves towards the centre. These findings suggest that the anions get rid of their hydration shell before getting translocated through the channel. In fact, the high specificity of naturally occurring Cl^- channels results from recognition sites where the anion is entirely desolvated and held only by hydrogen bonds.⁴³ Furthermore, the diameter of the channels (3.91 ± 0.12 Å for **1a** and 3.65 ± 0.16 Å for **2a**), calculated from electrophysiological experiments, are suitable for the recognition of dehydrated Cl^- ion having a diameter of 3.62 Å. Taken together, it appears that the hydration energy and radii of anions work together to influence the order of their selectivity. The cation selectivity studies using lucigenin assay, as described above, excludes the possibility of M^+/Cl^- cotransport during the course of ion transport. We further performed a valinomycin coupled lucigenin assay to get a direct insight into the operative ion transport mechanism.³⁶ The transport activities of both **1a** and **2a** were checked in the absence and presence of valinomycin, a K^+ selective transporter, in KCl buffer. In the case of a symport (i.e., M^+/Cl^- co-transport), valinomycin will not participate in the transport process as the transporter alone is self-sufficient in carrying a cation along with the Cl^- and thereby maintaining the charge neutrality. However, valinomycin is an electrogenic K^+ transporter and it results in a change in net charge. So, in the case of antiport (i.e., Cl^-/A^- exchange), an increment in the Cl^- influx rate is expected in the presence of valinomycin along with the transporter to maintain the overall charge neutrality. We observed a multifold enhancement in the quenching of fluorescence intensity of the entrapped lucigenin when we applied valinomycin along with the transporter molecule compared to the transporter alone (Figure 2.3F, G), which indicates

the synergistic effect of the transporters and valinomycin. The result confirms that the transport process of both **1a** and **2a** occurs through Cl^-/A^- antiport mode.

2.2.5 Evidence of Ion Channel Formation

To validate the active ion channel formation, electrical conductance was measured^{3, 18, 20, 31, 44} for the most active compounds **1a** (from ester series) and **2a** (from alcohol series) across the planar lipid bilayer membrane, composed of 1,2-diphytanoyl-*sn*-glycerol-3-phosphocholine (DPhPC) lipid, which isolates the *cis* and *trans* chamber filled with 1.0 M KCl solution. Upon addition of either of the channel forming compounds **1a** (2 μM) or **2a** (20 μM) in the *trans* chamber, followed by the application of different holding potentials between the two compartments, resulted in distinct single-channel opening and closing events confirming the active ion channel formation at the planar bilayer by the transporters (Figure 2.4A, C). The single-channel conductance values were found to be 16 ± 0.9 pS for **1a** and 14 ± 0.6 pS for **2a**. The diameter of the channels was also calculated to be 3.91 ± 0.12 Å for **1a** and 3.65 ± 0.16 Å for **2a**, following Hille's equation including the Sansom correction factor 5.61.⁴⁴⁻⁴⁷ Current-voltage (*I-V*) plots in the presence of the KCl symmetric solution (1.0 M each) revealed linear trends, reflecting an ohmic behavior for both **1a**, and **2a** (Figure 2.4B, D). This linearity is expected due to the non-dipolar nature of the channels. On the other hand, the ion selectivity of the channels was measured using asymmetric KCl solutions (0.5 M in the *trans* chamber and 1.0 M KCl in the *cis* chamber), and the permeability ratio ($P_{\text{Cl}^-}/P_{\text{K}^+}$) was calculated to be 2.55 for **1a**, and 2.21 for **2a**, which confirmed that both the channels are selective for anions.⁴⁸ (Figure 2.4B, D).

To gain additional support for channel formation, we monitored the Cl^- influx by **1a** and **2a** using lucigenin-trapped vesicles prepared from dipalmitoylphosphatidyl choline DPPC-LUV at 25 °C and 45 °C. DPPC undergoes a gel–liquid phase transition at 41 °C. Ion transport activity was found to be temperature-independent for both **1a** and **2a**, suggesting that it occurs through a channel formation⁴⁹ (Figure 2.26).

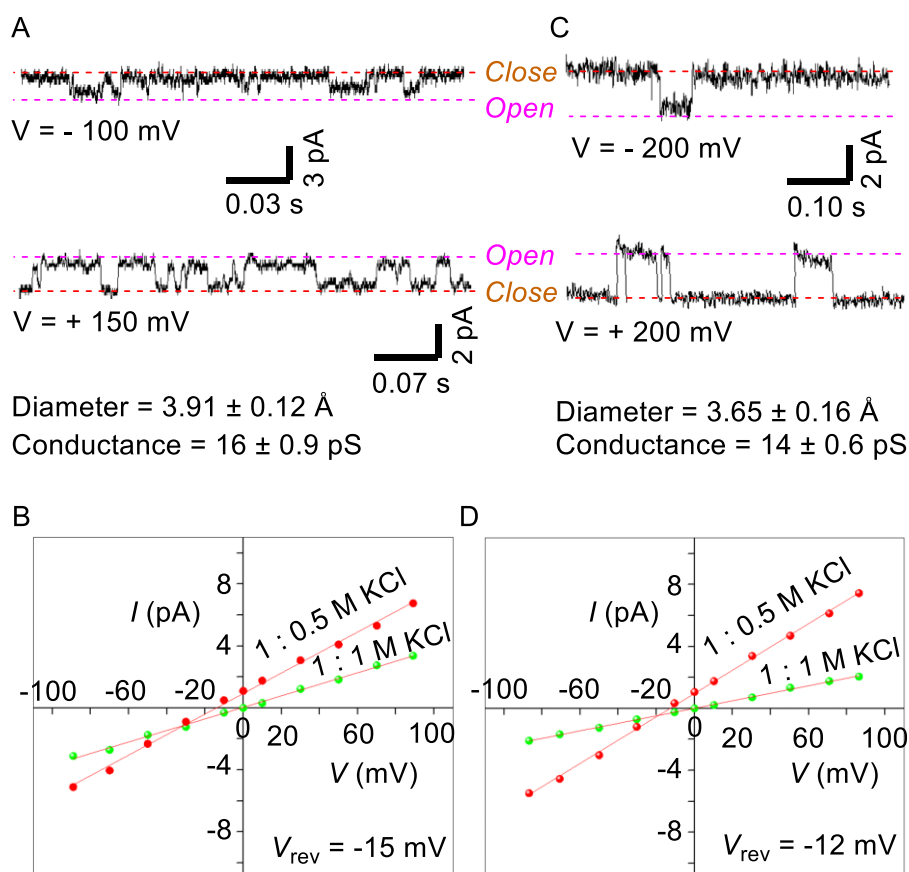


Figure 2.4 The single-channel current traces by **1a** (c = 2 μM) recorded at -100 mV, $+150$ mV, under symmetrical KCl solution (A). The current vs voltage plot of **1a** under symmetrical and unsymmetrical concentrations of KCl (B). The single-channel current traces by **2a** (c = 20 μM), recorded at -200 mV, $+200$ mV, under symmetrical KCl solution (C). The current vs voltage plot of **2a** under symmetrical and unsymmetrical concentrations of KCl (D).

2.2.6 Molecular Modeling of the Ion Channels

Computational studies were performed to get an atomistic insight into the channel architecture and the mode of Cl^- ion passage. The pattern of H-bonded self-assembled structure obtained from the crystal structure comprising six repetitive layers of **1a** and **2a** was used for the molecular modeling (Figure 5A and 5B for the **1a** and **2a** channels, respectively embedded within membrane separating two compartments containing an aqueous solution of salt). Initially, the coordinates of the channels of six component layers were minimized using MAESTRO.⁵⁰ Further, these minimized structures were used to build the channel/membrane complex constitution by employing a membrane builder in CHARMM-GUI.^{51, 52} The protocol included six consecutive steps described by Jo et al.:⁵³ Objects reading followed by

its orientation, system size determination, the building of a lipid bilayer, assembling the lipid bilayer, and finally, the system equilibrium. The general AMBER force field (GAFF)⁵⁴⁻⁵⁶ is employed for modelling the 12-mer alcohol channel and 6-mer ester channel arranged in six consecutive layers. The DPhPC lipid and ion parameters were taken from the CHARMM36m force field.⁵⁷⁻⁵⁹ The CHARMM TIP3P water model⁶⁰ is used for simulation. The free energetics of ion transport through each channel system is computed via umbrella sampling simulation. From the free energy profile (Figure 2.5C), it is evident that for both channels, the energy reaches its maximum when the anion resides at the center of the channel, and the free energy decreases considerably as the anion travels towards either end of the channel system which gives rise to fairly symmetric free energy profile on both the sides. The progress of the ion becomes more sluggish at the channel core, which contributes to the increased free energy barrier compared to when the ion reaches the bulk water interface. The lack of water molecules inside each channel, especially at its middle, provides a comparatively less favorable environment to the anion, relative to the presence of ions at the bulk, due to multiple factors elucidated later. However, compared with the free energetics of anion transport in the neat membrane (i.e., devoid of the channel), it is observed that the presence of the channel significantly reduces the free energy penalty for the movement of ions. In fact, both channels (i.e. **1a** and **2a**) efficiently provide lesser free energy barrier (ΔG is 10 and 12 kCal/mol for **1a** and **2a** channel, respectively) for Cl^- transport than neat membrane and thus facilitate easier passage of ions across the membrane than that through the membrane with no ion channel (ΔG is 20 kCal/mol). A close comparison of the free energy barrier between both the channel systems suggests that the channel constituted by **1a** acts as a relatively better ion channel for transporting Cl^- ion over the membrane as ΔG is larger for the channel constructed by **2a** (ΔG is 12 kCal/mol) in comparison to the channel of the ester components, **1a** (ΔG is 10 kCal/mol). The presence of fine curvatures in the free energy curve and also the periodic nature of those signifies a pronounced effect of the chemical structure of each of the channel systems on the ion transport energetics.

Further, to understand the molecular mechanism of Cl^- ion transport and how this is sensitive to the chemical nature of the channel, the fundamental interactions responsible for the

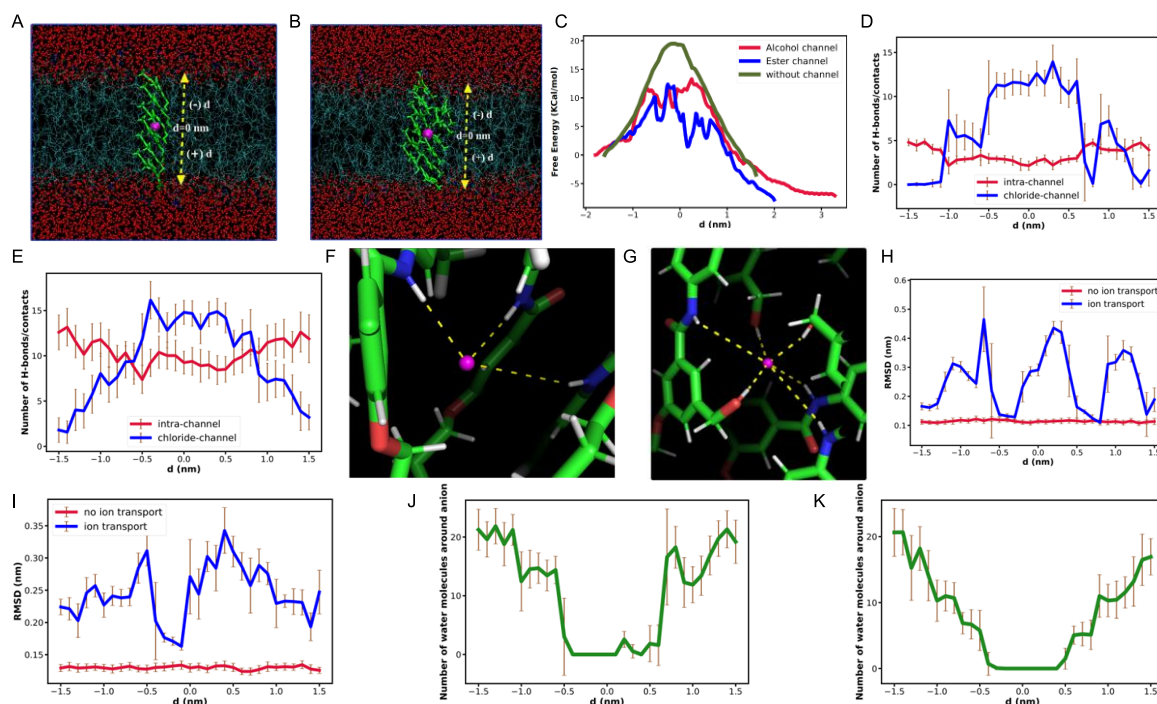


Figure 2.5 A representative snapshot of the channels **1a** (A) and **2a** (B) embedded within the membrane that separates two compartments containing an aqueous salt solution. The description of the collective variable, d , employed in the umbrella simulation is shown in yellow color. Free energy profiles corresponding to the Cl⁻ transport through the channels constructed by **1a**, **2a**, and through the membrane devoid of any channel inside are shown (C). Number of H-bonding among the components of a channel (red curve) and the number of contacts of Cl⁻ with the channel residues (blue curve) with the variation of d as the Cl⁻ ion passes through the channel starting from the mouth of the channel to the end of it for the channel formed by **1a** (D), and **2a** (E). Possible interactions between the passing Cl⁻ ion with the channel residues for **1a** (F) and **2a** (G) Root mean square deviation of the channel with respect to the crystal structure during Cl⁻ transport (blue) and when no Cl⁻ is transporting (red) through the channel by **1a** (H), and **2a** (I). The number of water molecules near Cl⁻ ion with the progression of Cl⁻ ion along the channel is plotted for **1a** (J) and **2a** (K).

movement of ions through the channel are analyzed for both the ion channel complexes. The average number of intra-channel hydrogen bonds (among the residue components of the channel itself) were analyzed with the progress of the Cl⁻ ion through the channel. The extent of the intra-channel H-bonding (Figure 2.5D, E) gets slightly reduced when the ion reaches near the center of the channel. This suggests that the movement of the ion towards the center of the channel results in a decrease in intra-channel H-bonds. In contrast, the contact of the Cl⁻ ion gradually increases to a maximum as the ion reaches the center of the channel core. The analysis suggests that the ion moves through the channel by disrupting the existing H-

bonds between the channel components and making its own favorable contact with the channel. The more the rigidity of the core of the channel (existence of a large number of H-bonds), the Cl^- ion would have to face a higher free energy penalty to encounter more of it (breaking of H-bonds) in making its own contact with the channel for its favorable transport across the membrane. Since the channel formed by **1a** consists of fewer intra-channel hydrogen bonds than the other channel, made up of alcohols, it would offer less hindrance to the Cl^- ion in its way to travel from one compartment to the other via the channel. This is clearly reflected in the relatively lower free energy barrier of the Cl^- ion transport through the channel of **1a** compared to the channel formed by **2a**. The snapshots (Figure 2.5F, G) represent the interactions experienced by the anion in the core of the channel.

The profile of root mean square deviation (RMSD) (with respect to the original crystal structure) of the channel as a function of the position of Cl^- ion inside the channel (Figure 2.5H, I) indicates that none of the channels deviates considerably from its crystal structure when no ion moves through it. On the other hand, a large RMSD of the structure of the channels is observed as Cl^- ion passes along the core of the channels. This is mostly accounted for by the disruption of the pre-existent intra-channel H-bonds by the Cl^- ion and due to the formation of anion-contact with the channel as it progresses through the channel; the complex tries to be deformed to allow the transport of the ion favorably. The presence of a lower number of H-bonds in **1a** nanochannel assembly renders the channel more flexible, which is reflected in the comparatively higher RMSD of channel **1a** (Figure 2.5H). The relatively high flexibility of channel **1a** allows a more favorable ion transport process. On the other hand, channel **2a** tries to maintain its rigidity (lower RMSD in figure 2.5I) due to the scope of extensive H-bonding interaction and thus lacks the required flexibility for the ion passage, resulting in a higher energy barrier than channel **1a**.

Along with investigating these fundamental interactions involving the requirement of perturbing intra-channel H-bonding and making favorable ion contact with the channel, the abundance of water molecules associated with the Cl^- ion was also examined with the passage of Cl^- along the channel lumen (Figure 2.5J, K). Nevertheless, for both channels, no considerable amount of water molecules was observed inside the channel as the Cl^- ion moves towards the center, suggesting that the ion transport mechanism in both channels is not mediated by solvent. Instead, the gradual decrease of water molecules with the progression of

the Cl^- ion to the center of the channel could be correlated with the increase of the associated free energy due to decreasing the solvation ability of the ion at the center of the channel. The Eisenman hypothesis suggests that strong anion binding offsets the cost of dehydration due to the strong H-bonding interactions with anion binding sites in **1a** and **2a**.^{39, 40} In nature as well, the high specificity of Cl^- channels results from recognition sites where the anion is entirely desolvated and held only by hydrogen bonds^{43, 61}. Furthermore, the diameter of the channels (3.91 ± 0.12 Å for **1a** and 3.65 ± 0.16 Å for **2a**), calculated from electrophysiological experiments, are suitable for the recognition of dehydrated Cl^- ion having a diameter of 3.62 Å. These findings suggest that the anions have to get rid of their hydration shell before getting translocated through the channel. It is because of these reasons, no considerable amount of water molecules was observed inside both channels as the Cl^- ion moved towards the center.

2.2.7 Effect on Cellular Systems

To investigate whether **1a** and **2a** can transport Cl^- ions across the cell membranes, we monitored the intracellular Cl^- ion concentration change using a cell-permeable Cl^- sensitive dye N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE).^{18, 21, 62, 63} In this experiment, non-tumorigenic epithelial cells (MCF10A) were incubated with MQAE (5 mM) for 3 h, followed by dose-dependent treatment with **1a** and **2a**. A significant quenching of the MQAE fluorescence ($\lambda_{\text{ex}} = 350$ nm and $\lambda_{\text{em}} = 460$ nm) demonstrates the ability of **1a** and **2a** to transport Cl^- ions across cell membranes (Figure 2.6A). Finally, to explore the potential of the Cl^- channels **1a** and **2a** for applications in biomedical science, we have assessed the viability of different epithelial cell lines such as noncancerous breast epithelial MCF-10A (Figure 2.6B), human lung adenocarcinoma epithelial A549 (Figure 2.6C), and human breast cancer MCF-7 (Figure 2.6D). Cells were incubated with compounds **1a** and **2a** for 48 hours, followed by an evaluation of cell viability by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.¹⁸ Interestingly, the results revealed that the compounds **1a** and **2a** were very well tolerated by the cells even at a loading concentration of 100 μM , resulting in the nontoxic behavior of **1a** and **2a**.

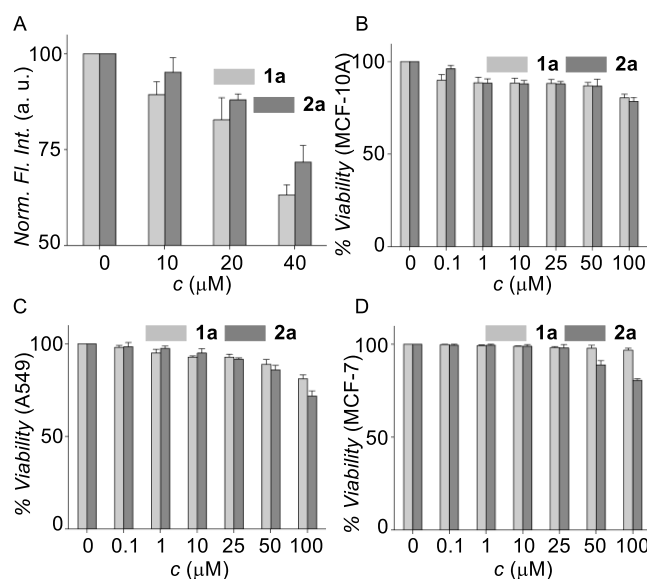


Figure 2.6 Normalized fluorescence intensity of MCF10A cells treated with **1a** and **2a** (0–40 μM) after 3 hours of incubation with MQAE (5 mM). (A). Percent cell viability of MCF10A cells (B), A549 cells (C), and MCF-7 cells (D), upon incubation with either **1a** or **2a**.

2.3 Conclusion

In conclusion, we introduced synthetically simple isophthalic acid-based small organic molecules **1a** and **2a**, which self-assemble to render supramolecular channels that allow selective transmembrane transport of Cl^- ions, confirmed by planar bilayer conductance measurement studies. The self-assembly was mainly governed by intermolecular and intramolecular hydrogen bonding interactions between the channel-forming molecules, as evident from the single-crystal X-ray diffraction analysis of **1a** and **2a**. Further, mechanistic ion transport studies demonstrated that the transport process happens through an antiport mechanism. The MD simulation studies for both **1a** and **2a** confirmed the formation of stable Cl^- channel assembly inside the bilayer membrane at ambient temperature and pressure. The symmetric nature and periodicity in free energy profiles of **1a** and **2a** also corroborate the adequate chemical structural specifications of the self-assembled channels for efficient Cl^- transport through it.

The MQAE assay showed the efficacy of compounds **1a** and **2a** in delivering Cl^- ions into cells, thereby increasing the intracellular Cl^- ion level. Viability assays proved that the compounds **1a** and **2a** are nontoxic to cells even at a loading concentration of 100 μM . Altogether, the current work presents synthetic small molecule-based Cl^- ion channels

capable of efficiently transporting Cl^- ions into the cells while being nontoxic at the same time; a combination which is rarely found. It has the realistic potential for treating diseases related to the dysfunctions in Cl^- channels.

2.4 Experimental Details

2.4.1 General Methods

All reagents and solvents for synthesis were purchased commercially and used without further purification. Dichloromethane (CH_2Cl_2) was pre-dried over calcium hydride and then distilled. Column chromatographies were performed on Merck silica gel (100 – 200 mesh). Thin layer chromatographies (TLCs) were carried out with E. Merck silica gel 60-F254 plates. Egg yolk phosphatidylcholine (EYPC) was obtained from Avanti Polar Lipids as a solution in CHCl_3 (25 mg/mL). HEPES buffer, HPTS dye, Triton X-100, NaOH and inorganic salts were purchased of molecular biology grade from Sigma. Large unilamellar vesicles (LUV) were prepared by using a mini extruder, equipped with a polycarbonate membrane of 100 and 200 nm pore size, obtained from Avanti Polar Lipids.

2.4.2 Physical Measurements

All NMR spectra were recorded either on 400 MHz Bruker Ascend™ 400 spectrometer using either residual solvent signals as an internal reference the δ scale. The chemical shifts (δ) were reported in ppm and coupling constants (J) in Hz. The following abbreviations were used: m (multiplet), s (singlet), d (doublet), t (triplet) dd (doublet of doublet). High-resolution mass spectra were obtained on a MicroMass ESI-TOF MS spectrometer equipped with a Micromass Z-Spray electrospray ionization (ESI) source (Waters Co., Synapt G2, France). All melting points were measured in open glass capillary, and values are uncorrected. Fluoromax-4 from JobinYvon Edison-equipped with an injector port and a magnetic stirrer, was used for recording the fluorescence spectra. For pH adjustment, a Helmer pH meter was used. All data from fluorescence studies were processed by Origin 8.5 program.

2.4.3 Synthesis Procedures

Procedure for the synthesis of dimethyl 5-butoxyisophthalate 5: In a 100 mL round bottom flask, a solution of 5-hydroxyisophthalic acid **3** (1.00 g, 5.5 mmol) in MeOH (30 mL)

was prepared, followed by the addition of catalytic H_2SO_4 (1.2 mmol). The resultant solution was then stirred for 4 hours under reflux conditions (using an oil bath). After completion of the reaction (monitored by TLC), MeOH was evaporated in vacuo under reduced pressure, and the residue was dissolved in EtOAc (100 mL). The solution in EtOAc was then washed using saturated NaHCO_3 (1×50 mL), followed by H_2O (2×50 mL), and finally with brine solution (1×50 mL). The organic extract was dried over Na_2SO_4 and evaporated in vacuo to obtain pure ester **4** (100 %). In a 100 mL round bottom flask, a suspension of dimethyl 5-hydroxyisophthalate **4** (1.10 g, 5.2 mmol) and K_2CO_3 (2.16 g, 15.7 mmol) in dry DMF (25 mL) was heated under reflux for 1 h. Butylbromide (0.66 mL, 6.28 mmol) was added to the reaction mixture and heated under reflux for 12 h. After cooling the reaction mixture to room temperature, it was poured into cold water (100 mL) and extracted with EtOAc (3×100 mL). The combined organic phase was washed with water (2×50 mL), followed by brine solutions (1×50 mL), dried over Na_2SO_4 , and removed under reduced pressure to obtain a viscous colorless oil. The crude oil was then purified through silica gel column chromatography using EtOAc:Hexane (10:90 (v:v)) to obtain pure compound **5** as a white crystalline solid (1.32 g, 95%).

Characterization data of 5: ^1H NMR (400 MHz, CDCl_3): δ 8.24 (t, $J = 1.5$ Hz, 1H), 7.73 (d, $J = 1.5$ Hz, 2H), 4.03 (t, $J = 6.4$ Hz, 2H), 3.93 (s, 6H), 1.86 – 1.72 (m, 2H), 1.58 – 1.42 (m, 2H), 0.98 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ 166.2, 159.2, 131.6, 122.7, 119.8, 68.3, 52.3, 31.1, 19.1, 13.8. **HRMS (ESI):** m/z : $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{14}\text{H}_{18}\text{O}_5$ 267.1227; found: 267.1238.

Procedure for the synthesis of 3-butoxy-5-(methoxycarbonyl)benzoic acid 6: To a suspension of dimethyl 5-butoxyisophthalate **5** (1.2 g, 4.5 mmol) in CH_3OH (30 mL) and water (10 mL) was added NaOH (162 mg, 4.02 mmol). The mixture was heated to reflux for 5 hours. After cooling the reaction mixture to room temperature, it was poured into cold water (100 mL) and the pH of the solution was adjusted to 1 with concentrated HCl. The crude product was extracted with EtOAc (3×100 mL). The combined organic phase was washed with water (2×50 mL), followed by brine solutions (1×50 mL). The organic solvent was then dried over Na_2SO_4 , and removed under reduced pressure. The residue was then purified through silica gel column chromatography using MeOH/ CHCl_3 (1:99 (v:v)) to obtain pure compound **6** as a white solid (0.9 g, 78%).

Characterization data of 6: $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.34 (t, J = 1.5 Hz, 1H), 7.80 (d, J = 1.5 Hz, 2H), 4.07 (t, J = 6.5 Hz, 2H), 3.95 (s, 3H), 1.80 (ddd, J = 8.9, 7.5, 6.3 Hz, 2H), 1.59 – 1.45 (m, 2H), 0.99 (t, J = 7.4 Hz, 3H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3): δ 166.7, 165.8, 159.3, 133.2, 131.8, 122.3, 119.8, 119.2, 68.3, 52.9, 31.0, 19.1, 14.1. **HRMS (ESI):** m/z : $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{13}\text{H}_{16}\text{O}_5$ 253.1071; found: 253.1084.

General procedure for the synthesis of methyl 3-butoxy-5-(arylcabamoyl)benzoate acid

1a–1c: In a 50 mL round bottom flask, to a solution of 3-butoxy-5-(methoxycarbonyl)benzoic acid **6** (0.25 g, 0.99 mmol) and arylamine (0.99 mmole) in DMF (15 mL) was added hydroxybenzotriazole (1.5 mmol) followed by 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC·HCl) (1.5 mmol). The reaction mixture was then stirred for 15 hours at room temperature. After completion (monitored by TLC), it was poured into cold water (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic phase was washed with water (2 x 10 mL) followed by brine solutions (1 x 10 mL), dried over Na_2SO_4 , and removed under reduced pressure to obtain white solids. The crude solids were then purified through silica gel column chromatography using EtOAc:Hexane to obtain pure compounds **1a–1c** as a white solid (49-90%).

Characterization data of 1a: Purified using EtOAc:hexane (7:93 (v:v)); white solid, Yield 0.22 g (49%), $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.21 (s, 2H), 8.18 (s, 1H), 8.02 (t, J = 1.5 Hz, 1H), 7.76 (dd, J = 2.7, 1.3 Hz, 1H), 7.67 (dt, J = 3.3, 1.6 Hz, 2H), 4.07 (t, J = 6.5 Hz, 2H), 3.96 (s, 3H), 1.86 – 1.75 (m, 2H), 1.55 – 1.45 (m, 2H), 0.99 (t, J = 7.4 Hz, 3H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3): δ 166.1, 164.8, 159.8, 139.2, 135.2, 132.5 (q, $J_{\text{C-F}}$ = 33.7 Hz), 131.9, 123.0 (q, $J_{\text{C-F}}$ = 272.9 Hz), 119.9, 119.5, 119.0, 118.2, 117.9, 68.4, 52.6, 31.0, 19.1, 13.7. **IR (Neat, v/cm^{-1}):** 3738, 3279, 2934, 1723, 1652, 1552, 1463, 1375, 1333, 1274, 1169, 1125, 1028, 938, 894, 843, 747, 691, 622. **HRMS (ESI):** m/z : $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{21}\text{H}_{19}\text{F}_6\text{NO}_4$ 464.1291; found: 464.1288.

Characterization data of 1b: Purified using EtOAc:hexane (9:91 (v:v)); white solid, Yield 0.30 g (77%), $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 8.49 (s, 1H), 7.96 (s, 1H), 7.79 (d, J = 5.4 Hz, 2H), 7.68 – 7.55 (m, 4H), 3.97 (t, J = 6.4 Hz, 2H), 3.88 (s, 3H), 1.75 (p, J = 6.5 Hz, 2H), 1.47 (h, J = 7.4 Hz, 2H), 0.96 (t, J = 7.4 Hz, 3H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3): δ 166.1, 165.0, 159.6, 140.9, 135.9, 131.7, 126.5, 126.2 (q, $J_{\text{C-F}}$ = 3.8 Hz), 124.0 (q, $J_{\text{C-F}}$ = 271.6 Hz), 119.9, 119.13 (q, $J_{\text{C-F}}$ = 35.9 Hz), 118.9, 118.2, 68.3, 52.5, 31.0, 19.1, 13.7. **IR (Neat, v/cm^{-1}):** 3740,

3280, 2948, 1654, 1599, 1515, 1451, 1402, 1332, 1258, 1219, 1157, 1114, 1061, 1025, 979, 835, 754, 694. **HRMS (ESI):** m/z : $[M + H]^+$ calculated for $C_{20}H_{20}F_3NO_4$ 396.1417; found: 396.1422.

Characterization data of 1c: Purified using EtOAc:hexane ((10:90 (v:v))); white solid, Yield 0.29 g (90%). **1H -NMR (400 MHz, $CDCl_3$):** δ 8.02 (s, 1H), 7.91 (s, 1H), 7.71 (m, 1H), 7.67 – 7.65 (m, 3H), 7.39 (t, J = 7.9 Hz, 2H), 7.17 (t, J = 7.4 Hz, 1H), 4.06 (t, J = 6.5 Hz, 2H), 3.95 (s, 3H), 1.86 – 1.73 (m, 2H), 1.52 (dt, J = 14.9, 7.4 Hz, 2H), 0.99 (t, J = 7.4 Hz, 3H). **^{13}C NMR (101 MHz, $CDCl_3$):** δ 166.3, 164.7, 159.7, 137.8, 136.6, 131.7, 129.1, 124.8, 120.3, 119.2, 118.8, 118.3, 68.4, 52.5, 31.1, 19.2, 13.9. **IR (Neat, v/cm^{-1}):** 3733, 3240, 2944, 1655, 1561, 1502, 1425, 1390, 1325, 1250, 1212, 1140, 1105, 1050, 1021, 980, 840, 751, 677. **HRMS (ESI):** m/z : $[M + H]^+$ calculated for $C_{19}H_{21}NO_4$ 328.1543; found: 328.1554.

General procedure for the synthesis of 3-butoxy-5-(hydroxymethyl)-N-arylbenzamide 2a–2c: To a solution of **1a–1c** (0.26 mmol), in THF at 0 °C was added $NaBH_4$ (2.34 mmol). The reaction mixture was then refluxed using an oil bath for 15 min, followed by the dropwise addition of MeOH. The resultant solution was kept under reflux conditions for 15 hours. After completion (monitored by TLC), THF was evaporated in vacuo. The crude residue was dissolved in EtOAc (50 mL) and washed with water (2×10 mL) followed by brine solution (1×10 mL). The collected organic phase was dried over Na_2SO_4 , and the solvent was removed under reduced pressure to obtain white solids. The crude solids were then purified through silica gel column chromatography using EtOAc:Hexane to obtain pure compounds **2a–2c** as a white solid (60-67%).

Characterization data of 2a: Purified using EtOAc:hexane (40:60 (v:v)); white solid, Yield 68 mg (49 %), **1H -NMR (400 MHz, $DMSO-d_6$):** δ 8.19 (s, 2H), 8.13 (s, 1H), 7.65 (s, 1H), 7.40 (s, 1H), 7.36 – 7.32 (m, 1H), 7.11 (m, 1H), 4.76 (d, J = 5.1 Hz, 2H), 4.04 (t, J = 6.5 Hz, 2H), 1.92 (t, J = 5.6 Hz, 1H), 1.84 – 1.73 (m, 2H), 1.50 (dt, J = 14.7, 7.5 Hz, 2H), 0.99 (t, J = 7.4 Hz, 3H). **^{13}C NMR (101 MHz, $DMSO-d_6$):** δ 166.5, 159.1, 145.3, 141.6, 135.3, 131.1 (q, J_{C-F} = 32.6 Hz), 123.8 (q, J_{C-F} = 272.8 Hz), 122.4, 120.4, 118.3, 116.4, 112.6, 67.9, 63.0, 31.2, 19.2, 14.2. **IR (Neat, v/cm^{-1}):** 3271, 2922, 2852, 1711, 1652, 1599, 1552, 1445, 1379, 1313, 1276, 1214, 1166, 1129, 1057, 1026, 1002, 945, 889, 843, 805, 761, 697, 679. **HRMS (ESI):** m/z : $[M + H]^+$ calculated for $C_{20}H_{19}F_6NO_3$ 436.1342; found: 436.1346.

Characterization data of 2b: Purified using EtOAc:hexane ((45:55 (v:v))); white solid, Yield 62 mg (65%), **¹H-NMR (400 MHz, DMSO-*d*₆):** δ 10.52 (s, 1H), 8.01 (d, *J* = 8.6 Hz, 2H), 7.72 (d, *J* = 8.7 Hz, 2H), 7.50 (s, 1H), 7.42 – 7.32 (m, 1H), 7.12 (s, 1H), 5.34 (t, *J* = 5.8 Hz, 1H), 4.56 (d, *J* = 5.8 Hz, 2H), 4.05 (t, *J* = 6.4 Hz, 2H), 1.79 – 1.66 (m, 2H), 1.46 (dq, *J* = 14.7, 7.4 Hz, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). **¹³C NMR (101 MHz, DMSO-*d*₆):** δ 166.3, 159.1, 145.2, 143.3, 136.0, 126.4 (q, *J*_{C-F} = 3.6 Hz), 124.1 (q, *J*_{C-F} = 32.2 Hz), 123.9, 120.6, 118.4, 116.1, 112.5, 67.9, 63.0, 31.2, 19.2, 14.2. **IR (Neat, v/cm⁻¹):** 3283, 2940, 1655, 1594, 1511, 1458, 1405, 1335, 1258, 1223, 1156, 1117, 1068, 1037, 1019, 982, 941, 880, 832, 791, 764, 746, 685. **HRMS (ESI):** *m/z*: [M + H]⁺ calculated for C₁₉H₂₀F₃NO₃ 368.1468; found: 368.1474.

Characterization data of 2c: Purified using EtOAc:hexane (40:60 (v:v)); white solid, Yield 52 mg (67%), **¹H-NMR (400 MHz, CDCl₃):** δ 7.92 (s, 1H), 7.69 – 7.56 (m, 2H), 7.42 – 7.28 (m, 4H), 7.21 – 7.10 (m, 1H), 7.09 – 7.01 (m, 1H), 4.70 (s, 2H), 4.00 (t, *J* = 6.5 Hz, 2H), 2.16 (s, 1H), 1.83 – 1.70 (m, 2H), 1.55 – 1.41 (m, 2H), 0.98 (t, *J* = 7.4 Hz, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 165.8, 159.0, 145.0, 139.6, 136.5, 129.0, 124.1, 120.9, 118.3, 115.8, 112.3, 67.8, 63.0, 31.2, 19.2, 14.2. **IR (Neat, v/cm⁻¹):** 3272, 2925, 1740, 1650, 1594, 1542, 1494, 1436, 1326, 1250, 1160, 1041, 863, 747, 682. **HRMS (ESI):** *m/z*: [M + H]⁺ calculated for C₁₈H₂₁NO₃ 300.1594; found: 300.1603.

2.4.4 Single Crystal X-ray Crystallographic Analysis

Compounds **1a** and **2a** were crystallized from acetonitrile:methanol (1:1) at room temperature. Bruker KAPPA APEX II CCD Duo diffractometer (operated at 1500 W power: 50 kV, 30 mA) using graphite-monochromated Mo K α radiation (λ = 0.71073 Å) was used to collect single-crystal X-ray data at 100 K. SAINT software was used to integrate and reduce the data. SHELXTL was used to solve all the structures and refined on *F*² by a full-matrix least-squares technique using the SHELXL-97 program built in the Apex-3 package.⁶⁴ Hydrogen atoms were constructed in geometric positions to their parent atoms.⁶⁵ *Adsym* subroutine of PLATON was used to confirm that there is no additional symmetry possible. The CCDC numbers for compounds **1a** and **2a** are 2181445 and 2181450, respectively. These data are available at The Cambridge Crystallographic Data Centre.

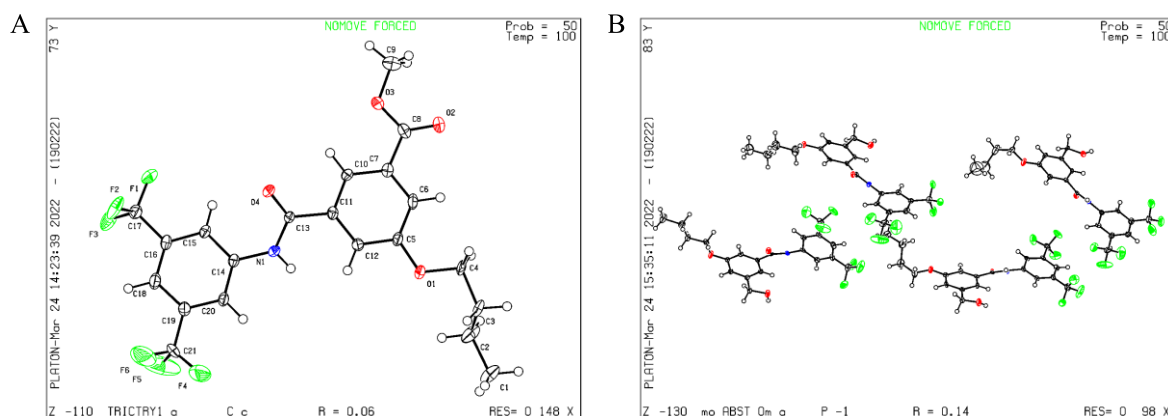


Figure 2.7 ORTEP diagrams of the structures **1a** (A) and **2a** (B) with 50% probability ellipsoids established by single X-ray crystallography.

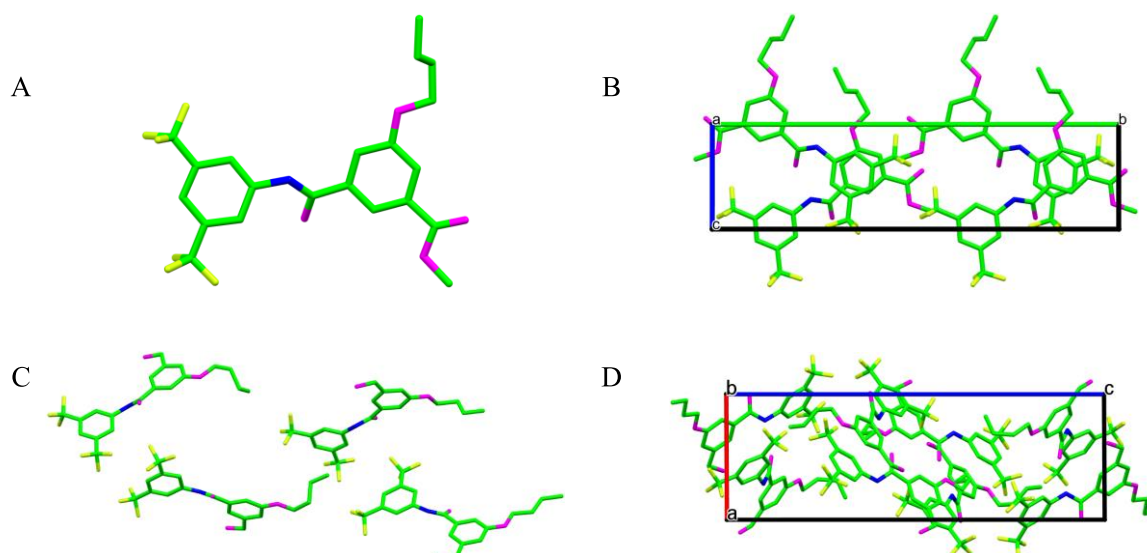


Figure 2.8 Asymmetric unit (A, C) and the unit-cell packing diagram (B and D) of **1a** and **2a**, respectively, at 100K.

2.4.5 Field Emission Scanning Electron Microscopy (FESEM) Images

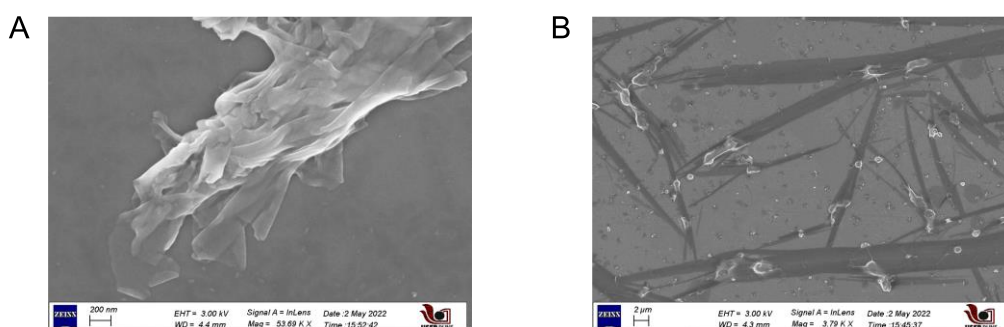


Figure 2.9 FESEM images of **1a** (A) and **2a** (B) showing distinct aggregation patterns recorded in methanol.

2.4.6 Dynamic Light Scattering (DLS) Experiments:

To determine the dimension of the aggregation, DLS experiments were performed. A stock solution of compound **1a** and **2a** were prepared in methanol and during the experiment, compound **1a** or **2a** from this stock was added to water keeping a final concentration of 100 μ M. The experiment provided with a mean size of 3.37 nm for **1a** and 5.23 nm for **2a**.

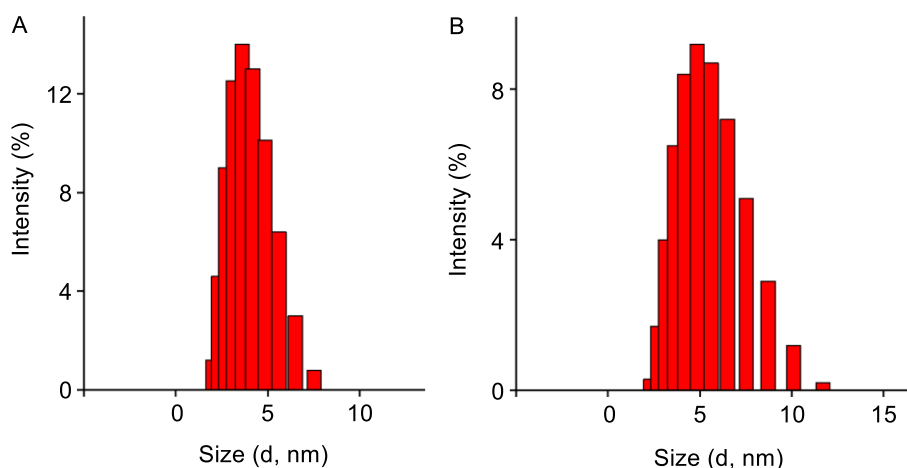


Figure 2.10 Size distribution of the aggregate by **1a** (A) and **2a** (B) using DLS experiment.

2.4.7 Concentration-Dependent NMR Studies

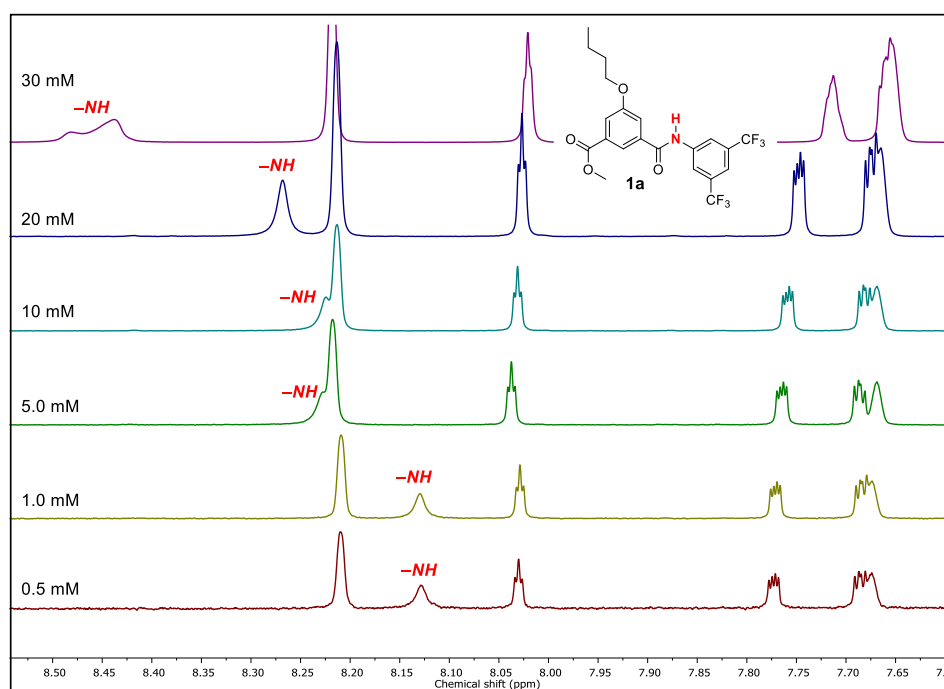
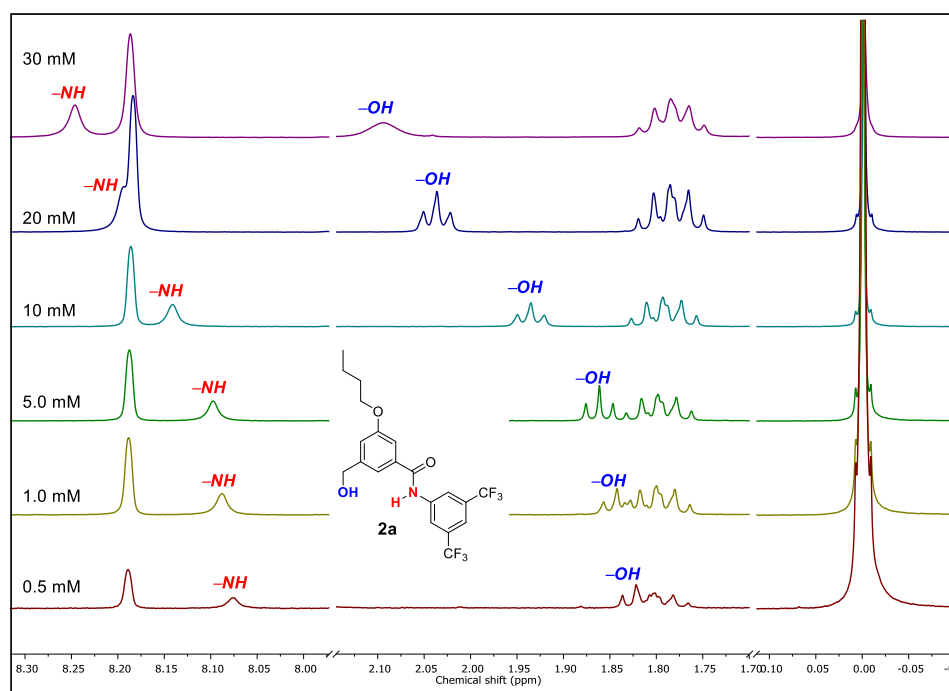
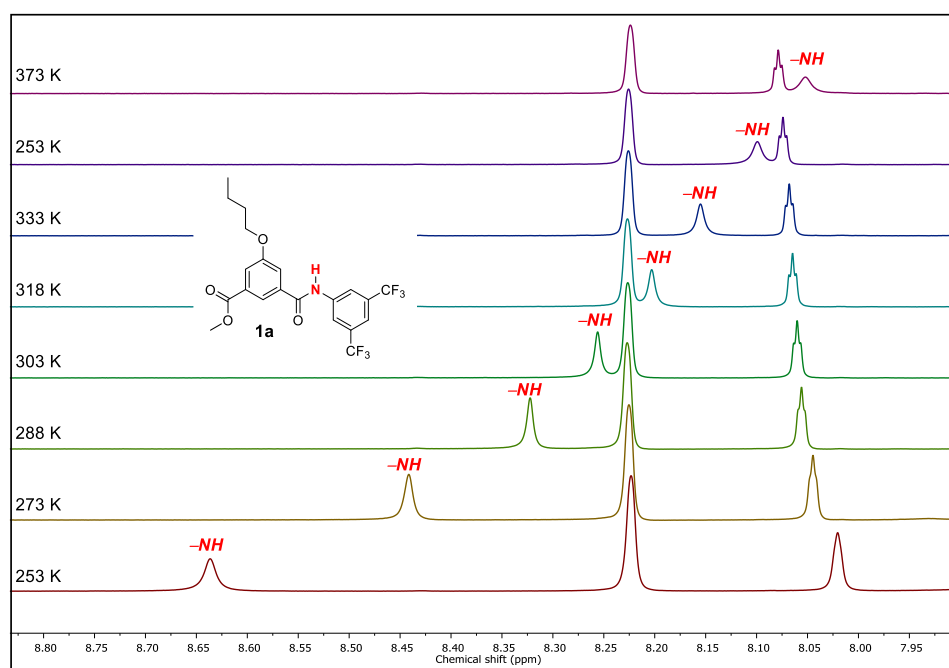


Figure 2.11 Partial ^1H -NMR spectra of **1a** in CDCl_3 at different concentrations (0.5 – 30 mM).**Figure 2.12** Partial ^1H -NMR spectra of **2a** in CDCl_3 at different concentrations (0.5 – 30 mM).

2.4.8 Variable Temperature NMR Experiment

**Figure 2.13** Partial ^1H -NMR spectra of **1a** (20 mM) in $\text{C}_2\text{D}_2\text{Cl}_4$ at variable temperatures (253 K – 373 K).

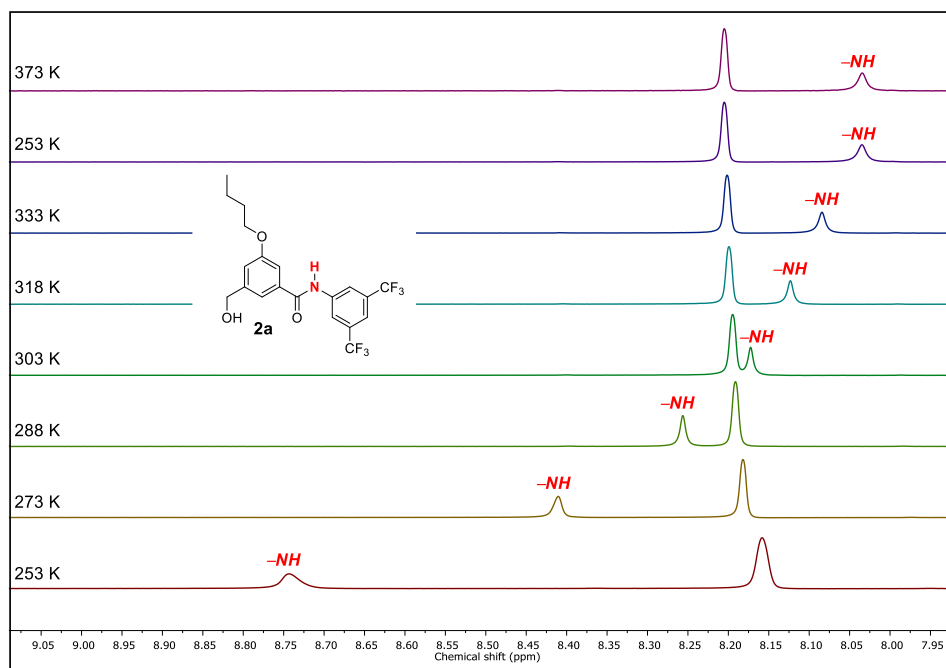


Figure 2.14 Partial ^1H -NMR spectra of **2a** (20 mM) in $\text{C}_2\text{D}_2\text{Cl}_4$ at variable temperatures (253 K – 373 K).

2.4.9 Ion Binding Studies

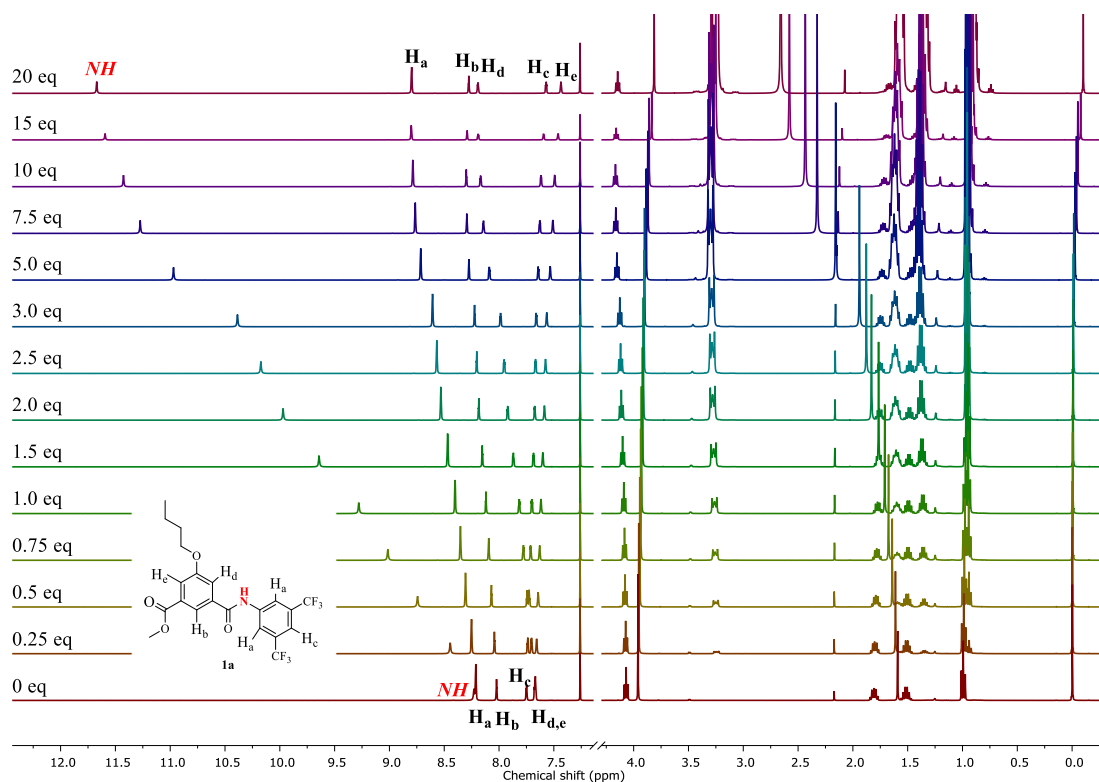


Figure 2.15 ^1H NMR titration of **1a** (20 mM) in CDCl_3 at 25 °C upon addition of increasing equivalents of tetrabutylammonium chloride.

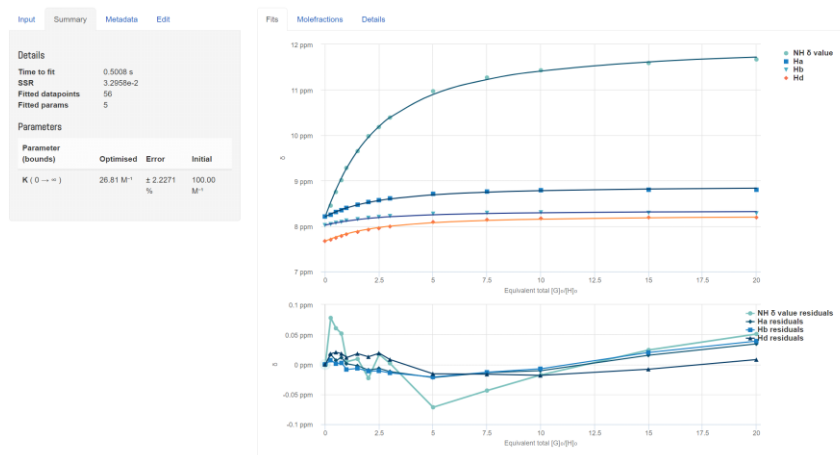


Figure 2.16 Screenshot of the fitted data plot for compound **1a** from supramolecular.org. The binding constant was found to be 26.81 M⁻¹ ± 2.22 % in the 1:1 receptor-to-anion binding model. The changing pattern of chemical shift and chemical shift residuals with the increasing equivalent of TBACl is shown. The Bindfit URL for this experiment is <http://app.supramolecular.org/bindfit/view/a1c5cd3b-bda0-454d-9002-884bf208814d>.

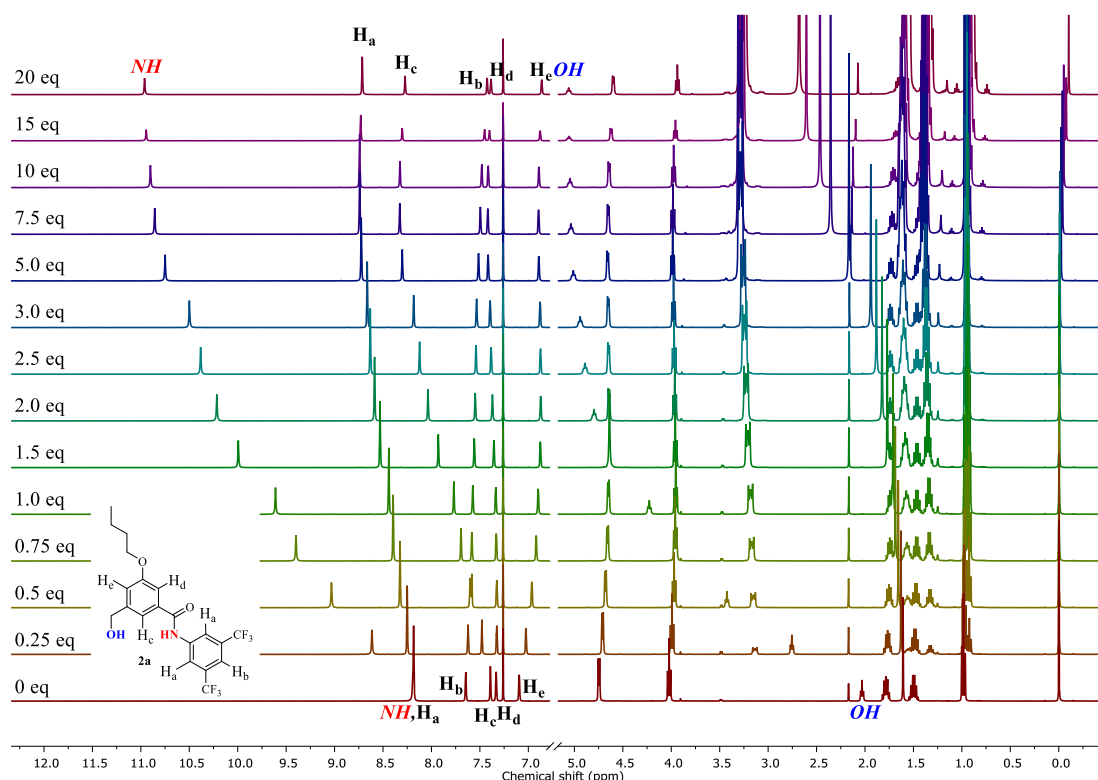


Figure 2.17 ^1H NMR titration of **2a** (20 mM) in CDCl_3 at 25 °C upon addition of increasing equivalents of tetrabutylammonium chloride.

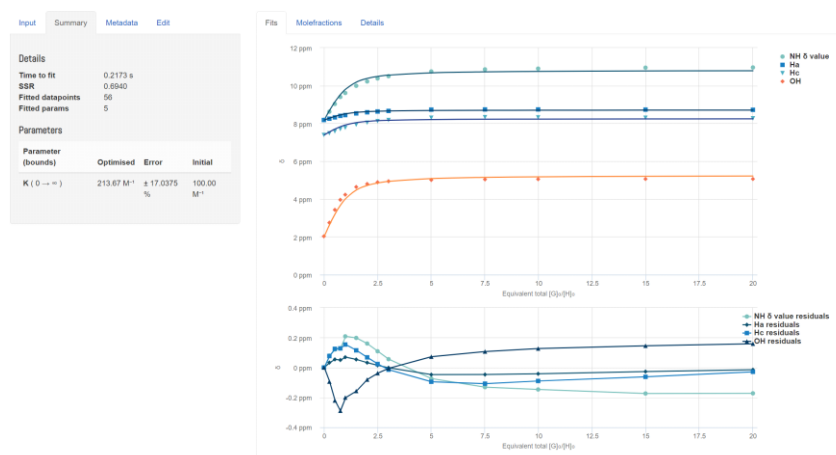


Figure 2.18 Screenshot of the fitted data plot for compound **1a** from supramolecular.org. The binding constant was found to be $213.67 \text{ M}^{-1} \pm 17.03 \%$ in the 1:1 receptor-to-anion binding model. The changing pattern of chemical shift and chemical shift residuals with the increasing equivalent of TBACl is shown. The Bindfit URL for this experiment is <http://app.supramolecular.org/bindfit/view/703b5f46-11fd-48e5-bdd8-1592f0129c2b>.

2.4.10 Ion Transport Activity

Chloride influx across EYPC-LUVs $\supset$ lucigenin:

Preparation of EYPC-LUVs $\supset$ lucigenin: Vesicles were prepared following a reported protocol.¹⁸ Final conditions: $\sim 5 \text{ mM}$ EYPC; *inside*: 200 mM NaNO_3 , 1 mM lucigenin; *outside*: 200 mM NaNO_3 .

Dose dependent chloride influx experiments: $1975 \mu\text{L}$ of NaNO_3 solution (200 mM NaNO_3) was taken in a clean and dry fluorescence cuvette, followed by the addition of $25 \mu\text{L}$ of EYPC-LUVs $\supset$ lucigenin. The cuvette was then kept in a fluorescence instrument (Fluoromax plus, Horiba Scientific). A magnetic stirrer is furnished with the instrument, which provides a slow stirring condition. The emission intensity of lucigenin fluorescence, F_t , was monitored at $\lambda_{\text{em}} = 535 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$). At $t = 20 \text{ s}$, a chloride gradient was created between the intra and extra vesicular media by adding $33.3 \mu\text{L}$ of 2 M NaCl to the cuvette. Transporter **1a–1c** and **2a–2c** were added separately at $t = 100 \text{ s}$ in different concentrations. Finally, at $t = 300 \text{ s}$, $25 \mu\text{L}$ of 10% Triton X-100 was added to lyse the vesicles leading to the

complete destruction of the chloride concentration gradient. The collected data was then normalized following a reported protocol to plot the change in fluorescence intensity with respect to time.¹⁸

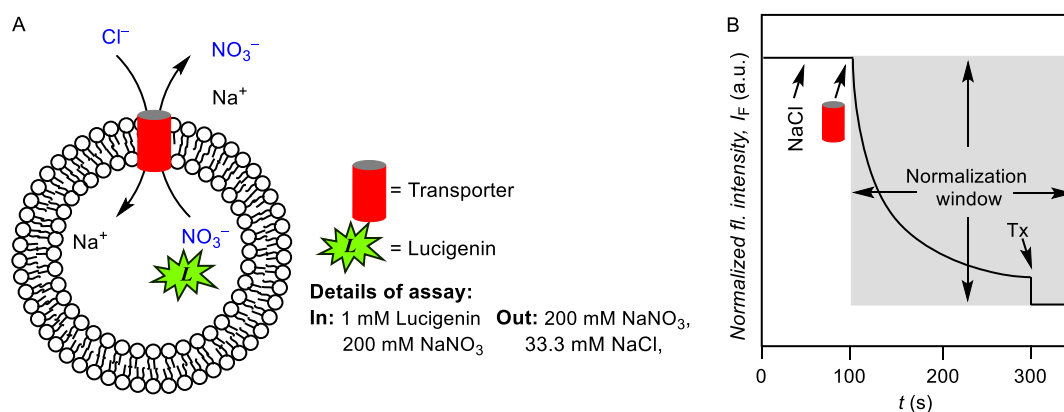


Figure 2.19 The depiction of fluorescence-based ion transport activity assay using EYPC-LUVs+lucigenin (A). Chloride influx kinetics illustrated with normalization window. (B).

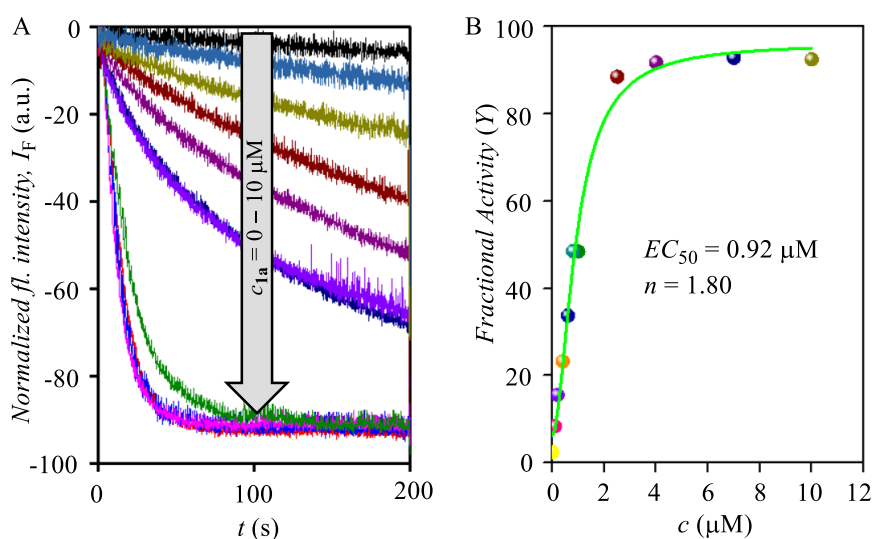


Figure 2.20 Dose-dependent chloride influx activity of **1a** (0 – 10 μM) (A). Hill plot of **1a** (B).

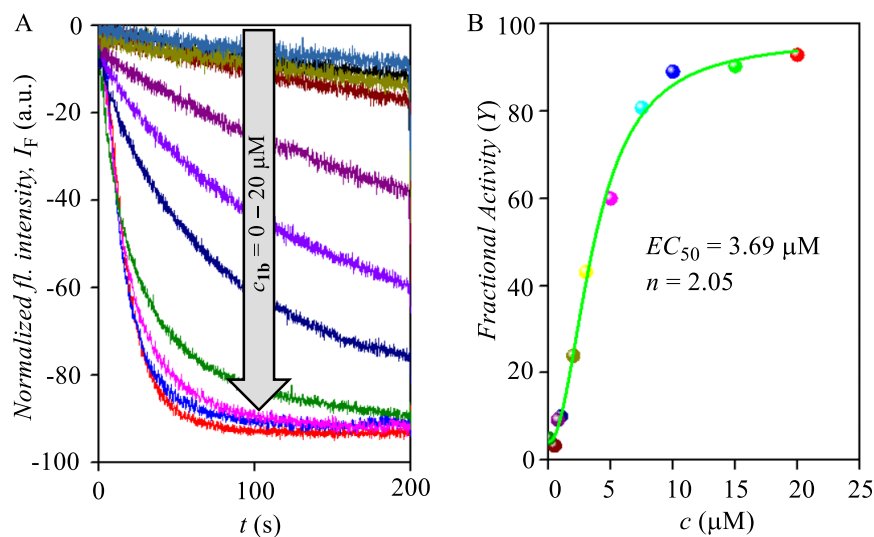


Figure 2.21 Dose-dependent chloride influx activity of **1b** (0 – 20 μM) (A). Hill plot of **1b** (B).

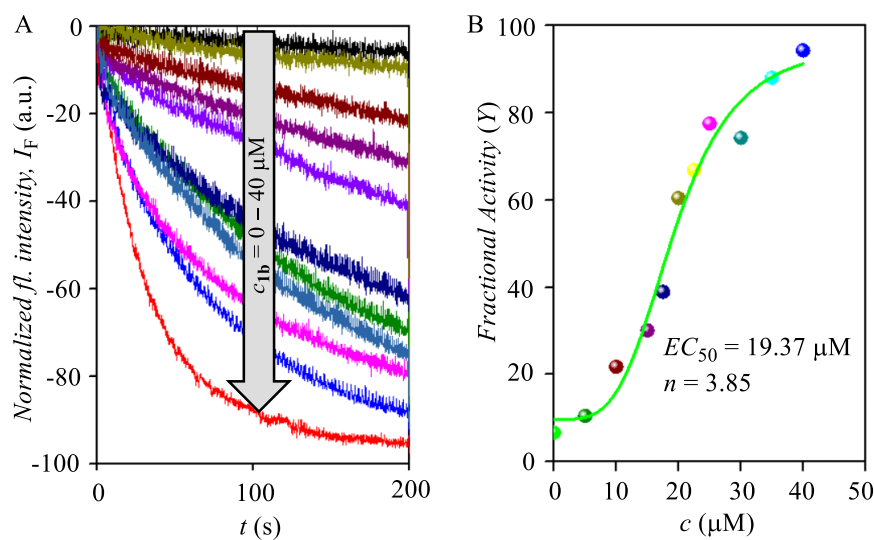


Figure 2.22 Dose-dependent chloride influx activity of **2a** (0 – 40 μM) (A). Hill plot of **2a** (B).

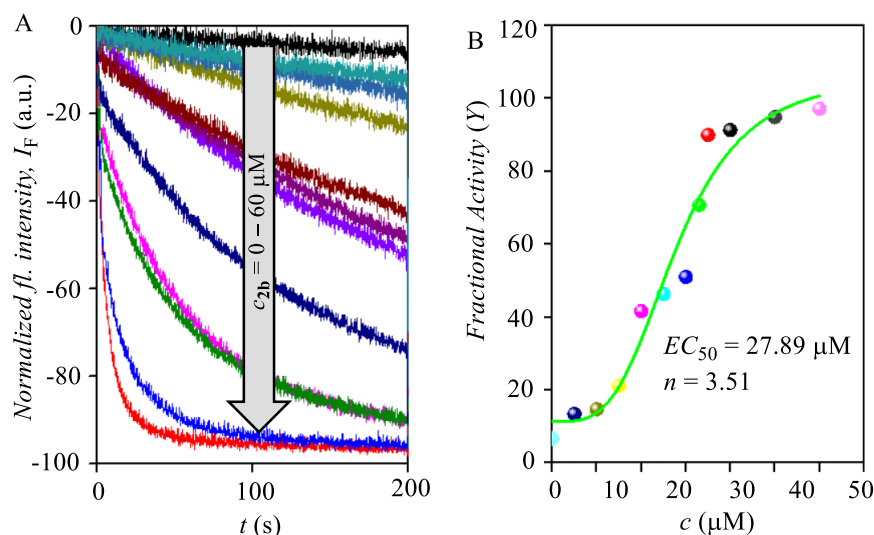


Figure 2.23 Dose-dependent chloride influx activity of **2b** (0 – 60 μM) (A). Hill plot of **2b** (B).

Table 2.1 EC_{50} and Hill coefficient values (n) for **1a-1c** and **2a-2c**.

Compound	EC_{50} /)		Hill coefficient (n)
	μM	mol%*	
1a	0.92	1.35	1.80
1b	3.69	5.41	2.05
1c	ND	ND	ND
2a	19.37	28.42	3.85
2b	27.89	40.92	3.51
2c	ND	ND	ND

*compound to lipid molar ratio

Effect of extravesicular cations in chloride influx:

Preparation of EYPC-LUVs $\Rightarrow$ lucigenin: The aforesaid lucigenin vesicles were used for the experiments.

Cation selectivity using lucigenin assay: 1975 μL of NaNO_3 solution (200 mM NaNO_3) was taken in a clean and dry fluorescence cuvette, followed by the addition of 25 μL of EYPC-LUVs $\supset$ lucigenin. The cuvette was then kept in a fluorescence instrument (Fluoromax plus, Horiba Scientific). A magnetic stirrer is furnished with the instrument, which provides a slow stirring condition. The emission intensity of lucigenin fluorescence, F_t , was monitored at $\lambda_{\text{em}} = 535 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$). At $t = 20 \text{ s}$, the ion gradient was created between the intra and extra vesicular media by adding 33.3 μL of 2 M MCl ($\text{M}^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+, \text{and Cs}^+$). Transporter **1a** and **2a** were added separately at $t = 100 \text{ s}$ in different concentrations. Finally, at $t = 300 \text{ s}$, 25 μL of 10% Triton X-100 was added to lyse the vesicles leading to the complete destruction of the chloride concentration gradient. The collected data was then normalized following a reported protocol to plot the change in fluorescence intensity with respect to time.¹⁸

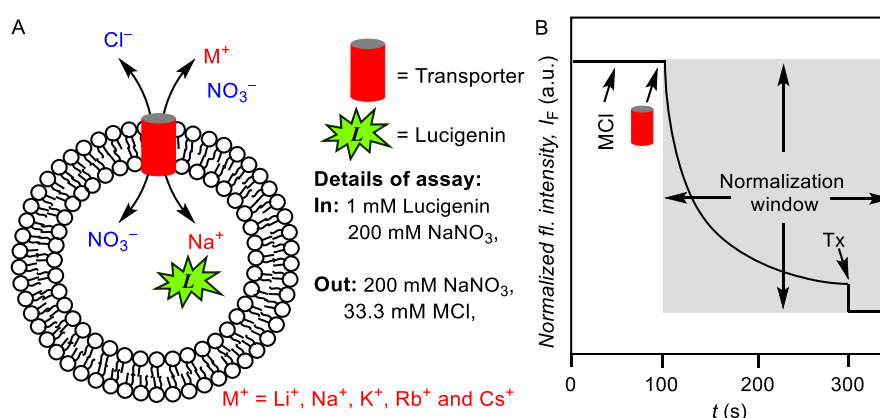


Figure 2.24 The illustration of fluorescence-based ion transport activity assay using EYPC-LUVs $\supset$ lucigenin in the presence of extravesicular alkali metal cations (A). Chloride influx kinetics illustrated with normalization window. (B).

Anion selectivity by HPTS assay:

Preparation of EYPC-LUVs $\supset$ HPTS: Vesicles were prepared following a reported protocol.¹⁸

Final conditions: EYPC $\sim 5 \text{ mM}$. **Inside:** 10 mM HEPES, 100 mM NaCl , 1 mM HPTS, pH 7.0; **Outside:** 10 mM HEPES, 100 mM NaX , (where, $\text{X}^- = \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{OAc}^-, \text{and NO}_3^-$), pH 7.0.

Anion selectivity experiments: 1975 μL of buffer (10 mM HEPES, 100 mM NaX, pH 7.0; where, $X^- = \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{OAc}^-, \text{and } \text{NO}_3^-$) was taken separately in a clean and dry fluorescence cuvette, followed by the addition of 25 μL of EYPC-LUV $\supset$ HPTS vesicles. The cuvette was then kept in a fluorescence instrument (Fluoromax plus, Horiba Scientific). A magnetic stirrer is equipped with the instrument which provided a slow stirring condition. The emission intensity of HPTS fluorescence, F_t , was monitored at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$). At $t = 20 \text{ s}$, the pH gradient of 0.8 was created between the intra and extra vesicular media by adding 20 μL of 0.5 M NaOH. Transporter **1a** or **2a** in DMSO was added at $t = 100 \text{ s}$ in different concentrations. Finally, at $t = 300 \text{ s}$, 25 μL of 10% Triton X-100 was added to lyse the vesicles leading to complete destruction of the pH gradient. The collected data was then normalized following a reported protocol to plot the change in fluorescence intensity with respect to time.¹⁸

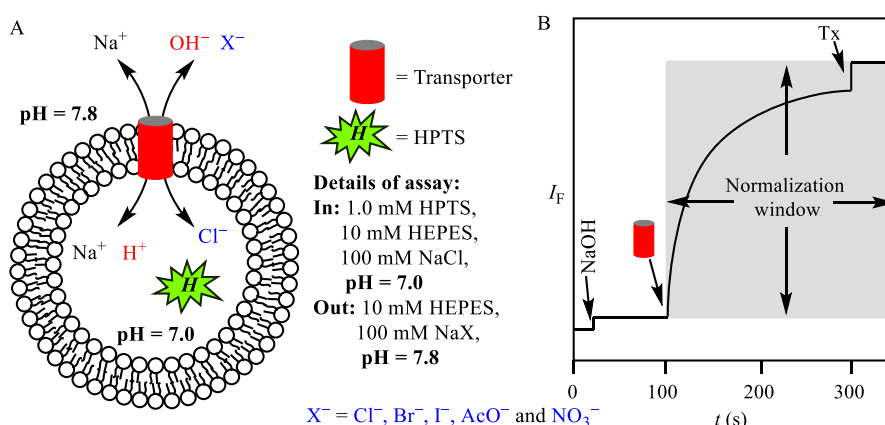


Figure 2.25 The fluorescence-based ion transport activity assay using EYPC-LUVs is represented in the figure (A) and ion transport kinetics illustrated with normalization window. (B).

Ion transport activity in the presence of valinomycin:

Preparation of EYPC-LUVs $\supset$ lucigenin: The aforesaid lucigenin vesicles were used for the experiments.

Description of the assay: 1975 μL of NaNO_3 solution (200 mM NaNO_3) was taken in a clean and dry fluorescence cuvette, followed by the addition of 25 μL of EYPC-LUVs $\supset$ lucigenin. The cuvette was then kept in a fluorescence instrument (Fluoromax plus, Horiba Scientific). A magnetic stirrer is furnished with the instrument which provided a slow stirring condition. The emission intensity of lucigenin fluorescence, F_t , was monitored at λ_{em}

= 535 nm ($\lambda_{\text{ex}} = 450$ nm). At $t = 20$ s, the ion gradient was created between the intra and extra vesicular media by adding 33.3 μL of 2 M KCl. At 50 s, valinomycin (1.0 μM) was added to the cuvette. Transporter **1a** and **2a** were added at $t = 100$ s in different concentrations, separately. Finally, at $t = 300$ s, 25 μL of 10% Triton X-100 was added to lyse the vesicles leading to complete destruction of the chloride concentration gradient. The collected data was then normalized following a reported protocol to plot the change in fluorescence intensity with respect to time.³⁶

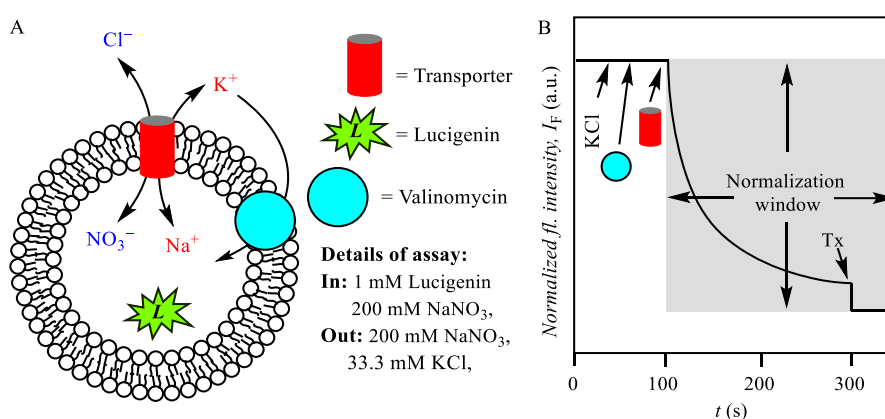


Figure 2.26 The fluorescence-based ion transport activity assay using EYPC-LUVs $\supset$ lucigenin in the presence and absence of valinomycin is depicted in the figure (A). Chloride influx kinetics illustrated with normalization window. (B).

DPPC Assay

Preparation of DPPC-LUVs $\supset$ lucigenin: Vesicles were prepared following a reported protocol.⁴⁹

Description of the assay: 1975 μL of NaNO₃ solution (200 mM NaNO₃) was taken in a clean and dry fluorescence cuvette, followed by the addition of 25 μL of DPPC-LUVs $\supset$ lucigenin. Required temperatures were acquired using a temperature controller equipped with the instrument. The cuvette was then kept in a fluorescence instrument (Fluoromax plus, Horiba Scientific). A magnetic stirrer is furnished with the instrument, which provides a slow stirring condition. The emission intensity of lucigenin fluorescence, F_t , was monitored at $\lambda_{\text{em}} = 535$ nm ($\lambda_{\text{ex}} = 450$ nm). At $t = 20$ s, the ion gradient was created between the intra and extra vesicular media by adding 33.3 μL of 2 M NaCl. Transporter **1a** and **2a** were separately added at $t = 100$ s. Finally, at $t = 300$ s, 25 μL of 10% Triton X-100

was added to lyse the vesicles leading to the complete destruction of the chloride concentration gradient. The collected data was then normalized following the abovementioned protocol to plot the change in fluorescence intensity with respect to time.

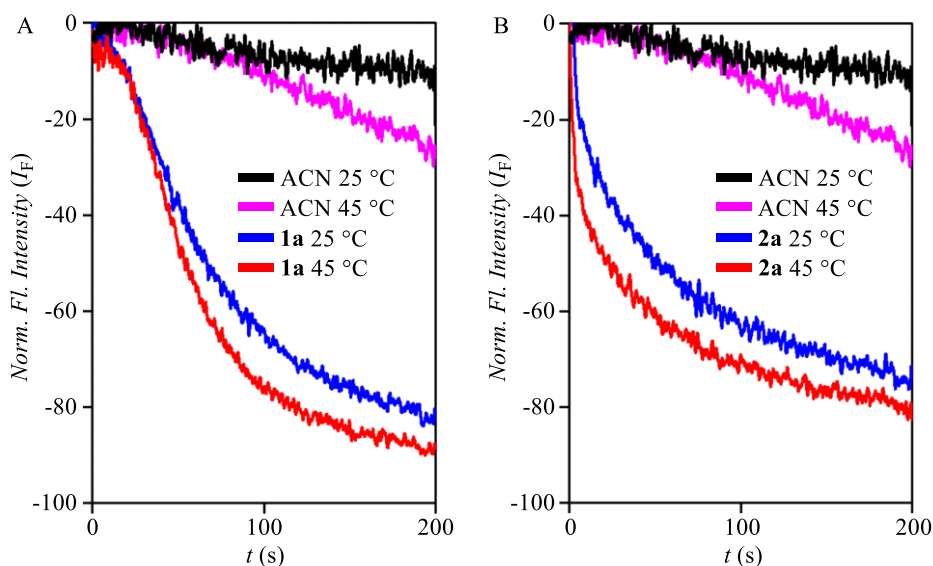


Figure 2.27 Cl^- transport by **1a** (A) and **2a** (B) in lucigenin-trapped DPPC-LUV at 25 °C and 45 °C.

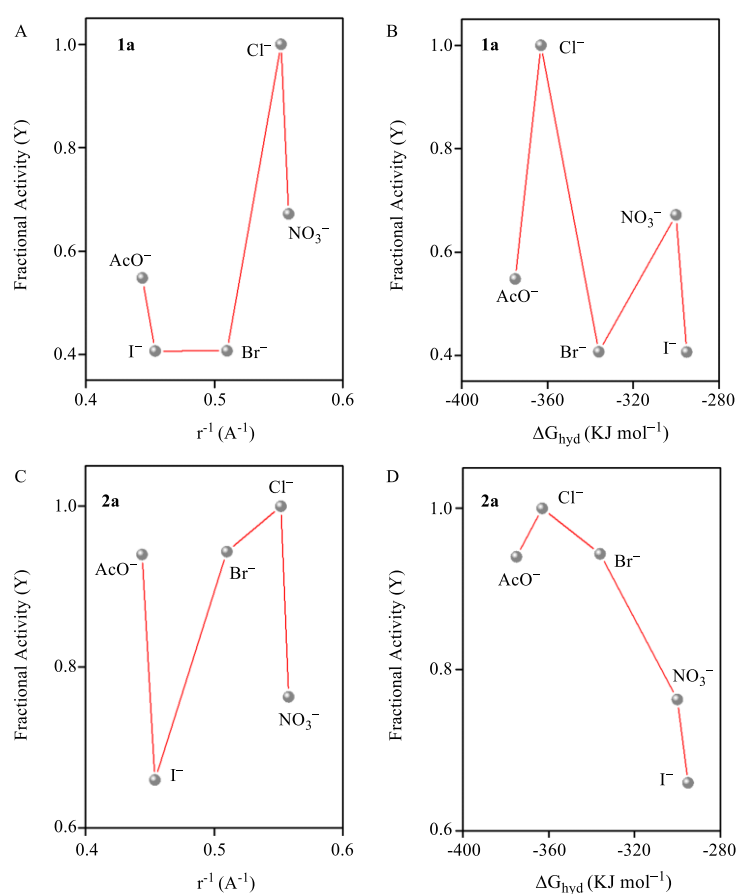


Figure 2.28 Anion selectivity presented as a fractional activity Y (relative to Cl^-) with respect to the reciprocal anion radius (A, C), and fractional activity Y (relative to Cl^-) with respect to the anion hydration energy (B, D).

2.4.11 Planner bilayer conductance measurement

The conductance measurement across the bilayer lipid membrane was done with the help of Warner instrument (USA). For that, a solution of diphytanoylphosphatidylcholine lipid (DPhPC; Avanti Polar Lipids) was prepared by dissolving it in *n*-decane of concentration 20 mg/mL. Then a bilayer membrane was formed by using this solution at the 150 μm diameter aperture within a cup made of polystyrene (Warner Instrument, USA). In both chambers (*cis* and *trans*) of the instrument, 1 M KCl was symmetrically injected. The setup was made in such a way that the *trans* compartment was held at virtual ground. The BC 535 head-stage (Warner Instrument, USA) connects the *cis* compartment through Ag-AgCl electrodes. Then the channel-forming molecule **1a** (2 μM), and **2a** (20 μM) was added to the *trans* chamber. An inbuilt magnetic stirrer was used to stir the solution for 20 min. Channel formation through the lipid bilayer was confirmed from channel opening and closing events upon application of different voltages using pClamp11 software (Molecular probes, USA). An analogue-to-digital converter (Digidata 1440A, Molecular Devices) and pClamp11 software (Molecular probes, USA) were used to low pass filter the currents at 1 kHz.

The channel opening and closing events at positive, as well as negative potentials, were analyzed by pClamp software (v. 10.6). A complete trace comprised of a series of opening and closing events for the time period of 2 h from where a small fraction is presented in the manuscript.

The channel diameter was calculated using the equation (Hille equation) mentioned below.⁴⁶

$$1/g = (l + \pi d/4) (4\rho/\pi d^2) \quad \text{Eq. 2.1}$$

where, g is the corrected conductance, calculated by multiplying measured single-channel conductance with Sansom's correction factor⁴⁷ 5.61; l is the length of the ion channel (34 Å), and ρ signifies resistivity of the KCl solution ($\rho = 10.1 \Omega\cdot\text{cm}$).

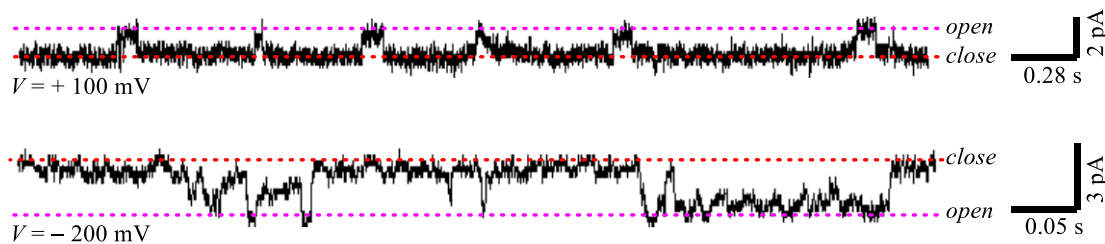


Figure 2.29 Single-channel current traces by **1a** ($c = 2 \mu\text{M}$) recorded at +100 mV, -200 mV, under symmetrical KCl solution.

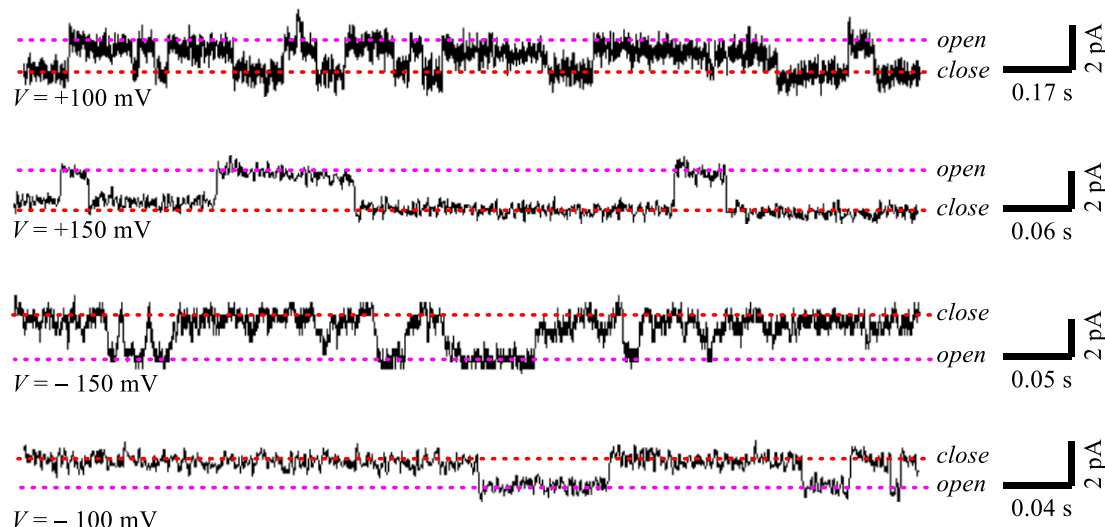


Figure 2.30 Single-channel current traces by **2a** ($c = 20 \mu\text{M}$) recorded at various negative and positive potentials under symmetrical KCl solution.

Determination of anion selectivity permeability ratio by Planar Bilayer Conductance

Measurements: The *cis* and *trans* chambers were filled with unsymmetric solutions of KCl. The *cis* chamber was filled with 1.0 M KCl solution and the *trans* chamber was filled with 0.5 M KCl. The compounds **1a** ($2 \mu\text{M}$), and **2a** ($20 \mu\text{M}$) were added to the *trans* chamber and stirred for 5 minutes. The permeability ratio ($P_{\text{Cl}^-}/P_{\text{K}^+}$) was calculated by using the Goldman-Hodgkin-Katz equation (Equation 2.2).⁴⁶

$$\frac{P_{\text{Cl}^-}}{P_{\text{K}^+}} = \frac{a_{\text{K}^+}^{\text{cis}} - a_{\text{K}^+}^{\text{trans}} \times \exp\left(-\frac{Z \times V_{\text{rev}} \times F}{R \times T}\right)}{a_{\text{Cl}^-}^{\text{cis}} \times \exp\left(-\frac{Z \times V_{\text{rev}} \times F}{R \times T}\right) - a_{\text{Cl}^-}^{\text{trans}}} \quad \text{Equation 2.2}$$

where, $P_{\text{Cl}^-}/P_{\text{K}^+}$ = anion/cation permeability ratio; $a_{\text{K}^+}^{\text{cis}}$ = K^+ activity in the *cis* chamber; $a_{\text{K}^+}^{\text{trans}}$ = K^+ activity in the *trans* chamber; $a_{\text{Cl}^-}^{\text{cis}}$ = Cl^- activity in the *cis* chamber; $a_{\text{Cl}^-}^{\text{trans}}$ = Cl^- activity in the *trans* chamber⁶⁶; $Z_{\text{K}^+} = +1$, $Z_{\text{Cl}^-} = -1$, V_{rev} = reversal potential; F = Faraday constant; R = gas constant; T = temperature (K).

2.4.12 Molecular Modeling Studies

System setup and simulation model: Both the crystal structures of the self-assembled architectures built from the constituent alcohol and ester molecules individually, were considered for atomistic Molecular Dynamics simulation. Initially, the coordinates of the channels of six component layers were minimized using MAESTRO (<https://www.schrodinger.com/products/maestro>). Further, these minimized structures were used to build the channel/membrane complex constitution by employing a membrane builder in CHARMM-GUI.⁵¹ The protocol included six consecutive steps described by Jo et al.:⁵³ object reading followed by its orientation, system size determination, the building of a lipid bilayer, assembling the lipid bilayer, and finally, the system equilibrium. In this study, the H-bonded self-assembled structures comprising six repetitive layers of solely alcohol or ester molecules, were placed in the center of a bilayer membrane which was composed of 144 molecules of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) lipids (72 molecules in each leaflet of membrane). The membrane was then placed in a rectangular box which was slightly extended in height (along Z-direction). The entire system was solvated with enough water on both sides of the bilayer leaflet and a particular ion concentration was maintained throughout the simulation (see table S8).

Table 2.2: Details of equilibrium simulation with the channel of **1a** and **2a** individually.

System	Box volume (nm ³)	No. of water molecules	No. of K ⁺ ions	No. of Cl ⁻ ions
1a	7.5 x 7.5 x 8.5	9193	163	163

2a	7.6 x 7.6 x 8.5	9374	166	166
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The same system composition was maintained for equilibrium simulation of a channel-embedded membrane system, inserted into 1M KCl salt concentration in each upper and lower solution compartment.

The general AMBER force field (GAFF)^{54, 56, 67} was employed for modelling the 12-mer alcohol channel and 6-mer ester channel arranged in six consecutive layers. The DPhPC lipid and ion parameters were taken from the CHARMM36m force field.⁵⁷⁻⁵⁹ The CHARMM TIP3P water model⁶⁰ was used for simulation.

Simulation method: Each of the simulations was performed employing periodic boundary conditions (PBC) in all three dimensions. For maintaining long-range electrostatic interactions, the particle mesh Ewald (PME) method⁶⁸ with cubic interpolation was utilized, while the cut-off of 1.2 nm was practiced for electrostatic interactions at a short-range. At every 20 steps, the neighbor lists were updated in the simulation. All the bonds, including hydrogen atoms of the lipids and the channel monomers, were constrained by the LINCS algorithm⁶⁹ and water molecules were kept rigid by applying the SETTLE⁷⁰ approach. The Verlet cut-off scheme⁷¹ with Lenard Jones interaction extending to 1.2 nm with dispersion corrections was employed for simulation. Initially, the system with restraints was subjected to energy minimization using the steepest-descent algorithm. Then the energy minimized system was allowed to be equilibrated well in successive six restrained MD simulations of 10 ns each, but the restrain is gradually reduced in each consecutive step. Throughout the equilibration process, the average temperature was maintained at 298.15 K using Berendsen thermostat⁷² and the pressure was kept fixed at an average value of 1.0 bar with the help of Berendsen barostat during the equilibration simulations. Finally, 1.0 microsecond long production MD simulation was performed with the equilibrated system in the NPT ensemble under slight restrain applied to the ion channels. During production simulation, the temperature was kept constant at 298.15 K via individual coupling of the channel, membrane, and solvent under Nose-Hoover thermostat⁷³ with a relaxation constant of 1.0 ps. The Parrinello-Rahman barostat⁷⁴ was used for maintaining the pressure at 1.0 bar with a coupling constant of 5 ps and compressibility of 4.5×10^{-5} bar under semi-isotropic coupling (coupled separately XY and Z directions) of the system to retain a tensionless bilayer during the

simulation. The simulation (production run) time step was set to 2 fs using the leapfrog integrator. The last 155 ns trajectory was used for the required analysis. A similar protocol of equilibrium simulation was followed for each of the synthetic channel systems composed of alcohol or ester subunits.

Free energy simulation:

The free energetics of ion transport through each of the channel system was computed via umbrella sampling simulation. To understand the mechanism of chloride ion transport, one single chloride ion was allowed to pass through the channel. A set of configurations spanning the position of the anion at diverse location of channel, starting with entry of the anion into the mouth of the channel, followed by its final exit via the other end, were saved in respective configurations to initiate umbrella sampling. One K^+ ion is incorporated for charge-neutralizing the system. The box dimension and number of water molecules in the umbrella sampling simulation were kept the same as the equilibrium simulation for each of the channel systems (**1a** and **2a**).

For a comparison with a reference system, a control free energy simulation of anion passage through channel-less membrane of similar lipid composition was performed. Similar to the abovementioned protocol, for this simulation also, the initial configurations for each umbrella window were generated by positioning the anion at multiple locations of the bilayer along the Z-direction across the membrane bilayer. For membrane-only umbrella sampling, the simulation system consists of the DPhPC lipid bilayer solvated with 8975 water molecules. The setup included one K^+ and one Cl^- ion and the box size was kept fixed at 7.6 x 7.6 x 8.5 nm³.

The Z-direction of the chloride anion with respect to the channel (excluding hydrogen atoms), d , serves as the collective variable (CV) for umbrella sampling of anion transport. The negative value of d refers to the location of the ion at the upper half of the channel (the passage towards the mouth region of the channel from the center of the same), while positive d signifies that the anion is situated at the lower half of the channel (the way from the center of the channel to the end of it). $d = 0$ nm means the mid-position (at the center of the channel system) of the anion in the channel core.

Configurations were generated corresponding to the CV ranging from -1.7 nm to 3.2 nm for the alcohol channel and -1.5 nm to 2.0 nm for the channel of ester with the spacing of 0.1 nm of d for both channel systems. In each of the umbrella sampling windows, a force constant ranging from 5000 to 6300 kJ/mol/nm² was applied to maintain the respective position of the anion. Each of the configurations (traditionally referred as ‘windows’) were energy minimized by applying a steepest-descent algorithm followed by 500 ps of NVT equilibration (simulation time step of 1 fs), leading to 10 ns of final production run (with 2 fs time step) in NPT ensemble in each of the windows. The temperature and pressure were maintained at 298.15 K and 1 bar using the same thermostat and barostat employed for equilibration and production run, as was used for equilibrium simulation. The weighted histogram analysis method (WHAM)⁷⁵ was invoked for calculating the respective equilibrium free energy profile via S32 combination of the data corresponding to each independent trajectory of every window. GROMACS software of version 20x was utilized to execute all simulations.

Besides calculating the free energy profile of anion transport, the molecular level insight of the interaction of anion and the channel components were acquired by H-bonding analysis and measurement of root mean square deviation (RMSD) of the channel. To know whether there is any considerable role of the water in the channel core for assisting ion passage, the number of water molecule(s) around the chloride ion was calculated as the anion moves through the channel.

2.4.12 *In Vitro* Studies:

Cell culture protocol:

DMEM media were used to culture human epithelial breast cancer MCF-7 cells. Triple negative breast cancer cells MDA-MB-231, and human lung cancer cells A549 were cultured in RPMI media as monolayers. 10% FBS, 100 µg/ml penicillin and 100 µg/ml streptomycin were used as cell supplements. Cells were incubated at 37°C, 5% CO₂ atmosphere in a humid condition. DMEM/F-12 supplemented with 10% horse serum was used to culture normal breast epithelial cells MCF10A.

Measurement of intracellular Cl⁻ concentrations: Time-dependent influx data of Cl⁻ were measured in normal breast epithelial MCF10A cells by using chloride selective cell-permeable fluorescent dye *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide

(MQAE).²¹ Cells were seeded in 96-well flat-bottom tissue culture-treated plates (Corning) at a density of 3000 cells/well (per 100 μ L) and incubated at 37 °C, 5% CO₂ for 24 h. MQAE was added to each well by maintaining the final concentration as 5 mM for 3.0 h. Extracellular dye was removed by washing with PBS (twice), compounds **1a** and **2a** were separately added in DMEM at various concentrations, and cells were incubated for 24 h. The MQAE fluorescence was measured using a fluorescence microplate reader (λ_{ex} = 350 nm and λ_{em} = 460 nm).

MTT-based cytotoxicity assay:

Cells were grown in 96 well plates (3000 cells per well) for 24 h. The cells were then treated with the compounds (**1a** and **2a** separately) with an increasing concentration (0, 0.1, 1, 10, 25, 50 and 100 μ M) for 44 h. The cells were then incubated with the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide)] for 4 h. A solution of 100 % isopropanol, 3.6×10^{-3} N HCl and 0.1 % Triton X – 100 was used to dissolve the resultant formazan, and then the reading (OD) was taken in the ELISA reader (Multiscan GO, Thermo Scientific) at 570 nm.

2.4.13 NMR Spectra

NMR Studies:

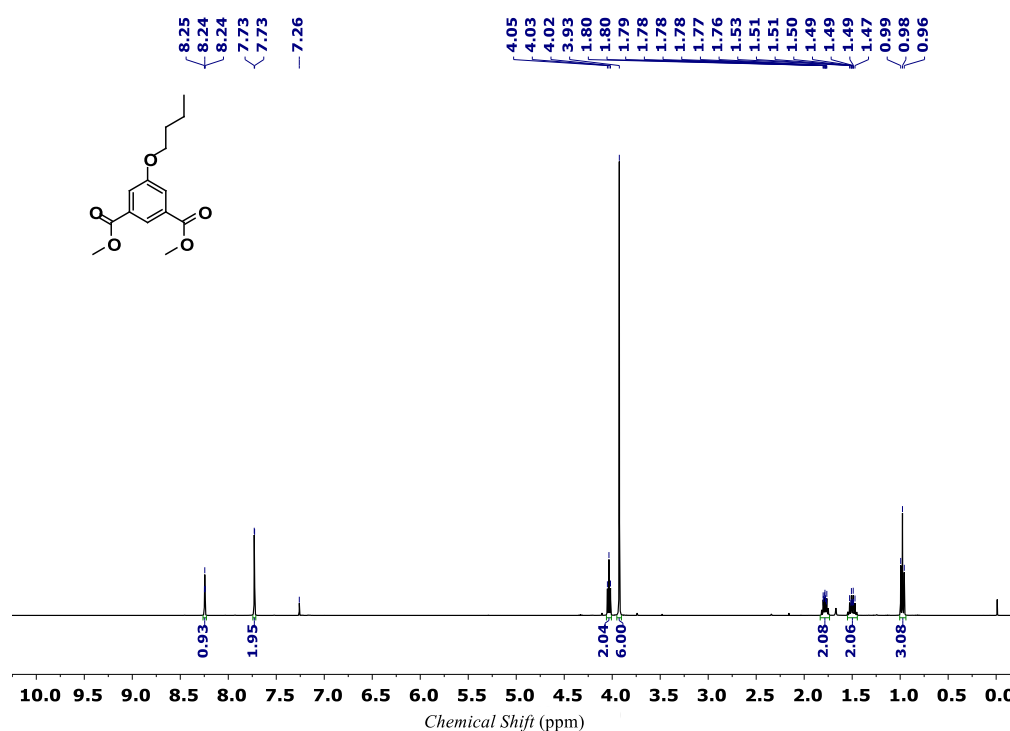
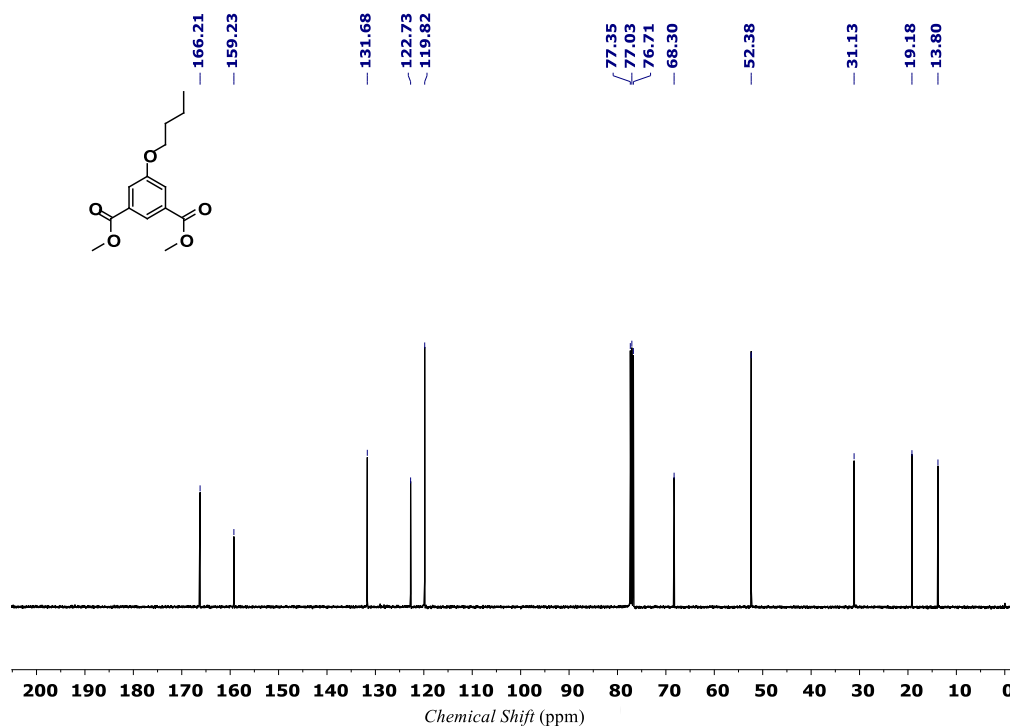
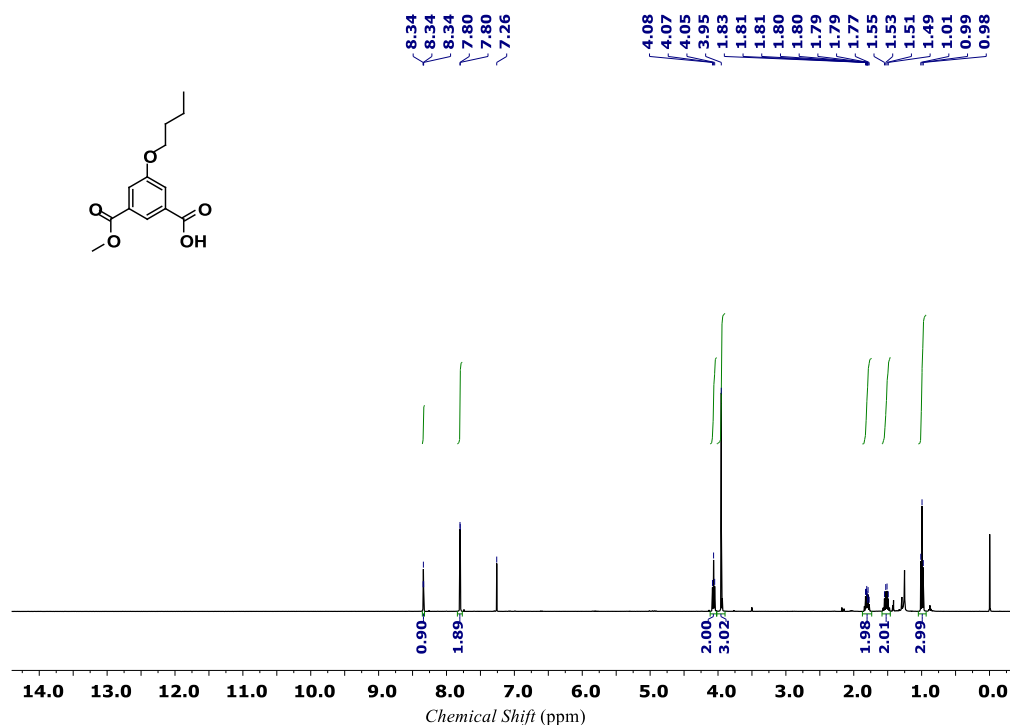


Figure 2.31 400 MHz ^1H NMR of compound **5** in CDCl_3 .**Figure 2.32** 101 MHz ^{13}C NMR of compound **5** in CDCl_3 .**Figure 2.33** 400 MHz ^1H NMR of compound **6** in CDCl_3 .

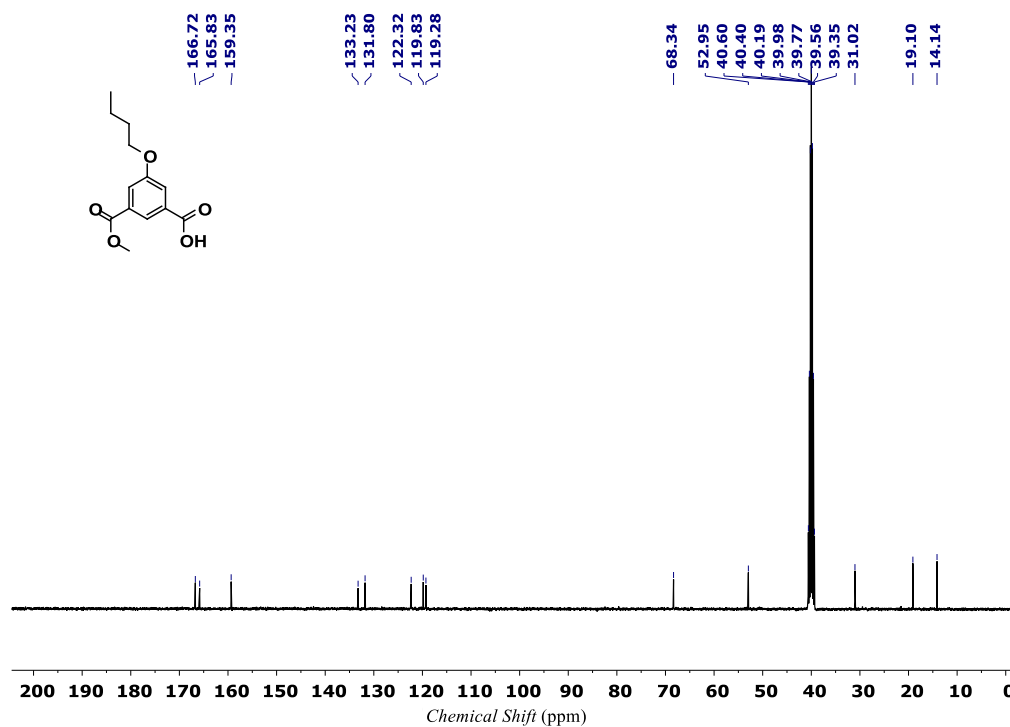


Figure 2.34 101 MHz ^{13}C NMR of compound **6** in DMSO-d_6 .

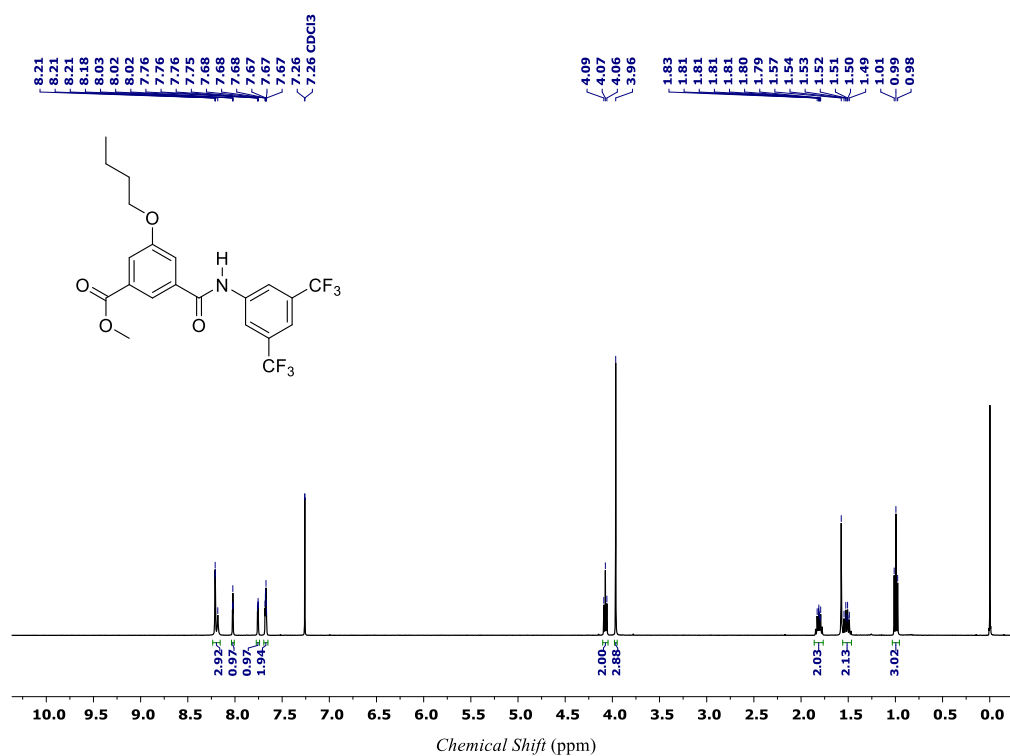


Figure 2.35 400 MHz ^1H NMR of compound **1a** in CDCl_3 .

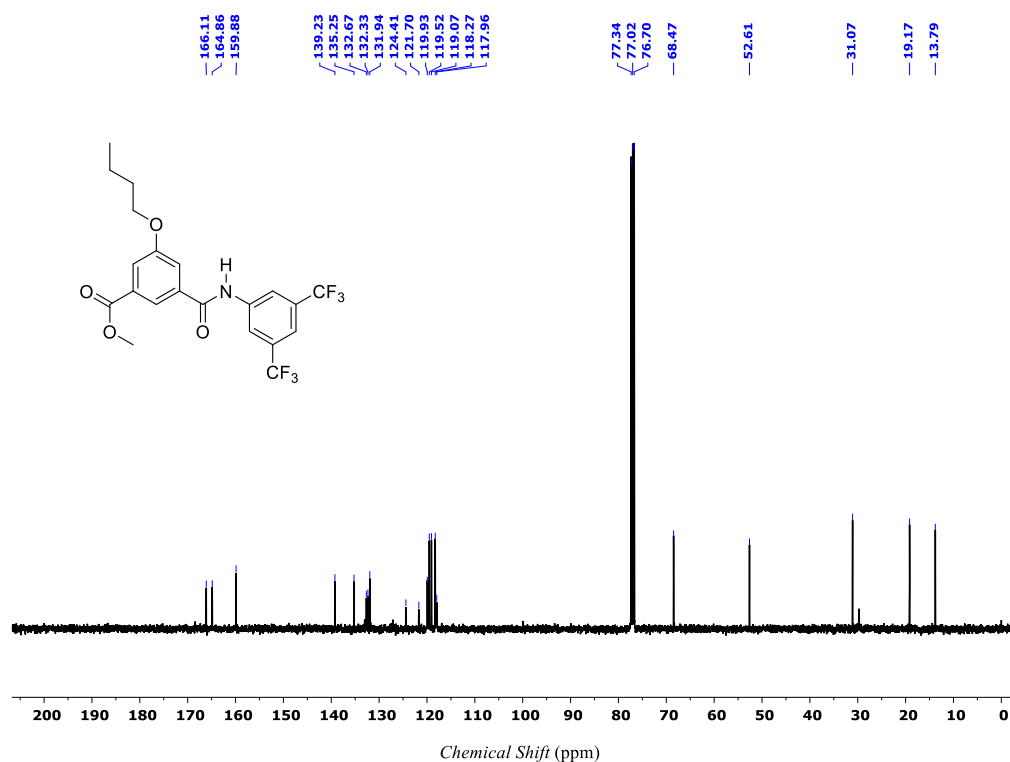


Figure 2.36 101 MHz ¹³C NMR of compound **1a** in CDCl₃.

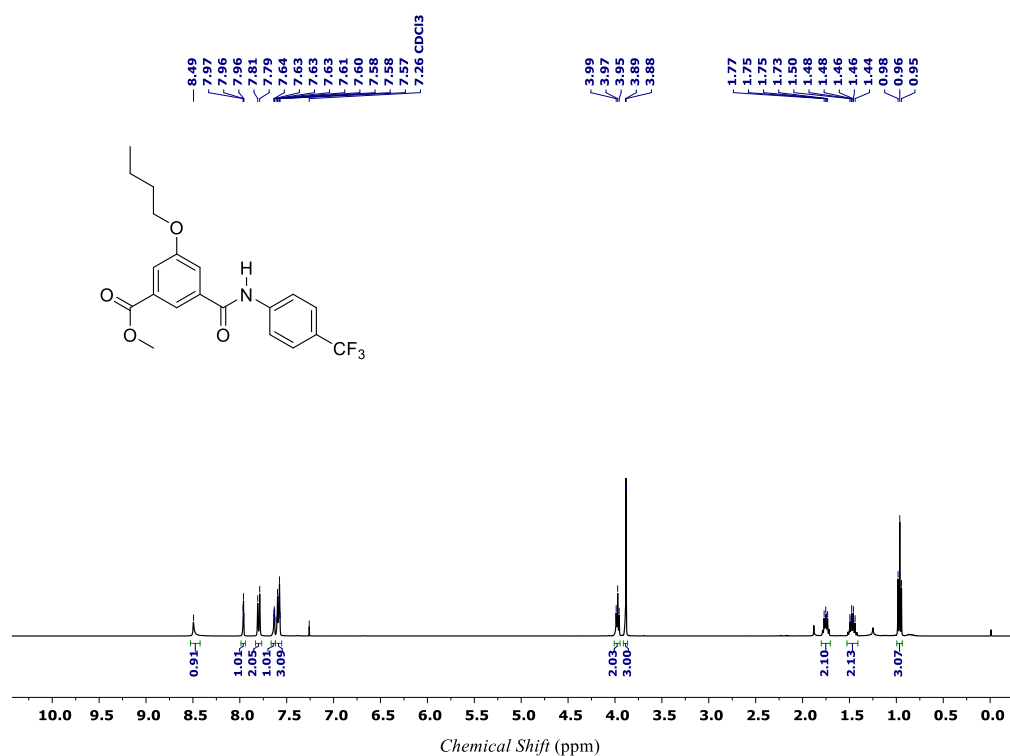


Figure 2.37 400 MHz ¹H NMR of compound **1b** in CDCl₃.

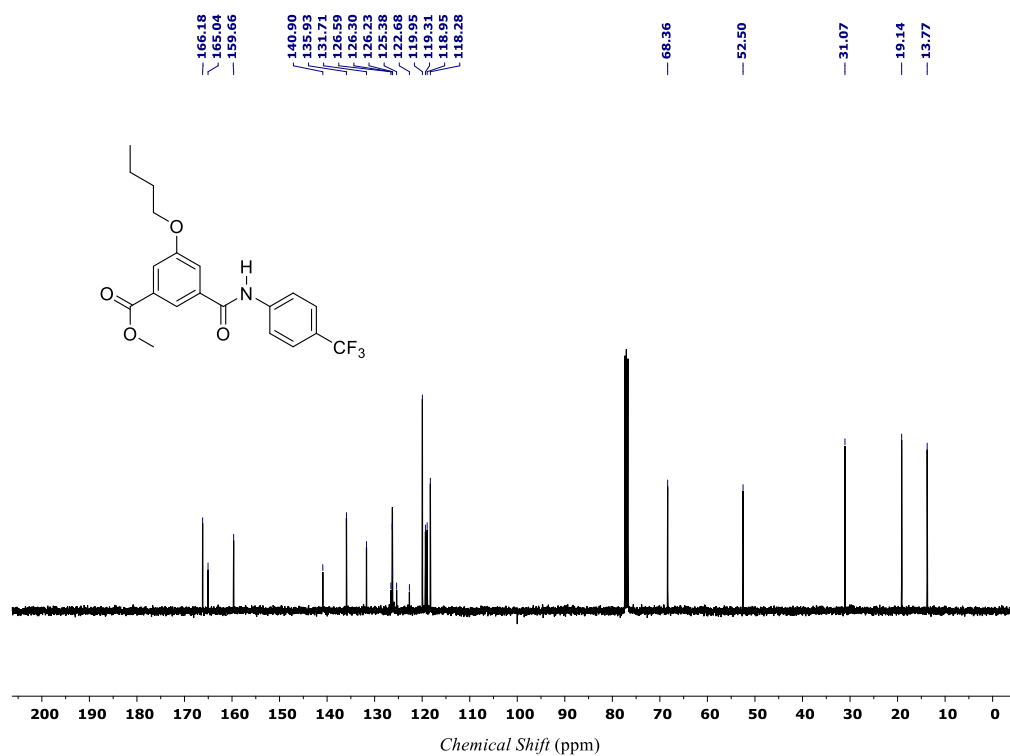


Figure 2.38 101 MHz ^{13}C NMR of compound **1b** in CDCl_3 .

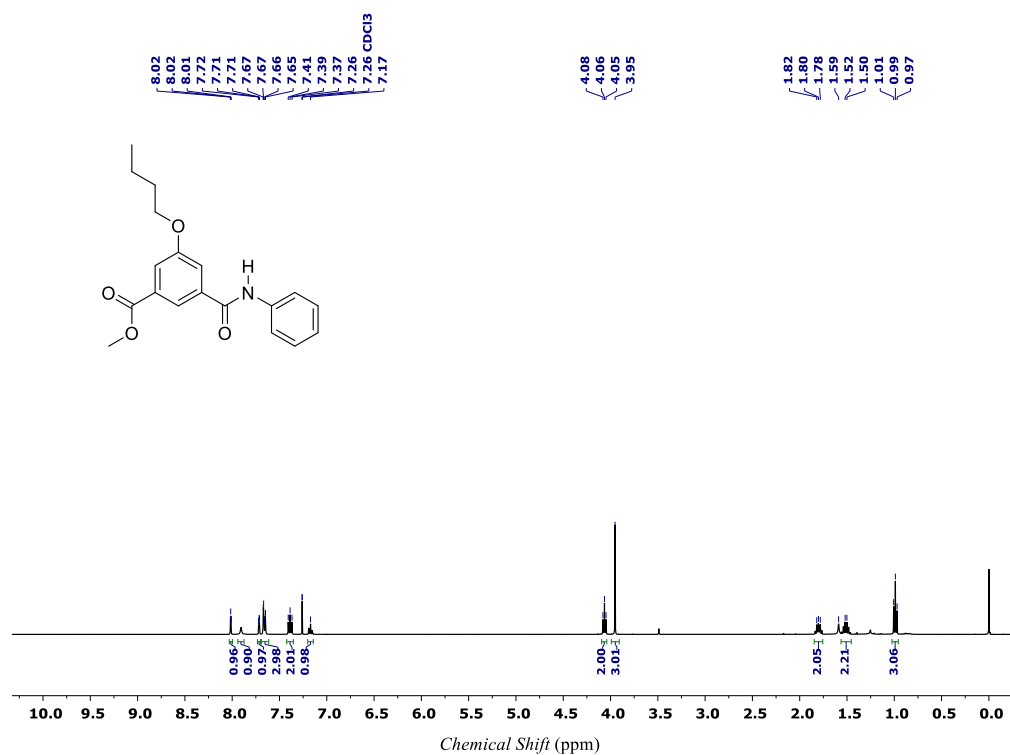


Figure 2.39 400 MHz ^1H NMR of compound **1c** in CDCl_3 .

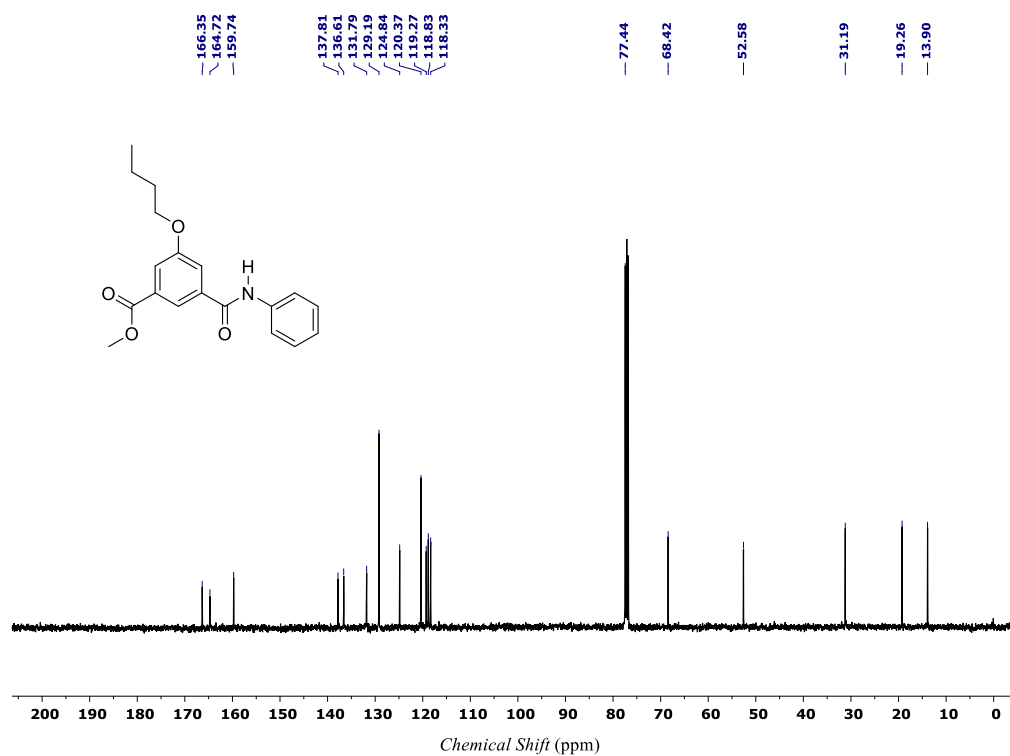


Figure 2.40 101 MHz ¹³C NMR of compound **1c** in CDCl₃.

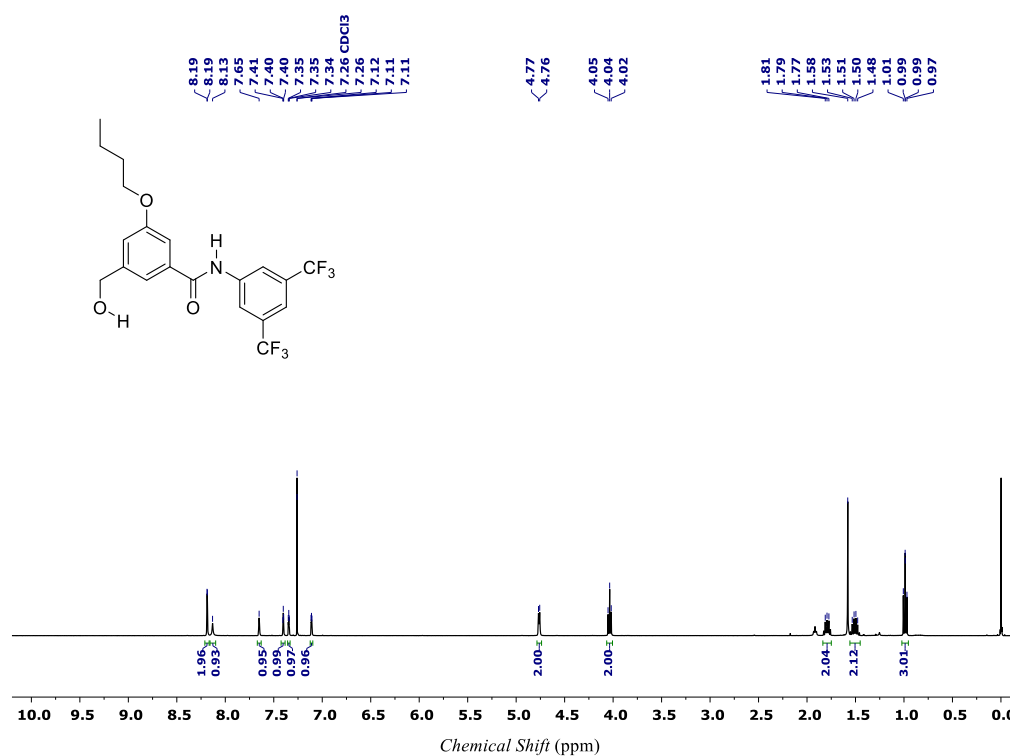


Figure 2.41 400 MHz ¹H NMR of compound **2a** in CDCl₃.

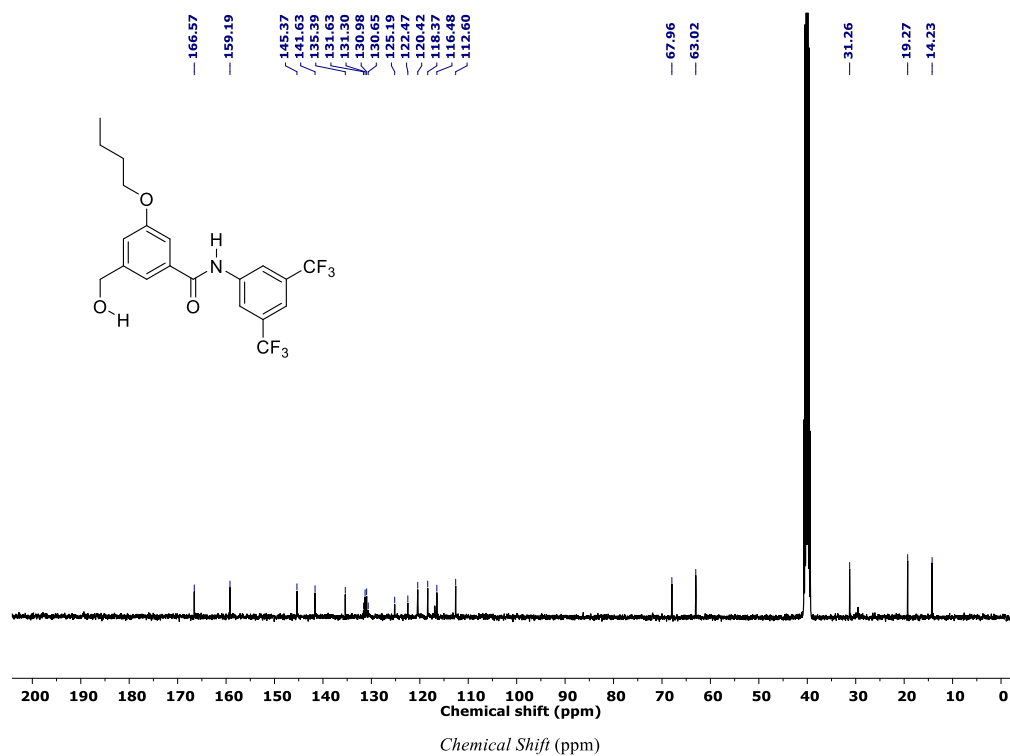


Figure 2.42 101 MHz ¹³C NMR of compound **2a** in DMSO-d₆.

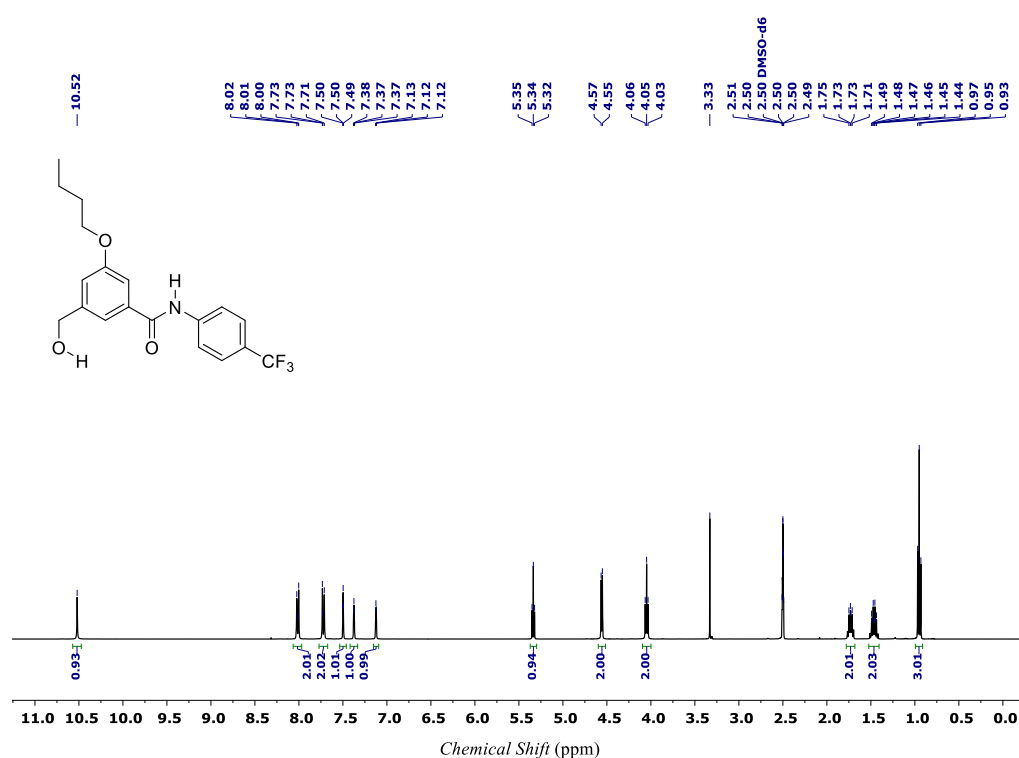


Figure 2.43 400 MHz ¹H NMR of compound **2b** in DMSO-d₆.

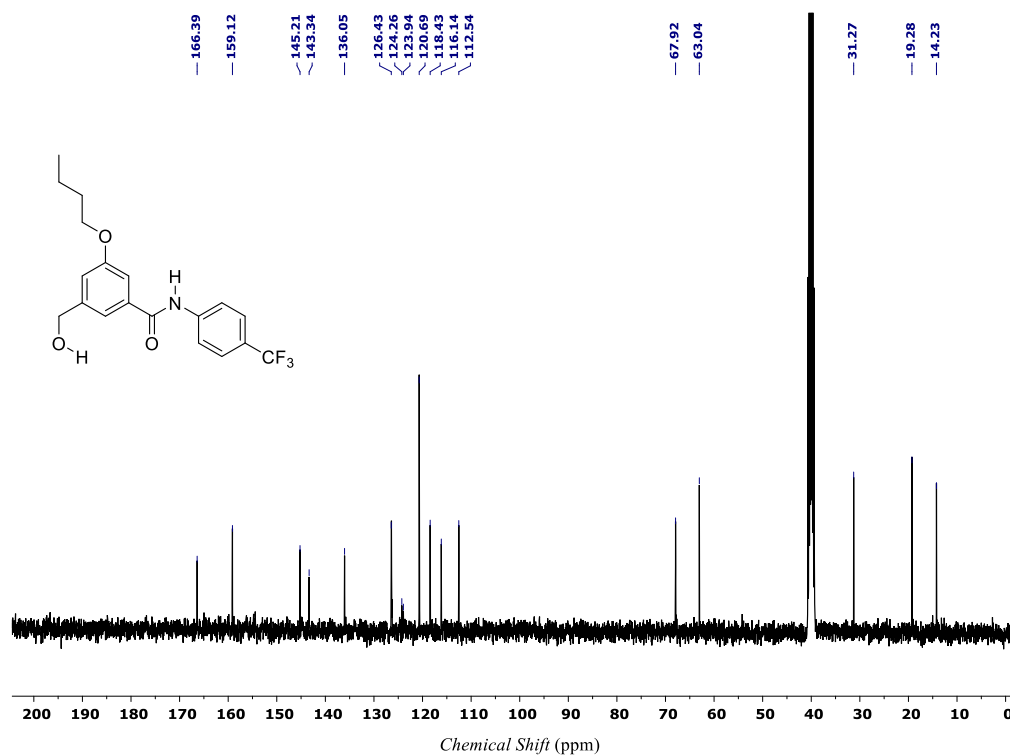


Figure 2.44 101 MHz ¹³C NMR of compound 2b in DMSO-d₆.

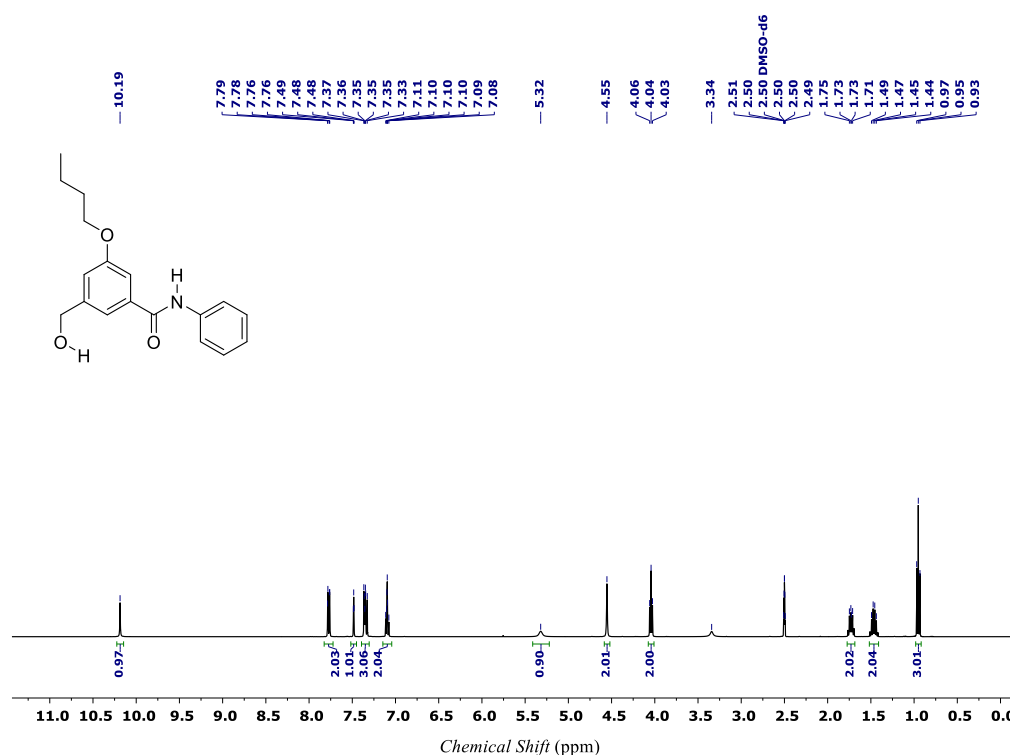


Figure 2.45 400 MHz ¹H NMR of compound 2c in DMSO-d₆.

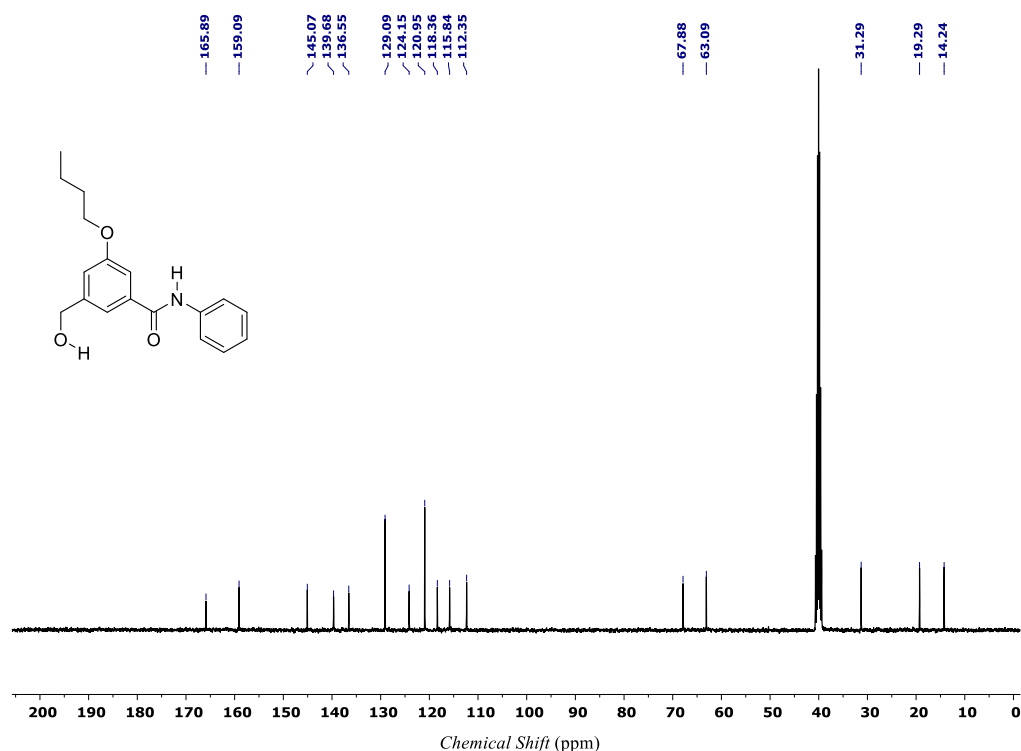


Figure 2.46 101 MHz ^{13}C NMR of compound 2c in DMSO- d_6 .

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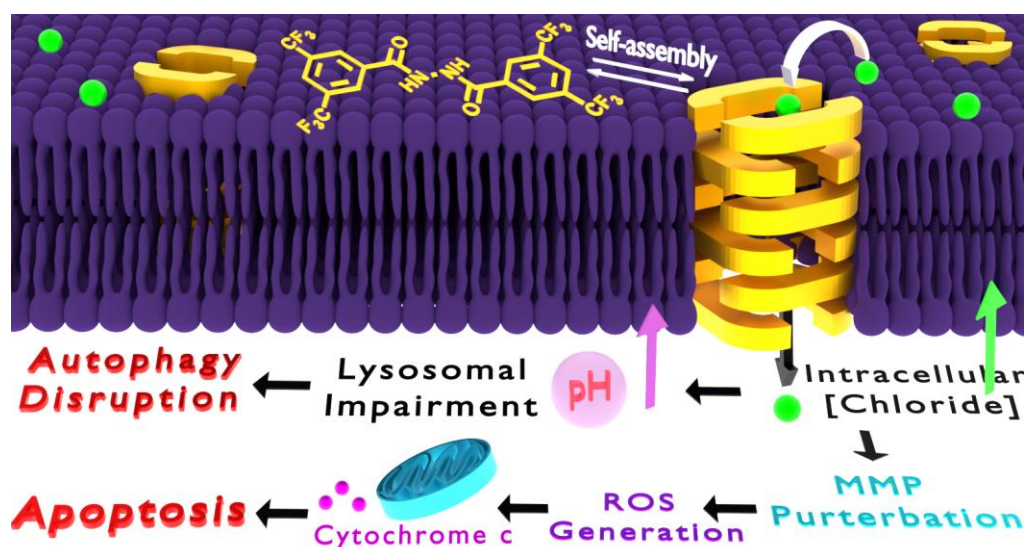
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Chapter 3

A Benzohydrazide-Based Artificial Ion Channel that Modulates Chloride Ion Concentration in Cancer Cells and Induces Apoptosis by Disruption of Autophagy



3.1 Introduction

As part of detoxification, roughly 10^{11} cells die each day and are replaced by new ones in healthy adults.¹ Three significant mechanisms, namely, apoptosis, necrosis, and autophagy, are involved in removing old and unwanted cells.² While on the one hand, apoptosis is a vigilantly controlled process involving programmed cell death, wherein the entire cell breaks down without affecting its surrounding cells; necrosis is an uncontrolled process where cells burst, resulting in spillage of cellular contents causing damage to the neighboring cells. In addition to necrosis and apoptosis, autophagy – a self-degradative process where cytoplasmic components are engulfed in double-membraned vesicles and delivered to the lysosome for degradation – is the most common mechanism of removing old and unwanted cells.³ Nevertheless, both apoptosis and autophagy play essential roles in living organisms to maintain cellular homeostasis.⁴ The loss of apoptotic control in cancer cells induces uncontrolled growth and proliferation.⁵ Autophagy can, however, play dual roles: it can act as a tumor suppressor early on in the process of tumorigenesis through its role in the removal of potential oncogenic molecules; or can promote the survival of tumor cells at advanced stages by alleviating stress in the microenvironment.⁶ Along these lines, identifying compounds/small molecules that can induce apoptosis and disrupt the autophagic processes to target cancer cells from two different angles become necessary to combat cancers.

The concentration of chloride (Cl^-) ion in the extracellular matrix is much higher (~ 120 mM) than that in the cytosol (~ 5 -40 mM).⁷ The membrane-embedded protein channels typically maintain these ionic concentrations.⁸ It is known in the literature that alterations in the ionic gradients across the phospholipid bilayer induce apoptosis.⁹ It is important to note that the cytosolic Cl^- ions play an essential role in regulating the lysosomal pH. An imbalance in the Cl^- ion gradient leads to the deacidification of lysosomes, which eventually culminates in the disruption of autophagic processes in cells.¹⁰ Hence, the development of synthetic systems that mimic the function of naturally occurring membrane-embedded chloride channels and artificially modulate Cl^- concentrations in the cytosol as well as in the lysosome is of

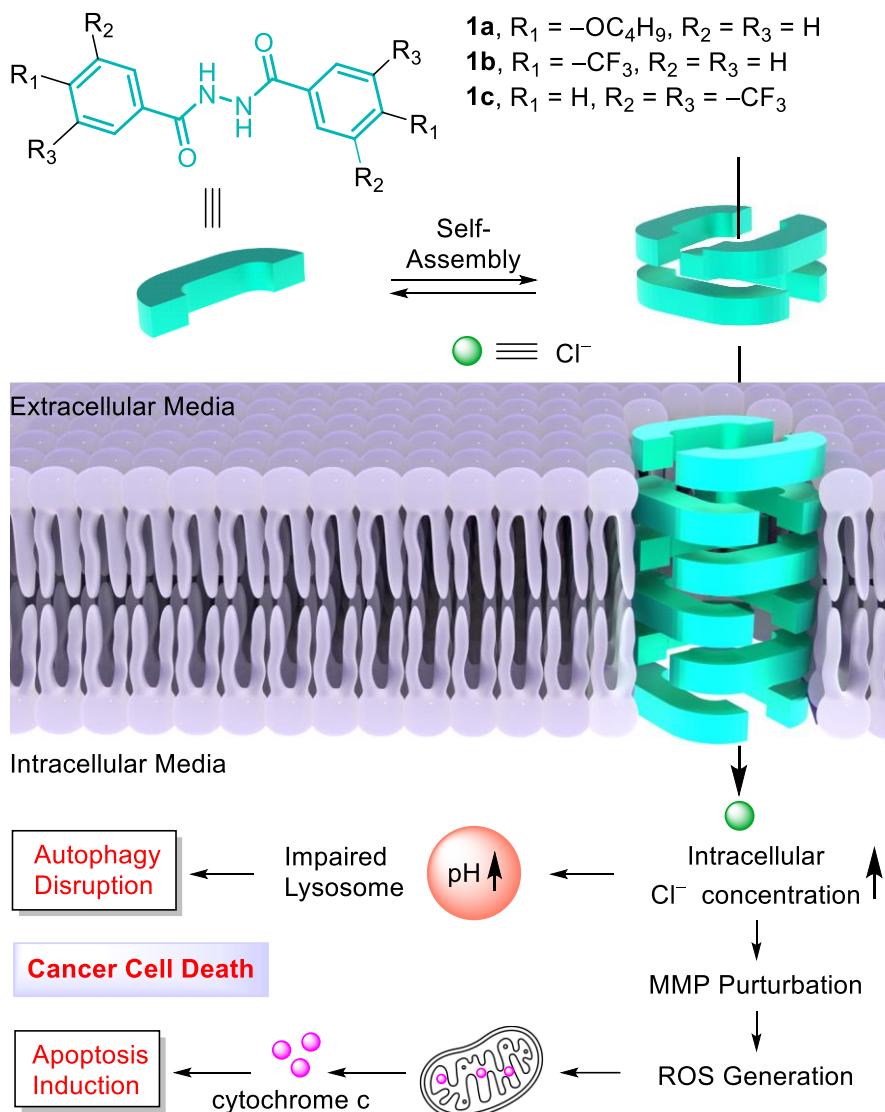


Figure 3.1 Structures of benzohydrazide derivatives **1a-1c**, and schematic representation of self-assembly of these molecules.

paramount importance because they could potentially play a dual role in inducing apoptosis as well as disrupting autophagy in cancer cells.

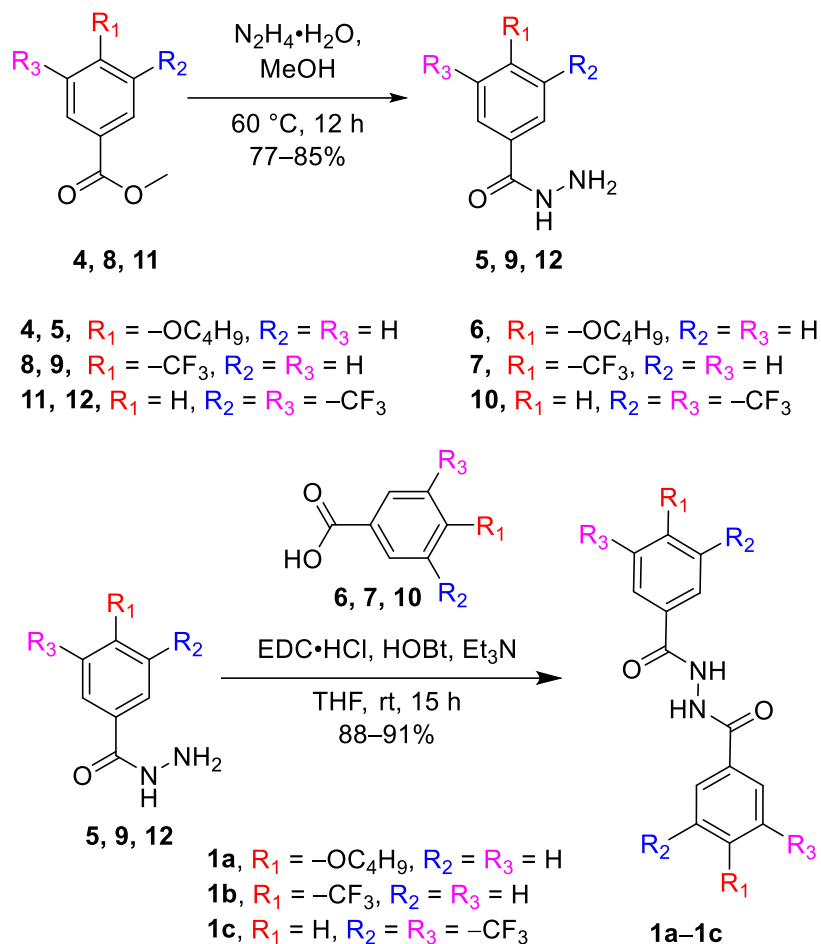
The use of synthetic ion transporters is one of the attractive approaches currently being explored as a new avenue in anticancer drug discovery due to their ability to induce apoptosis.¹¹⁻¹³ Such systems also have the potential for addressing multi-drug resistance in cancer because their actions are independent of cancer-causing proteins, receptors, enzymes, and genes.¹⁴ However, most of the synthetic ion transporters are non-specific in action and can mediate damage to healthy cells as well.¹³ Moreover, reports on synthetic ion transporters that can induce apoptosis while disrupting autophagy in cancer cells are rare.¹⁵⁻¹⁷

Molecular and supramolecular conformational stability is one of the most important factors controlling the selectivity and efficiency of transport.¹⁸ Facile synthesis, functional tunability, stability, and flexibility contribute to the advantages of structurally simple small molecules.¹⁹ Nevertheless, developing a self-assembled framework from a “small molecule” is challenging because, often, the monomer unit lacks adequate functions or pre-organization to provide the dimensions and binding sites for the assembly of ion channels.²⁰ A recent report from our lab demonstrated that the hydrazide backbone is highly influential in acting as a selectivity filter for Cl^- ions.²¹ In an effort to develop a structurally simple and low molecular weight Cl^- ion selective supramolecular nanochannel assembly, we chose the hydrazide backbone and linked it to simple aryl moieties (Figure 3.1). We anticipated that the intermolecular $\text{C}=\text{O}\cdots\text{H}-\text{N}$ hydrogen bonding interactions would aid in forming a preorganized geometry for anion recognition, and the $\pi-\pi$ stacking interactions would lead to aggregation resulting in the formation of an active ion channel across the membrane. Here, we report a benzoylbenzohydrazide compound **1c** which self-assembles to form a supramolecular nanochannel assembly that selectively mediates the translocation of Cl^- ions across cell membrane, perturbs the cellular ionic homeostasis, eventually leading to apoptosis in cancer cells. On the other hand, marginal toxicity was observed when **1c** was added to cell lines of non-cancerous origin. Interestingly, **1c** supplementation led to the deacidification of lysosomal pH and disruption of the autophagic processes in cancer cells.

3.2 Results and Discussions

3.2.1. Synthesis

Synthesis of compounds **1a-1c** was started from derivatives of methyl benzoate **4**, **8**, and **11**, which were prepared following known protocols from the literature (Scheme S1). Compounds **4**, **8**, and **11** were then treated with hydrazine hydrate to obtain the corresponding hydrazides **5**, **9**, and **12**. Finally, the coupling reactions of **5**, **9**, and **12** with the corresponding benzoic acid derivatives **6**, **7**, and **10** using EDC·HCl coupling reagent provided the final compounds **1a-1c** (Scheme 3.1).



Scheme 3.1 Synthetic scheme for the preparation of **1a–1c**.

3.2.2 Self-assembly Behavior

The crystal suitable for single crystal X-ray diffraction analysis of **1c** was successfully obtained by allowing n-pentane to diffuse slowly into the solution of **1c** in chloroform/methanol (9:1). From the crystal structure, it is evident that several monomer units of the compound **1c** self-aggregate via intermolecular $\text{C}=\text{O}\cdots\text{H}-\text{N}$ hydrogen bonding interactions and $\pi-\pi$ stacking interactions among the aryl moieties, thereby resulting in the formation of a stacked antiparallel framework. Two such skeletons further connect through a methanol bridge to render a supramolecular assembly with a hollow nanotubular cavity (Figure 3.2). It is interesting to note that the hydrophobic aryl moieties containing the $-\text{CF}_3$ groups face outwards. On the other hand, the polar groups are directed inwardly towards the cavity, which is optimal for the compound to sit and span the lipid bilayer membrane resulting in a static hydrophilic path across it. Furthermore, we also recorded field emission scanning electron microscopy (FESEM) data for **1c** by preparing the sample in methanol

(Figure 3.10). Interestingly, a well-distinct multilayer sheet-type morphology was observed, which validates the substantial self-assembly property of the hydrazide **1c**.

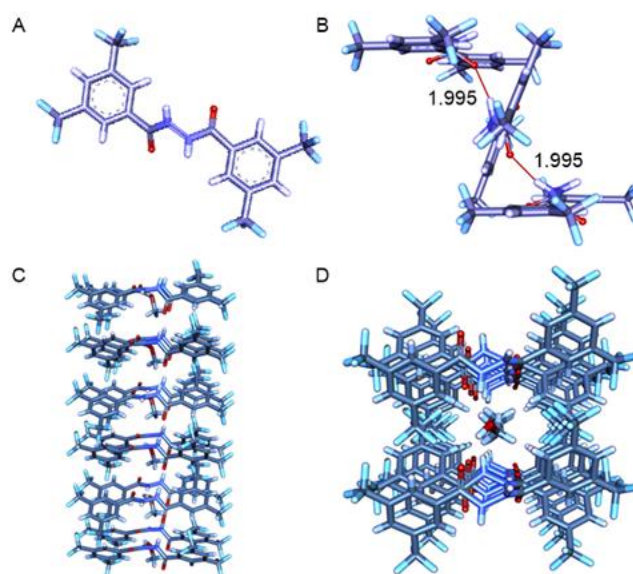


Figure 3.2 Crystal structure of compound **1c** (A). Inter-molecular hydrogen bonding interactions among the monomer units of **1c** (B). Side view (C) and the top view (D) of the self-assembled architecture of **1c** in the presence of methanol.

3.2.3 Ion Transport Activities

Fluorometric analyses were conducted to determine the ion transport activities of **1a–1c** across large unilamellar egg yolk phosphatidylcholine vesicles (EYPC-LUVs). These vesicles were prepared by entrapping 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) dye, followed by the application of a pH gradient prior to the addition of the transporters.^{22–27} The comparison of ion transport activity of compounds **1a–1c** at 20 μM concentration provided the sequence: **1c** $\gg$ **1b** $>$ **1a** (Figure 3.3A). The activity sequence suggests that $-\text{CF}_3$ groups on the aryl moiety of compound **1c** play a crucial role in increasing the lipophilicity of the compound, which in turn favors the efficient membrane permeation of the self-assembled channel.²⁸ The concentration-dependent activity profile followed by Hill analysis provided the effective concentration at 50% activity, $EC_{50} = 0.44 \mu\text{M}$, and Hill coefficient $n = 2.28$. The Hill coefficient suggests that a dimeric structure is responsible for constructing the basic unit of the supramolecular nanotubular assembly. Further ion transport studies were carried out with the most active derivative, **1c**.

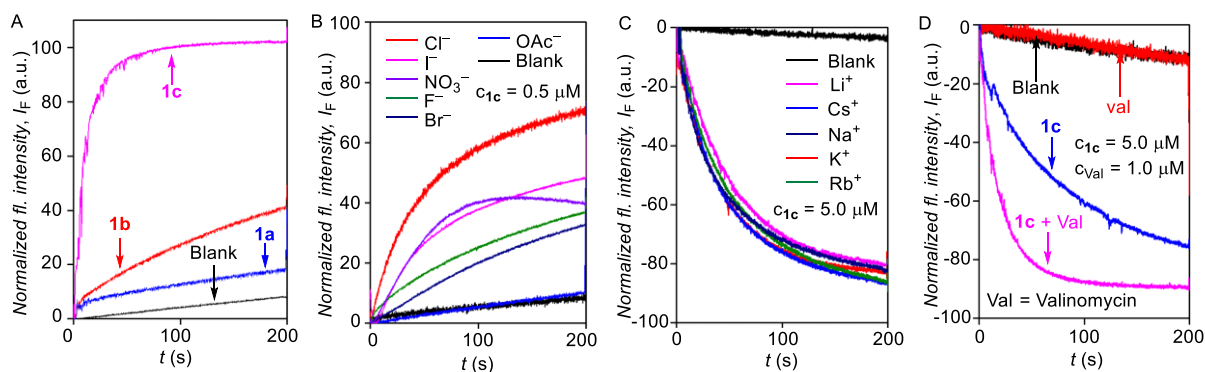


Figure 3.3 The comparison of ion transport activities of **1a–1c** in the HPTS assay at 20 μM transporter concentration (A). The ion transport activity of **1c** in the HPTS assay by varying the extravesicular anions, i.e., F^- , Cl^- , Br^- , I^- , NO_3^- , and OAc^- (B). The ion transport activity of **1c** in the lucigenin assay by varying the extravesicular cations, i.e., Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+ (C). The ion transport activity of **1c** in the lucigenin assay in the absence and the presence of valinomycin (D).

To study the anion selectivity of compound **1c**, different extravesicular buffer solutions containing Na^+ salts of varied anions (i.e., Cl^- , Br^- , I^- , F^- , OAc^- , and NO_3^-) and intravesicular buffer solutions containing NaCl were used, and the change in the fluorescent intensity was monitored using the aforementioned HPTS assay. The change of anions resulted in the sequence: $\text{Cl}^- > \text{I}^- > \text{NO}_3^- > \text{F}^- > \text{Br}^- \gg \text{OAc}^-$ with the highest selectivity toward Cl^- (Figure 3.3B). Therefore, we evaluated the influx of Cl^- across EYPC-LUVs having entrapped lucigenin dye.^{22, 29} Upon creation of a gradient of Cl^- in the extravesicular buffer versus NO_3^- in the intravesicular buffer, followed by the addition of **1c**, a significant influx of Cl^- ions was noted based on the observed quenching of the lucigenin fluorescence intensity (Figure 3.3C). Such Cl^- influx was not influenced by the variation of alkali metal cations (i.e., Li^+ , Na^+ , K^+ , Rb^+ and Cs^+) in the extravesicular buffer (Figure 3.3C), ruling out the role of cations in the overall transport process.

The lucigenin-based cation selectivity assay helped us to rule out the possibility of an M^+/Cl^- symport mechanism. To understand the operative ion transport mechanism further, valinomycin-coupled lucigenin assays were performed.³⁰ Valinomycin is an electrogenic K^+ selective transporter, i.e., it leads to a change in the net charge across the bilayer membrane during the transport process. In a symport mechanism (i.e., M^+/Cl^- co-transport), the transporter molecule alone can transport a cation along with Cl^- maintaining the charge neutrality, and hence the addition of valinomycin along with the transporter does not increase the rate of the transport process. However, in an antiport mechanism (i.e., Cl^-/A^- exchange),

there should be an enhancement in the rate of Cl^- influx in the presence of valinomycin as compared to the transporter alone so as to maintain the overall charge neutrality. Adding KCl to the extravesicular buffer, followed by adding valinomycin and transporter **1c** sequentially, a faster Cl^- influx was observed compared to the experiment that excluded valinomycin (Figure 3.3D). These results suggest the synergistic effect of the transporter **1c** and valinomycin, which confirms that compound **1c** transports via the Cl^-/A^- antiport mechanism.

3.2.4 Ion Channel Formation

To confirm the formation of an active ion channel by the transporter **1c**, the electrical conductance across the planar lipid bilayer membrane comprised of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) lipid was recorded.^{22, 31-33} The lipid was painted at the orifice isolating the *cis* and *trans* compartments of the instrument filled with 1.0 M KCl solution. When compound **1c** (2 μM) was added to the *trans* chamber followed by the application of either positive or negative voltage, distinct and periodic channel opening and closing events were observed (Figure 3.4A), which confirms the active ion channel formation at the black lipid membrane (BLM). From these current traces, the single channel conductance value was calculated to be 25.0 ± 0.7 pS, and the channel diameter was found to be 4.92 ± 0.11 Å using Hille's equation.³⁴ In the presence of the symmetric KCl solutions (1.0 M each), the variation of current with voltage (I-V plot) showed a linear trend, indicating ohmic behavior for the channel formed by **1c** (Figure 3.4B), as would be expected given its non-dipolar nature. On the other hand, when

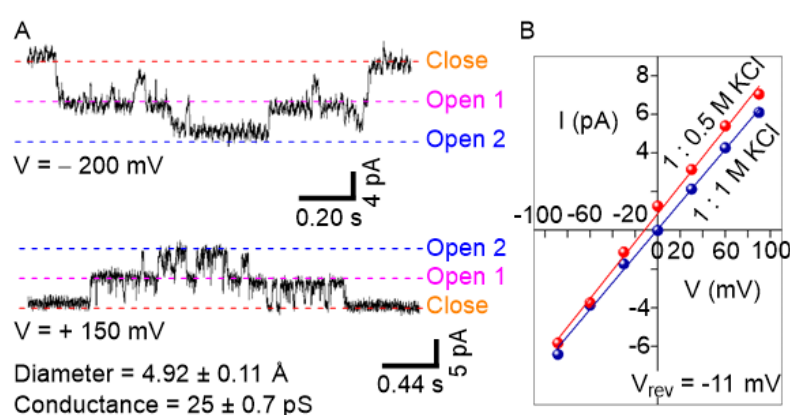


Figure 3.4 Current traces were recorded with **1c** ($c = 2.0$ μM) at -200 mV and $+150$ mV in a symmetrical KCl solution (A). Current–voltage relationship of **1c** in both symmetrical and asymmetrical KCl concentrations (B).

the ion selectivity was assessed using asymmetric solutions of KCl in the two compartments (0.5 M in *trans* and 1.0 M KCl in *cis*), and the reversal potential was found to be -11 mV (Figure 3.4B), which confirms that the conducting ion is Cl^- . The permeability ratio $P_{\text{Cl}^-}/P_{\text{K}^+} = 2.1$ was calculated following Goldman-Hodgkin-Katz equation³⁴, which supports the anion selectivity of the channel formed by **1c**.³⁵

3.2.5 Molecular Modeling of the Ion Channel

To have an atomistic perception of the nanochannel framework and the mode of translocation of the Cl^- ions, in-depth theoretical studies were performed. The self-assembled structure composed of six repetitive layers of compound **1c**, obtained from the crystal structure, was used for the molecular dynamics simulations (Figure 3.5A). To explore the structural dynamics and permeation of ions through the channel, the complex was inserted into the diphytanoylphosphatidylcholine (DPhPC) lipid membrane with the help of a membrane builder in CHARMM-GUI^{36, 37}. The general AMBER force field (GAFF)³⁸ was employed in modeling the ion channel formed by **1c** comprising twelve monomers arranged in six repetitive layers of the channel framework. For the DPhPC lipid bilayer, the parameters were adopted from the CHARMM36m force field.³⁹⁻⁴¹ The CHARMM TIP3P water model⁴² was employed for the water molecules present in the simulation.

An umbrella sampling simulation was performed to explore the free energy associated with the ion movement through the **1c** channel. To evaluate the mechanism and the free energetics related to the Cl^- transport through the synthetic channel, the movement of one Cl^- ion along the core of the channel from top to bottom of the self-assembled system was simulated. The free energy profile (Figure 3.5B) suggests that when the anion gets inserted into the channel to reach a compartment separated by a membrane, it experiences higher free energy (ΔG is 7 kcal/mol) compared to when it meets the water level. As the anion approaches either end of the channel, the free energy starts to decrease, which gives rise to an almost symmetric free energy profile of the anion transport, suggesting that there is a less entropic contribution of the ion channel system to the anion when it sits within the channel. Since the anion becomes less mobile within the channel as it provides structural confinement, it needs to pay more energy penalty to pass through the channel. Instead, when the ion resides in the water interface of the bilayer, the corresponding free energy decreases as it

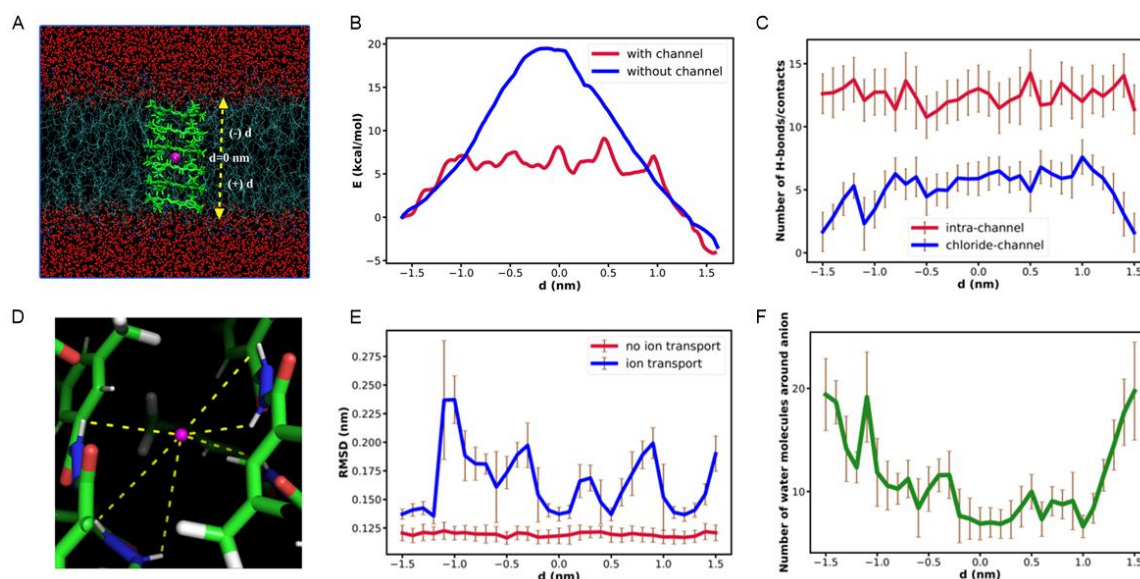


Figure 3.5 Snapshot of channel **1c** displaying the collective variable, d , used in umbrella simulation (A). Free energy profile corresponding to Cl^- transport via the channel and across the membrane without any channel embedded (B). The number of H-bonding among channel components (red curve) and the number of Cl^- interactions with channel residues (blue curve) with respect to d (C). Key interactions between the moving Cl^- ion and the channel residues (D). The channel's root mean square deviation from the crystal structure during Cl^- transport (blue) and when no Cl^- is transporting (red) across the channel (E). The number of water molecules adjacent to Cl^- ions against its movement through the channel (F).

serves solvation to the ion. In addition to the ion flexibility issue, the lesser availability of water within the core of the channel also stands as a reason for the rise in the free energy barrier. In the water compartment, the ion can be surrounded by ample water molecules, which would provide it with stabilization via the H-bonding interaction with water. This is the reason why the free energy associated with the ion transport gets enhanced as the ion enters the ion channel of certain confinement and with an environment of fewer water molecules. However, when the free energetics of anion transport was compared with the neat membrane, i.e., without the channel inside (ΔG is 20 kcal/mol), it was found that the free energy barrier for the movement of ion gets significantly reduced in the presence of the channel formed by **1c**. Thus, the ion chose to travel via the channel-embedded membrane. Again, the identification of fine curvatures of the free energy profile and its periodic nature

could be correlated with the pronounced effect of the chemical structure of the channel on the ion transport energetics.

To investigate the fundamental interactions responsible for the movement of ions, the intra-channel hydrogen bonding interactions (among the residue components of the channel itself) during the transport of the ions through the channel were analyzed (Figure 3.5C). It was observed that the H-bond forming components get aligned along the edges of the channel, i.e., there is no considerable amount of hindrance near the central core region of the channel for the translocation of ions. In fact, the channel core remains sufficiently broad to allow the smooth passage of the ions through it. Hence, the ions do not need to pay much penalty to break the existing H-bonds of the channel to widen the path of its favorable transport. To elucidate this, we calculated the interactions among the residues of the channel itself and with the passing anions, which may act as the key interactions behind the migration of ions through the supramolecular channel structure. Figure 3.5C shows that the intra-channel H-bonds do not get substantially affected when the ion travels through the channel. Also, no appreciable change in the intra-channel H-bonding was observed with the progression of the ions; instead, the ion proceeded by forming its own contacts with the channel residues (Figure 3.5D).

To explore the structural deformation of the channel framework due to the formation of new ion contact with the channel residues, the root mean square deviation of the channel was calculated with respect to the corresponding crystal structure. No noticeable deformation of the channel was detected with respect to its crystal structure when no anion traveled through it (Figure 3.5E). Further, to understand the direct effect of the ions on the structural framework of the channel, the RMSD during the movement of the ions was compared with the RMSD of the channel where no ions were passing through (in equilibrium simulation). The change in RMSD was found to be low (Figure 3.5E), and the channel did not lose its integrity throughout the process of transport, which indicates that the supramolecular assembly gets somewhat distorted for favorable interactions with the ions facilitating their smooth transport. Next, we investigated the influence of water molecules surrounding the anions within the ion channel during the transport process. An ample amount of water molecules near the anions within the channel were traced (Figure 3.5F). Since the core of the channel is broad enough, it could accommodate water molecules through its interior. Although going from either end of the channel to the center of it, the number of water

molecules embracing the anion decreased gradually; however, the path of the ion transport did not become fully dehydrated by the narrow confinement of the channel. This, in turn, could maintain a comfortable hydrated environment around the ions to ease their transport through the core of the channel.

3.2.6 Effect on Cellular Systems

Prompted by the excellent ion transport property of compound **1c** in vesicular systems, we went ahead to investigate the effect of transporter **1c** in cellular systems. Recent studies revealed that synthetic Cl^- ion transporters are capable of inducing apoptosis in cells by disrupting ionic homeostasis.^{11, 43-46} To explore the potential application of channel **1c** in biomedical science, MTT assays were conducted on various human-derived cancerous and human/non-human-derived non-cancerous cell lines of different origins. These included human lung adenocarcinoma epithelial cells-A549, human breast cancer cells-MCF7, triple-negative human breast cancer cells-MDA-MB-231, human osteosarcoma cells-U2OS, colon adenocarcinoma cells-SW480, human cervical cancer cells-HeLa; and the non-cancerous human embryonic kidney cells-HEK293T, and mouse embryonic fibroblasts (MEFs). As can be observed from Figures 3.6A and 3.16, compound **1c** showed significant toxicity in all cancerous cell lines, with the highest toxicity observed in MDA-MB-231 cells ($IC_{50} = 9.6 \mu\text{M}$). Interestingly, a significant increase in the percentage of cells in the G1 phase was observed in **1c**-treated MDA-MB-231 cells compared to control cells (Figure 3.17). In contrast, marginal toxicity was observed in the non-cancerous HEK293T cells and MEFs. Since maximum toxicity was observed in MDA-MB-231 cells, all further experiments were performed using these cells.

To understand the mechanism by which compound **1c** was mediating cytotoxicity in MDA-MB-231 cells, further studies were performed, which focused on studying the efficacy of compound **1c** in transporting Cl^- ions into the intracellular matrix.^{11, 16, 17, 22, 45, 47, 48} The fluorescence of MQAE – a cell-permeable Cl^- ion-sensitive dye – gets quenched in the presence of Cl^- ions, thereby indicating intracellular Cl^- transport. A time-and-dose-dependent quenching of MQAE fluorescence ($\lambda_{\text{ex}} = 350 \text{ nm}$ and $\lambda_{\text{em}} = 460 \text{ nm}$) was observed (Figure 6B). These results indicate that the artificial ion channel formed by compound **1c** is indeed capable of transporting Cl^- ions across the cell membrane.

To further assess the role of Cl^- ion transport in inducing cytotoxicity in cancer cells, the viability of MDA-MB-231 cells was measured using extracellular media containing or lacking Cl^- ions in the presence of compound **1c**. To achieve the same, two different HBSS buffers (Hank's balanced salt solution)^{45, 49} with and without Cl^- were prepared. The cells were incubated with compound **1c** for 24 h in the presence of these buffers. As can be seen from Figure 3.6C, higher cytotoxicity was observed in MDA-MB-231 cells treated with compound **1c** in HBSS buffer containing Cl^- ions, thereby confirming the role of Cl^- transport in mediating cytotoxicity.

Previous reports from our group⁵⁰ and others^{13, 51} have demonstrated that disruption of cellular Cl^- ion homeostasis is associated with the induction of apoptotic pathways in cells. To gain further insights into the cytotoxicity mediated by **1c**, mitochondrial membrane potential (MMP) was monitored in MDA-MB-231 cells using JC1 dye. JC1 is an MMP-sensitive probe that shows red fluorescence emission due to the formation of *J*-aggregates in cells with healthy mitochondrial membranes; while depolarised mitochondrial membranes do not accumulate JC-1 and as a result, the dispersion of the dye in the cytosol leads to green fluorescence emission.^{52, 53} Figure 3.6D represents quantitative data depicting a dose-dependent decrease in the ratio obtained from red/green fluorescence measurements for JC1 dye when MDA-MB-231 cells were preincubated with **1c** (0, 5, 10, and 20 μM). This data is supported by live cell images of JC-1-stained MDA-MB-231 cells which showed red fluorescence under control conditions (Figure 3.6E). On the other hand, treatment of these cells was associated with a sharp decrease in the red fluorescence along with a concomitant increase in the green fluorescence, thereby indicating a disruption in MMP by **1c** in these cells (Figure 3.6F).

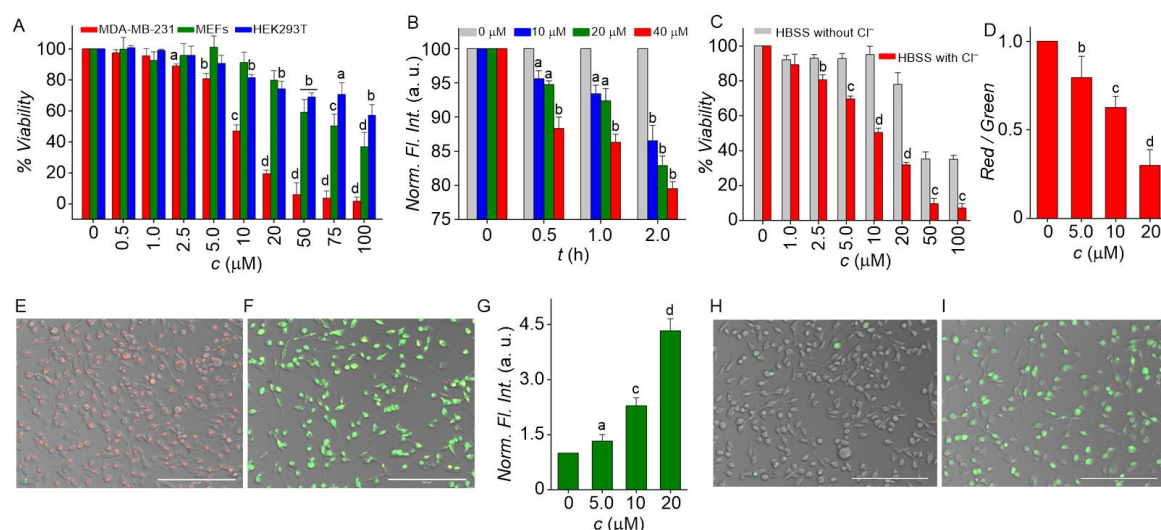


Figure 3.6 Compound **1c** affects cellular viability. Assessment of cellular viability of cancer and non-cancerous cells in the presence of **1c** by MTT assay (A). Cl^- uptake by MDA-MB-231 cells as estimated by MQAE fluorescence-based assay in the presence of **1c** (B). Viability of MDA-MB-231 cells upon treatment with **1c** in the presence/absence of Cl^- ions in the extracellular medium (C). Mitochondrial membrane potential as assessed by fluorescence intensity of JC-1 dye in the presence of **1c** in MDA-MB-231 cells (D). Representative live-cell images of MDA-MB-231 cells upon treatment with 0 μM (E) and 10 μM of **1c** (F) followed by staining with JC-1 dye. Red and green channel images were merged to generate the displayed image. Mitochondrial ROS levels, as estimated by MitoSox red in the presence of **1c** in MDA-MB-231 cells (G). The representative live-cell images of H_2DCFDA dye-stained MDA-MB-231 cells upon treatment with 0 μM (H), 10 μM (I) of **1c** have been shown. *a* represents $p < 0.05$; *b* represents $p < 0.01$; *c* represents $p < 0.001$; and *d* represents $p < 0.0001$ using Tukey HSD test when compared to untreated control.

Perturbation in mitochondrial membrane potential and dynamics has been associated with increased oxidative stress and build-up of reactive oxygen species (ROS).^{54, 55} Indeed, there was increased mitochondrial ROS levels (as measured by MitoSox red; Figure 3.6G) and cellular ROS levels (as depicted by increased green fluorescence using H_2DCFDA probe; Figure 6H, I) in MDA-MB-231 cells treated with **1c**.

A build-up of ROS levels in the cells leads to the release of apoptotic factors, which eventually rupture the electron transport chain in the mitochondrial respiratory cycle.^{56 55, 57} Along these lines, the release of cytochrome c from the mitochondria into the cytosol was studied by immunostaining MDA-MB-231 cells with cytochrome c specific primary antibody. Treatment of these cells with **1c** led to the release of cytochrome c from the mitochondria to the cytosol (Figure 3.7B, C) compared to the untreated cells (Figure 3.7A).

This was associated with a dose-dependent increment in the cleaved poly(ADP-ribose) polymerase (PARP) (86 kDa), a hallmark of apoptosis (Figure 3.7D, E).⁵⁸ All these results confirmed that **1c** induces apoptosis in MDA-MB-231 cells.

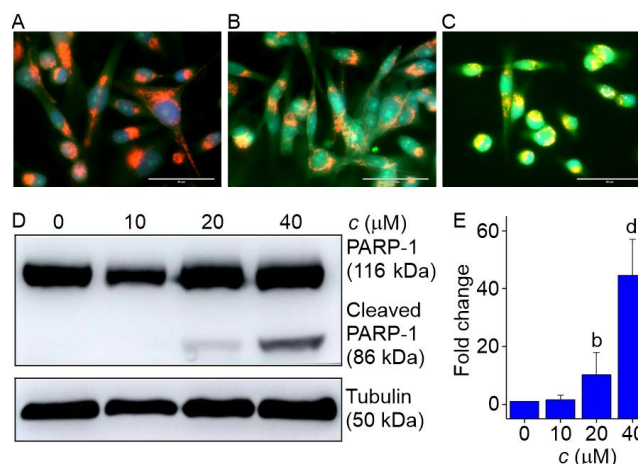


Figure 3.7 Representative live-cell images of MDA-MB-231 cells treated with 5 μM (B), and 10 μM (C) of **1c** for 24 h, and untreated cells (A) analyzed for cytochrome c release by immunostaining with cytochrome c specific primary antibody (green). Cells were counterstained with phalloidin (red) and DAPI (blue). Immunoblot assay for PARP-1 in MDA-MB-231 cells, after 24 h incubation with different concentrations of **1c** (D). Densitometric analysis (N = 3) of the expression of cleaved PARP-1 with respect to tubulin (used as reference gene) using ImageJ (E). *a* = *, *b* = **, *c* = ***, *d* = **** (* represents $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$) when compared to untreated control.

Typically, the pH of the lumen of the lysosomes is maintained at ~ 5.0 under physiological conditions. This feat is accomplished by maintaining the Cl^- concentrations of ~ 80 mM with the help of lysosomal Cl^- ion transporters.⁷ A reduction in lysosomal activity is observed when the lysosomal pH exceeds 5.0 and has been associated with a disruption in the activity of lysosomal enzymes and inefficient autophagy.^{7, 10} Since in our experiments **1c** showed efficient Cl^- ion transport activity, its effect on the lysosomal pH and autophagy was studied. Acridine orange (AO) is a membrane-permeable pH-sensitive dye which, when accumulated in the acidic compartment of the cellular organelles such as lysosomes, emits orange fluorescence while showing green emission at a higher pH, such as in cytosol.⁴⁴ A decline in the red fluorescence with an increase in the green fluorescence was observed (Figure 3.8A, B) when MDA-MB-231 cells were treated with **1c** (10 μM) followed by the incubation with AO. These results suggest that **1c** mediated Cl^- transport induced alkalization of lysosomal pH. This was associated with increased expression of microtubule-associated protein 1 light

chain 3-II (LC3-II) and p62 by immunoblot assays in **1c** treated cells (Figure 3.8C, D), thereby indicating disruption of autophagic processes in these cells.⁵⁹

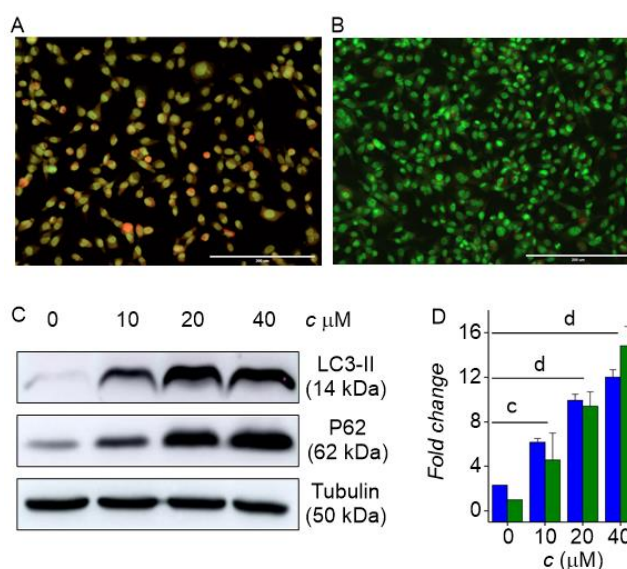


Figure 3.8 Representative live-cell images of MDA-MB-231 cells upon treatment with 10 μM (B) of **1c** for 8 h and untreated cells (A) followed by staining with AO dye. Red and green channel images were merged to generate the displayed image. Immunoblot assay for p62 and LC3-II in MDAMB-231 cells after 24 h incubation with different concentrations of **1c** (C). Densitometric analysis (N = 3) of the expression of P62 (Blue) and LC3-II (Green) with respect to tubulin using ImageJ (D). *a* = *, *b* = **, *c* = ***, *d* = **** (* represents $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$).

3.3 Conclusion

In summary, we introduced a benzoylbenzohydrazide compound **1c** which self-assembles via intermolecular H bonding and π - π stacking interactions to form an oligomeric supramolecular channel lumen that allows efficient and selective transmembrane translocation of Cl^- ions. In-depth ion transport studies revealed the Cl^-/A^- antiport mechanism as the operative mode of action. In addition to electrophysiological experiments, detailed MD simulations were also carried out to confirm the formation of a stable ion channel across the membranes at ambient temperatures and pressures.

Cellular studies using cell lines of cancerous and non-cancerous origin from human and nonhuman sources showed that the Cl^- ion transport mediated by compound **1c** imparted toxicity in cancerous cells but was relatively non-toxic in cell lines of non-cancerous origin. The increased intracellular Cl^- ion concentrations induced by compound **1c** altered the

mitochondrial membrane potential, which was associated with increased ROS levels in MDA-MB-231 cells. This resulted in the release of cytochrome c from the mitochondria into the cytosol, thereby initiating the process of apoptosis. In addition to this, autophagic processes in the cancer cells were disrupted due to alteration in lysosomal pH. Taken together, our results suggest that transporter **1c** is capable of inducing apoptosis as well as disrupting autophagy, a combination seldom seen in cancer-targeting drugs. Research on the development of small molecule ion transporters that inhibit autophagy, pioneered by Prof. Shin, Sessler, Gale, and colleagues, would be significantly advanced by this work on self-assembled artificial ion channels.

3.4 Experimental Section

3.4.1 General Methods

All chemicals, solvents and fluorescent dyes were purchased from commercial sources (Sigma-Aldrich, Alfa Aesar, Fisher Scientific, Spectrochem, and Avra). All commercial reagent grade chemicals were used as received without further purification unless otherwise specified. All deuterated solvents, HPTS dye, HEPES buffer, Triton X-100, sodium hydroxide and inorganic salts of molecular biology grade were obtained from Sigma-Aldrich. Egg-yolk phosphatidylcholine (EYPC) lipid as a solution in CHCl_3 (25 mg/mL), polycarbonate membrane (100 nm and 200 nm pore size), and mini-extruder used for vesicle preparation was obtained from Avanti Polar Lipids. All the Stock solutions were prepared in either HPLC grade DMSO, methanol or acetonitrile.

3.4.2 Physical Measurements

^1H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra were recorded at 101 MHz. All the spectra were calibrated with respect to the residual solvent peak. The following abbreviations were used to describe peak patterns wherever appropriate: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants are reported in hertz (Hz). High-resolution mass spectra (HRMS) were performed on an electron spray ionization time-of-flight (ESI-TOF). Melting points were measured with a micro melting point apparatus. Fluorescence spectra were recorded from Fluoromax-4 from JobinYvon Edison equipped with an injector port and a magnetic stirrer. Measurements of pH were done using a Hanna pH meter. All data from fluorescence studies were processed by Origin 8.5 software. Planar

bilayer conductance (BLM) measurements were done on a BLM instrument from Warner Instrument, USA.

3.4.3 Synthesis procedures

Synthesis of compound 3, 8, and 11: Synthesis of these compounds were done according to known protocol.⁶⁰

Synthesis of compound 4: Synthesis of compound 4 was done according to known protocol.⁶⁰

General procedure for the synthesis of compounds 5, 9, and 12: Aryl benzohydrazide (2.94 mmol) in methanol (10 mL) and 56% hydrazine hydrate (33 equivalents) were heated using an oil bath at 60 °C for 12 hours in a 50 mL round bottom flask. After cooling to room temperature, the precipitate was filtered off and washed several times with water and dried to give buff white solids (77–85%). Because of the instability of the benzohydrazides, the products thus obtained were used in subsequent reactions without further purification.

Synthesis of compound 6: Synthesis of compound 6 was done according to known protocol.⁶¹

General Procedure for the synthesis 1a-1c: In a 50 mL round bottom flask, to a solution of aryl benzohydrazides (0.99 mmol) and aryl acids (0.99 mmol) in THF (15 mL) was added hydroxybenzotriazole (1.5 mmol) followed by 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC·HCl) (1.5 mmol). The reaction mixture was then stirred for 15 hours at room temperature. After completion (monitored by TLC), THF was evaporated in vacuo, and the crude residue was dissolved in EtOAc (50 mL). It was then washed with water (2 × 25 mL) followed by brine solution (25 mL), and the collected organic phase was dried over Na₂SO₄. The solvent was then removed under reduced pressure to obtain solid residues. The crude solids were then purified through silica gel column chromatography using EtOAc:Hexane to obtain pure compounds **1a–1c** as a white solid (88–91%).

Characterization data of 1a: Purified using EtOAc:Hexane (10:90 (v/v)); White solid, Yield 90%, 178 mg, ¹H-NMR (400 MHz, CDCl₃): δ 9.54 (s, 2H), 7.97 – 7.62 (m, 4H), 6.99 – 6.74 (m, 4H), 3.99 (t, *J* = 6.5 Hz, 4H), 1.84 – 1.71 (m, 4H), 1.56 – 1.43 (m, 4H), 0.98 (t, *J* =

7.4 Hz, 6H). **¹³C NMR (101 MHz, CDCl₃):** δ 164.9, 162.4, 129.4, 123.5, 114.4, 68.0, 31.2, 19.3, 13.9. **IR (Neat, v/cm⁻¹):** 3736, 3196, 2955, 2930, 2870, 1742, 1679, 1640, 1602, 1576, 1506, 1452, 1391, 1286, 1244, 1184, 1168, 1107, 1067, 1025, 1000, 966, 898, 835, 800, 753, 691, 660, 621. **HRMS (ESI):** m/z : [M + H]⁺ calculated for C₂₂H₂₈N₂O₄ 385.2122; found: 385.2125.

Characterization data of 1b: Purified using EtOAc:Hexane (15:85 (v/v)); White solid, Yield 91%, 168 mg, **¹H-NMR (400 MHz, CDCl₃):** δ 10.87 (s, 2H), 8.12 (d, J = 8.1 Hz, 4H), 7.94 (d, J = 8.2 Hz, 4H), **¹³C NMR (101 MHz, CDCl₃):** δ 165.1, 136.6, 132.25 (q, J_{C-F} = 31.9 Hz), 128.8, 126.1, 124.3 (q, J_{C-F} = 272.6 Hz). **IR (Neat, v/cm⁻¹):** 3730, 3390, 1740, 1615, 1575, 1485, 1445, 1350, 1270, 1166, 1120, 845, 744, 687, 671, 619. **HRMS (ESI):** m/z : [M + H]⁺ calculated for C₁₆H₁₀F₆N₂O₂ 377.0719; found: 377.0724.

Characterization data of 1c: Purified using EtOAc:Hexane (5:95 (v/v)); White solid, Yield 88%, 166 mg, **¹H-NMR (400 MHz, CDCl₃):** δ 11.30 (s, 2H), 8.58 (d, J = 1.7 Hz, 4H), 8.45 (s, 2H), **¹³C NMR (101 MHz, CDCl₃):** δ 162.9, 134.2, 130.82 (d, J_{C-F} = 33.5 Hz), 128.3, 123.03 (q, J_{C-F} = 273.2 Hz), 125.9. **IR (Neat, v/cm⁻¹):** 3739, 3195, 1743, 1627, 1590, 1490, 1451, 1366, 1280, 1178, 1122, 909, 847, 759, 744, 699, 680, 625. **HRMS (ESI):** m/z : [M + H]⁺ calculated for C₁₈H₈F₁₂N₂O₂ 513.0467; found: 513.0484.

3.4.4 Single Crystal X-ray Crystallographic Study

Compound **1c** was crystallized from methanol at room temperature. Bruker KAPPA APEX II CCD Duo diffractometer (operated at 1500 W power: 50 kV, 30 mA) using graphite-monochromated Mo K α radiation (λ = 0.71073 Å) was used to collect single-crystal X-ray data at 100 K. SAINT software was used to integrate and reduce the data. SHELXTL was used to solve all the structures and refined on F^2 by a full-matrix least-squares technique using the SHELXL-97 program built in the Apex-3 package⁶². Hydrogen atoms were constructed in geometric positions to their parent atoms.⁶³ *Adsym* subroutine of PLATON was used to confirm that there is no additional symmetry possible. The CCDC number for compound **1c** is 2236157. This data is available at The Cambridge Crystallographic Data Centre.

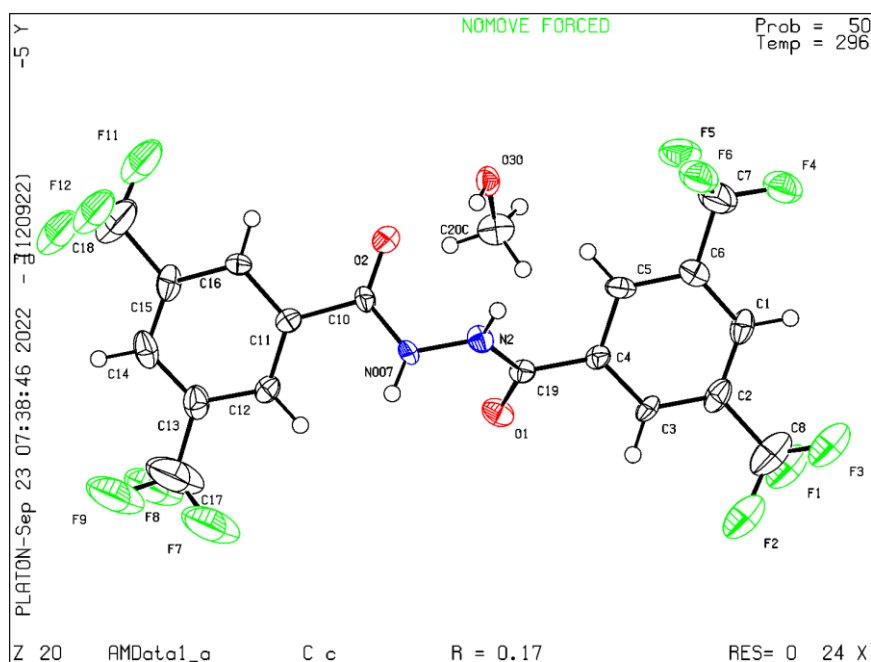


Figure 3.9 ORTEP diagrams of the structure **1c** with 50% probability ellipsoids established by single X-ray crystallography.

Table 3.1. Data collection parameters for **1c**.

Chemical formula	C ₁₉ H ₁₂ N ₂ O ₃ F ₁₂
Formula weight	544.31 g/mol
Temperature	296(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	Cc
Unit cell dimensions	$a = 31.664(4)$ Å
	$b = 6.8399(9)$ Å

	$c = 9.6409(13) \text{ \AA}$
	$\alpha = 90^\circ$
	$\beta = 98.888(4)^\circ$
	$\gamma = 90^\circ$
Volume	$2063.0(5) \text{ \AA}^3$
Z	4
Density (calculated)	1.753 g/cm^3
Absorption coefficient	0.190 mm^{-1}
$F(000)$	1088
Theta range for data collection	$2.604 \text{ to } 28.392^\circ$
Index ranges	$-42 \leq h \leq 42,$ $-7 \leq k \leq 9,$ $-12 \leq l \leq 12$
Reflections collected	16369
Independent reflections	5060 [$R(\text{int}) = 0.0341$]
Coverage of independent reflections	98.3%
Function minimized	$\Sigma w(F_o^2 - F_c)^2$
Data / restraints / parameters	5060 / 2 / 263
Goodness-of-fit on F^2	2.375

Δ/σ max	0.082
Final R indices	4314 data; $[I > 2\sigma(I)]$ $R_1 = 0.1674$, $wR_2 = 0.4321$
	all data , $R_1 = 0.1814$, $wR_2 = 0.4448$
Largest diff. peak and hole	1.180 and $-0.789 \text{ e}\text{\AA}^{-3}$
R.M.S. deviation from mean	$0.135 \text{ e}\text{\AA}^{-3}$

3.4.5 Field Emission Scanning Electron Microscopy (FESEM) Images

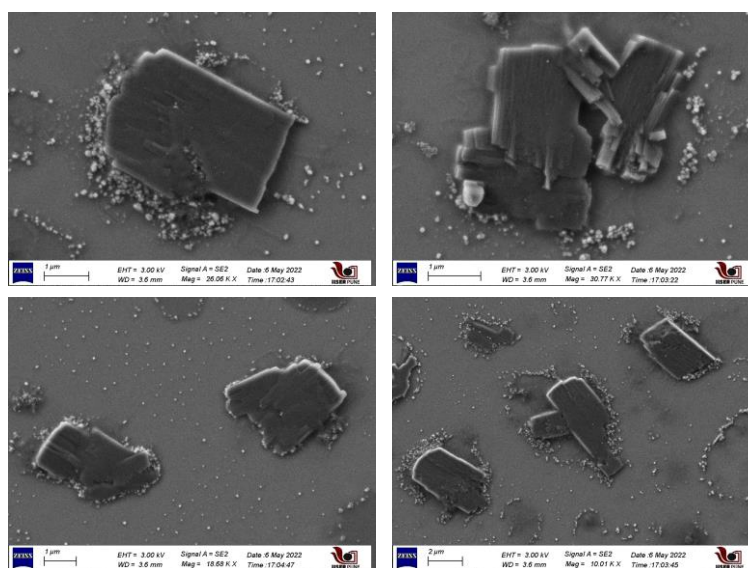


Figure 3.10 FESEM images of **1c** showing distinct multi-layered aggregation patterns recorded in methanol.

3.4.6 Dynamic Light Scattering Experiment (DLS):

To determine the dimension of the aggregation, DLS experiments were performed. A stock solution of compound **1c** was prepared in methanol and during the experiment, compound **1c** from this stock was added to water keeping a final concentration of $100 \mu\text{M}$. The experiment provided with a mean size of 6.28 nm .

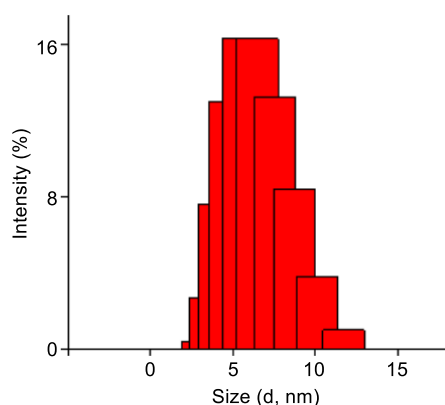


Figure 3.11 Size distribution of the aggregate by **1c** using DLS experiment.

3.4.7 Ion Transport Experiments

Ion Transport Activity and Selectivity

A. Ion transport across EYPC-LUVs $\Rightarrow$ HPTS:

Preparation of EYPC-LUVs $\Rightarrow$ HPTS: Vesicles were prepared following a reported protocol.²²

Final conditions: EYPC $\sim$ 5 mM. **Inside:** 10 mM HEPES, 100 mM NaCl, 1 mM HPTS, pH 7.0; **Outside:** 10 mM HEPES, 100 mM NaCl, pH 7.0.

Ion transport activity by HPTS assay: In a clean and dry fluorescence cuvette, 1975 μ L of buffer (10 mM HEPES, 100 mM NaCl, pH 7.0) was added, followed by the addition of 25 μ L EYPC-LUV $\Rightarrow$ HPTS vesicles. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The change in HPTS fluorescence intensity at $\lambda_{em} = 510$ nm ($\lambda_{ex} = 450$ nm) was monitored as a function of time. During the experiment, base (20 μ L, 0.5 M NaOH to create pH gradient) was added at $t = 20$ s, and transporter **1c** in DMSO was added at $t = 100$ s. Finally, 10% Triton X-100 (25 μ L) was added at $t = 300$ s to lyse the vesicles to achieve complete destruction of the pH gradient. The fluorescence intensity was monitored up to 350 s.²²

The time data were normalized according to Equation 3.1 and then the fluorescence intensity data was normalized to the percentage change in fluorescence intensity as a course of time using Equation 3.2:

$$t = t - 100 \quad (\text{Equation 3.1})$$

$$I_F = [(I_t - I_0) / (I_\infty - I_0)] \times 100 \quad (\text{Equation 3.2})$$

where, I_F is the normalized fluorescence intensity in percentage, I_0 is the initial intensity, I_t is the intensity at time t , and I_∞ is the final intensity after the addition of a Triton X-100.

For concentration-dependent ion transport data of each compound, the I_F values at 150 s after the addition of the transporter were plotted against respect concentration values. Subsequently, the plot was fitted with the Hill equation (Equation 3.3):

$$Y = Y_\infty + (Y_0 - Y_\infty) / [1 + (c / EC_{50})^n] \quad (\text{Equation 3.3})$$

where, Y_0 is the fluorescence intensity just before the transporter addition (at $t = 0$ s), Y_∞ is the fluorescence intensity with excess transporter concentration, c is the concentration of transporter compound, and EC_{50} is the effective concentration required to reach the 50% of the maximum activity.

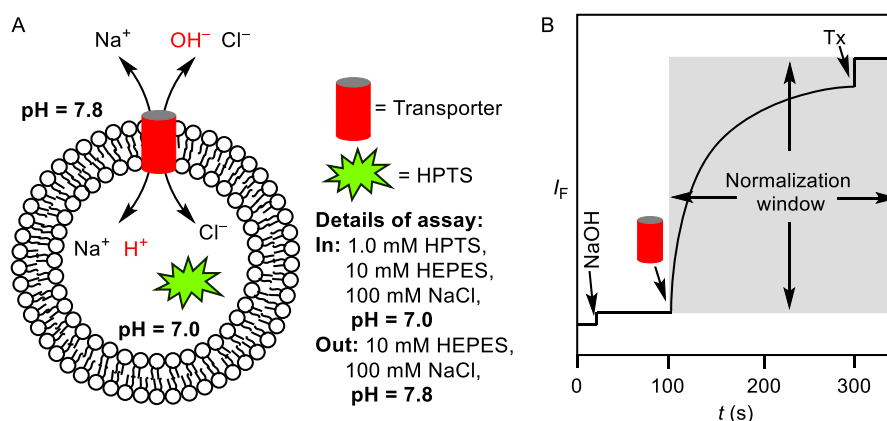


Figure 3.12 Representations of fluorescence-based ion transport activity assay using EYPC-LUVs (A) and representative ion transport kinetics showing normalization window (B).

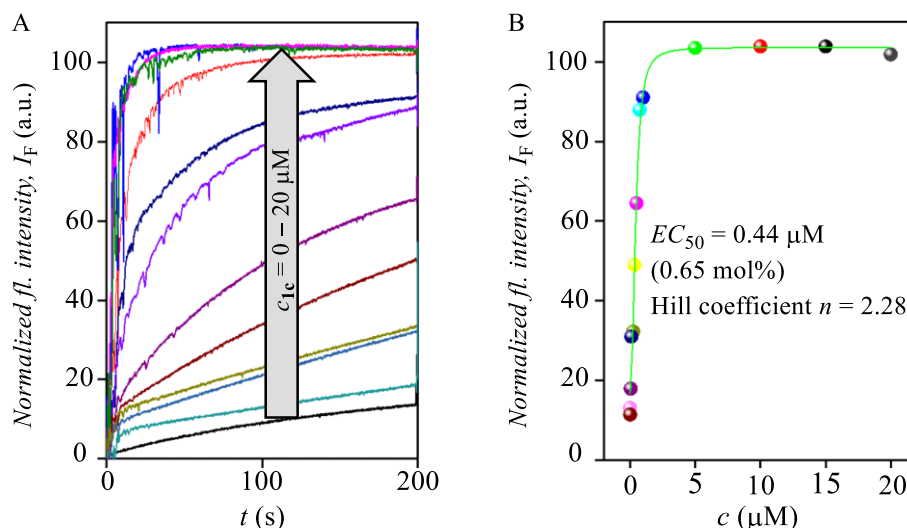


Figure 3.13 Ion transport activities of **1c** at concentrations 0–20 μM (A). Hill plot of compound **1c** (B).

Anion selectivity by HPTS assay:

Preparation of EYPC-LUVs $\supset$ HPTS: Vesicles were prepared following a previously reported protocol.²²

Final conditions: EYPC $\sim 5 \text{ mM}$. **Inside:** 10 mM HEPES, 100 mM NaCl, 1 mM HPTS, pH 7.0; **Outside:** 10 mM HEPES, 100 mM NaX, (where, $\text{X}^- = \text{F}^-, \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{OAc}^-, \text{and } \text{NO}_3^-$), pH 7.0.

Anion selectivity experiments: In a clean and dry fluorescence cuvette 1975 μL of buffer (10 mM HEPES, 100 mM NaX, pH 7.0; where, $\text{X}^- = \text{F}^-, \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{OAc}^-, \text{and } \text{NO}_3^-$) was added followed by the addition of 25 μL EYPC-LUV $\supset$ HPTS vesicles. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The change in HPTS fluorescence intensity at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$) was monitored as a function of time. During the experiment, base pulse (20 μL , 0.5 M NaOH to create a pH gradient of 0.8) was given at $t = 20 \text{ s}$, and transporter **1c** in DMSO was added at $t = 100 \text{ s}$. Finally, 10% Triton X-100 (25 μL) was added at $t = 300 \text{ s}$ to lyse the vesicles resulting in the complete destruction of the pH gradient. The fluorescence intensity was monitored up to 350 s. The collected data was then normalized according to Equations 3.1 and 3.2 to get the percentage change in fluorescence intensity as a function of time.²²

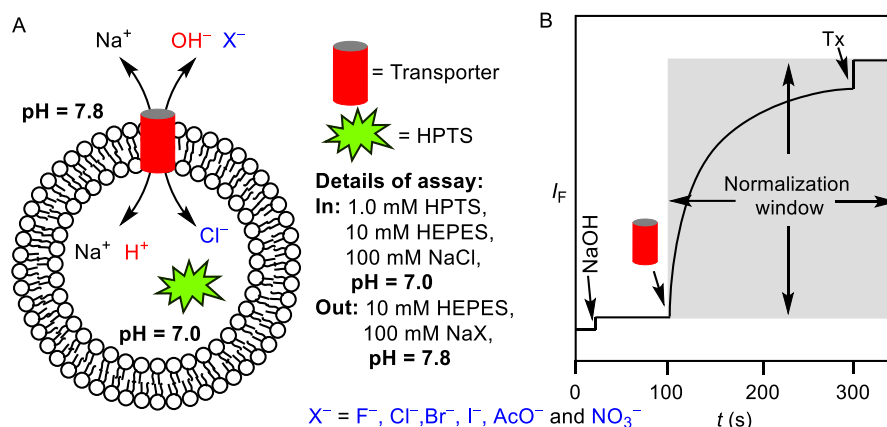


Figure 3.14 Representations of fluorescence-based ion transport activity assay using EYPC-LUVs (A) and representative ion transport kinetics showing normalization window (B).

Effect of extravesicular cations in chloride influx:

Preparation of EYPC-LUVs $\supset$ lucigenin: Vesicles were prepared following a reported protocol.²² Final conditions: ~ 3.3 mM EYPC; *inside*: 200 mM NaNO_3 , 1 mM lucigenin; *outside*: 200 mM NaNO_3 .

Cation selectivity using lucigenin assay: In a clean and dry fluorescence cuvette, 1975 μL of NaNO_3 solution (200 mM) was added, followed by the addition of 25 μL of EYPC-LUVs $\supset$ lucigenin. The cuvette was placed in the fluorescence instrument in a slow stirring condition by a magnetic stirrer equipped with the instrument (at $t = 0$ s). The time course of lucigenin fluorescence emission intensity, F_t was observed at $\lambda_{\text{em}} = 535$ nm ($\lambda_{\text{ex}} = 455$ nm). 33.3 μL of 2 M MCl ($\text{M}^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+ \text{ and } \text{Cs}^+$) was added to the cuvette at $t = 25$ s to create the chloride gradient between the intra and extra vesicular system. Transporter **1c** was added at $t = 100$ s, and finally, 25 μL of 10% Triton X-100 was added at $t = 300$ s to lyse those vesicles resulting in the complete destruction of the chloride gradient. The collected data was then normalized according to Equations S1 and S2 to get the percentage change in fluorescence intensity as a function of time.²²

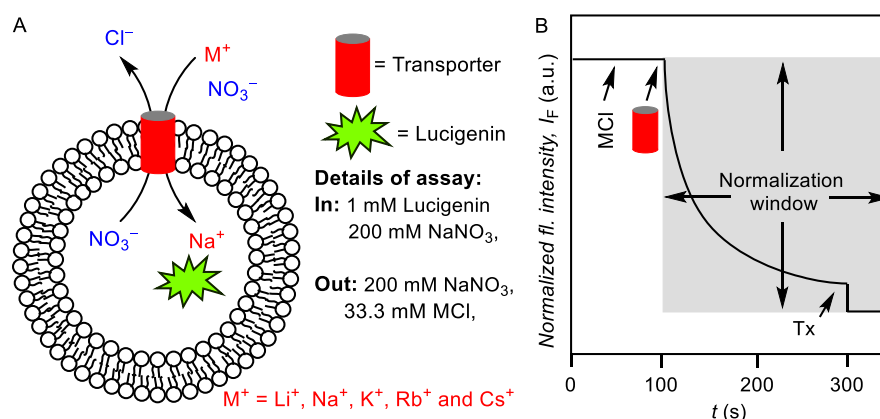


Figure 3.15 Representations of chloride influx activity across EYPC-LUVs containing lucigenin in the presence of different extravesicular cations (A). Illustration of chloride influx kinetics showing normalization window (B).

Ion transport activity in the presence of valinomycin:

Preparation of EYPC-LUVs containing lucigenin: The aforementioned lucigenin vesicles were used for the experiments.

Description of the assay:

In a clean and dry fluorescence cuvette, 1975 μL of NaNO₃ solution was added, followed by the addition of 25 μL of EYPC-LUVs containing lucigenin. The cuvette was placed in a fluorescence instrument in a slow stirring condition by a magnetic stirrer equipped with the instrument (at $t = 0$ s). The time course of lucigenin fluorescence emission intensity, F_t was observed at $\lambda_{\text{em}} = 535$ nm ($\lambda_{\text{ex}} = 450$ nm). 33.3 μL of 2 M KCl was added to the cuvette at $t = 25$ s to create the chloride gradient between the intra and extra vesicular system. Valinomycin (1.0 μM) was added to the cuvette at 50 s. After that **1c** was added at $t = 100$ s and finally, at $t = 300$ s 25 μL of 10% Triton X-100 was added to lyse those vesicles resulting in complete destruction of the chloride gradient. The collected data was then normalized according to Equations 3.1 and 3.2 to get the percentage change in fluorescence intensity as a function of time.

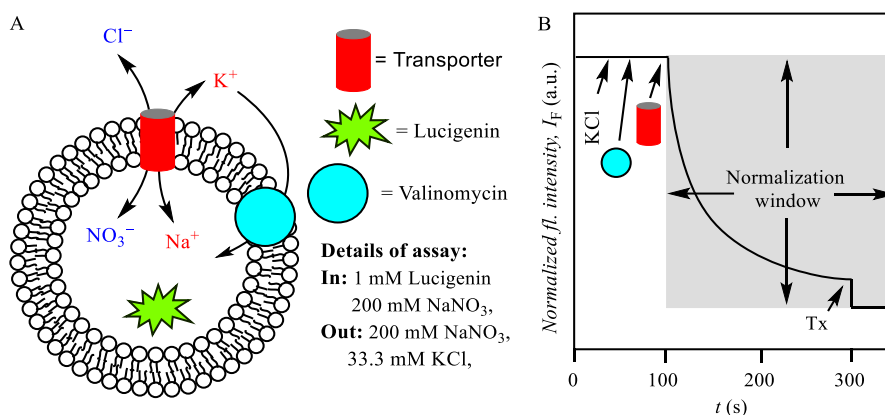


Figure 3.16 Representations chloride influx activity across EYPC-LUVs $\Rightarrow$ lucigenin in the presence and absence of valinomycin (A). Illustration of chloride influx kinetics showing normalization window (B).

3.4.8 Planner bilayer conductance measurement

The conductance measurement across the bilayer lipid membrane was done with the help of Warner instrument (USA). For that, a solution of diphytanoylphosphatidylcholine lipid (DPhPC; Avanti Polar Lipids) was prepared by dissolving it in *n*-decane of concentration 20 mg/mL. Then a bilayer membrane was formed by using this solution at the 150 μm diameter aperture within a cup made of polystyrene (Warner Instrument, USA). In both chambers (*cis* and *trans*) of the instrument, 1 M KCl was symmetrically injected. The setup was made in such a way that the *trans* compartment was held at virtual ground. The BC 535 head-stage (Warner Instrument, USA) connects the *cis* compartment through Ag-AgCl electrodes. Then the channel-forming molecule **1c** (2 μM) was added to the *trans* chamber. An inbuilt magnetic stirrer was used to stir the solution for 20 min. Channel formation through the lipid bilayer was confirmed from channel opening and closing events upon application of different voltages using pClamp11 software (Molecular probes, USA). An analogue-to-digital converter (Digidata 1440A, Molecular Devices) and pClamp11 software (Molecular probes, USA) were used to low pass filter the currents at 1 kHz.

The channel opening and closing events at positive as well as negative potentials were analyzed by pClamp 11 software. A complete trace comprised of a series of opening and closing events for the time period of 2 h from where a small fraction is presented in the manuscript.

The channel diameter was calculated using the equation (Hille equation) mentioned below.³⁴

$$1/g = (l + \pi d/4) (4\rho/\pi d^2) \quad \text{Eq. 3.4}$$

where, g is the corrected conductance, calculated by multiplying measured single-channel conductance with the Sansom's correction factor⁶⁴ 5.61; l is the length of the ion channel (34 Å), and ρ signifies resistivity of the KCl solution ($\rho = 10.1 \Omega \cdot \text{cm}$).

Determination of anion selectivity permeability ratio by Planar Bilayer Conductance

Measurements: The *cis* and *trans* chambers were filled with unsymmetric solutions of KCl. The *cis* chamber was filled with 1.0 M KCl solution and the *trans* chamber was filled with 0.5 M KCl. The compounds **1c** (2 μM) were added to the *trans* chamber and stirred for 5 minutes. The permeability ratio ($P_{\text{Cl}^-}/P_{\text{K}^+}$) was calculated by using the Goldman-Hodgkin-Katz equation (Equation 3.5).³⁴

$$\frac{P_{\text{Cl}^-}}{P_{\text{K}^+}} = \frac{a_{\text{K}^+ \text{ cis}} - a_{\text{K}^+ \text{ trans}} \times \exp\left(-\frac{zV_{\text{rev}} \times F}{R \times T}\right)}{a_{\text{Cl}^- \text{ cis}} \times \exp\left(-\frac{zV_{\text{rev}} \times F}{R \times T}\right) - a_{\text{Cl}^- \text{ trans}}} \quad \text{Eq 3.5}$$

where, $P_{\text{Cl}^-}/P_{\text{K}^+}$ = anion/cation permeability ratio; $a_{\text{K}^+ \text{ cis}}$ = K^+ activity in the *cis* chamber; $a_{\text{K}^+ \text{ trans}}$ = K^+ activity in the *trans* chamber;⁶⁵ $Z_{\text{K}^+} = +1$, $Z_{\text{Cl}^-} = -1$ $a_{\text{Cl}^- \text{ cis}}$ = Cl^- activity in the *cis* chamber; $a_{\text{Cl}^- \text{ trans}}$ = Cl^- activity in the *trans* chamber; V_{rev} = reversal potential; F = Faraday constant; R = gas constant; T = temperature (K).

3.4.9 MD Simulations:

Simulation Model and Method

System setup: Atomistic Molecular Dynamics simulation was performed for understanding the molecular mechanism of ion permeation through the ion channel framed by the self-assembly of hydrazide monomers. The study was done on the synthetic ion channel, which was built with six layers of hydrazide molecules, constituting two molecules in each layer (the channel structure comprises a total of 12 molecules in it). Prior to starting the simulation, the coordinates of the mentioned channel architecture were minimized by utilizing MAESTRO (<https://www.schrodinger.com/products/maestro>). To identify the structural dynamics and permeation of ion through the channel, the complex was inserted into the 1,2-

diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) lipid membrane with the help of a membrane builder in CHARMM-GUI.^{36, 66} With this protocol, we designed the system as membrane-embedded channel system surrounded with salt-water, by following six connective steps (described by Jo et al.⁶⁷) involving the reading of objects and then its orientation, system size determination, construction of lipid bilayer followed by further assembling of it, and finally the system equilibrium. The membrane network was constructed with total 144 molecules of DPhPC lipids distributed equally in each of the leaflets (72 molecules per leaflet of the membrane). The simulation box dimension was fixed at 7.6 x 7.6 x 8.5 nm³, and a total of 9408 water molecules were inserted on both sides of the membrane. 1.0 M KCl salt concentration (168 K⁺ and 168 Cl⁻ ions all total) was maintained in the water level of the ion-channel system.

The general AMBER force field (GAFF)^{66, 68} was employed in modelling the hydrazide ion channel comprising twelve monomers arranged in six repetitive layers of the channel framework. For the PhPC lipid bilayer, the parameters were adopted from the CHARMM36m force field.³⁹⁻⁴¹ The CHARMM TIP3P water model⁴² was employed for the water molecules present in the simulation.

Simulation protocol: In performing each of the simulations, periodic boundary condition (PBC) was used in all three dimensions. For handling long-range electrostatic interactions, Particle Mesh Ewald (PME) summation⁶⁹ combined with cubic interpolation was used, and 1.2 nm cutoff was applied to maintain short-range electrostatic interactions. At every 20 steps of the simulation, the neighbor list was updated. In constraining the bonds involving hydrogen atoms of the lipids and the channel residues, the LINCS algorithm³⁹ was occupied. The rigidity of water molecules was managed with the help of SETTLE⁷⁰ algorithm. The Lenard Jones interaction (up to 1.2 nm) were taken into account with the Verlet cutoff scheme⁷¹ combined with dispersion corrections. The simulation system was first energy minimized with the steepest-decent algorithm under sufficient restrain. Then the energy minimized system was subjected to six-step of restrained (with a continuous step-by-step decrease of restrain) equilibration for long 60 ns (10 ns in each step). For maintaining the temperature at 298.15 K during equilibration, the Berendsen thermostat⁷² was used, and pressure was kept constant at 1 bar by employing the Berendsen barostat. Finally, one microsecond long NPT production run was performed with the equilibrated system under a slight restrain for maintaining the integrity for the channel embedded in the lipid bilayer.

During the production run, 298.15 K of an average temperature was maintained by individual coupling of the channel, membrane, and solvent with the assistance of Nose-Hoover thermostat⁷³ under a relaxation constant of 1.0 ps. The Parrinello-Rahman barostat⁷⁴ was occupied for keeping the pressure fixed at 1 bar. For pressure coupling the time constant of 5 ps and compressibility of 4.5×10^{-5} bar was employed under semi-isotropic coupling (coupled separately along XY and Z directions) of the system in settling the lipid-bilayer to be tensionless. Leapfrog integrator utilized in the simulation (production run) with the time step of 2 fs. The last 155 ns of the entire trajectory was considered for performing the required analysis on the ion transport mechanism.

To explore the free energy associated with the ion movement through the hydrazide channel, we performed an umbrella sampling simulation. To evaluate the mechanism and the free energetics related to the anion (Cl^-) transport through the synthetic channel complex, the movement of one chloride ion along the core of the channel from top to bottom of the self-assembled system was simulated. To achieve this, the final equilibrated structure was taken and all the ions of the simulation system were removed, keeping only a pair of ions (one K^+ and one Cl^-) intact. Other than that, the simulation box size, the dimension of the channel and the number of water molecules were left unaltered. The initial configurations for each of the umbrella windows were generated by carrying the anion through the channel core along the Z-direction, starting from the mouth to the end of the channel. The Z-direction of the anion within the core of the channel (the whole channel without any hydrogen atom), d , is treated as the collective variable (cv) for umbrella sampling. The negative value of d means the ion initial is at the upper part of the channel (d would become more negative with the progress of the ion towards the mouth of the channel structure from the center of the same), while the position of the anion in the lower half of channel results in the positive value of d (the ion movement from the channel-center towards its end leads to increase in d) and $d = 0$ nm represents the presence of the anion at the center of the channel.

We noticed that the harmonic potential of force constants ranging between 5000 and 6300 kJ/mol/nm² were optimum at umbrella windows of cv in the range of -1.5 nm to 1.5 nm (with 0.1 nm spacing) for exploring the desired windows and sufficient overlap among the adjacent windows. For sampling each of the windows of umbrella simulation, energy minimization was done for each of the respective configurations by employing the steepest-decent algorithm. After energy minimization of the system, 500 ps of NVT equilibration (with 1 fs

simulation time step) was performed, followed by 20 ns of final production run (with a time step of 2 fs) in NPT ensemble. Similar to the above-mentioned equilibrium simulation, the temperature and the pressure in umbrella sampling also was controlled by the similar thermostat and barostat (in equilibration and production run) to maintain the temperature and pressure at 298.15 K and 1 bar, respectively. Finally, the weighted histogram analysis method (WHAM)⁷⁵ was applied for estimating the unbiased histograms and calculating the corresponding free energies by a combination of the data obtained from each independent trajectory of every umbrella window. Gromacs software of version 2018, 2018.2 and 2019 were used in performing the simulations.

Following a similar simulation protocol, another set of umbrella sampling simulations was carried out with the same composition of lipid membrane but devoid of any channel within it. In a similar way of generating configurations of previous umbrella simulations, here also, the initial configurations of each umbrella window were created by gradually taking the anion along Z-direction through the center of the bilayer from one end to the other end of the channel free membrane. For umbrella sampling of the membrane without a channel, the simulation system was incorporated with the same DPhPC lipid bilayer solvated with 8975 water molecules. At this time also, one K⁺ and one Cl⁻ ion was included with the system, the box size was kept constant at 7.6 x 7.6 x 8.5 nm³ and the same extent of force constant was used for sampling each of the windows.

Together with the computation of the transport process free energy corresponding to the synthetic channel, a comparison was made with the anion transport across the membrane, which lacked any ion channel embedded within it. Further studies were performed on the relevant interactions of anion and the channel components by calculating the average number of H-bonding and estimating of root mean square deviation (RMSD) of the ion channel (with respect to its original crystal structure).

3.4.10 Biological Studies

Cell lines used: Different human and nonhuman origin cell lines of cancerous and non-cancerous origin were used for the study. The human cancerous cell lines included: lung

adenocarcinoma – A549 (basal epithelial cell); breast cancer – MCF-7; triple negative breast cancer – MDA-MB-231; osteosarcoma – U2OS; colon adenocarcinoma – SW480; cervical cancer – HeLa; and the non-cancerous human line: HEK293T. In addition to these, mouse embryonic fibroblasts (MEFs) were also used as a control.

Cell culture: All cell lines used for the study were grown in high glucose containing Dulbecco's Modified Eagle Medium (DMEM; Lonza) containing 10% fetal bovine serum (FBS; Invitrogen), 2.0 mM L-glutamine (Invitrogen) and 100 units/mL penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were maintained in tissue culture-treated T25 flasks (Corning) at 37 °C in a humidified 5% CO₂ incubator (Thermo Scientific).⁷⁶ Cells were subcultured every 2 days once the flask attained 80% confluency.

MTT-based cytotoxicity assay: For assessment of cellular viability under the influence of **1c**, MTT assays were performed. Briefly, cells were dispersed in 96-well flat-bottom tissue culture-treated plates (Corning) at the density of 8×10^4 cells/well (per 100 µL) in complete DMEM media and were incubated at 37 °C in a 5% CO₂ incubator overnight for recovery. Post-recovery, the cells were treated with increasing concentrations of compound – **1c** (0 to 100 µM) by maintaining a maximum amount of DMSO at 2 µL/well and were further incubated for 24 h. Post incubation, the media containing the compounds was replaced with 100 µL of MTT solution (0.5 mg MTT/mL of PBS) and incubated 37 °C for 4 h. After 4 h, 100 µL of solubilization buffer (10% SDS containing 0.01 N HCl in PBS) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a multi-mode plate reader (HiDex). All experiments were performed in triplicates, and the relative cell viability (%) was expressed as a percentage of untreated cells (set as 100%).⁷⁶ The cellular viability has been depicted in Figure S8. Since maximum cytotoxicity was observed in MDA-MB-231 cells, all further experiments were carried out using these cells.

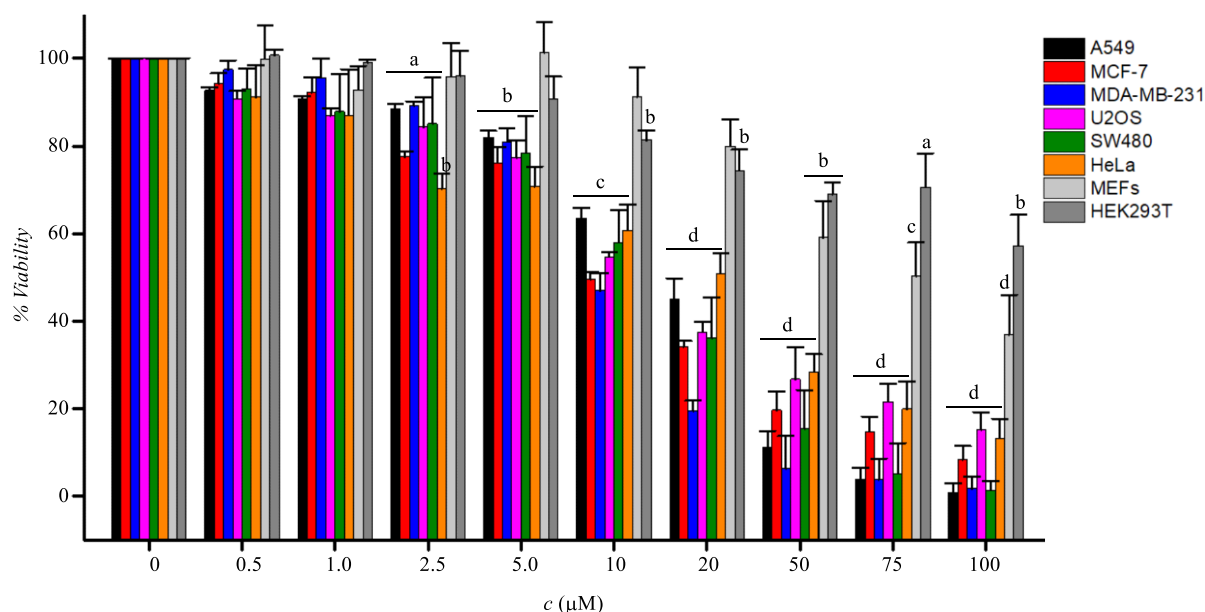


Figure 3.17 Cellular viability (as assessed by MTT assays) of A549 (Black), MCF-7 (Red), MDA-MB-231 (Blue), U2OS (Pink), SW 480 (Green), HeLa (orange), MEFs (light grey), HEK293T (dark grey) in response to compound **1c**. *a* represents $p < 0.05$ = *, *b* represent $p < 0.01$ = **, *c* represents $p < 0.001$ = ***, and *d* represents $p < 0.0001$ = **** (* represents $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$).

Table 3.2: IC_{50} values for compound **1c** against cell lines of cancerous and noncancerous origins.

Cell lines	A549	MCF-7	MDA-MB-231	U2OS	SW480	HeLa	MEFs	HEK293T
IC_{50} (μM)	14.5	11.1	9.6	14.0	12.1	14.5	> 50	> 50

Measurement of intracellular Cl^- ion concentration: Time-dependent influx data of Cl^- ions was measured in MDA-MB-231 cells by using chloride selective cell-permeable fluorescent dye *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE). Cells were dispersed in 96-well flat-bottom tissue culture-treated plates (Corning) at a density of 8×10^4 cells/well (per 100 μL) in complete DMEM media and were incubated at 37 $^\circ\text{C}$ in a 5% CO_2 incubator overnight for recovery. Post-recovery, MQAE was added to each well by maintaining the final concentration as 5 mM for a period of 3.0 h. The extracellular dye was removed by washing with 1X PBS (twice) and the plate was replenished with DMEM

containing compound **1c** at various concentrations (0, 10, 20 and 40 μM). The plates were further incubated for a total duration of 2 h at 37 °C in a 5% CO_2 incubator. MQAE fluorescence was measured using a multi-mode microplate reader (HiDex, $\lambda_{\text{ex}} = 350 \text{ nm}$ and $\lambda_{\text{em}} = 460 \text{ nm}$) at different time intervals (0, 0.5, 1 and 2 h).¹¹

Chloride mediated cytotoxicity assay

HBSS buffer solution. Hank's balanced salt solution (HBSS with Cl^-) was prepared with the following compositions: 136.9 mM NaCl, 5.5 mM KCl, 0.34 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 0.81 mM MgSO_4 , 1.25 mM CaCl_2 , 5.5 mM D-glucose, 4.2 mM NaHCO_3 and 10 mM HEPES (pH 7.4). Chloride-free HBSS was prepared by mixing 136.9 mM Na-gluconate, 5.5 mM K-gluconate, 0.34 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 0.81 mM MgSO_4 , 1.25 mM Ca-gluconate, 5.5 mM D-glucose, 4.2 mM NaHCO_3 and 10 mM HEPES (pH 7.4).¹¹

MDA-MB-231 cells were dispersed in 96-well flat bottom tissue culture-treated plates (Corning) at a density of 8×10^4 cells/well (per 100 μL) in complete DMEM media and were incubated at 37 °C in a 5% CO_2 incubator overnight for recovery. Post-recovery, media was replaced by HBSS buffer (either with Cl^- or without Cl^-) containing 10% FBS and 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen). Compound **1c** was added to each well in increasing concentrations (0 – 100 μM) by maintaining the maximum amount of DMSO at 2 $\mu\text{L}/\text{well}$ and incubated for 24 h. Post incubation, 10 μL of MTT (5 mg MTT/mL of PBS) was added to each well and the plates were incubated at 37 °C for 4 h. After 4h, 100 μL of solubilization buffer (10% SDS containing 0.01 N HCl in PBS) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a multi-mode plate reader (HiDex).

Cell Cycle Analysis:

MDA-MB-231 cells were dispersed in 35 mm tissue culture-treated plates (Corning) at the density of 8×10^4 cells/ml in complete DMEM media and were incubated at 37 °C in a 5% CO_2 incubator overnight for recovery. Post-recovery, the cells were treated with 0, 10, and 20 μM of **1c** for 24 h. After incubation, cells were harvested by trypsinization, washed with ice-cold 1X PBS and fixed with 70% methanol at –20 °C overnight. Subsequently, the cells were resuspended in PBS buffer containing 1 μL RNase A (stock 20 mg/ml; Himedia) and incubated for 20 min at room temperature. The cells were then stained with 50 $\mu\text{g}/\text{mL}$

propidium iodide (Stock-1mg/ml; Himedia) for 30 min in the dark at room temperature. The cells were analyzed by acquiring 10,000 events using a BD FACS Celesta™ flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FloJo software (Ashland, OR). The representative overlay of histograms has been depicted in Figure 3.20A, 3.20B and the quantitation of cells in the different phases of the cell cycle has been depicted in Figure 3.20C.

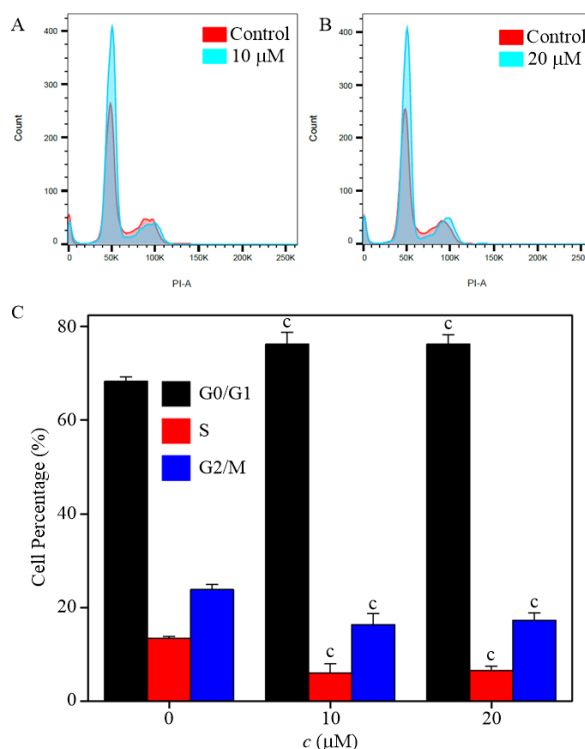


Figure 3.18 Representative cell cycle profiles of MDA-MB-231 cells treated with 10 μM (A) and 20 μM (B) of **1c** (cyan) when compared to the untreated cells (red). The quantitation of cells in the different phases of cell cycle phase has been depicted in (C). *a* represents $p < 0.05 = *$, *b* represents $p < 0.01 = **$, *c* represents $p < 0.001 = ***$, and *d* represents $p < 0.0001 = ****$ (* represents $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$).

Measurement of Reactive oxygen species (ROS) levels:

MDA-MB-231 cells were dispersed in 96-well flat bottom tissue culture-treated plates (Corning) at a density of 8×10^4 cells/well (per 100 μL) in complete DMEM media and were incubated at 37 °C in a 5% CO₂ incubator overnight for recovery. Compound **1c** was added to each well in increasing concentrations (0, 5, 10 and 20 μM) by maintaining the maximum amount of DMSO at 2 μL and incubated for 24 h. In separate experiments, H₂DCFDA

(cellular ROS probe) or MitoSox Red (mitochondrial superoxide indicator) was added to each well at a final concentration 5 μM and the plates were incubated further for 20 min in dark. This was followed by 2 washes with 1X ice-cold PBS. For quantitation of MitoSox, fluorescence intensities were read using a multimode plate reader (Hidex) at (excitation 510 nm/emission 580 nm). All experiments were performed in triplicate, and the relative emission intensity was normalized with respect to the untreated cells. For H₂DCFDA assay, fluorescence imaging was performed by acquiring images in the green channel using EVOS FL auto imaging system (Life Technologies).⁷⁶ The representative images from untreated cells (control) and cells treated with **1c** (10 and 20 μM) are depicted in Figure 3.18.

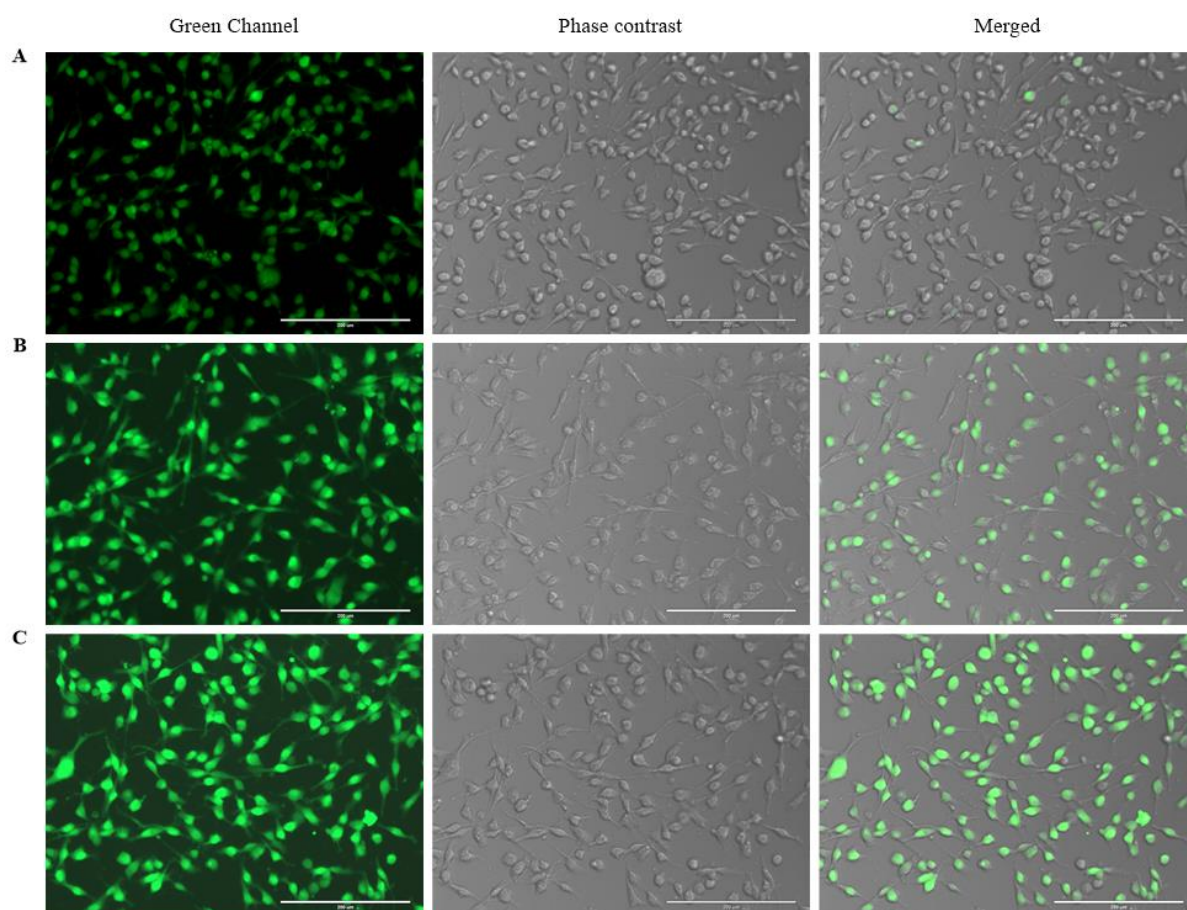


Figure 3.19 Representative live cell images of H₂DCFDA stained MDA-MB-231 cells under control conditions (A) and after treatment with **1c** at 10 μM (B), and 20 μM (C) concentrations.

Mitochondrial membrane potential measurement

MDA-MB-231 cells were dispersed in 96-well flat bottom tissue culture-treated plates (Corning) at a density of 8×10^4 cells/well (per 100 μ L) in complete DMEM media and were incubated at 37 °C in a 5% CO₂ incubator overnight for recovery. After recovery, cells were incubated with 0, 5, 10 and 20 μ M of **1c** for 24 h. The cells were incubated with JC-1 (Invitrogen), a dye used to assess mitochondrial membrane potential, at the final concentration of 2 μ g/mL for 30 min. After incubation, the cells were washed with ice-cold 1X PBS and fluorescence was measured at 485 nm/530 nm (green) and 510 nm/580 nm (red) using a multi-mode plate reader (HiDex) for quantitation. For qualitative analysis, fluorescent images were acquired after PBS washes in red and green channels using EVOS FL auto imaging system (Life Technologies). The representative images from untreated cells (control) and cells treated with 0, 10 and 20 μ M **1c** have been depicted in Figure 3.19.

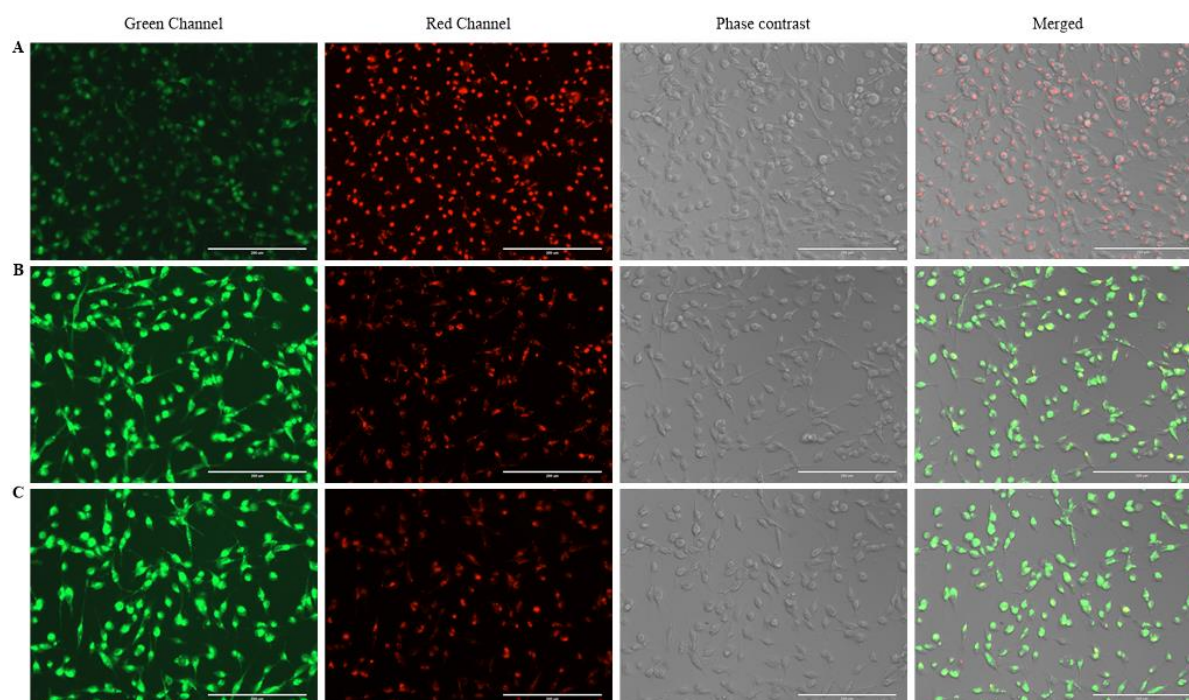


Figure 3.20 Representative live cell images of MDA-MB-231 cells stained with JC1 upon treatment with **1c** at 10 μ M (B), and 20 μ M (C) and in untreated cells (A).

Acridine orange staining:

MDA-MB-231 cells were dispersed in 24-well flat bottom tissue culture treated plates (Corning) at a density of 8×10^4 cells/well (per 100 μ L) in complete DMEM media and were incubated at 37 °C in a 5% CO₂ incubator overnight for recovery. Post-recovery, cells were

incubated with DMEM containing compound **1c** (10, and 20 μM) at 37 $^{\circ}\text{C}$ for 8 h. Post incubation, the cells were washed with ice-cold 1X PBS and incubated with Acridine Orange solution (5.0 $\mu\text{g/mL}$; Sigma) for an additional 0.5 h. The media was subsequently removed and the plates were washed with ice-cold 1X PBS. The cells were maintained in DMEM and observed in the red and green channels using EVOS FL auto imaging system (Life Technologies).²⁶ The representative images have been depicted in Figure 3.20.

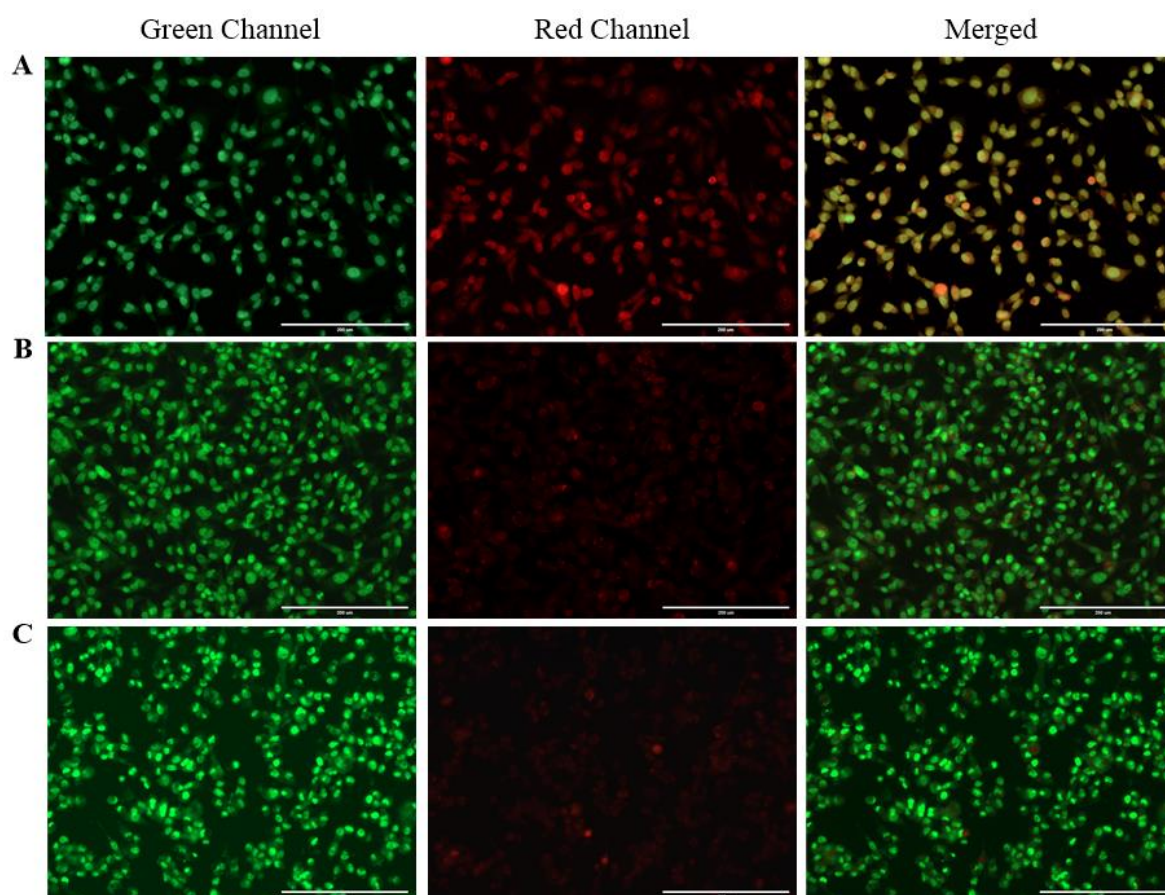


Figure 3.21 Representative live-cell images of MDA-MB-231 cells stained with Acridine Orange upon treatment with **1c** at 10 μM (B), and 20 μM (C) when compared to untreated cells (A).

Immunofluorescence analysis for cytochrome c release:

MDA-MB-231 cells were dispersed at a density of 2×10^4 cells/mL on glass coverslips (Micro-Aid, India) in 24 well plates (Corning) and were allowed to recover overnight at 37 $^{\circ}\text{C}$ in a 5% CO_2 incubator. Post recovery, the cells were allowed to incubate in the presence of **1c** (0, 5 and 10 μM) for 24 h. Post-incubation, the cells were washed with ice-cold 1X

PBS, fixed using 4% p-formaldehyde (Sigma) for 30 min and were permeabilised using 0.25% Triton X-100 (Sigma) for 10 min at RT. Following this, the cells were blocked with 10% (v/v) FBS (Invitrogen) for 1 h, stained with primary antibody (cytochrome *c* antibody; sc13561, 1:250 dilution in blocking buffer) overnight and then incubated with secondary antibody (goat anti-rabbit AlexaFluor-488) for 1 h. Cells were counterstained with FITC-conjugated Phalloidin (1:300; P5282, Sigma) and mounted onto glass slides (Micro-Aid, India) using Vectashield mounting medium with DAPI (Vector). Cell images were taken on EVOS FL auto imaging system (Life Technologies) using a 60X oil-immersion objective (Figure 3.21).¹¹

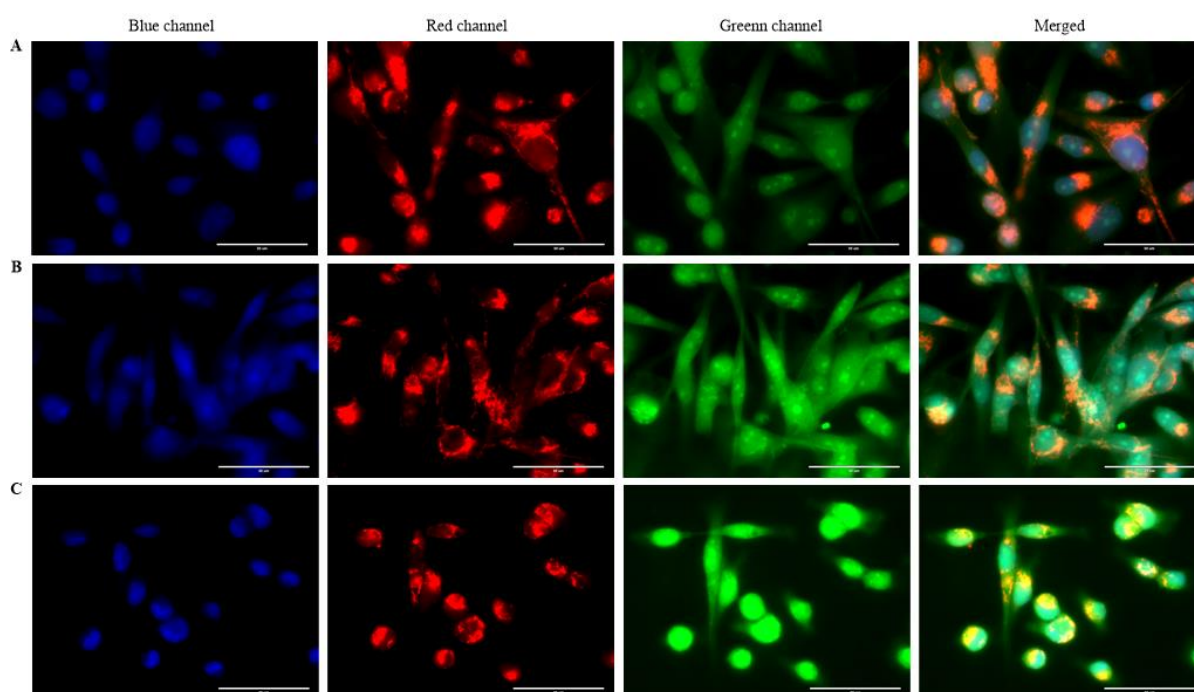


Figure 3.22 Representative live-cell images MDA-MB-231 cells treated with 5 μM (B) and 10 μM (C) of **1c** for 24 h and untreated cells (A). Immunostaining for cytochrome *c* release was performed using cytochrome *c* specific primary antibody (green). Cells were counterstained with phalloidin (red) and DAPI (blue).

Immunoblot analysis:

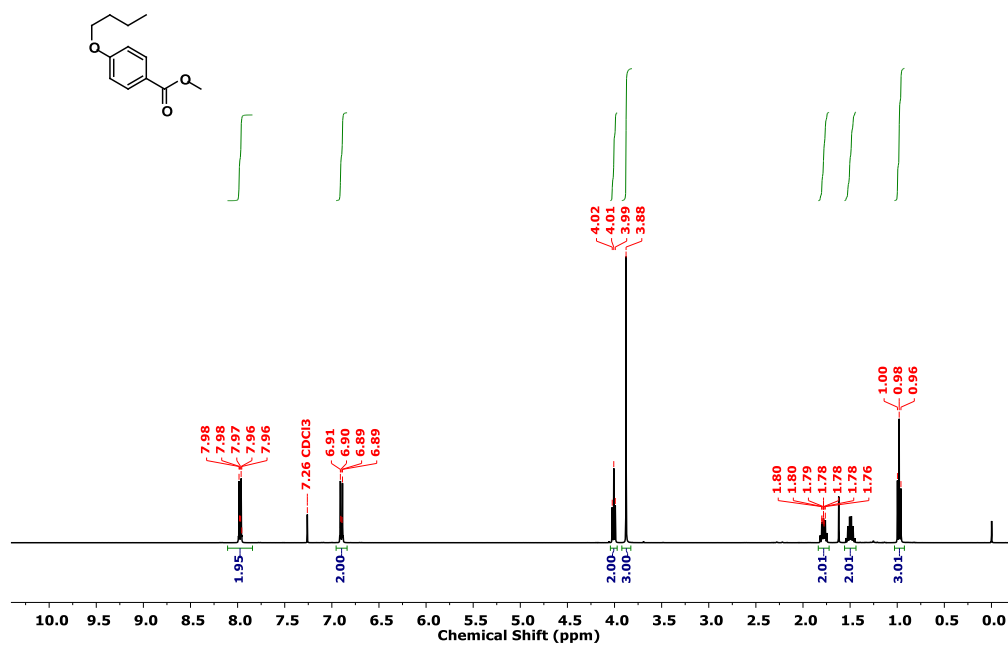
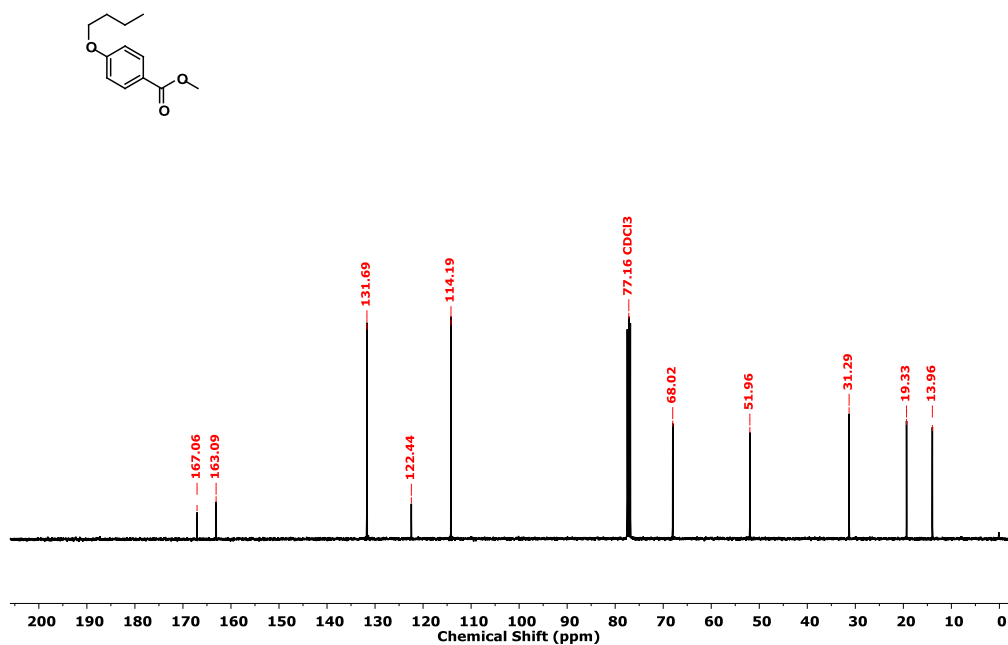
MDA-MB-231 cells were dispersed at a density of 8×10^4 cells/ ml media in 60 mm tissue culture-treated dishes (Corning) in complete DMEM media and maintained at 37 °C for 24 h. Cells were then treated with **1c** for 24 h at different concentrations (0, 10, 20, and 40 μM). Control cells were treated with an equivalent volume of DMSO. After 24 h treatment, media

was aspirated and cells were washed twice with ice-cold 1X PBS and lysed in RIPA lysis buffer (Himedia) containing protease inhibitors (Roche). Cell lysates were resolved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. Blocking was performed in 5% (w/v) skimmed milk (SACO Foods, USA) prepared in 1X Tris-buffered saline (TBS) containing 0.1% Tween 20 (1X TBS-T) for 1 h at room temperature. Blots were incubated overnight at 4 °C in primary antibodies (PARP; (46D11), P62; (5114S), LC3; (D3U4C), Cell Signaling, and Tubulin; (T9026), Sigma) diluted in blocking buffer. Following washes, blots were incubated with peroxidase-conjugated secondary antibody (Sigma) prepared in 5% (w/v) skimmed milk in 1X TBS-T for 1 h at room temperature, following which blots were developed using the WesternBright ECL kit (Advansta) and visualized using ImageQuant LAS 4000 (GE Healthcare).¹¹ Quantitation of the intensities for the bands was measured using densitometric scanning (ImageJ; NCBI).

Statistical Analysis:

All cellular experiments were performed in triplicate, unless stated otherwise. Data has been represented as Mean $\pm$ SEM, where SEM represents the standard error of the mean and is expressed as the standard deviation divided by the square root of sample size. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey HSD post-hoc test. $p \leq 0.05$ was considered to be statistically different when comparing different groups.

3.4.11 NMR Studies

Figure 3.23 400 MHz ¹H NMR of compound **4** in CDCl₃.Figure 3.24 101 MHz ¹³C NMR of compound **4** in CDCl₃.

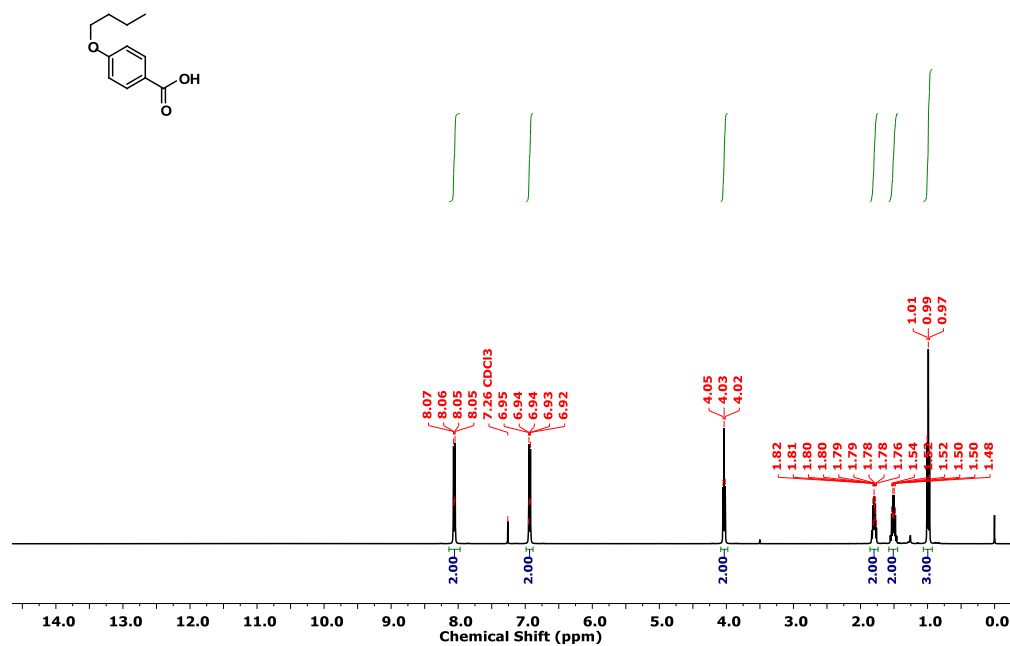


Figure 3.25 400 MHz ¹H NMR of compound 6 in CDCl₃.

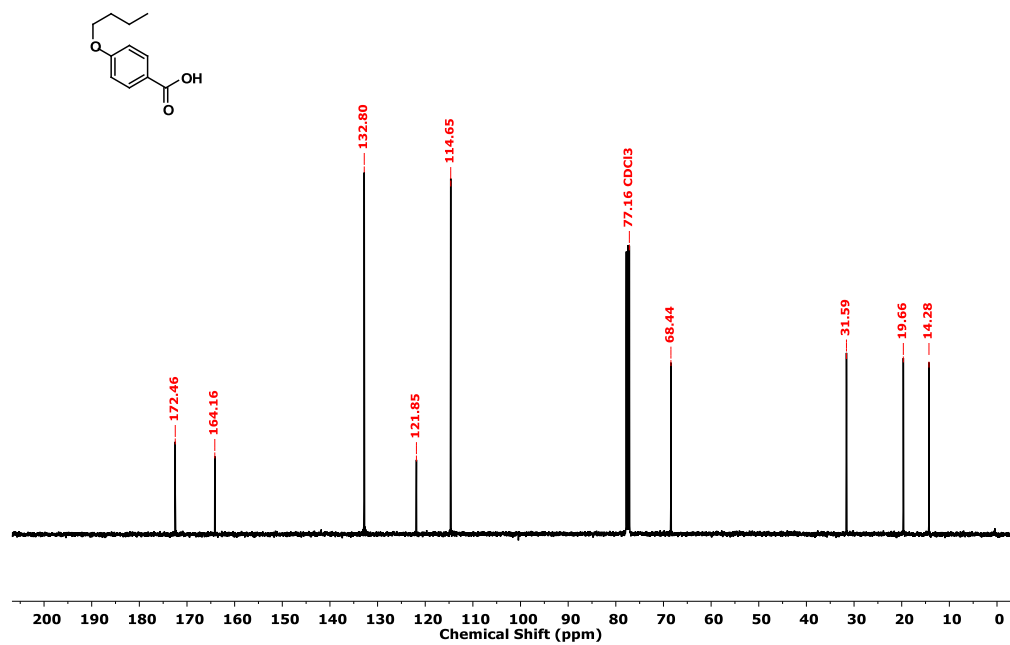


Figure 3.26 101 MHz ¹³C NMR of compound 6 in CDCl₃.

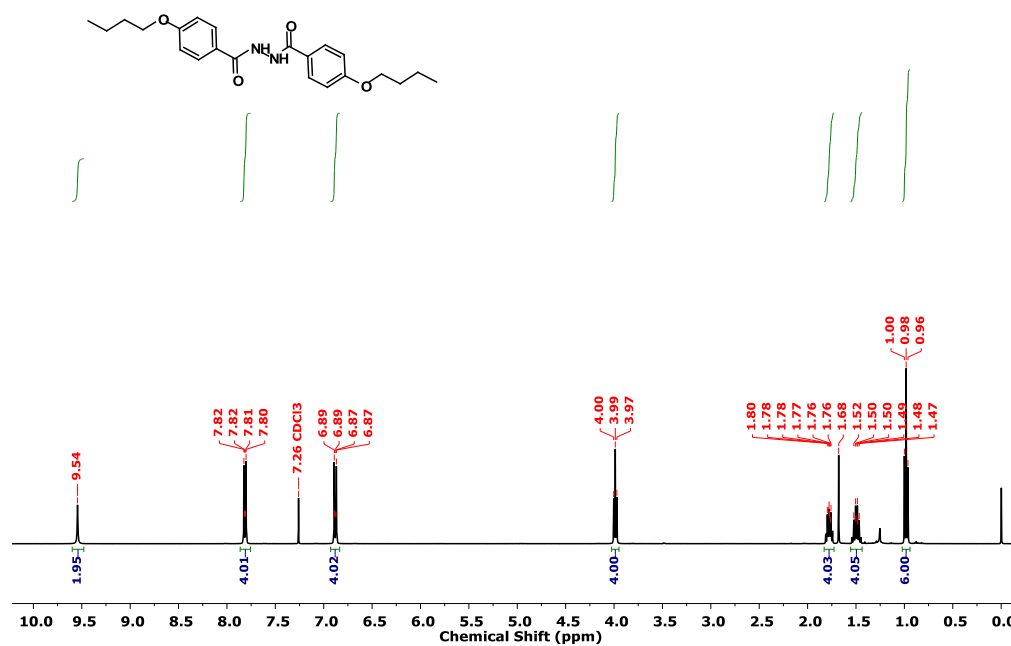


Figure 3.27 400 MHz ¹H NMR of compound **1a** in CDCl₃.

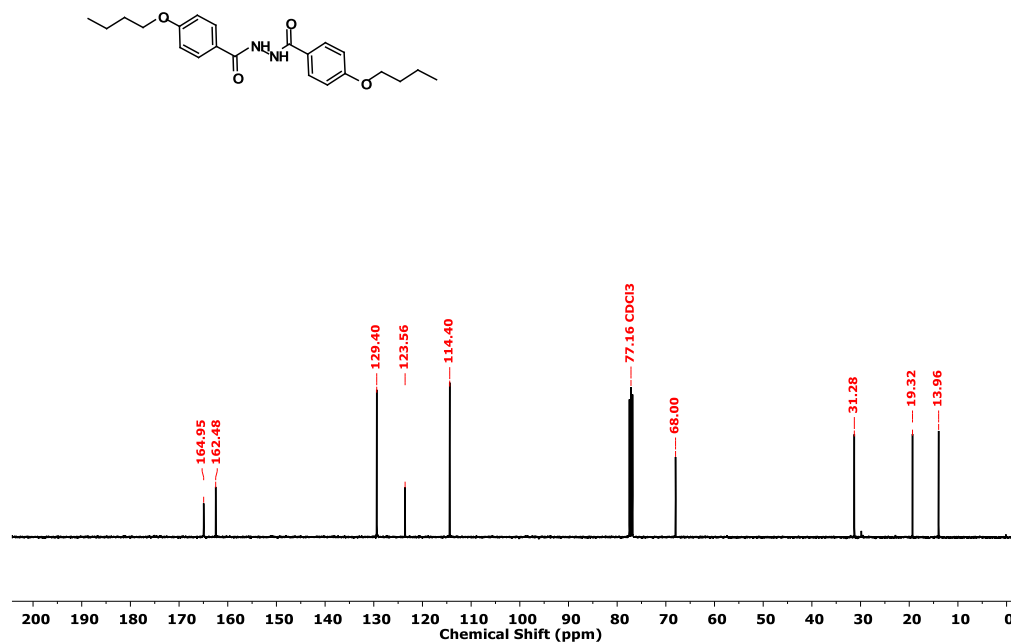


Figure 3.28 101 MHz ¹³C NMR of compound **1a** in CDCl₃.

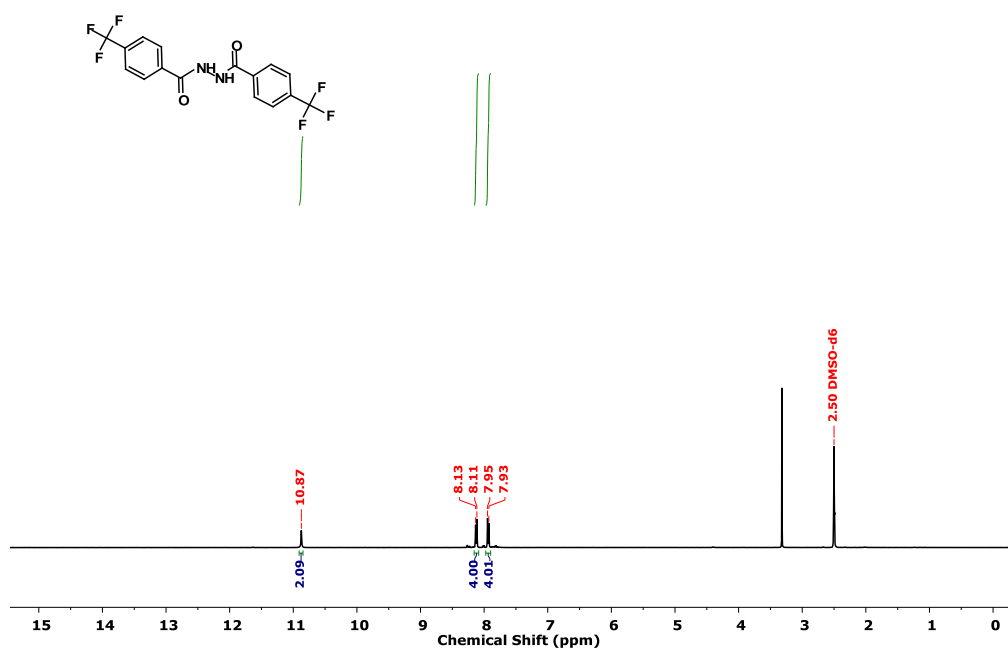


Figure 3.29 400 MHz ¹H NMR of compound **1b** in DMSO-*d*₆.

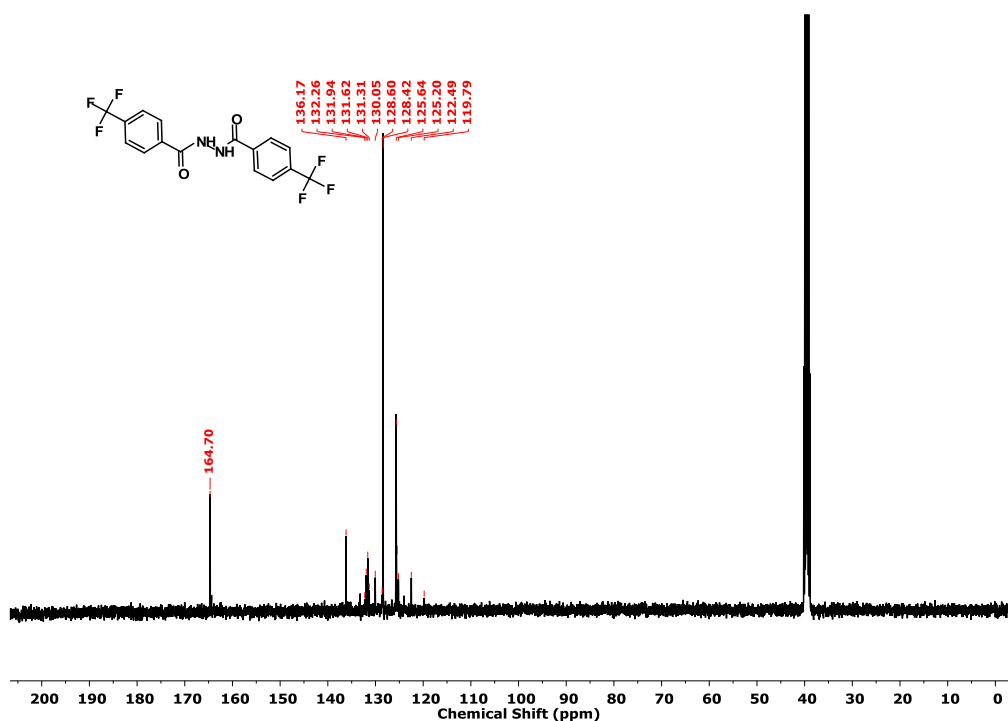


Figure 3.30 101 MHz ¹³C NMR of compound **1b** in DMSO-*d*₆.

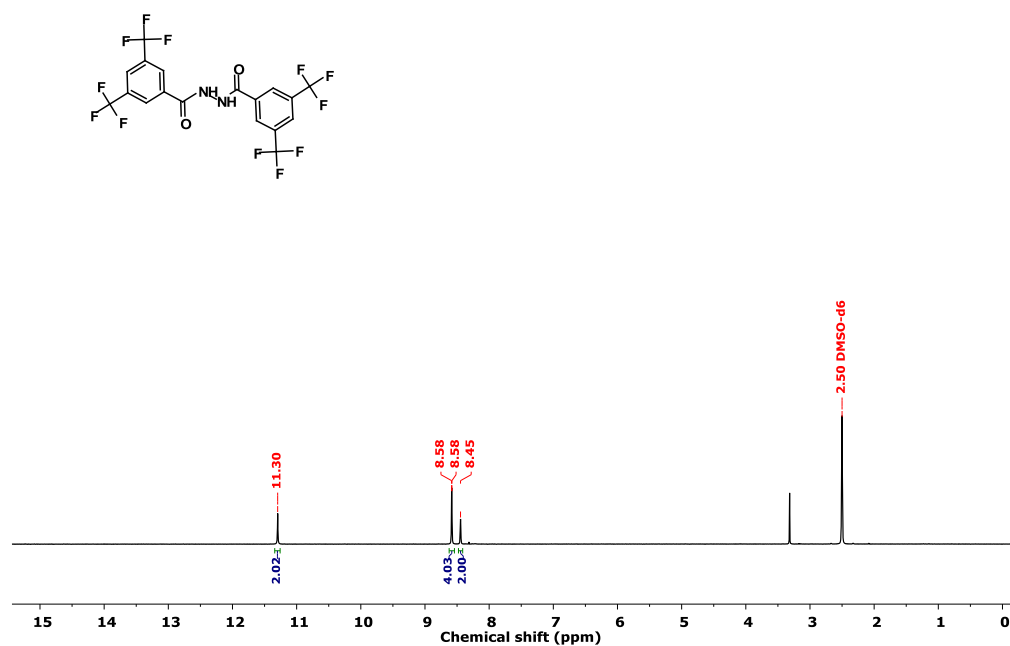


Figure 3.31 400 MHz ¹H NMR of compound **1c** in DMSO-*d*₆.

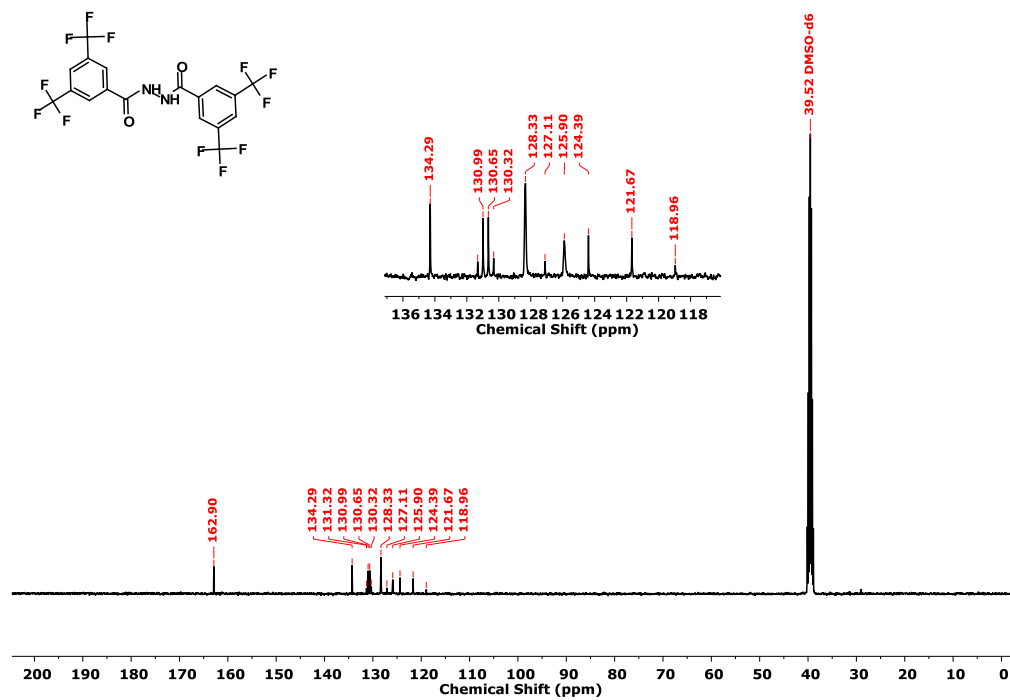


Figure 3.32 101 MHz ¹³C NMR of compound **1c** in DMSO-*d*₆.

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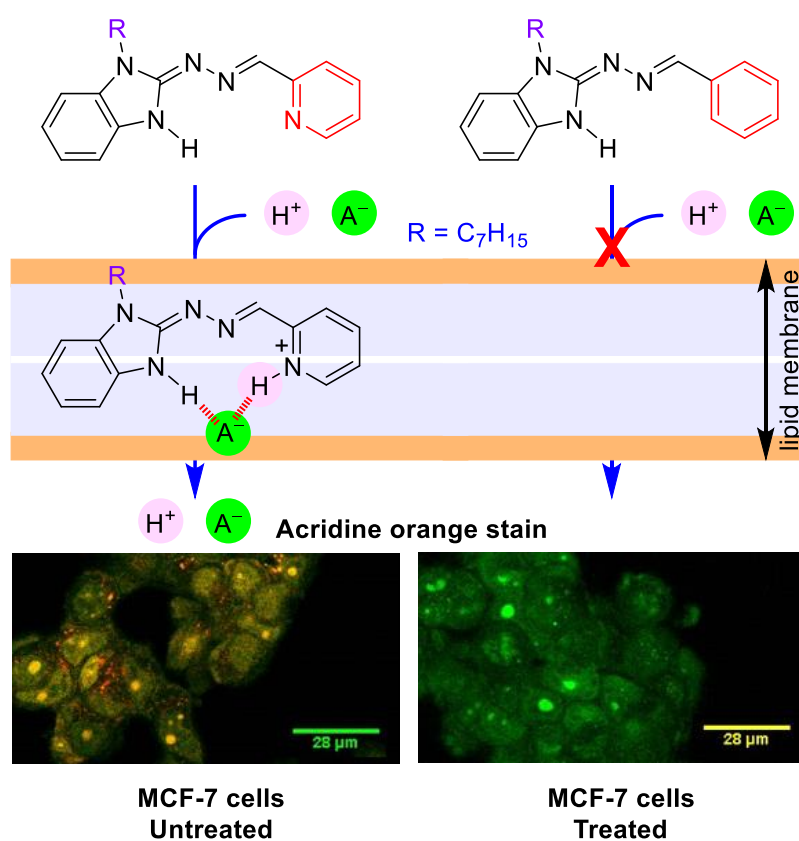
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Chapter 4

A Pyridyl-Linked Benzimidazolyl Tautomer Facilitates Prodigious H^+/Cl^- Symport through a Cooperative Protonation and Chloride Ion Recognition



4.1 Introduction

Synthetic molecules capable of transporting ions across lipid bilayers have been studied extensively over several years.¹⁻⁹ Remarkable progress in the understanding of gene mutations to the physiology of Cl^- transport proteins has led to wide therapeutic opportunities for synthetic Cl^- transporters¹⁰ in the diseases such as cystic fibrosis, Bartter syndrome, etc.¹¹ Recently, apoptosis-inducing activity of synthetic Cl^- transporters against different cancer cell lines were also demonstrated⁸ paving a plausible approach for looking into anticancer drug development. However, this advancement is still beyond any control to keep normal cells safe. Dysregulated pH is an adaptive feature of most cancer cells as they show a 'reversed' pH gradient (i.e., $\text{pH}_i = 7.12\text{--}7.68$ and $\text{pH}_e = 6.2\text{--}6.9$) due to the excretion of excess lactic acid produced in the abnormal anaerobic glycolysis process.¹² The acidic microenvironment of tumor tissues resists the cellular uptake of many commonly used weakly basic cancer drugs, which get protonated at lower pH. On that account, chemical substances having the ability to neutralize the pH gradient can restore drug sensitivity synergistically. Prodigiosin and other similar classes of H^+/Cl^- cotransporters such as tambjamines,¹³ perenosins,¹⁴ calix-[4]arene amide,¹⁵ imidazole-linked pyrrole amide,¹⁶ trenamide,¹⁷ tris-ureas, and tris-thioureas¹⁸ are active across the pH range of 4–7. Moreover, no synthetic H^+/Cl^- cotransporter is achieved to date, which is more active compared to prodigiosin.

Gale and coworkers demonstrated that tautomeric equilibrium between two N_1H and N_3H on benzimidazole urea-based receptors plays an important role in switching anion binding selectivity.¹⁹ They have further shown that the equilibrium between the amino and imino tautomeric forms of amide/urea-linked benzimidazoles can also change the anion binding mode, thereby affecting anion selectivity. Moreover, Medvedeva and coworkers demonstrated that the imino form of benzimidazolyl hydrazone derivative is more stable than its amino form²⁰ and this observation led us to realize that the N_3H can be used for the recognition of HCl if a basic functional group is connected to benzimidazole moiety at an optimal distance using an adequate linker. We selected pyridine as the basic group ($\text{pK}_a = 4.38\text{--}7.45$) for the protonation at physiological pH and the N_3H of benzimidazole for anion

recognition. Such proton-chloride recognition can be favored by cooperative and microenvironmental effects. Therefore, we designed three pyridyl-linked benzimidazolyl hydrazones **1a-1c** with R = ethyl, butyl, and heptyl with increasing calculated cLogP values as obtained from ChemDraw 19.0.0.22 program (Figure 4.1). The Tautomer Generator Plugin of ChemAxon also predicted that these molecules have the imino tautomer as the major form (Table 4.2). So, the HCl binding by each receptor would occur via protonation of the N₁₁ of the pyridyl ring and anion binding between the N₁₁-H and N₃-H (Figure 1C). Therefore, transport of the bound proton-anion pair across the lipid bilayer membrane can easily be realized. To understand the importance of the pyridyl ring in HCl recognition, we designed a phenyl-linked benzimidazolyl hydrazone **1d** that cannot bind HCl.

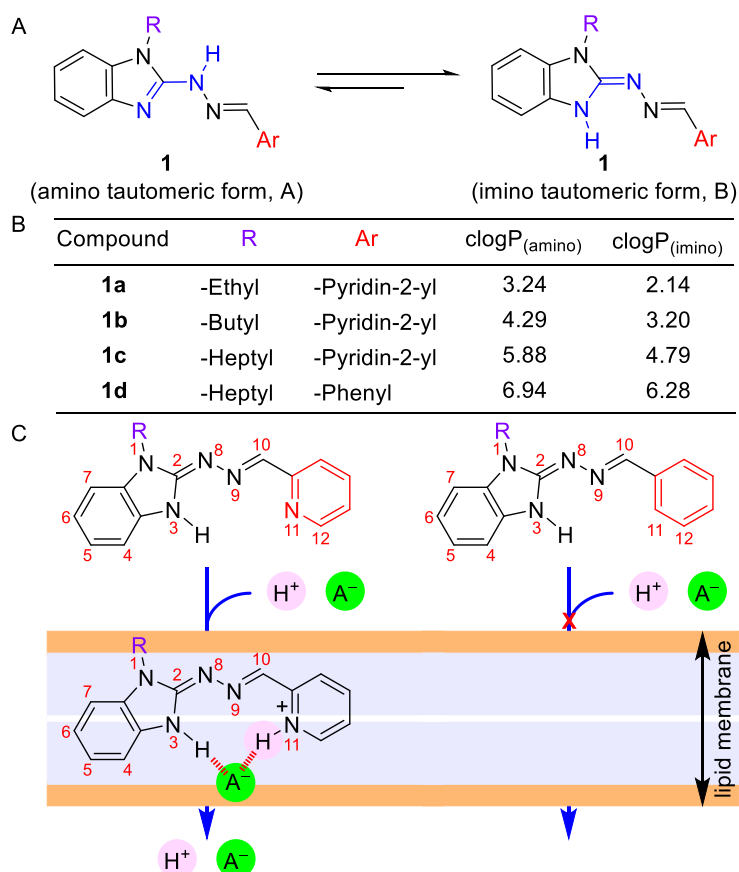
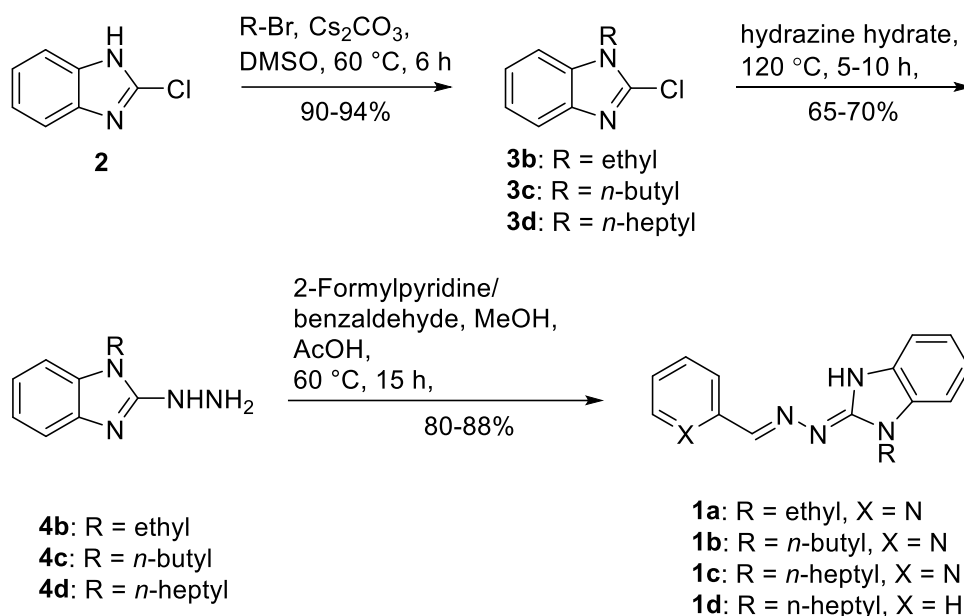


Figure 4.1 Amino-imino tautomeric equilibrium of benzimidazolyl hydrazones **1a-1d** (A). Description of the molecules and calculated clogP values (B). The mode of HCl transport by the transporters (C).

4.2 Results and Discussions

4.2.1. Synthesis

Synthesis of the receptors **1a-1d** was carried out from **2**, which was synthesized from the reaction of benzene-1,2-diamine with urea followed by chlorination by POCl₃ (Scheme 4.1). The reaction of compound **2** with different alkyl bromides gave **3a-3c** in 90-94% yield. Then the reaction of **3a-3c** with hydrazine hydrate produced **4a-4c** in 65-70% yield. Further reaction of **4a-4c** with 2-formylpyridine in the presence of catalytic acetic acid provided **1a-1c** in 80-88% yield. The reaction of **4c** with benzaldehyde in the presence of catalytic acetic acid produced **1d** in 86% yield.



Scheme 4.1 Synthesis of benzimidazolyl hydrazone compounds **1a-1d**.

4.2.2 Conformational Analysis

The crystal structure of **1b** indicated a shorter C₂-N₈ bond = 1.309 Å compared to C₂-N₁ and C₂-N₃ bond lengths of 1.373 Å and 1.370 Å, respectively (Figure 4.5), indicating the presence of imino-tautomeric form in the solid state. The ¹H-¹H 2D NOESY spectrum of **1c** DMSO-d₆ confirmed the existence of imino-amino tautomeric equilibrium in the solution phase (Figure 4.32). The data also showed the presence of **Conf-3**, a rotamer of the imino-tautomeric form. We also observed a line-broadening for the protons. The line-width (full width at half maximum) for H_i proton decreased from 5.9 Hz (c_{1d} = 15 mM) to 2.9 Hz (c_{1d} = 1.5 mM) and 2.4 Hz (c_{1d} = 0.75 mM), (Figure 4.34) suggesting the solution-phase self-assembly of **1c**, which becomes negligible beyond 1.5 mM. The self-assembly was also confirmed as a minor population by the cross peak between H_a and H_d (i.e., cross peak 8, Figure 4.33) in the NOESY spectrum. The solution state

exchange events of the molecule were further characterized by Carr-Purcell-Meiboom-Gill (CPMG) ^1H relaxation dispersion NMR experiment. The global fit of the CPMG data of four protons was carried out, and exchange parameters were derived (Figure 4.2, Figure 4.35, and Table 4.1). The transverse relaxation rates obtained for the protons were suggestive of a large molecular size indicating the self-assembled state of the molecule. The determined lifetime of 394 μs is suggestive of an intermediate exchange process with respect to the NMR measurement timescale, which could be a result of two processes: (1) an exchange between self-assembled and the monomer, and (2) an exchange between amino and imino tautomeric form (Figure 4.2).

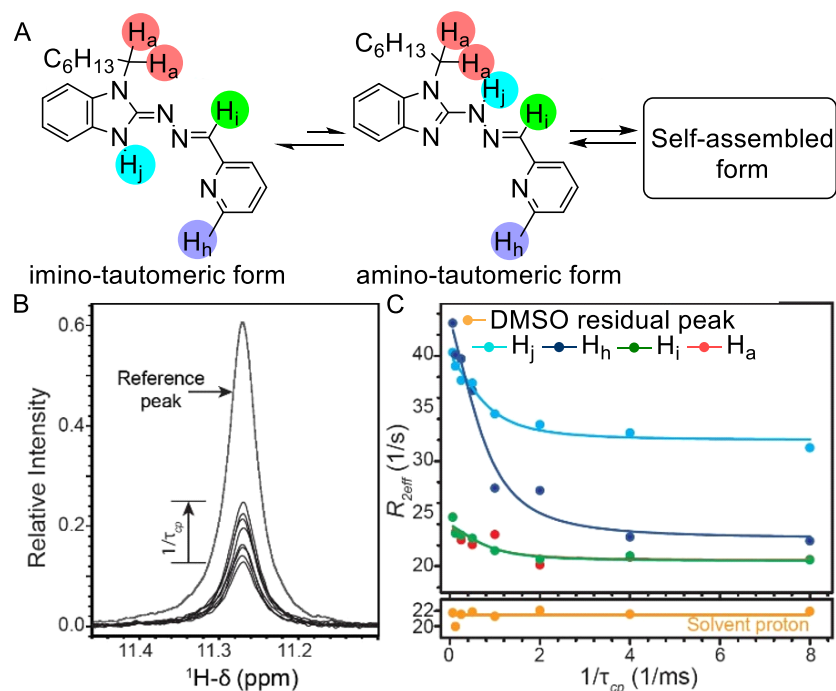


Figure 4.2 The representation of the equilibrium between the imino-tautomer, amino-tautomer, and self-assembled forms of **1c** (A). Raw CPMG ^1H relaxation dispersion data for H_j proton showing reference peak intensity and intensity variation of the peak as a function of $1/\tau_{cp}$ (B). Effective transverse relaxation rates (R_{2eff}) are plotted against $1/\tau_{cp}$ for all four sets of protons (C).

4.2.3 Ion Transport Activity

Ion transport properties of **1a-1c** were measured across large unilamellar vesicles (LUVs) prepared from egg-yolk phosphatidylcholine (EYPC) lipids by entrapping 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) and after applying a pH gradient ($\text{pH}_{in} = 7.2$ and $\text{pH}_{out} = 7.8$).²¹ Their ion transport activity at concentration $c = 50$ μM provided the sequence: **1c** > **1b** > prodigiosin > **1a** (Figure 3A). The Hill plot analysis based on the concentration

profiles of **1a**, **1b**, and **1c** and prodigiosin provided effective concentration at 50% activity, EC_{50} = 0.00017, 0.000055, 0.000022, 0.00011 mol% (i.e., 117.80, 36.64, 14.90, and 79.48 pM), respectively (Table S1). This data suggest that the alkyl groups play an important role in increasing the $cLogP$ values of the benzimidazolyl hydrazones and thereby, improving the membrane permeability. Interestingly, the calculated transporter-to-lipid ratio at the EC_{50} values of **1b** and **1c** are better than the naturally occurring HCl transporter prodigiosin. To the best of our knowledge, these compounds also show better activity than all reported synthetic HCl transporters because the highest active synthetic HCl transporter hitherto reported in literature trails the transport activity of prodigiosin by a small margin.²¹ The calculated Hill coefficient n values of 1.17, 0.99, and 1.00 for **1a**, **1b**, and **1c**, respectively, indicate that one molecule is associated with forming the active transporter.

Further studies were carried out with the most efficient transporter **1c**. At first, the influence of cations in the ion transport across EYPC–LUVs $\supset$ HPTS was evaluated for **1c** (c = 15 pM) by varying the cations (*e.g.*, Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+) in the extravesicular buffer.²² Very similar rates of HPTS fluorescence intensity increase observed in these experiments confirm that the alkali metal cations are not involved in the ion transport process (Figure 4.13). In contrast, the variation of anions (F^- , Cl^- , Br^- , I^- , OAc^- , and NO_3^-) in the extravesicular buffer provided a sequence with the highest selectivity toward Cl^- (Figure 4.14). Compound **1c** also showed a concentration-dependent influx of Cl^- across EYPC–LUVs $\supset$ lucigenin (Figure 4.15). There was no significant influence in the influx of the anion when cations were varied in the extravesicular MCl buffer (M^+ = Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+) of EYPC–LUVs $\supset$ lucigenin, ruling out the alkali metal cation binding, and hence cation transport by **1c** (Figure 4.16).

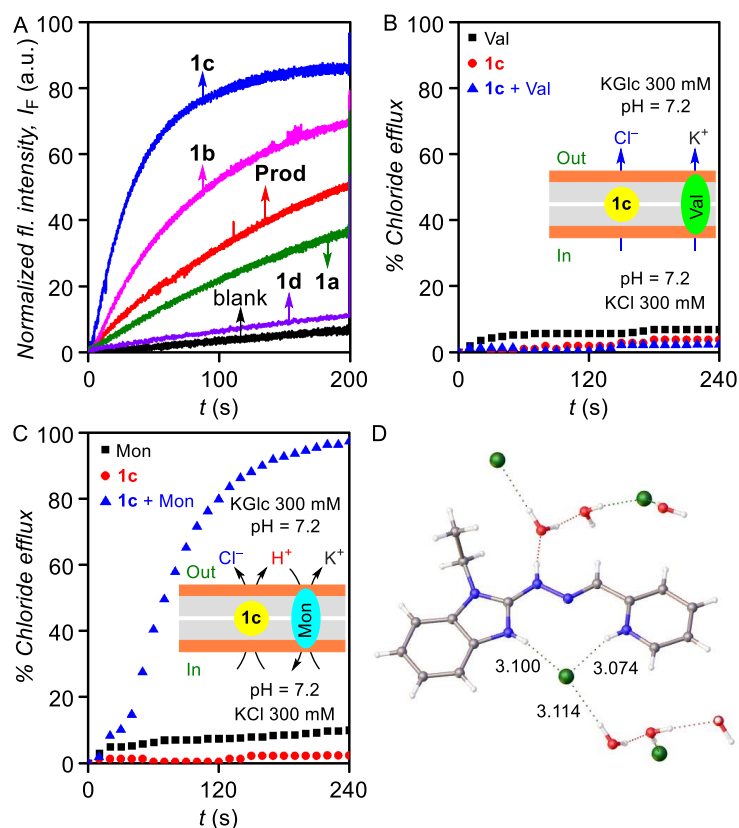


Figure 4.3 Comparison of ion transport activities of **1a-1d** and prodigiosin (50 pM each) across EYPC-LUVs $\Rightarrow$ HPTS (A). Chloride efflux activity of **1c** from EYPC-LUV in the absence and presence of valinomycin (B) and in the absence and presence of monensin (C). X-ray crystal structure of molecule **1a** complexed with HCl (D).

To investigate the operative ion transport mechanism, we adopted a cationophore-coupled assay recently reported by Gale and coworkers.²³⁻²⁵ For a transporter, the transport can be either an electrogenic (i.e., there would be a change of the net charge across the membrane) or an electroneutral (i.e., there would not be any change in the net charge) or a combination of both processes. Valinomycin is an electrogenic cationophore, which transports K^+ causing a change in net charge without affecting the transmembrane pH gradient.¹² On the other hand, monensin behaves as an M^+/H^+ antiporter, i.e., an electroneutral cationophore, which dissipates the transmembrane pH gradient.²⁶

Notably, we have already ruled out the contribution of various alkali metal cations in the transmembrane transport process by performing HPTS and lucigenin-based cation selectivity assays. The lucigenin assay has also eliminated the possibility of M^+/Cl^- symport mechanism ($\text{M}^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+, \text{and } \text{Cs}^+$). So, we tried to understand whether the transport happens

in an electrogenic or electroneutral manner. The valinomycin and monensin coupled experiments were carried out across EYPC–LUVs by monitoring the concentration of effluxed Cl^- using a chloride ion-selective electrode. When **1c** was examined in these assays, we found that Cl^- efflux is only observed in the presence of monensin but not valinomycin (Figure 3B and 3C). It signifies that K^+/H^+ antiport by monensin cooperatively couples to H^+/Cl^- cotransport promoted by the **1c**, leading to overall KCl efflux from the liposomes. Particularly, no chloride efflux in the presence of valinomycin explains that the H^+ and Cl^- transport facilitated by **1c** is an inseparable process. It suggests that the hydrazone **1c** behaves like prodigiosin where chloride cannot be transported without a proton also being transported, and the protonated **1c** cannot diffuse through the lipid bilayer without a counteranion. The H^+/Cl^- symport nature of **1c** was further verified by investigating the effect of valinomycin on the Cl^- influx activity of **1c** across EYPC–LUVs $\supset$ lucigenin, which showed no significant difference in the activity in the absence and presence of the K^+ carrier (Figure 4.17).²⁷

4.2.4 Theoretical Studies

The crystal of compound **1a**, complexed with HCl, was obtained by purging dry HCl gas into a solution of the compound in acetonitrile/water (9:1), followed by slow evaporation of the solvent. The crystal structure (Figure 3D) revealed the 1:1 kosmotropic complexation of **1a** with HCl, stabilized by water molecules. It is evident from the crystal structure that **1a** gets protonated at the N_{11} center of pyridine. Moreover, a second protonation also occurs at the N_8 center. Further, the cavity formed by benzimidazole and pyridine moieties accommodates a Cl^- through hydrogen bonding interactions, which was evident from $\text{N}_3 \cdots \text{Cl}^- = 3.100 \text{ \AA}$ and $\text{N}_{11} \cdots \text{Cl}^- = 3.074 \text{ \AA}$ distances featuring a perfect consequence of positive cooperativity. The Cl^- is additionally stabilized by $\text{HOH} \cdots \text{Cl}^-$ hydrogen bond at a distance of $3.114 \text{ \AA}$. Additionally, the Hirshfield surface analysis also confirmed these hydrogen-bonding interactions (Figure 4.7).

Furthermore, we compared the ion transport activities of **1c** with **1d**, which has a phenyl ring in place of the pyridyl ring. A bare minimum activity of compound **1d** (50 pM) supports the importance of the protonation of pyridyl moiety in the transport process (Figure 3A). Based on the information received from the crystal structure, geometry optimization for the $[\text{1c} + \text{H}^+ + \text{Cl}^-]$ was performed by Gaussian 09 program using B3LYP functional and 6-

31G(d,p) basis set. The geometry-optimized structure of [**1c**+H⁺+Cl⁻] complex indicated that the protonated form of the molecule (protonated at N₁₁) binds to Cl⁻ via N₁₁H⁺⋯Cl⁻, N₃H⁺⋯Cl⁻ and C₁₂H⁺⋯Cl⁻ (Figure 4.36).

4.2.5 Biological Studies

The excellent H⁺/Cl⁻ cotransport activity of **1c** inspired us to evaluate its effect on biological systems. The compound was incubated in epithelial human breast cancer cells MCF-7 for 24 h, and the cell viability was checked by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The results showed that the compound is very toxic to the cells, and the dose-dependent cell viability studies for **1c** provided the $IC_{50} = 1.0 \mu\text{M}$ (Figure 4.4A). To gain a direct insight in support of Cl⁻ ion mediated cell death, we measured the cytotoxicity of **1c** towards MCF-7 cells in the presence and absence of Cl⁻ ions (Figure 4.4C). Compound **1c** showed remarkably higher cytotoxicity in the presence of Cl⁻ ions in the extracellular media strongly suggests that Cl⁻ ion mediated cell death is one of the major factors responsible for the cytotoxic effects. It is known in the literature that such Cl⁻ ion-mediated cell death triggers the apoptotic pathways in live cells.²⁸

Since the compound displayed the H⁺/Cl⁻ cotransport across the bilayers, we anticipated that it might have a direct impact on the lysosomal pH. To investigate this phenomenon, acridine orange (AO, a cell-permeable pH-sensitive dye) was used to monitor the lysosomal pH changes in cells treated with **1c**. AO shows a characteristic orange emission when accumulated in acidic compartments like lysosomes (Figure 4.4D) but shows a green fluorescence at higher pH, such as in cytosol.²⁹ When **1c** was incubated in MCF-7 cells for 1 h, followed by treatment with AO for 30 minutes, the emergence of green fluorescence, with concomitant disappearance of orange fluorescence, was observed (Figure 4.4E). The data confirms the dissipation of lysosomal pH by **1c**, which is plausible because of the H⁺/Cl⁻ cotransport from lysosomes to the cytoplasm due to the higher concentration of Cl⁻ in lysosomes. Thereby, this work has also disclosed a plausible mechanism that remained unknown with regard to the apoptosis-inducing activity of this novel class of benzimidazolyl hydrazones.³⁰

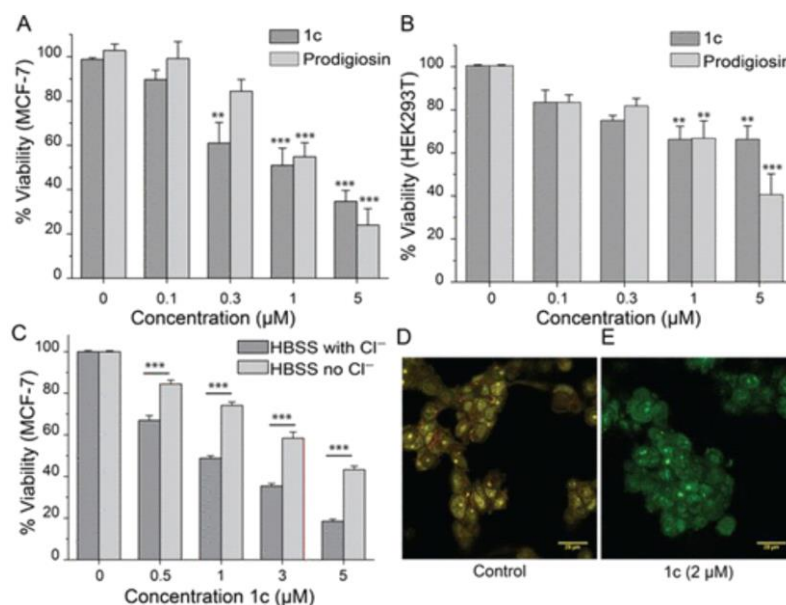


Figure 4.4 Cellular viability (assessed by the MTT assays) for **1c** and prodigiosin in (A) MCF-7 and (B) HEK293T cells. **, $p < 0.001$, and ***, $p < 0.0001$, when compared to untreated cells. The viability of MCF-7 cells was assessed in the presence and absence of chloride anions in extracellular media (HBSS buffer) for **1c** (C). MCF-7 cells stained with acridine orange after incubation at 0 μM (D) and 2.0 μM (E) compound **1c** for 24 h.

4.3 Conclusion

In conclusion, we have introduced a benzimidazolyl hydrazone system **1a-1c** as nonelectrogenic ion transporters that remain in the imino-amino tautomeric equilibrium in the solution phase and provide a suitable H^+/Cl^- binding site. The presence of the tautomeric equilibrium was confirmed by ^1H - ^1H 2D NOESY NMR of **1c**. Moreover, **1b** and **1c** possess highly efficient ion transport properties with EC_{50} values as low as 0.000055 and 0.000022 mol%, respectively, which are better than prodigiosin as well as the highest active synthetic HCl transporter hitherto reported in the literature. Different cationophore-coupled Cl^- efflux assays were performed to confirm the nonelectrogenic H^+/Cl^- cotransport mechanism that mimics the transport mechanism of prodigiosin. The compound **1c** showed Cl^- mediated cytotoxicity in human breast cancer MCF-7 cells as well as deacidification of the acidic compartments of cellular organelles such as lysosomes. Further studies on the detailed cell-death mechanism are under progress.

4.4 Experimental Section

4.4.1 General Methods

All reagents and solvents for synthesis were purchased commercially and used without further purification. Dichloromethane (CH_2Cl_2) was pre-dried over calcium hydride and then distilled. Column chromatographies were performed on Merck silica gel (100 – 200 mesh). Thin layer chromatographies (TLCs) were carried out with E. Merck silica gel 60-F254 plates. Egg yolk phosphatidylcholine (EYPC) was obtained from Avanti Polar Lipids as a solution in CHCl_3 (25 mg/mL). HEPES buffer, HPTS, lucigenin, Triton X-100, NaOH and inorganic salts were purchased of molecular biology grade from Sigma. Large unilamellar vesicles (LUVs) were prepared by using a mini extruder, equipped with a polycarbonate membrane of 100 nm pore size, obtained from Avanti Polar Lipids.

4.4.2 Physical Measurements

All NMR spectra were recorded either on 400 MHz Bruker, or 400 MHz Bruker Ascend™ 400 spectrometer using residual solvent signals as an internal reference on the δ scale. The chemical shifts (δ) were reported in ppm and coupling constants (J) in Hz. The following abbreviations were used: s (singlet), br s (broad singlet), d (doublet), t (triplet) dd (doublet of doublet), m (multiplet). High-resolution mass spectra were obtained on a MicroMass ESI-TOF MS spectrometer equipped with a Micromass Z-Spray electrospray ionization (ESI) source (Waters Co., Synapt G2, France). FT-IR spectra were obtained using NICOLET 6700 FT-IR spectrophotometer and reported in ν (cm^{-1}). Fluorescence spectra were recorded from Fluoromax-4 from JobinYvon Edison-equipped with an injector port and a magnetic stirrer. Measurements of pH were done using a Helmer pH meter. All data from fluorescence studies were processed either by KaleidaGraph 3.51 or Origin 8.5 program. ChemBio Draw 16 Ultra software was used for drawing structures and processing figures.

4.4.3 Synthesis procedures

General Procedure for the synthesis of 2-chloro-1-alkyl-1*H*-benzo[*d*]imidazole (3a-3c):

In a 100 mL round bottom flask, to a solution of 2-chloro-1*H*-benzo[*d*]imidazole **2** (0.97 g, 6.36 mmol) in DMSO (15 mL) was added alkyl bromide (9.54 mmol) and Cs_2CO_3 (9.54 mmol). The reaction mixture was then stirred for 6 hours under reflux condition. After

completion of the reaction, (monitored by TLC), then extracted in EtOAc. The organic extract was dried over Na₂SO₄ and evaporated in vacuo to obtain a crude residue. The later on purification by column chromatography using EtOAc in hexane gave yellow liquid pure compound **3a-3c** (90-94%).

Characterization data of 3a: ¹H NMR (400 MHz, CDCl₃): δ 7.71 – 7.68 (m, 1H), 7.32 – 7.24 (m, 3H), 4.28 – 4.22 (q, *J* = 7.3 Hz, 2H), 1.45 – 1.41 (t, *J* = 7.3 Hz, 3H). The ¹H NMR data were matched with the data available in the literature.³⁰

Characterization data of 3b: ¹H NMR (400 MHz, CDCl₃): δ 7.70 – 7.67 (m, 1H), 7.30 – 7.25 (m, 3H), 4.18 (t, *J* = 7.3 Hz, 2H), 1.84 – 1.77 (m, 2H), 1.43 – 1.34 (m, 2H), 0.98 – 0.94 (t, *J* = 7.4 Hz, 3H). The ¹H NMR data were matched with the data available in the literature.³⁰

Characterization data of 3c: ¹H NMR (400 MHz, CDCl₃): δ 7.69 – 7.66 (m, 1H), 7.29 – 7.24 (m, 3H), 4.17 – 4.13 (t, 2H), 1.84 – 1.78 (dt, *J* = 14.7, 7.4 Hz, 2H), 1.35 – 1.22 (m, 8H), 0.87 – 0.84 (t, *J* = 6.9 Hz, 3H). The ¹H NMR data were matched with the data available in the literature.³⁰

General Procedure for the synthesis of 2-hydrazineyl-1-alkyl-1H-benzo[d]imidazole 4a-4c: 2-Chloro-1-alkyl-benzimidazole **3a-3c** (2.8 mmol) and 56% hydrazine hydrate (20 equivalents) was heated at 120 °C for 5-10 hours. After cooling to room temperature, the precipitate was filtered off and washed several times with water and dried to give buff white solids **4a-4c** (65-70%). Because of the instability of **4a-4c**, the product thus obtained was used in subsequent reactions without further purification.

General Procedure for synthesis (E)-1-alkyl-2-(2-(pyridine-2-ylmethylene)hydrazineyl)-1H-benzo[d]imidazole 1b-1e: A mixture of picolinaldehyde (benzaldehyde for compound **1d**) (2.9 mmol), the hydrazine **4a-4c** (2.9 mmol) and MgSO₄ (14.9 mmol) in methanol containing a catalytic amount of glacial acetic acid was heated to reflux until thin layer chromatography monitoring indicated no further conversion (approximately 15 h). The resulting hydrazone was then extracted in CH₂Cl₂. The organic extract was dried over Na₂SO₄ and evaporated in a vacuum to obtain a crude residue which was then subjected to purifying by column chromatography using MeOH in CHCl₃ to get the solid pure compounds **1a-1d** (80-88%).

Characterization data of 1a: Greenish Yellow Solid, ^1H -NMR (400 MHz, CDCl_3): δ 10.56 (s, 1H), 8.56 (dt, $J = 4.9, 1.4$ Hz, 1H), 8.37 (s, 1H), 7.66 – 7.64 (m, 2H), 7.16 (ddd, $J = 6.1, 4.9, 2.6$ Hz, 1H), 7.05 – 7.01 (m, 4H), 4.10 – 4.04 (q, $J = 7.2$ Hz, 2H), 3.56 (methanol solvent residue), 1.40 – 1.36 (t, $J = 7.2$ Hz, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 156.98, 154.72, 149.48, 146.34, 136.72, 132.16, 130.91, 122.63, 122.30, 121.35, 121.15, 107.51, 49.97, 36.72, 13.43. **IR (Neat, v/cm^{-1}):** 3060, 2975, 1611, 1531, 1465, 1398, 1351, 1269, 1134, 1053, 985, 922, 737. **HRMS (ESI):** m/z : $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{15}\text{H}_{15}\text{N}_5$ 266.1405; found: 266.1409.

Characterization data of 1b: Yellow Solid, ^1H NMR (400 MHz, CDCl_3): δ 10.08 (s, 1H), 8.58 – 8.57 (d, $J = 4.6$ Hz, 1H), 8.39 (s, 1H), 7.74 – 7.72 (dd, $J = 8.0$ Hz, 1H), 7.65 (dd, $J = 7.5$ Hz, 1H), 7.17 (dd, $J = 6.8, 5.1$ Hz, 1H), 7.16 – 6.99 (m, 4H), 4.00 (t, $J = 7.3$ Hz, 2H), 1.84 – 1.76 (m, 2H), 1.47 – 1.37 (dd, $J = 15.1, 7.5$ Hz, 2H), 0.95 (t, $J = 7.4$ Hz, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 175.96, 154.77, 149.41, 147.02, 136.61, 132.70, 127.84, 122.64, 121.82, 121.29, 121.24, 109.21, 107.74, 41.76, 30.25, 20.20, 13.88. **IR (Neat, v/cm^{-1}):** 3843, 3739, 3616, 3059, 2948, 2879, 2357, 1691, 1617, 1541, 1472, 1250, 1135, 1060, 1003, 921, 738. **HRMS (ESI):** m/z : $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{17}\text{H}_{19}\text{N}_5$ 294.1721; found: 294.1718.

Characterization data of 1c: Orange solid, ^1H NMR (400 MHz, CDCl_3): δ 9.93 (s, 1H), 8.59 – 8.57 (dd, $J = 3.0, 2.0$ Hz, 1H), 8.39 (s, 1H), 7.77 – 7.75 (d, $J = 7.9$ Hz, 1H), 7.66 (dd, 1H), 7.17 – 7.16 (m, 1H), 7.14 – 6.99 (m, 4H), 3.98 (t, $J = 7.1$ Hz, 2H), 1.85 – 1.78 (m, 2H), 1.27 (m, 8H), 0.87 (t, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 156.81, 154.95, 149.57, 147.20, 136.56, 132.70, 130.55, 122.65, 121.91, 121.29, 121.23, 108.92, 107.75, 50.16, 42.00, 31.85, 29.11, 26.95, 22.73, 14.21. **IR (Neat, v/cm^{-1}):** 3741, 3059, 2927, 2958, 1615, 1538, 1471, 1256, 1143, 1061, 737. **HRMS (ESI):** m/z : $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{20}\text{H}_{25}\text{N}_5$ 336.2188; found: 336.2194.

Characterization data of 1d: Yellowish green solid, ^1H NMR (400 MHz, CDCl_3): δ 8.86 (s, 1H), 8.35 (s, 1H), 7.74 – 7.72 (d, 2H), 7.37 (t, 2H), 7.30 (m, 1H), 7.03 (m, 4H), 3.96 (t, 2H), 1.82 (m, 2H), 1.37-1.28 (m, 8H), 0.88 (t, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 149.28, 148.86, 136.09, 132.87, 128.66, 128.51, 126.80, 121.12, 120.92, 107.46, 41.97, 31.76, 29.02, 28.06, 26.89, 22.62, 14.09. **IR (Neat, v/cm^{-1}):** 3841, 3608, 3052, 2918, 2855, 1609, 1538, 1483, 1434, 1353, 1249, 1134, 1032, 941, 846, 737, 683. **HRMS (ESI):** m/z : $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{21}\text{H}_{26}\text{N}_4$ 335.2278; found: 335.2236.

4.4.4 Single Crystal X-ray Crystallographic Analysis Study

Compounds **1c** was crystallized from acetonitrile at room temperature. The cocrystal of compound **1b** with HCl was obtained by purging dry HCl gas to a solution of the compound in acetonitrile/water (9:1) followed by slow evaporation of the solvent. Single-crystal X-ray data was collected at 200 K on a Bruker KAPPA APEX II CCD Duo diffractometer (operated at 1500 W power: 50 kV, 30 mA) using graphite-monochromated Mo K α radiation ($\lambda = 0.71073$ Å). The data integration and reduction were further processed with SAINT software. Multi-scan absorption correction was applied to the collected reflections. All those structures were solved by the direct method using SHELXTL and were refined on *F*² by a full-matrix least-squares technique using the SHELXL-97 program package within the WINGX program. All non-hydrogen atoms were refined anisotropically and all hydrogen atoms were located in the successive difference Fourier maps and were treated as riding atoms using SHELXL default parameters. The structures were examined by using the *Adsym* subroutine of PLATON to confirm that no additional symmetry could be applied to the models. CCDC numbers for compounds **1c** and **1b** complexed with HCl are 1949928 and 2022742 respectively. This data can also be obtained free of charge from The Cambridge Crystallographic Data Centre.

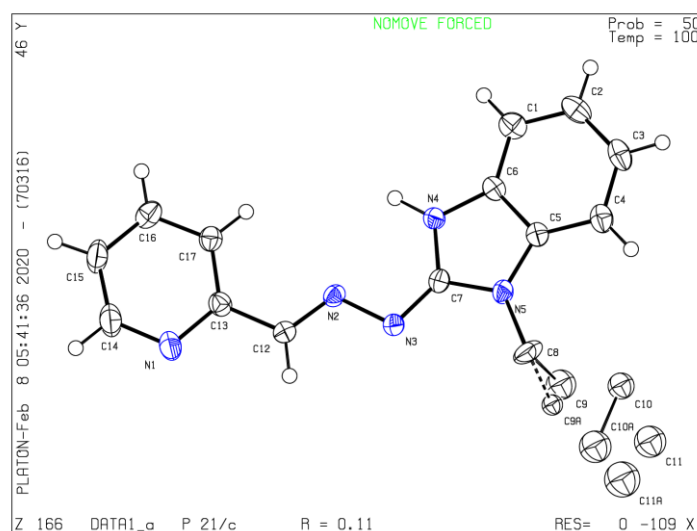


Figure 4.5. ORTEP diagram of the structure **1c** established by single X-ray crystallography.

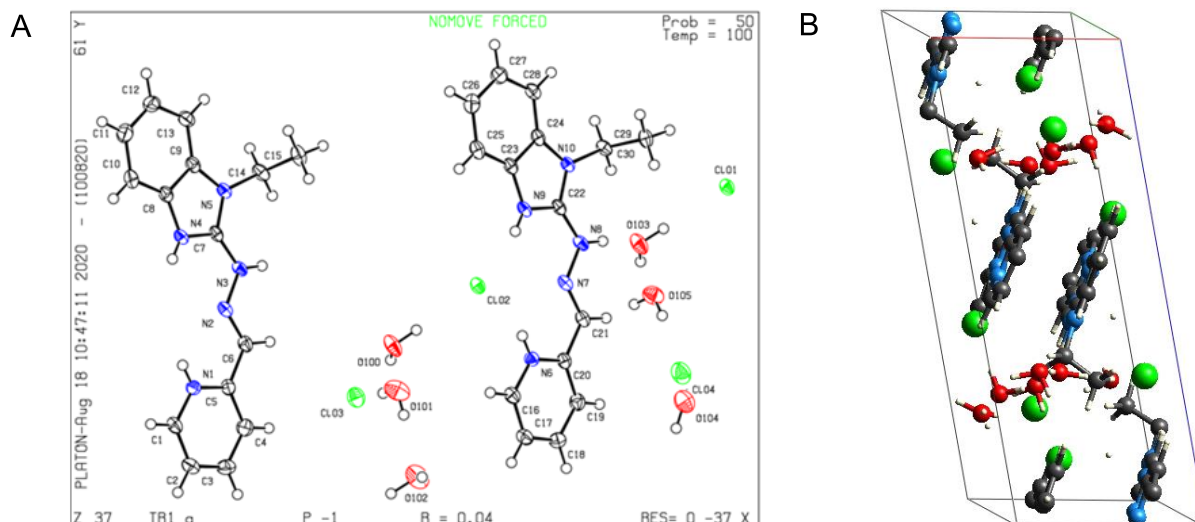


Figure 4.6. ORTEP diagram of the structure **1b** complexed with HCl established by single X-ray crystallography (A). Unit cell representation (B).

Hirshfeld surface analysis: The Hirshfeld surface analysis is a very useful tool to identify the intermolecular interactions in molecular crystals. The tool has been widely used for analyzing the interactions in crystal structures. The presence of hydrogen bonding interactions were visualized using the CrystalExplorer-17.5 software. The results of Hirshfeld surface analysis are shown in Figure 4.7. To understand the electron density distribution on the receptor molecule in cocrystal structure using the CrystalExplorer-17.5 program. The Hirshfeld surface was also generated for the Cl^- ions in the same software (Figure 4.7B, Figure 4.7C). The red, white, blue and color code indicates strong, medium and weak interactions, respectively. The results obtained are analogous to the previously predicted results obtained using Mercury 3.0 software. We have also generated the electrostatic potential of the receptor using the crystal data. The electrostatic potential on receptor surface generated in crystalExplorer17 software using the default program “Tonto”, method “DFT” and basis set “6-31G(d)” (Figure 4.7D). In this case, the blue and red colors indicate the electron-deficient and electron-rich areas on a molecule (Figure 4.7D). The analysis clearly indicates that the protonated benzimidazole NH, and pyridine NH are strongly electron-deficient, leading to the participation in hydrogen bonds.³¹

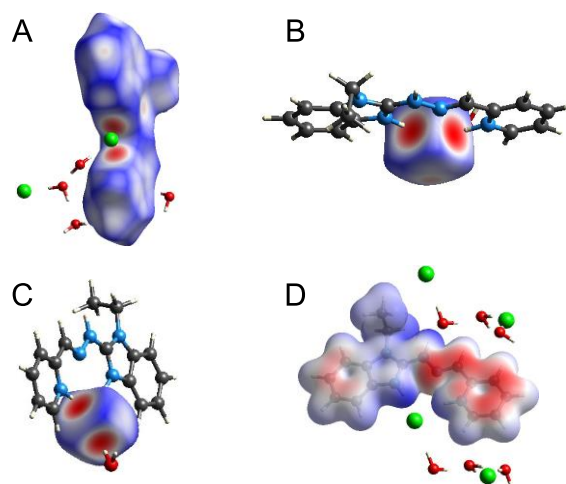


Figure 4.7 The d_{norm} mapped over the Hirshfeld surface using universal red, blue and white color code to show important interactions taking a neighboring molecule. Hirshfeld surface generated for the receptor (A) and Cl^- atoms, front view (B), side view (C). The red, white, blue and color code indicates strong, medium and weak interactions, respectively. The electrostatic potential on receptor surface generated in crystalExplorer17 software using the default program “Tonto”, method “DFT” and basis set 6-31G(d) (D). In electrostatic potential surfaces, the blue and red colors indicate the electron-deficient and electron-rich areas on a molecule.

4.4.5 Ion Transport Experiments

Ion Transport Activity and Selectivity

A. Ion transport across EYPC-LUVs $\supset$ HPTS:

Preparation of EYPC-LUVs $\supset$ HPTS: These vesicles were prepared following the protocol discussed in Chapter 2.

Ion transport activity by HPTS assay: In a clean and dry fluorescence cuvette, 1975 μL of buffer (10 mM HEPES, 100 mM NaCl, pH 7.2) was added, followed by the addition of 25 μL EYPC-LUV $\supset$ HPTS vesicles. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The change in HPTS fluorescence intensity at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$) was monitored as a function of time. During the experiment, base (20 μL , 0.5 M NaOH to create pH gradient) was added at $t = 20 \text{ s}$, and transporter **1b-1d** in DMSO was added at $t = 100 \text{ s}$. Finally, 10% Triton X-100 (25 μL) was added at $t = 300 \text{ s}$.

to lyse the vesicles to achieve complete destruction of the pH gradient. The fluorescence intensity was monitored up to 350 s.

The time data were normalized according to equation 1, and then the fluorescence intensity data was normalized to the percentage change in fluorescence intensity as a course of time using equation 2:

$$t = t - 100 \quad (\text{Equation 1})$$

$$I_F = [(I_t - I_0) / (I_\infty - I_0)] \times 100 \quad (\text{Equation 2})$$

where I_F is the normalized fluorescence intensity, I_0 is the initial intensity, I_t is the intensity at time t , and I_∞ is the final intensity after the addition of a Triton X-100.

For concentration-dependent ion transport data of each compound, the I_F values at 150 s after the addition of the transporter were plotted against respect concentration values. Subsequently, the plot was fitted with the Hill equation (Equation 3):

$$Y = Y_\infty + (Y_0 - Y_\infty) / [1 + (c / EC_{50})^n] \quad (\text{Equation 3})$$

where Y_0 is the fluorescence intensity just before the transporter addition (at $t = 0$ s), Y_∞ is the fluorescence intensity with excess transporter concentration, c is the concentration of transporter compound, and EC_{50} is the effective concentration required to reach the 50% of the maximum activity.

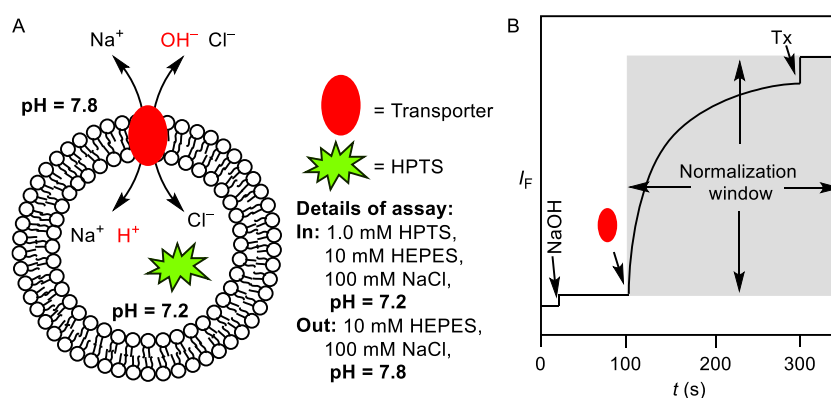


Figure 4.8 Representations of fluorescence-based ion transport activity assay using EYPC-LUVs (A) and representative ion transport kinetics showing normalization window (B).

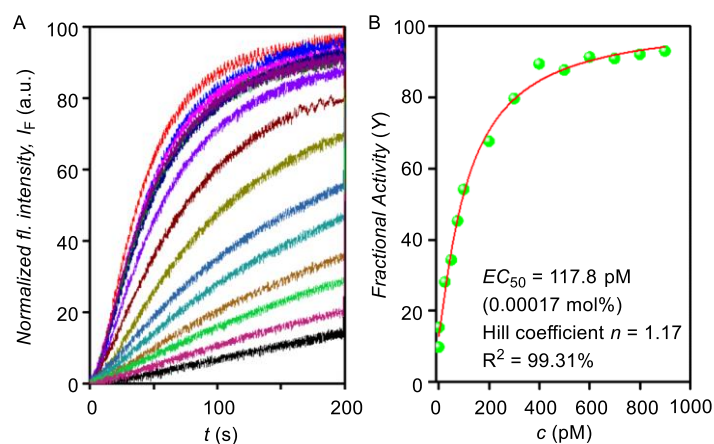


Figure 4.9 Ion transport activities of **1b** at concentrations 0-1000 pM (A). Hill plot of compound **1b** (B).

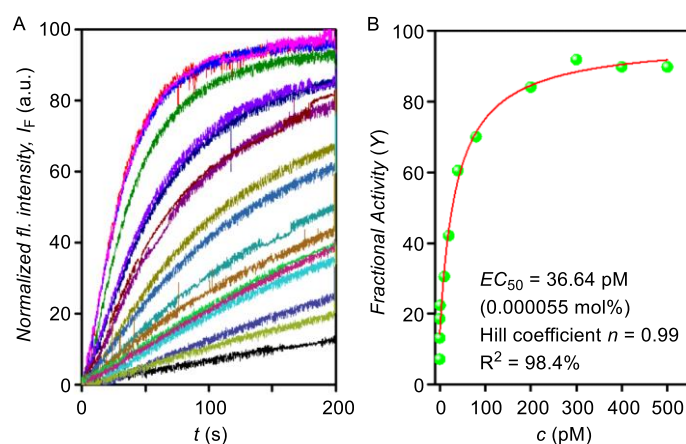


Figure 4.10 Ion transport activities of **1c** at concentrations 0-500 pM (A). Hill plot of compound **1c** (B).

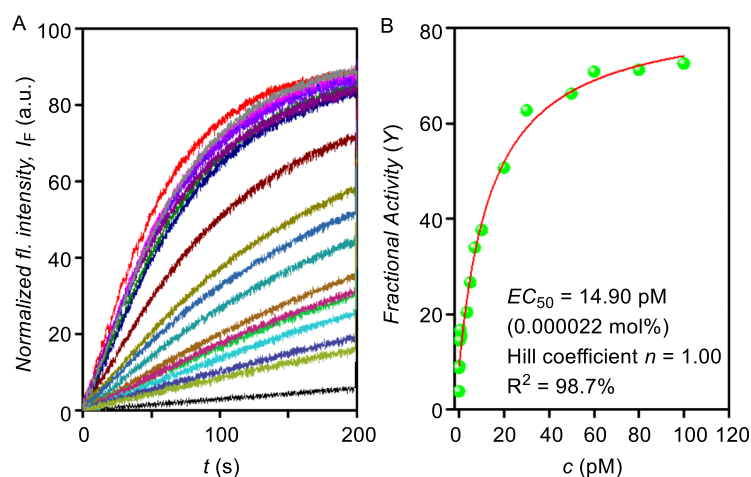


Figure 4.11 Ion transport activities of **1d** at concentrations 0-100 pM (A). Hill plot of compound **1d** (B).

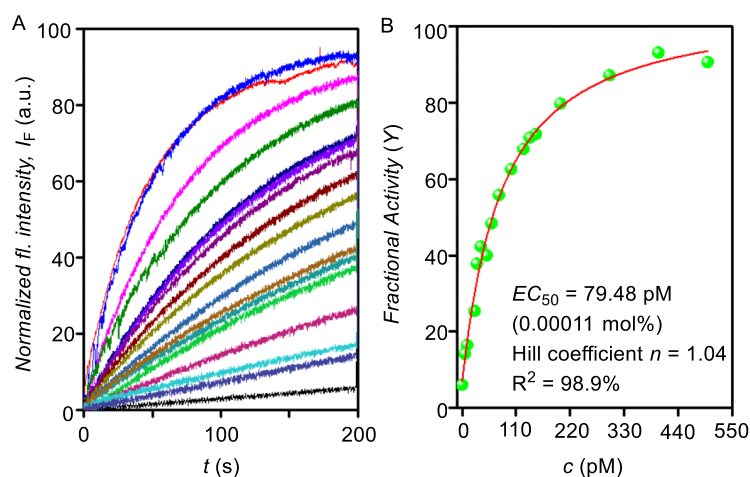


Figure 4.12 Ion transport activities of prodigiosin at concentrations 0-500 pM (A). Hill plot of prodigiosin (B).

Cation selectivity by HPTS assay:

Preparation of EYPC-LUVs>HPTS vesicles: EYPC-LUVs>HPTS with intravesicular NaCl were prepared according to the protocol discussed in Chapter 2.

Final conditions: EYPC ~ 5 mM. **Inside:** 10 mM HEPES, 100 mM NaCl, 1 mM HPTS, pH 7.2; **Outside:** 10 mM HEPES, 100 mM NaCl, pH 7.2.

Cation selectivity experiments: In a clean and dry fluorescence cuvette was added 1975 μ L of buffer containing 10 mM HEPES, 100 mM MCl (where, $M^+ = Li^+, Na^+, K^+, Rb^+$ and Cs^+)

at pH 7.2 followed by the addition of 25 μL EYPC-LUV $\supset$ HPTS in NaCl. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The change in HPTS fluorescence intensity at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$) was monitored as a function of time. During the experiment, base (20 μL , 0.5 M NaOH to create a pH gradient of 0.6) was added at $t = 20 \text{ s}$ and transporter **1d** in DMSO was added at $t = 100 \text{ s}$. Finally, 10% Triton X-100 (25 μL) was added at $t = 300 \text{ s}$ to lyse the vesicles to achieve complete destruction of the pH gradient. The fluorescence intensity was monitored up to 350 s. The collected data was then normalized according to Equations 1 and 2 to get the percentage change in fluorescence intensity as a function of time.

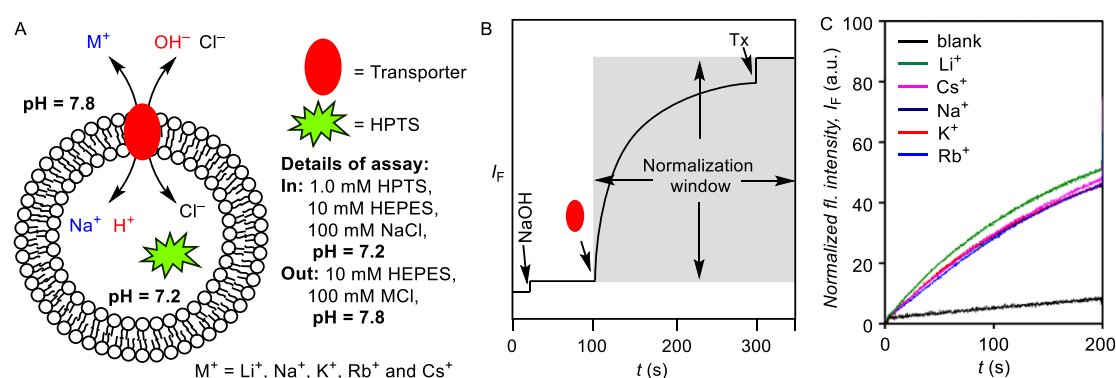


Figure 4.13 Representations of fluorescence-based ion transport activity assay using EYPC-LUVs (A) and representative ion transport kinetics showing normalization window (B). The cation selectivity of **1d** (15 pM) was measured by varying the external cations of EYPC-LUVs $\supset$ HPTS (C).

Anion selectivity by HPTS assay:

Preparation of EYPC-LUVs $\supset$ HPTS: In a small clean, dry round bottom flask, a transparent thin film of egg yolk phosphatidylcholine (EYPC) was prepared by drying a 1 mL of EYPC lipid (25 mg/ mL in CHCl_3) with continuous rotation and purging of nitrogen gas. The transparent film formed was kept in a high vacuum for 6 h to remove all the traces of CHCl_3 . The transparent lipid film was rehydrated with 1 mL of buffer (10 mM HEPES, 100 mM NaX, 1 mM HPTS, pH = 7.2, where $X^- = \text{Cl}^-, \text{NO}_3^-$). The resulting suspension was vortexed at an interval of 10 min for 60 min. The suspension was subjected to ≥ 10 freeze-thaw cycles (liquid N_2 , 55 $^\circ\text{C}$ water bath), and > 19 times (must be an odd number) extruded through a polycarbonate membrane (pore size 100 nm). Extravesicular dyes were removed by size exclusion chromatography using Sephadex G-50 (Sigma-Aldrich) by eluting with buffer

(10 mM HEPES, 100 mM NaX, pH = 7.2, where, $X^- = Cl^-$, NO_3^-), and finally diluted to 6 mL.

Final conditions: EYPC ~ 5 mM. **Inside:** 10 mM HEPES, 100 mM NaX, (where, $X^- = Cl^-$, NO_3^-), 1 mM HPTS, pH 7.2; **Outside:** 10 mM HEPES, 100 mM NaX, (where, $X^- = Cl^-$, NO_3^-), pH 7.2.

Anion selectivity experiments: In a clean and dry fluorescence cuvette 1975 μ L of buffer (10 mM HEPES, 100 mM NaX, pH 7.2; where, $X^- = F^-$, Cl^- , Br^- , I^- , OAc^- , and NO_3^-) was added followed by the addition of 25 μ L EYPC-LUV $\Rightarrow$ HPTS vesicles. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The change in HPTS fluorescence intensity at $\lambda_{em} = 510$ nm ($\lambda_{ex} = 450$ nm) was monitored as a function of time. During the experiment, base (20 μ L, 0.5 M NaOH to create a pH gradient of 0.6) was added at $t = 20$ s, and transporter **1d** (80 pM) in DMSO was added at $t = 100$ s. Finally, 10% Triton X-100 (25 μ L) was added at $t = 300$ s to lyse the vesicles to achieve complete destruction of the pH gradient. The fluorescence intensity was monitored up to 350 s. The collected data was then normalized according to Equations 1 and 2 to get the percentage change in fluorescence intensity as a function of time.

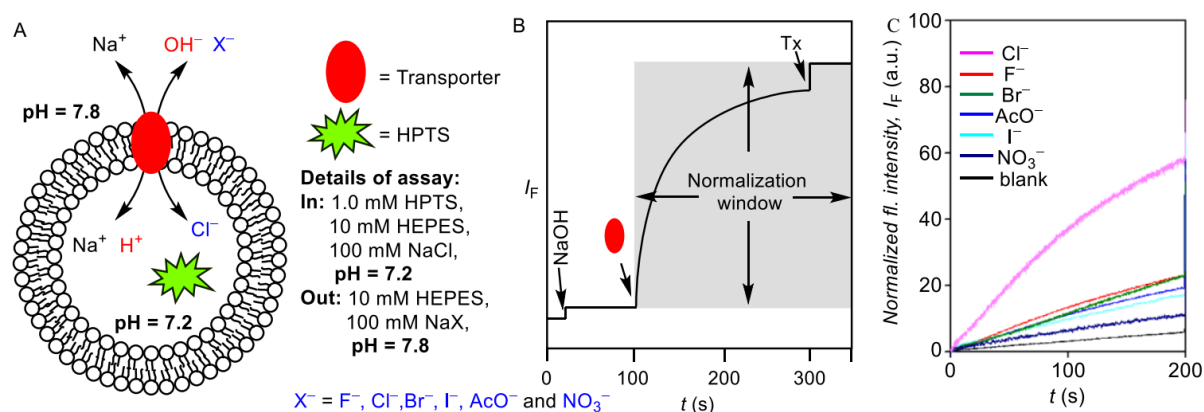


Figure 4.14 Representations of fluorescence-based ion transport activity assay using EYPC-LUVs (A) and representative ion transport kinetics showing normalization window (B). Cl^- versus NO_3^- selectivity of **1d** (80 pM) across EYPC-LUVs $\Rightarrow$ HPTS (C). Higher concentration of **1d** (80 pM) was used in the anion selectivity assay because, no transport was observed when NO_3^- was used as intravesicular and as well as extravesicular anion.

B. Chloride influx across EYPC-LUVs $\Rightarrow$ lucigenin:

Preparation of EYPC-LUVs $\Rightarrow$ lucigenin: The vesicles were prepared following the protocols discussed in Chapter 2.

Dose-dependent chloride influx experiments: In a clean and dry fluorescence cuvette, 1975 μL of NaNO_3 solution (200 mM NaNO_3) was added, followed by the addition of 25 μL of EYPC-LUVs $\Rightarrow$ lucigenin. The cuvette was placed in a fluorescence instrument under slow stirring conditions by a magnetic stirrer equipped with the instrument (at $t = 0$ s). The time course of lucigenin fluorescence emission intensity, F_t was observed at $\lambda_{\text{em}} = 535$ nm ($\lambda_{\text{ex}} = 450$ nm). 33.3 μL of 2 M NaCl was added to the cuvette at $t = 20$ s to create the chloride gradient between the intra and extra vesicular system. Transporter **1d** was added at $t = 100$ s in different concentrations (0 – 100 nM). Finally, 25 μL of 10% Triton X-100 was added at $t = 300$ s to lyse those vesicles resulting in the complete destruction of the chloride gradient. The collected data was then normalized according to Equations 1 and 2 to get the percentage change in fluorescence intensity as a function of time.

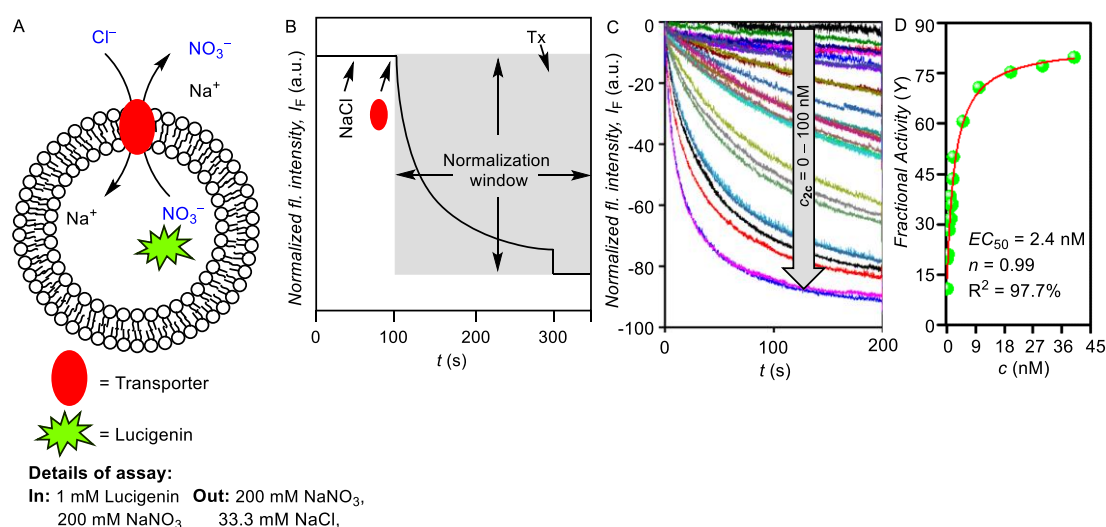


Figure 4.15 Representations chloride influx activity across EYPC-LUVs $\Rightarrow$ lucigenin (A). Illustration of chloride influx kinetics showing normalization window (B). Dose-dependent chloride influx activity of **1d** (0 – 100 nM) (C). Hill plot of **1d** (D).

Effect of extravesicular cations in chloride influx:

Preparation of EYPC-LUVs $\Rightarrow$ lucigenin: The aforesaid lucigenin vesicles were used for the experiments.

Cation selectivity using lucigenin assay: In a clean and dry fluorescence cuvette, 1975 μL of NaNO_3 solution (200 mM) was added, followed by the addition of 25 μL of EYPC-LUVs $\supset$ lucigenin. The cuvette was placed in the fluorescence instrument in a slow stirring condition by a magnetic stirrer equipped with the instrument (at $t = 0$ s). The time course of lucigenin fluorescence emission intensity, F_t was observed at $\lambda_{\text{em}} = 535$ nm ($\lambda_{\text{ex}} = 450$ nm). 33.3 μL of 2 M MCl ($\text{M}^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+$ and Cs^+) was added to the cuvette at $t = 25$ s to create the chloride gradient between the intra and extra vesicular system. Transporter **1d** (2.0 nM) was added at $t = 100$ s, and finally, 25 μL of 10% Triton X-100 was added at $t = 300$ s to lyse those vesicles resulting destruction of the chloride gradient. The collected data was then normalized according to Equations 1 and 2 to get the percentage change in fluorescence intensity as a function of time.

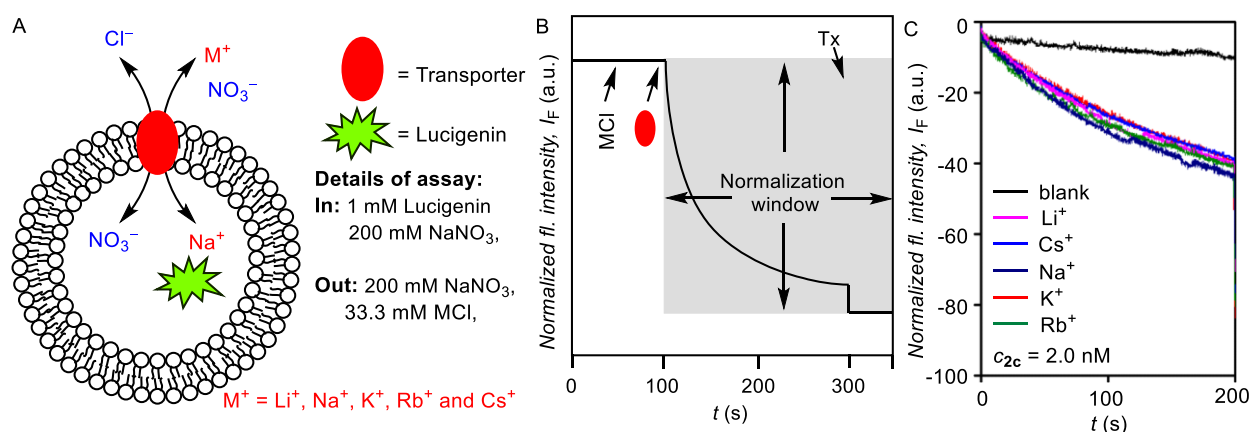


Figure 4.16 Representations of chloride influx activity across EYPC-LUVs $\supset$ lucigenin in the presence of different extravesicular cations (A). Illustration of chloride influx kinetics showing normalization window (B). Study of cation selectivity of **1d** at a concentration of 2.0 nM (C).

Ion transport activity in the presence of valinomycin:

Preparation of EYPC-LUVs $\supset$ lucigenin: The aforesaid lucigenin vesicles were used for the experiments.

Description of the assay:

In a clean and dry fluorescence cuvette, 1975 μL of NaNO_3 solution was added, followed by the addition of 25 μL of EYPC-LUVs $\supset$ lucigenin. The cuvette was placed in a fluorescence

instrument in a slowly stirring condition by a magnetic stirrer equipped with the instrument (at $t = 0$ s). The time course of lucigenin fluorescence emission intensity, F_t was observed at $\lambda_{em} = 535$ nm ($\lambda_{ex} = 450$ nm). 33.3 μ L of 2 M KCl was added to the cuvette at $t = 25$ s to create the chloride gradient between the intra and extra-vesicular system. Valinomycin (1.0 μ M) was added to the cuvette at 50 s. After that, **1d** (4.0 nM) was added at $t = 100$ s, and finally, at $t = 300$ s 25 μ L of 10% Triton X-100 was added to lyse those vesicles resulting destruction of the chloride gradient. The collected data was then normalized according to Equations 1 and 2 to get the percentage change in fluorescence intensity as a function of time.

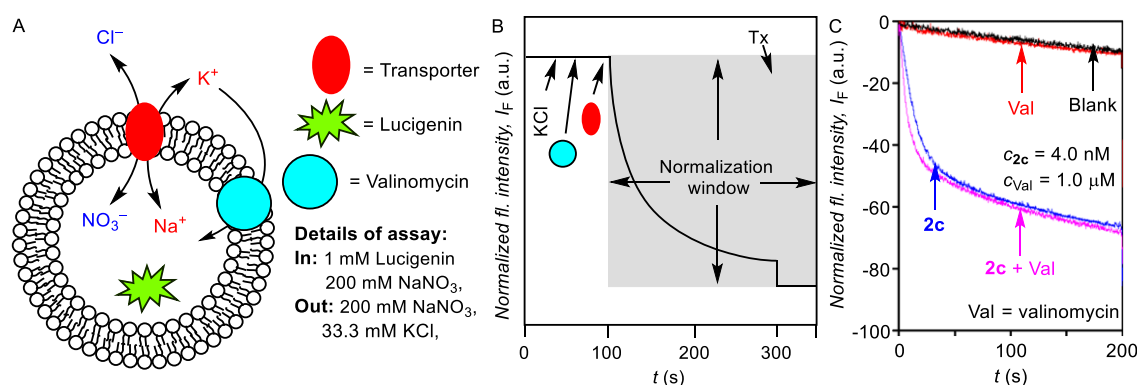


Figure 4.17 Representations of chloride influx activity across EYPC-LUVs $\supset$ lucigenin in the presence and absence of valinomycin (A). Illustration of chloride influx kinetics showing normalization window (B). Chloride influx by **1d** (4.0 nM) in the presence and absence of valinomycin (1.0 μ M) (C).

C. Ion transport across EYPC-LUVs by chloride ion selective electrode:

Transport mechanism by valinomycin and monensin coupled chloride screen:

Preparation of EYPC-LUVs:

A thin film of egg yolk phosphatidylcholine (EYPC) was formed from a chloroform solution (25mg/mL) with purging nitrogen and dried under a vacuum for at least 6 hours. The lipid film was rehydrated by vortexing with a metal chloride (KCl) salt solution and then buffered to pH 7.2 with sodium phosphate salts. The lipid suspension was then subjected to 10 freeze-thaw cycles. The suspension was alternatively allowed to freeze in a liquid nitrogen bath,

followed by thawing in a water bath (55 °C). The lipid suspension was allowed to age for 30 min at room temperature and was subsequently extruded 19 times through a 200 nm polycarbonate membrane. The resulting unilamellar vesicles were dialyzed (using membrane MWCO 1 kD) against KGlc (300 mM) in potassium phosphate buffer of pH 7.2 to remove unencapsulated Cl⁻ ions.

Internal solution: KCl 300 mM, KH₂PO₄ 10 mM, pH 7.2

External solution: KGlc 300 mM, KH₂PO₄ 10 mM, pH 7.2

Description of the experiments:

Chloride efflux during transport experiments was measured using a chloride-selective electrode (*Accumet*). The electrode was calibrated against potassium chloride solutions of known concentrations before the experiments. The lipid concentration in the cuvette was 1.0 mM. A DMSO solution of the anionophore (0.1 μM) was added to start the experiment, and the chloride efflux was monitored using the chloride-sensitive electrode. When a cationophore (valinomycin or monensin) was used, a DMSO solution of the cationophore (0.5 μM) was added to the liposome solution before adding the anionophore at 60 s. At 300 s, the vesicles were lysed with 25 μL of Triton X-100 (10% solution in water), and the total chloride reading was taken at 360 s. The value at 60 s was set at 0% chloride efflux, and the final chloride reading (at 360 s) was set as 100% chloride efflux to normalize the collected data from each measurement.

The time data was normalized according to equation 4. Then the fluorescence intensity data were normalized to the percentage change in fluorescence intensity over time using Equation 5:

$$t = t - 60 \quad \text{(Equation 4)}$$

$$E_F = [(E_t - E_0) / (E_\infty - E_0)] \times 100 \quad \text{(Equation 5)}$$

where E_F is the normalized chloride efflux, E_0 is the initial chloride reading, E_t is the chloride reading at time t , and E_∞ is the final chloride reading after the addition of a Triton X-100.

With KCl inside and potassium gluconate (KGlc) outside the vesicles, there is a Cl^- concentration gradient, which can be considered the main driving force for the transport process. However, with the addition of the anionophore alone chloride transport cannot occur, as there would be a buildup of membrane potential (in the case of an electrogenic Cl^- carrier) or a pH gradient (in the case of an H^+/Cl^- symporter or a Cl^-/OH^- antiporter). The addition of valinomycin would dissipate the membrane potential, and hence overall KCl efflux would occur upon adding anionophore if the anionophore is an electrogenic Cl^- transporter. In contrast, the addition of monensin would dissipate the pH gradient, and hence overall KCl transport would occur upon adding anionophore if the anionophore is of nonelectrogenic nature.

4.5 NMR Studies

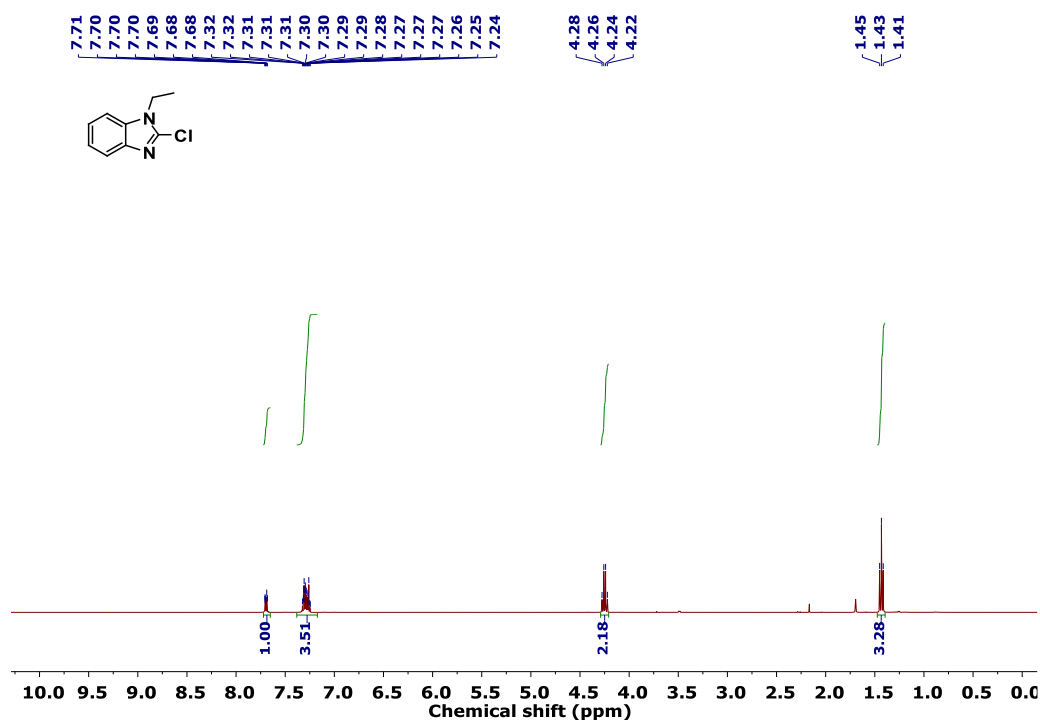


Figure 4.18 400 MHz ^1H NMR of compound **3b** in chloroform-*d*.

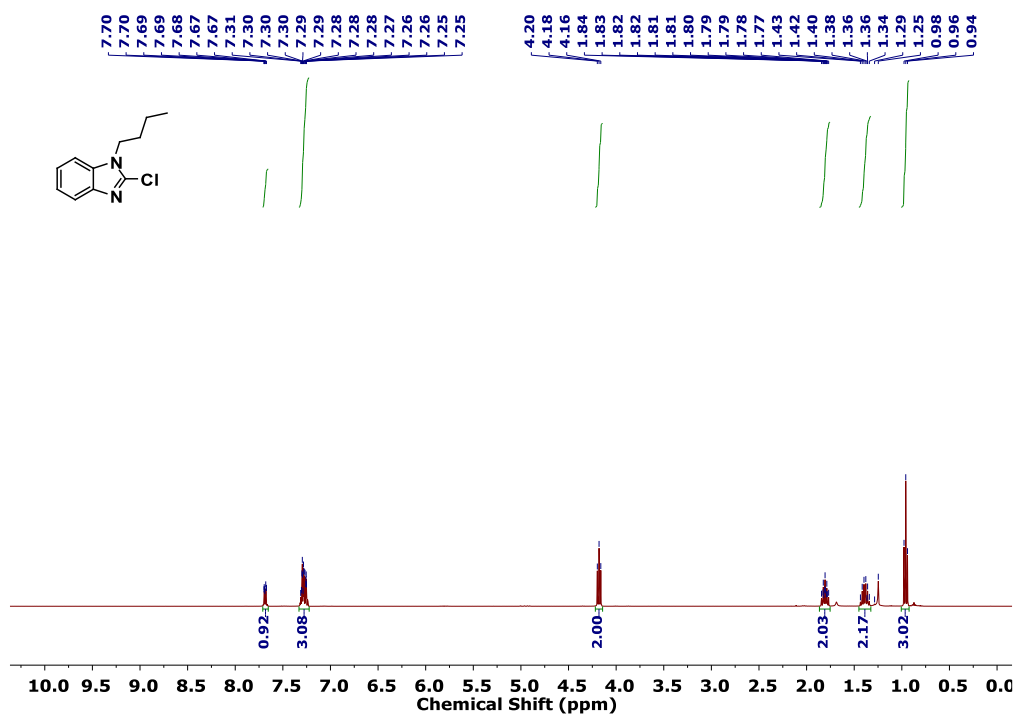


Figure 4.19 400 MHz ^1H NMR of compound **3c** in chloroform-*d*.

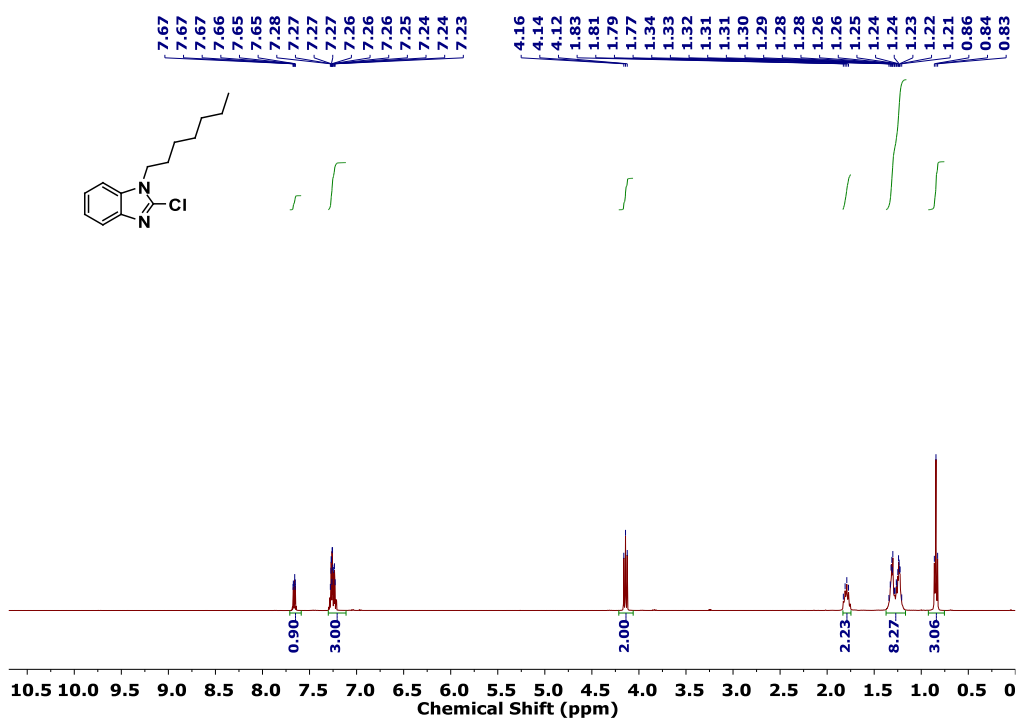


Figure 4.20 400 MHz ^1H NMR of compound **3d** in chloroform-*d*.

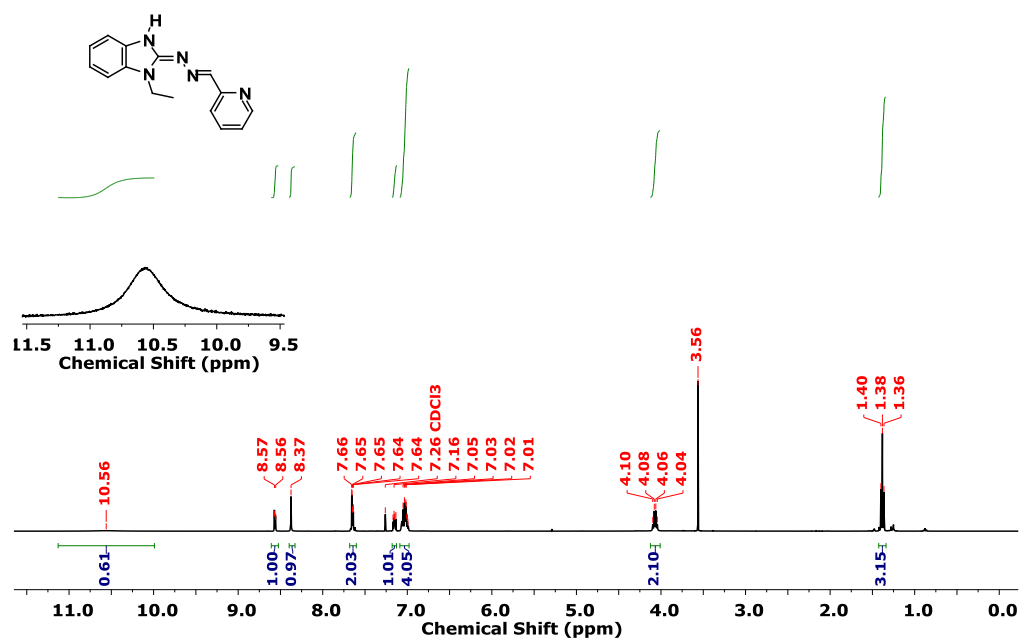


Figure 4.21 400 MHz ^1H NMR of compound **1b** in chloroform-*d*.

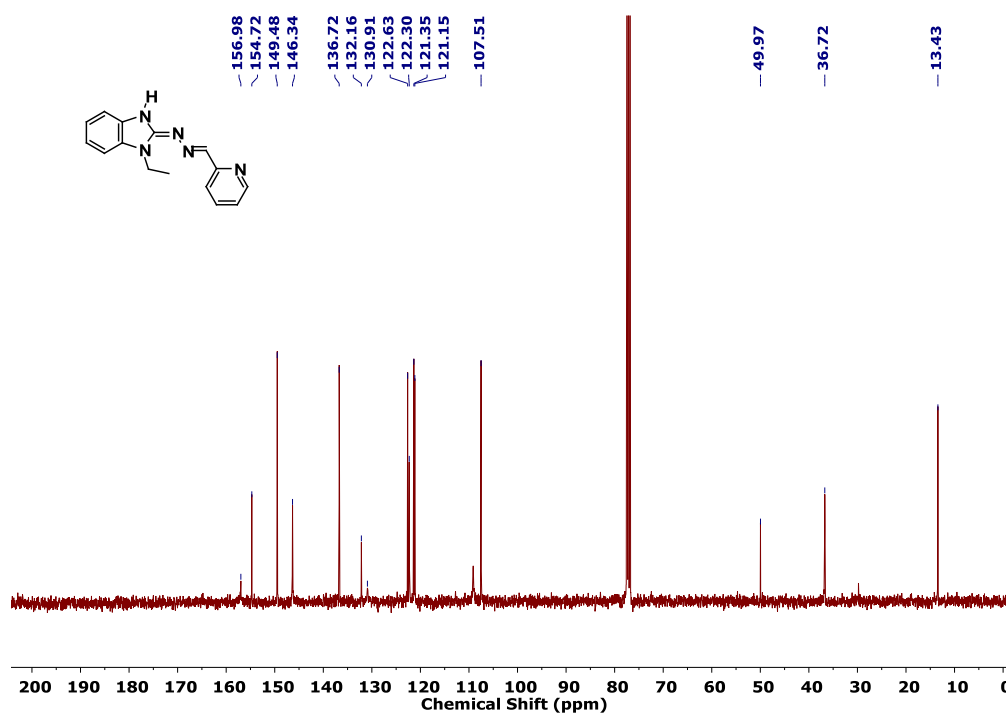


Figure 4.22 101 MHz ^{13}C NMR of compound **1b** in chloroform-*d*.

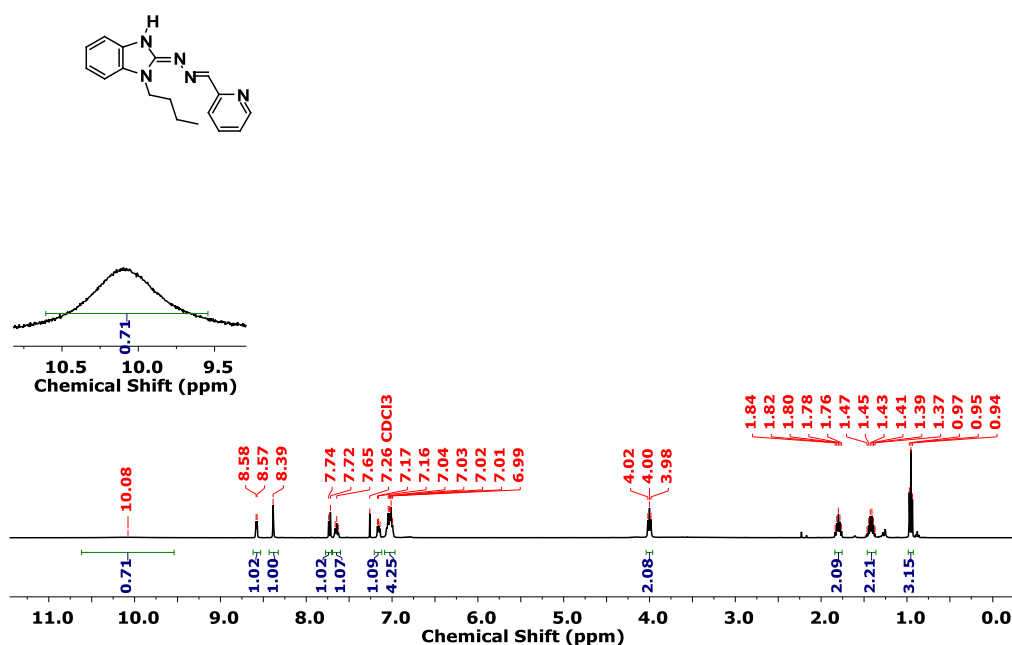


Figure 4.23 400 MHz ¹H NMR of compound **1c** in chloroform-*d*.

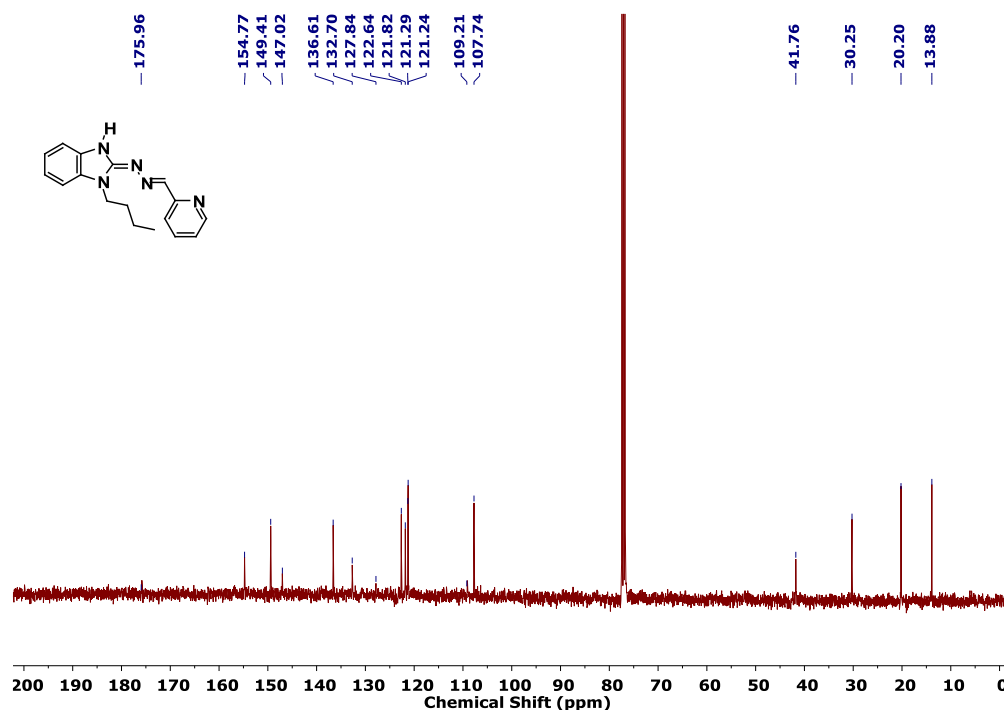


Figure 4.24 101 MHz ¹³C NMR of compound **1c** in chloroform-*d*.

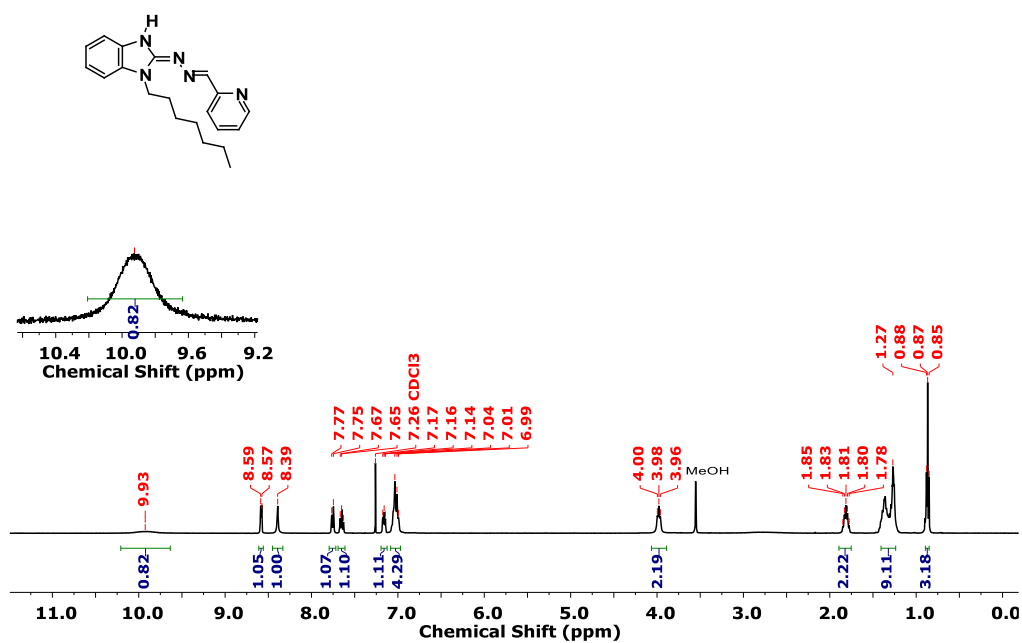


Figure 4.25 400 MHz ¹H NMR of compound **1d** in chloroform-*d*.

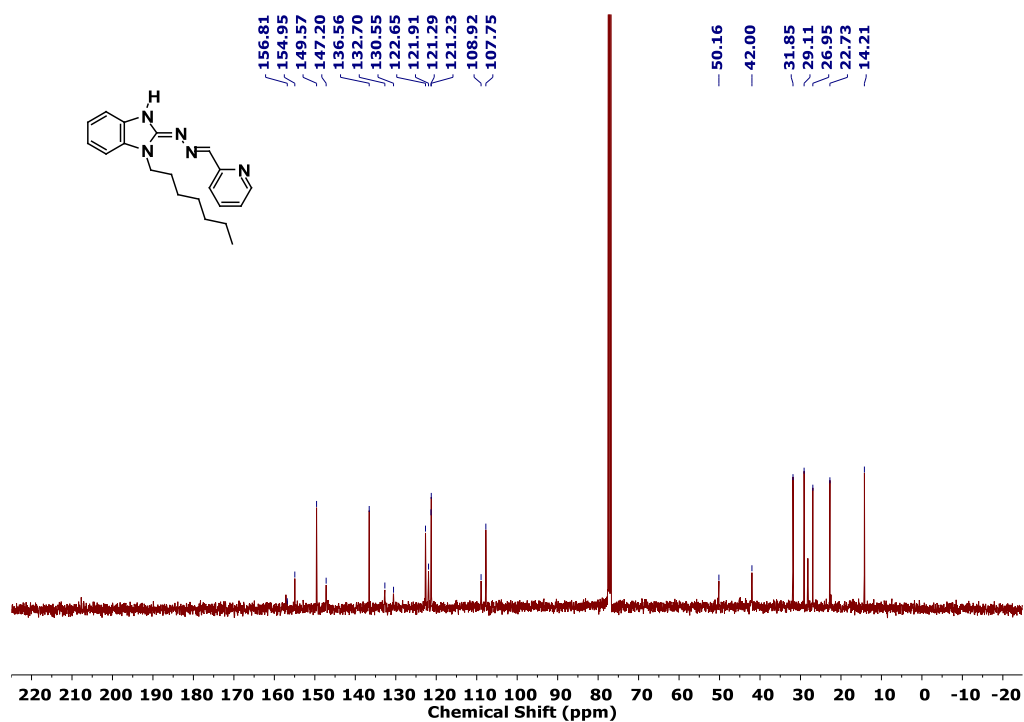


Figure 4.26 101 MHz ¹³C NMR of compound **1d** in chloroform-*d*.

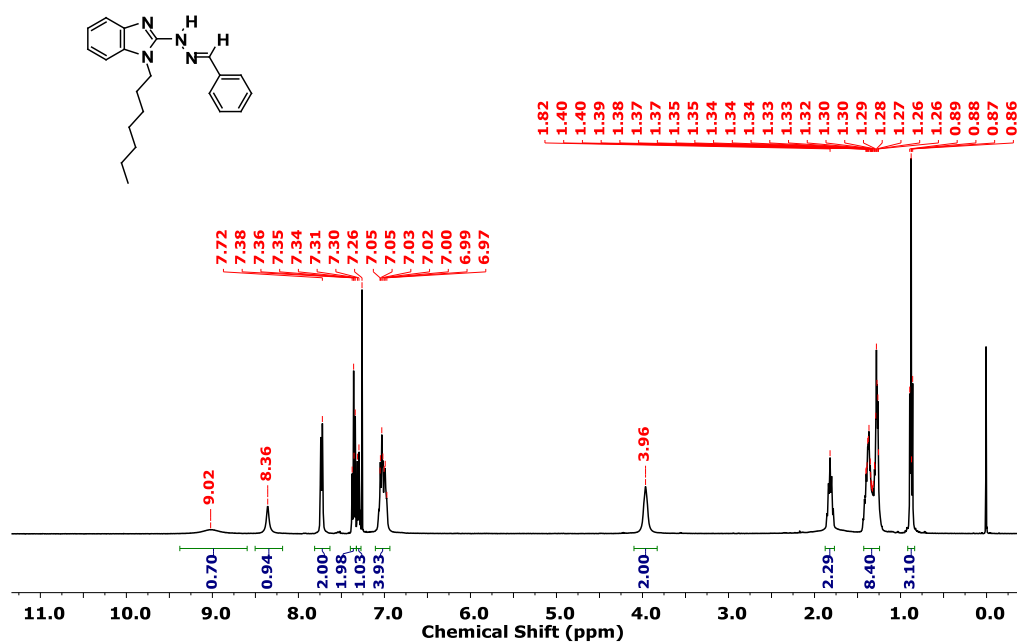


Figure 4.27 400 MHz ¹H NMR of compound **1e** in chloroform-*d*.

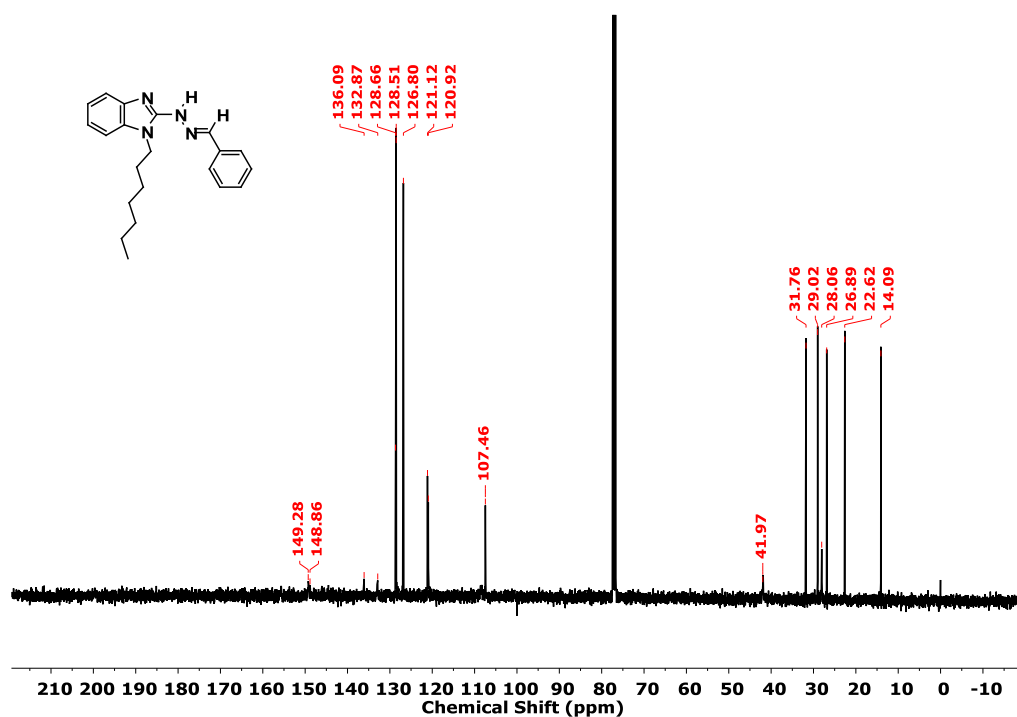


Figure 4.28 101 MHz ¹³C NMR of compound **1e** in chloroform-*d*.

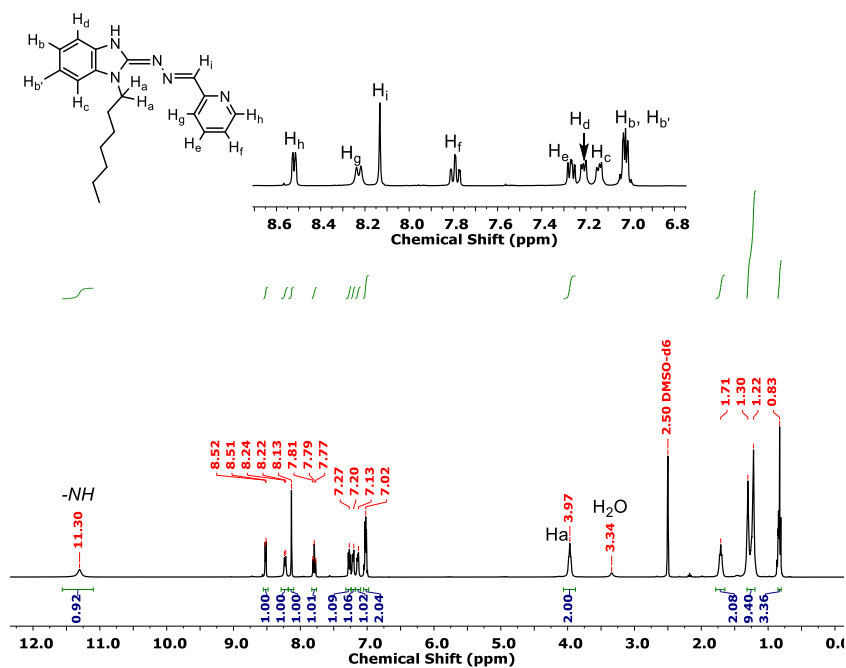


Figure 4.29 400 MHz 1H NMR of compound **1d** in $DMSO-d_6$.

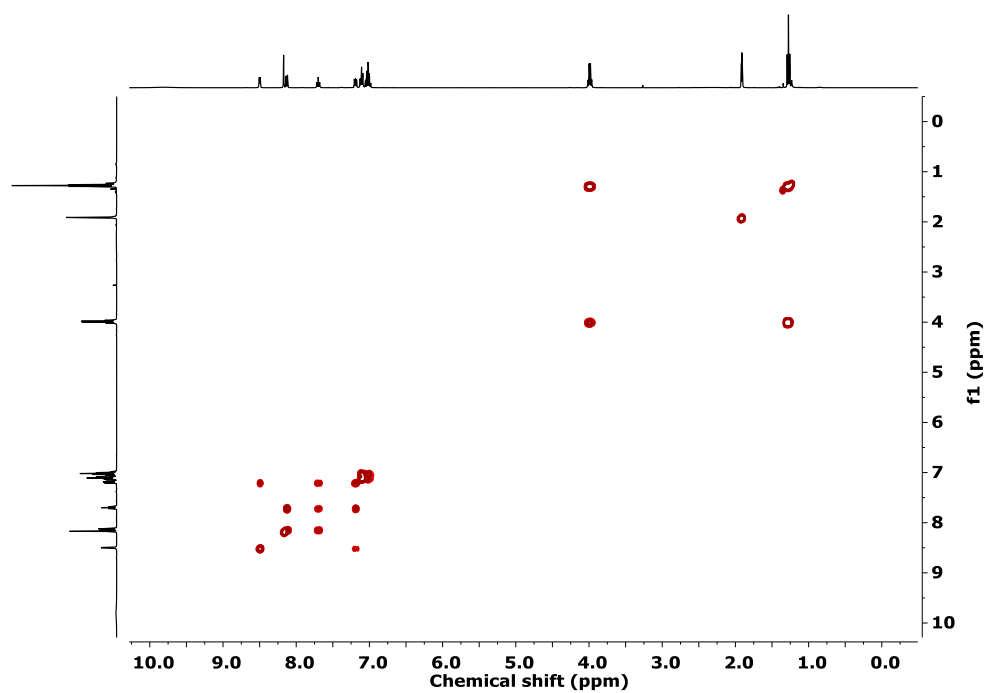


Figure 4.30 2D 1H - 1H COSY NMR spectrum of compound **1b** in $CDCl_3$.

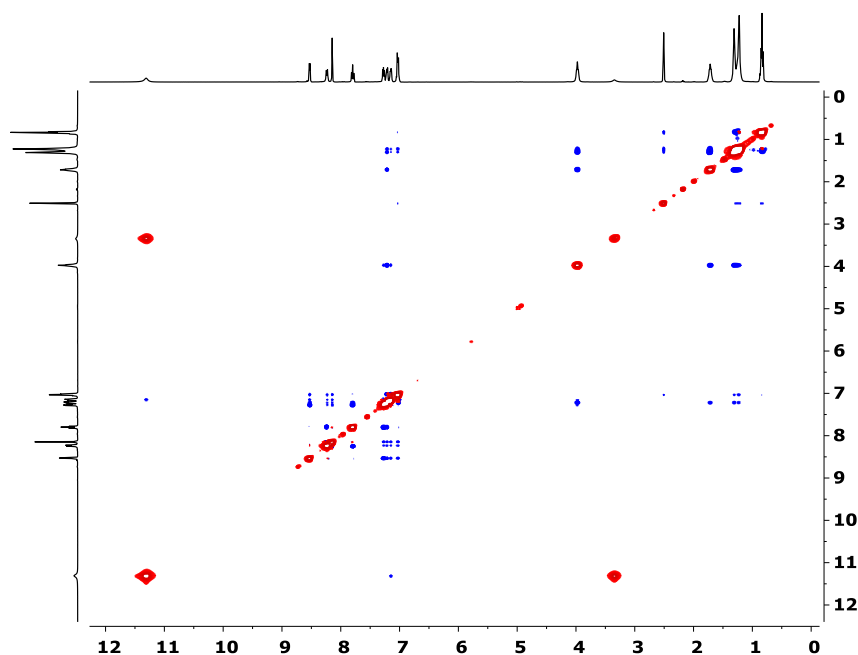


Figure 4.31 2D ^1H - ^1H NOESY NMR spectrum of compound **1d** in DMSO-d_6 .

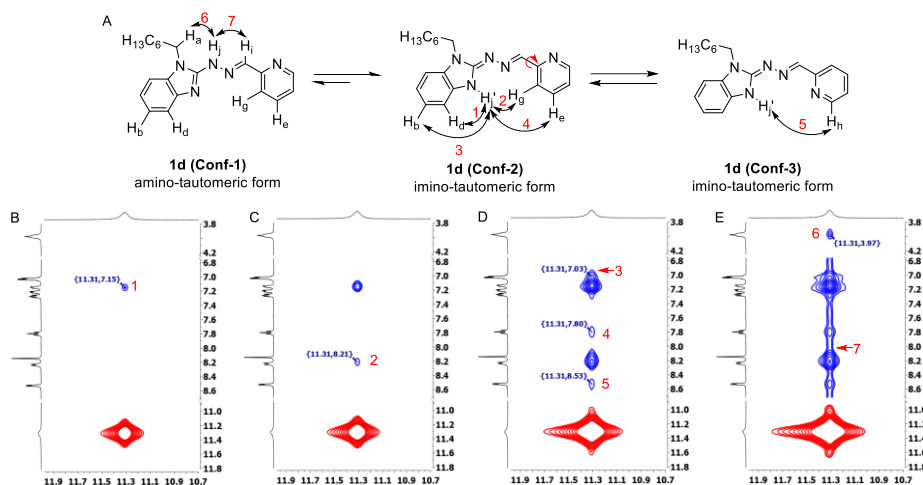


Figure 4.32 Equilibrium between the imino-amino tautomeric forms of **1d** (A). A section of the ^1H - ^1H 2D NOESY spectrum of **1d** in DMSO-d_6 recorded at gradually decreasing contour levels in the order shown in (B, C, D, E). Red peak shows the diagonal peak and blue peaks represent various cross-peaks labeled as 1-7.

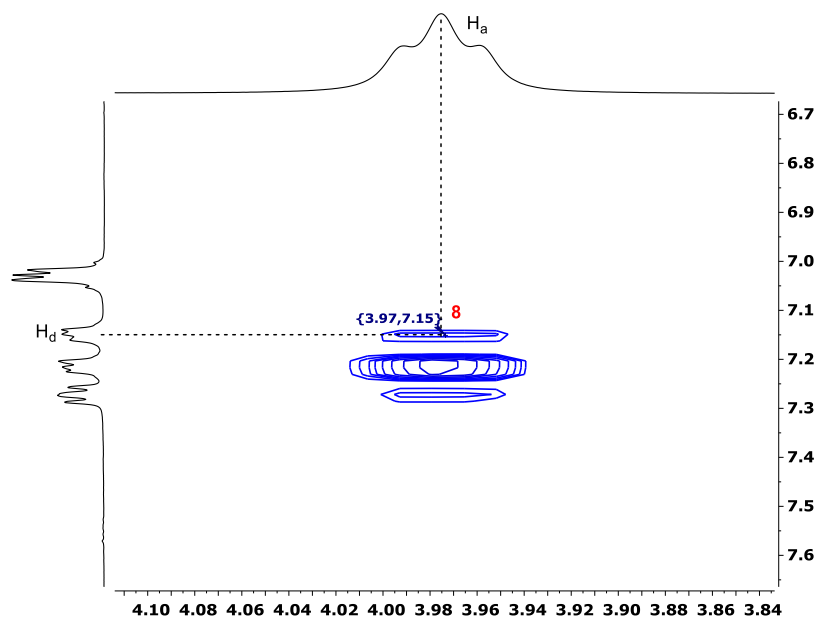


Figure 4.33 Cross peak between H_a and H_d (i.e., cross peak 8) in the 2D ^1H - ^1H NOESY NMR spectrum due to the self-assembly of compound **1d**. This cross peak was observed at a much lower contour level.

Interpretation of 2D ^1H - ^1H NOESY NMR data:

In the solution phase, the major population of imino tautomeric form is well supported with four different correlations i.e., cross peak 1 between H_j and H_d , cross peak 2 between H_j and H_g , cross peak 3 between H_j and H_b , and cross peak 4 between H_j and H_e appeared at higher contour levels (Figure S28). The appearance of the cross peak 5 at an intermediate contour level indicates the presence of **Conf-3**, which is a rotamer of **Conf-2**. The correlations observed between H_j and H_a (i.e., cross peak 6) as well as H_j and H_i (i.e., cross peak 7) appeared at much lower contour levels suggesting the presence of a minor populated **Conf-1** because the cross peak 6 can only appear if H_j proton resides on hydrazone nitrogen that is connected directly to the benzimidazole moiety.

Concentration-dependent ^1H NMR of **1d**:

All NMR spectra were recorded on Bruker AvanceTM III HD Ascend 400 MHz NMR spectrometer equipped with BB(F) double channel probe at 25 °C. ^1H NMR spectra of **1d** at three different concentrations of 15 mM, 1.5 mM and 0.75 mM were measured. The line width at half height of the peak corresponding to the H_i proton was determined in Topspin3.6 software.

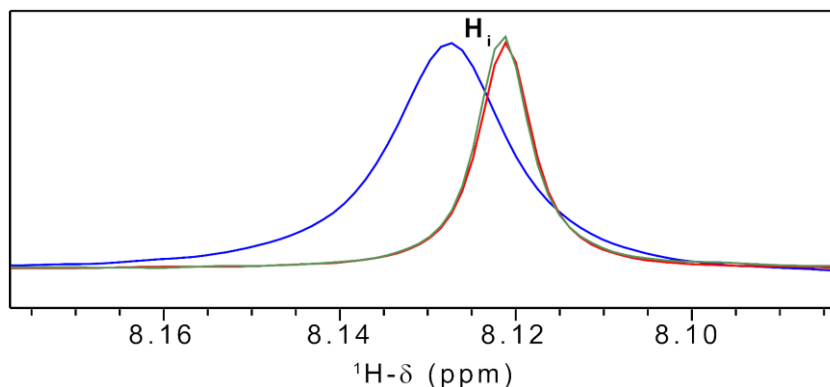


Figure 4.34 ^1H -NMR spectra for **1d** at concentrations of 15 mM (blue) and 1.5 mM (green) and 0.75 mM (red) indicating a decrease in line-width and chemical shift of the H_i proton on dilution that suggests the presence of self-assembly in solution.

CPMG experiment:

The ^1H CPMG experiment was recorded with a constant relaxation delay (T_{relax}) of 32 ms with a variable number of spin-echo blocks ($\tau_{\text{cp}}-180-\tau_{\text{cp}}$) applied. The values of τ_{cp} used are 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0 ms. The area under the curve of each peak was then measured by integration and is used to calculate effective relaxation rates (R_{2eff}) using equation 6:

$$R_{\text{2eff}} = \frac{1}{T_{\text{relax}}} \ln \left(\frac{I_0}{I_{\tau_{\text{cp}}}} \right) \quad (\text{Equation 6})$$

where, I_0 is the integral in the reference spectrum and $I_{\tau_{\text{cp}}}$ is the integral in the spectrum recorded with spin-echo block applied. The effective relaxation rates thus calculated were then fit to two state exchange model using Equation 7 as below in Origin 8.5 software:

$$R_{\text{2eff}} = R_2 + (R_{\text{ex}}) \left(1 - 2 * \tau_{\text{ex}} * \frac{1}{\tau_{\text{cp}}} * \tanh \left(\frac{1}{2 * \tau_{\text{ex}} * 1/\tau_{\text{cp}}} \right) \right) \quad (\text{Equation 7})$$

where, R_2 is the intrinsic relaxation rate of each proton, R_{ex} is the exchange contribution to the effective relaxation rate and τ_{ex} is the timescale of the exchange event.³¹

Table 4.1 Exchange parameters calculated from the global fit of ^1H CPMG-relaxation dispersion data.

Proton	τ_{ex} (ms)	R_2 (1/s)	R_{ex} (1/s)
H _i	0.39 ± 0.04	73.65 ± 1.32	18.62 ± 2.12
H _h	0.39 ± 0.04	47.38 ± 1.28	7.64 ± 2.13
H _j	0.39 ± 0.04	47.23 ± 1.28	7.99 ± 2.13
H _a	0.39 ± 0.04	52.16 ± 1.54	47.95 ± 2.13

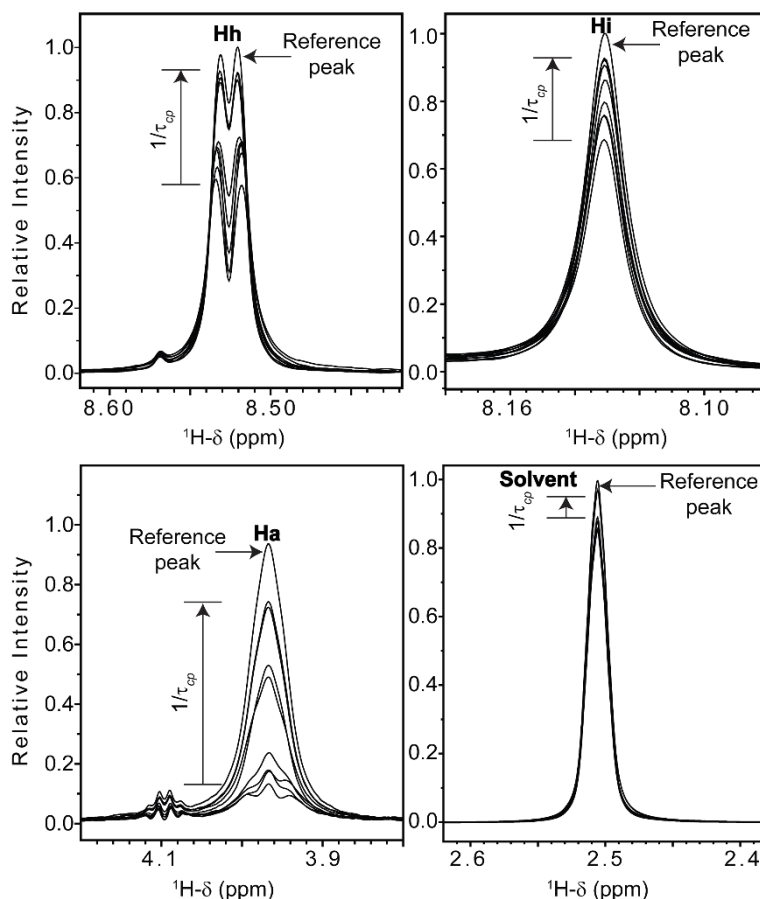
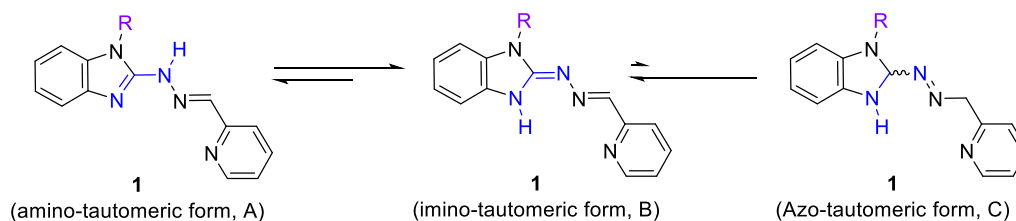


Figure 4.35 Raw ^1H CPMG relaxation dispersion data showing reference peak intensity and intensity variation of the peak as a function of $1/\tau_{\text{cp}}$. (Label in bold on top of each panel indicates the protons as marked in Figure S27). The bottom right panel for solvent proton showed no change in peak intensity on changing $1/\tau_{\text{cp}}$, indicating the absence of relaxation dispersion and hence the absence of any conformational/chemical exchange at this timescale.

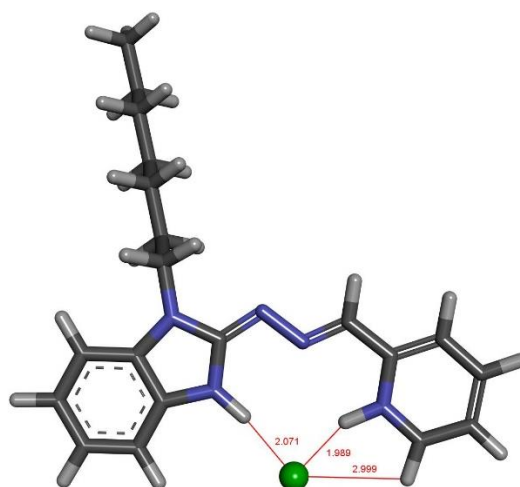
4.4.7 Theoretical Studies

Prediction of the tautomeric population: Theoretical prediction of the tautomeric population was acquired from the tautomer generator plugin of ChemAxon.

Table 4.2 Prediction of Tautomeric Equilibrium

Compound	R	Imino tautomer	Amino tautomer	Azo tautomer
1a	-Methyl	63%	33%	3%
1b	-Ethyl	63%	33%	4%
1c	-Butyl	63%	33%	4%
1d	-Heptyl	63%	33%	4%

Geometry optimization of the $[1d+H^++Cl^-]$ complex: The geometry-optimized structure of the $[1d+H^++Cl^-]$ complex (Figure 4.36) was obtained by Gaussian 09 program using B3LYP functional and 6-31G(d,p) basis set.

**Figure 4.36** Geometry optimized structure of $[1d+H^++Cl^-]$ complex showing the various hydrogen bond interactions involved in anion binding.**Table 4.3** Atomic coordinates of $[1d+H^++Cl^-]$ after geometry optimization by Gaussian 09 program using B3LYP functional and 6-31G(d,p) basis set.

Charge = 0
Multiplicity = 1

Atom #	Atom Type	x	y	z
1	C	0.3434	-0.6958	0.5143

2	N	0.5827	0.6056	0.7515
3	N	1.8456	0.8872	0.4463
4	C	2.2673	2.1098	0.6184
5	C	3.6325	2.3919	0.2789
6	N	4.4256	1.3909	-0.2006
7	C	-4.1926	0.7134	0.6145
8	C	5.7131	1.5647	-0.5445
9	C	6.3175	2.8004	-0.4269
10	C	5.5433	3.8674	0.0634
11	C	4.2193	3.6687	0.4121
12	N	-0.8843	-1.2556	0.7506
13	C	-0.8327	-2.6054	0.3988
14	C	0.4813	-2.8526	-0.0466
15	N	1.173	-1.6556	0.0387
16	C	-1.7897	-3.6164	0.4359
17	C	-1.3883	-4.8862	0.0136
18	C	-0.0787	-5.1312	-0.4293
19	C	0.8809	-4.1182	-0.4674
20	C	-2.0509	-0.5193	1.2299
21	H	2.1755	-1.5097	-0.2246
22	H	4.0589	0.3972	-0.3311
23	H	1.6347	2.9114	1.0029
24	H	6.201	0.6664	-0.9077
25	H	7.3553	2.9304	-0.7066
26	H	5.9852	4.8536	0.1694
27	H	3.6113	4.4825	0.791
28	H	-2.8035	-3.4307	0.7742
29	H	-2.1072	-5.6993	0.0276
30	H	0.1946	-6.1314	-0.7504
31	H	1.8959	-4.2925	-0.8066
32	C	-2.9464	-0.012	0.0932
33	H	-2.6077	-1.1847	1.8987
34	H	-1.6752	0.3179	1.8218
35	H	-3.2445	-0.8575	-0.5397
36	H	-2.3548	0.662	-0.5378
37	C	-5.0966	1.2404	-0.5066
38	H	-3.8859	1.5501	1.2577
39	H	-4.7706	0.0328	1.2562
40	C	-6.3553	1.9512	0.0052
41	H	-5.391	0.4046	-1.1571
42	H	-4.5215	1.9293	-1.1409
43	H	-6.0624	2.7865	0.6573
44	H	-6.9304	1.2608	0.6389
45	C	-7.2591	2.4783	-1.1159

46	C	-8.5189	3.1791	-0.5988
47	H	-7.5468	1.644	-1.7701
48	H	-6.6861	3.1723	-1.7456
49	H	-9.1422	3.5414	-1.4227
50	H	-8.2646	4.0408	0.0289
51	H	-9.1302	2.5001	0.0065
52	Cl	4.1575	-1.5267	-0.8263

4.4.8 UV-Vis Titration Experiments

UV titration experiment with compounds **1a**, **1b** and **1c** was carried out with HBF₄ followed by TBACl to determine the *K_a* value. The path length of the cells used for absorption studies was 1 cm. Working solutions of the host and guest were prepared from their respective stock solutions in acetonitrile. The concentration of host solution in acetonitrile was 4×10^{-5} M. All the absorbance spectra were taken after 3 minutes of mixing the host with guests.

At first, **1c** was titrated with HBF₄ (0, 0.2, 0.4, 0.6, 0.8 and 1.0 eq) in acetonitrile to monitor the changes in the UV-Vis absorption band of the compound (Figure 4.36).

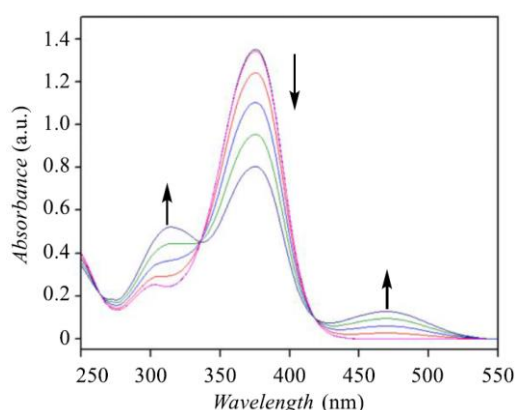


Figure 4.36 UV-Vis titration of compound **1c** with increasing equivalents of HBF₄ (0 – 1 equivalent).

Then in the solution of each compound, 1 eq HBF₄ was added to obtain the salt in situ. The resulting solution was titrated by adding TBACl to determine the *K_a* value. In each of these experiments, we have observed significant changes in wavelengths (a significant increase in absorbance at around 470 nm and a decrease in absorbance at about 312 nm) and absorbance values along with very distinct isosbestic points (Figure 4.37, 4.39, 4.41). Based on this data, we have calculated the binding constant values using Bindfit software (Figure 4.38, 4.40, 4.42).

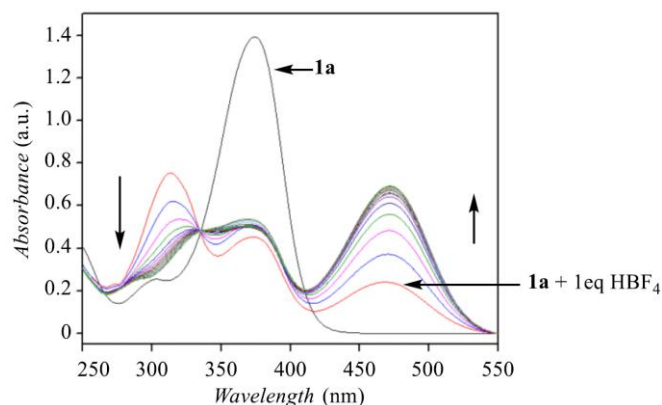


Figure 4.37 UV-Vis titration of compound **1a**•HBF₄ with increasing equivalents of TBACl (0 – 10 equivalents).

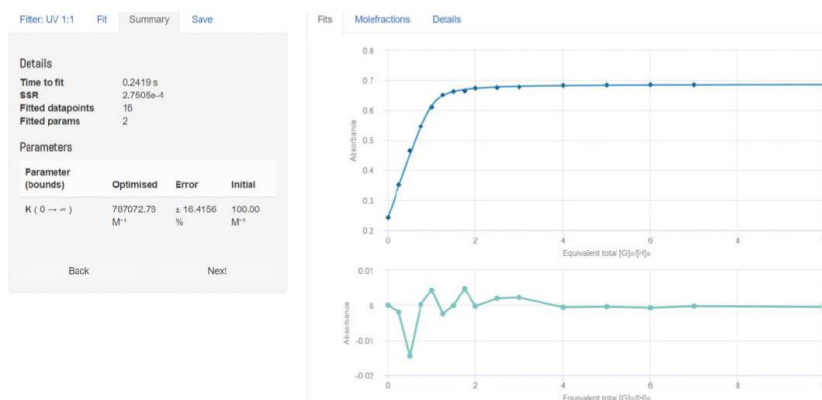


Figure 4.38 Screenshot of the fitted data plot for compound **1a**•HBF₄ from supramolecul.org. The binding constant was found to be 787072.78 M⁻¹ ± 16.4156 % in 1:1 receptor-to-anion binding model. The changing pattern of chemical shift and chemical shift residuals with the increasing equivalent of TBACl. The Bindfit URL for this experiment is

<http://app.supramolecular.org/bindfit/view/7edc9b6c-4c8a-4f26-9660-a77af4a0b2a3>.

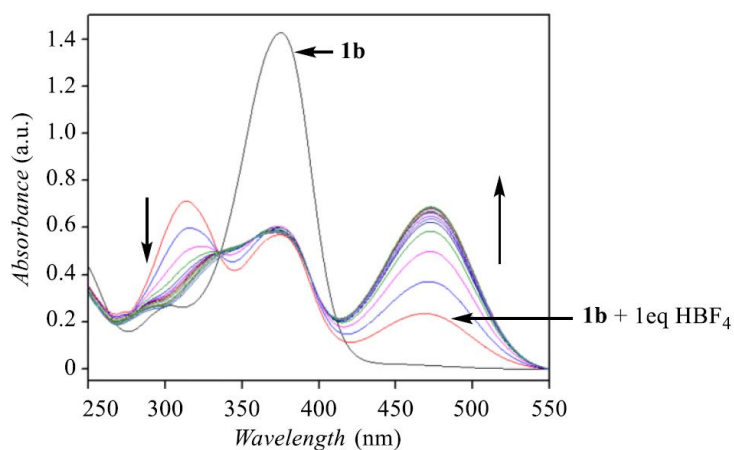


Figure 4.39 UV-Vis titration of compound **1b**•HBF₄ with increasing equivalents of TBACl.

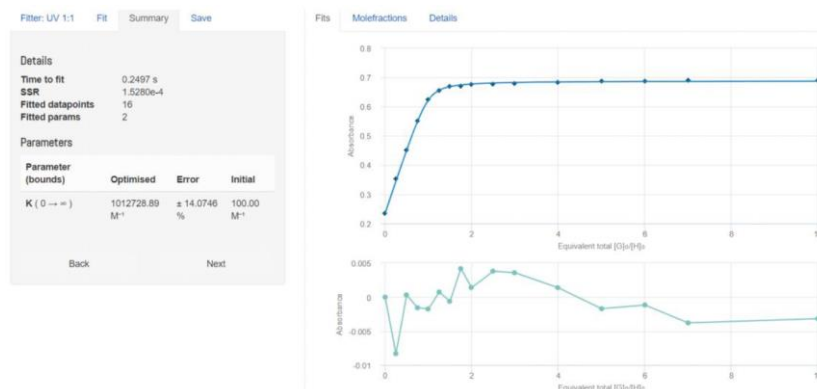


Figure 4.40 Screenshot of the fitted data plot for compound **1b**•HBF₄ from supramolecular.org. The binding constant was found to be 1012728 M⁻¹ ± 14.0746 % in 1:1 receptor-to-anion binding model. The changing pattern of chemical shift and chemical shift residuals with the increasing equivalent of TBACl. The Bindfit URL for this experiment is <http://app.supramolecular.org/bindfit/view/15923121-7ea4-4045-9aae-2551dcc0e1b0>.

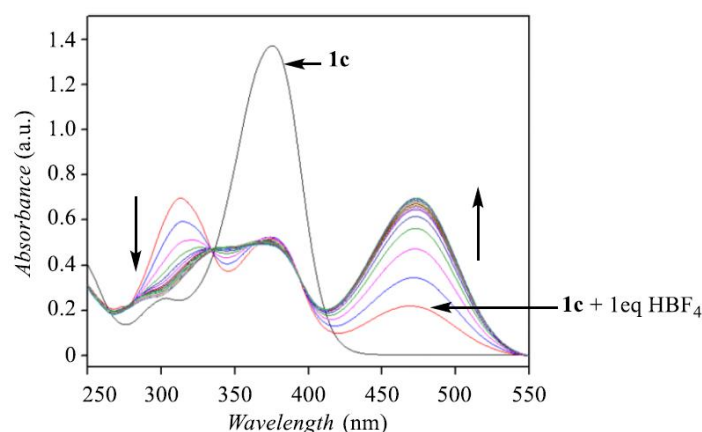


Figure 4.41 UV-Vis titration of compound **1c**•HBF₄ with increasing equivalents of TBACl.

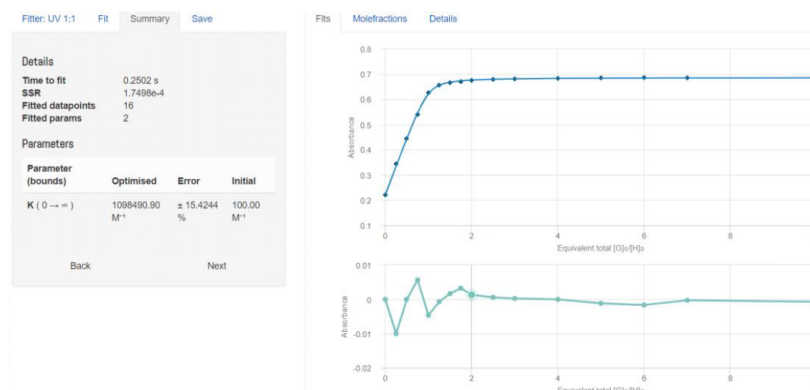


Figure 4.42 Screenshot of the fitted data plot for compound **1c•HBF₄** from supramolecular.org. The binding constant was found to be $1098490.90 \text{ M}^{-1} \pm 15.4244 \%$ in 1:1 receptor-to-anion binding model. The changing pattern of chemical shift and chemical shift residuals with the increasing equivalent of TBACl. The Bindfit URL for this experiment is

<http://app.supramolecular.org/bindfit/view/1fa55682-8208-4695-a457-21fabea831e4>.

We also noted a colour change from colourless to yellow when TBACl was added to a solution of **1c•HBF₄**

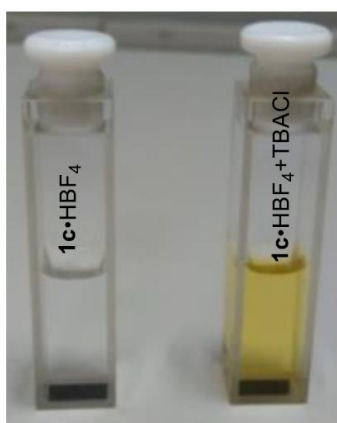


Figure 4.42 Image of **1c•HBF₄** salt solution in acetonitrile (left) and **1c•HBF₄+TBACl** (right).

4.4.9 U-Tube Experiments

In this experiment, we used a “U” shaped tube where 1 mM solution of **1c** in CHCl_3 was placed at the bottom of the U-tube. The left arm of the U-tube was filled with aqueous HCl (100 mm) solution (pH 1.05), while the right arm was filled with aqueous NaNO_3 (100 mm).

The outcome is such that two aqueous solutions never come in contact. The pH and Cl^- concentration changes were monitored in the right arm by pH and Cl^- selective electrodes, respectively, from time to time. The steady decrease in pH and increase in Cl^- concentration in the right arm of the U-tube within a very short period of time clearly proves that compound **1c** transports HCl through a mobile carrier mechanism. In contrast, no chloride concentration and pH change was observed in the absence of **1c**.

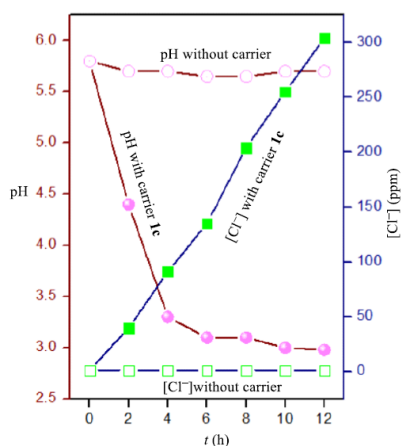


Figure 4.43 Transport of H^+/Cl^- by **1c**, measured across a U-tube using pH and Cl^- selective electrodes.

4.4.10 Planar Bilayer Conductance Measurements

A bilayer membrane was formed across an aperture of 150 μm diameter in a polystyrene cup (Warner Instrument, USA) with lipid diphytanoylphosphatidylcholine (Avanti Polar Lipids) dissolved in *n*-decane (20 mg/mL). Both chambers (cis and trans) were filled with a symmetrical solution containing 1.0 M KCl. The trans compartment was held at virtual ground, and the cis chamber was connected to the BC 535 head-stage (Warner Instrument, USA) via matched Ag- AgCl electrodes. Compound **1c** (20 μM) was added to the trans chamber, and the solution was stirred with a magnetic stirrer for 10 min. No channel opening and closing events were observed after applying voltages. Currents were low pass filtered at 1 kHz using pClamp9 software (Molecular probes, USA) and analog-to-digital converter (Digidata 1440, Molecular probes). The data were analyzed by the software pClamp 11.1. The complete data trace contained no opening and closing events at any point; hence, the channel mechanism in the transport process can be ruled out. From this entire trace, a small portion is presented below.



Figure 4.44 Current traces at the applied voltage of 200 mV under symmetrical KCl (1.0 M) conditions across the diphytanoylphosphatidylcholine (diPhyPC) bilayer membrane before (A) and after (B) addition of **1c** (20 μM).

4.4.11 Biological Studies

Cell culture protocol: The MCF 7 cells were grown in High Glucose Dulbecco's Modified Eagle Medium (DMEM; Lonza) containing 10% fetal bovine serum (FBS; Invitrogen), 2.0 mM L-glutamine (Invitrogen) and 100 units/mL penicillin-streptomycin (Invitrogen). Cells were maintained in 100 mm tissue culture-treated dishes (Corning) at 37 °C in humidified 5% CO₂ incubator (Thermo Scientific).

MTT-based cytotoxicity assay: The cells were dispersed in 96-well flat-bottom tissue culture-treated plates (Corning) at the density of 10⁴ cells/well (per 100 μL) and incubated at 37 °C in a 5% CO₂ incubator for 24 h. Compounds were added to each well in different concentrations by maintaining a maximum amount of DMSO at 2 μL and incubated for 24 h. DMEM solution containing compounds in each well was replaced with 110 μL of MTT-DMEM mixture (5 mg MTT/mL of DMEM) and incubated for 4 h in identical conditions. After 4 h, the MTT solution was removed and 100 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was recorded in a microplate reader (Varioskan Flash) at 570 nm. All experiments were performed in triplicates, and the relative cell viability (%) was expressed as a percentage of untreated cells.

Chloride mediated cell death studies.

HBSS buffer solution. Hank's balanced salt solution (HBSS with Cl⁻) was prepared with the following compositions: 136.9 mM NaCl, 5.5 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.81 mM MgSO₄, 1.25 mM CaCl₂, 5.5 mM D-glucose, 4.2 mM NaHCO₃ and 10

mM HEPES (pH 7.4). Chloride-free HBSS was prepared by mixing 136.9 mM Na-gluconate, 5.5 mM K-gluconate, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.81 mM MgSO₄, 1.25 mM Ca-gluconate, 5.5 mM D-glucose, 4.2 mM NaHCO₃ and 10 mM HEPES (pH 7.4).³²

Cells were dispersed in 96-well flat bottom tissue culture treated plates (Corning) at 104 cells/well (per 100 µL) density and incubated at 37 °C in 5% CO₂ for 16 h. Cellular media was replaced by an HBSS buffer (either with Cl⁻ or without Cl⁻) containing 10% FBS. Compounds were added to each well in different concentrations by maintaining a maximum amount of DMSO at 2 µL and incubated for 24 h. HBSS buffer solution of compounds in each well was replaced by 110 µL of MTT-DMEM mixture (0.5 mg MTT/mL of DMEM) and incubated for 4 h in identical conditions. The remaining MTT solution was removed after 4 h, and 100 µL of DMSO was added in each well to dissolve the formazan crystals. The absorbance was recorded in a microplate reader (Varioskan Flash) at 570 nm.

Acridine orange staining

Literature procedures^{29, 33, 34} were adopted to carry out acridine orange staining experiments. Specifically, MCF 7 cells were cultured for 24 h, and the cell plate was washed with PBS. Compound **1d** (2.0 µM) was added and incubated at 37 °C for 4 h. Then the cell plate was washed with PBS, and Acridine Orange solution (5.0 µg/mL) was added. Incubation was conducted for an additional 0.5 h. The supernatant was removed, and the cell plate was washed with PBS. Standard DMEM culture media (1.0 mL) was added, and the cells were examined by Leica sp8 confocal microscope in the red and green channels. The images were processed by ImageJ software.

4.5 References

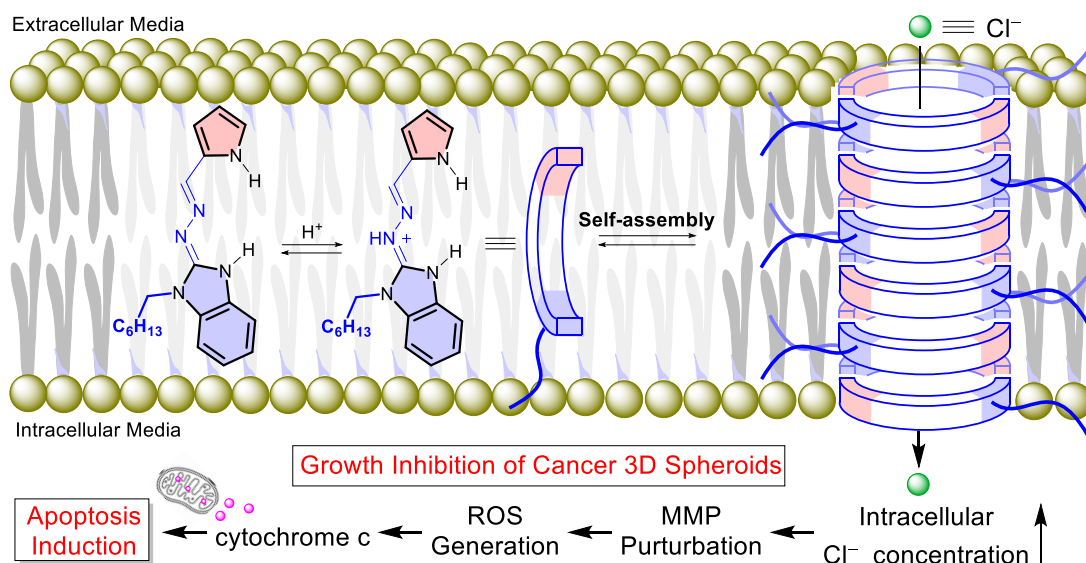
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Chapter 5

A Pyrrole-linked Benzimidazolyl Hydrazone Self-Assembles to Form HCl Channel and Induces Apoptosis in Cancer Cells



5.1 Introduction

One of the most critical processes in living cells is ion transport across membranes.^{1, 2} This critical action is provided by proteins that operate as ion channels or carriers.³ Any faults in these systems would result in specific diseases associated with ion flow abnormalities, known clinically as channelopathies.⁴⁻⁷ For example, cystic fibrosis (CF) is associated with poor Cl^- conductance along the CFTR channel due to unanticipated mutations.⁸ The effort to comprehend these proteins has prompted the creation of artificial anion channel models. Another reason for developing synthetic anion channels is that these substances may well govern anion exchange mechanisms in cells.⁹ In recent years, synthetic ion transporters have been developed as potential therapeutic options for replacing defective proteins with structurally simpler molecules to efficiently restore transport activities.¹⁰⁻¹² Moreover, recent research revealed that artificial ion transporters have the potential to cause death in cancer cells by altering intracellular pH or interrupting cellular ion homeostasis^{9, 13-15}, reigniting interest among scientists working in this realm to develop novel antitumor agents. Over the years, a significant amount of work has been invested in developing artificial transporters capable of selective ion recognition and transport. However, compared to well-documented Cl^- carriers that shuttle across membranes, examples of synthetic channels with hydrophilic interiors allowing subsequent HCl hopping via specific conducting pathways are rare.^{16, 17} Moreover, only limited headway has been made in transferring fundamental studies concerning transport processes into *in vitro* systems.

Throughout the last few decades, tremendous progress has been made in understanding cancer. Yet, cancer is the world's second leading cause of death.¹⁸ Nowadays, systemic medicines, such as chemotherapy, hormone therapy, and biological therapies, are often used to treat cancer. However, the uncontrolled killing of healthy cells, the toxicity of current chemotherapeutic medications, and the emergence of multi-drug resistance offer a motivation to search for alternative therapies based on new mechanisms of action. One such alternate strategy might be the use of artificial transmembrane ion transporters, which can cause apoptosis (also known as programmed cell death) in cancer cells by altering the intracellular pH or interfering with intracellular ion homeostasis.^{9, 19} Since such systems do not rely on cancer-causing proteins, receptors, enzymes, or genes, they may also be able to combat cancer's multi-drug resistance.

Synthetic ion channels have been built using both single-molecule and self-assembly techniques.^{9, 20, 21} The ability of basic building blocks to form useful structures makes self-

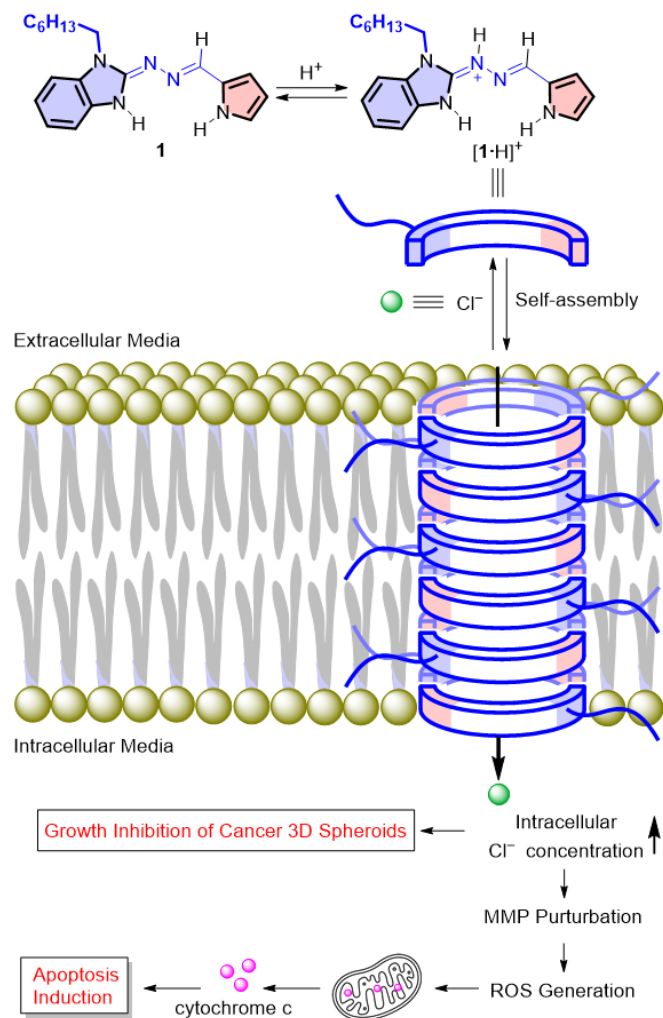


Figure 5.1 Structures of benzohydrazide derivatives **1** and schematic representation of self-assembly of these molecules.

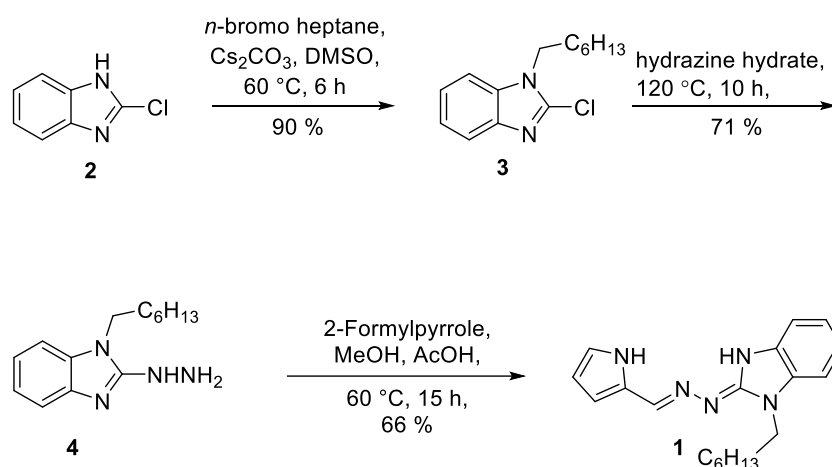
assembly an appealing method for creating channels within membranes. The crystal structures of the mechanosensitive and K^+ channels exemplify how spectacularly nature uses self-assembly to produce ion channels.^{22, 23} It is challenging to create a self-assembled framework from a "small molecule" since the basic building block frequently lacks the pre-organization or appropriate functionalities needed to give the dimensions and binding sites for the construction of ion channels.²⁴ In a recent work reported by our group,²⁵ we introduced a pyridyl-linked hydrazone that exists in its imino- tautomeric form and provides a suitable binding cavity for Cl^- ion through a cooperative protonation and Cl^- ion recognition. Interestingly, the transporter is the highest active HCl carrier reported to date in the literature.²⁵ When we examined the crystal structure of the protonated transporter, we found that the transporter lacks a proper stacking interaction, which hinders the formation of an ion channel across the membrane. In a quest to develop a channel for HCl transport, we anticipated that if the pyridyl group could be replaced with an electron-rich aryl moiety, such as pyrrole, there would be antiparallel stacking between the electron-rich pyrrole and electron-deficient benzimidazole ring. Two such stacked skeletons would further connect to each other in the presence of Cl^- ions to form a Cl^- ion channel across the membrane. Herein, we report a benzimidazolyl hydrazone that self-assembles to form a nanochannel assembly across the membrane for the selective translocation of HCl – a system, which is rarely reported in the

literature. We also demonstrate solid-state evidence that the compound self-assembles in the presence of HCl to produce organized arrays that include chloride-filled and water-filled channels. When tested on cellular systems, the compound showed extremely high toxicity toward cancerous cell lines while remaining relatively nontoxic to cell lines of non-cancerous origin. The compound also showed better efficacy when compared with the commercially available anticancer drug, Doxorubicin, in inhibiting the growth of MCF-7 3D spheroid cultures.

5.2 Results and Discussions

5.2.1 Synthesis

Synthesis of compound **1** was carried out from 1-heptyl-2-hydrazineyl-1*H*-benzo[*d*]imidazole **4**, which was synthesized following our previously reported protocol.²⁵ Compound **4** was subjected to a condensation reaction with 2-Formylpyrrole in the presence of a catalytic amount of glacial acetic acid to obtain the final product **1** in 66 % yield (Scheme 5.1).



Scheme 5.1 Synthetic scheme for the preparation of **1**.

5.2.2 X-Ray Crystal Structure Analysis

Compound **1** was crystallized by the slow evaporation of a solution of **1** in CH_2Cl_2 (Figure 5.2 A). Compound **1**·HCl was crystallized by slow diffusion of HCl into the solution of **1** in CH_2Cl_2 (Figure 5.2 C). The crystal structure of **1** revealed that compound **1** itself does not participate in self-assembly to form a channel-like architecture in the solid state as it lacks a proper stacking interaction for constructing a nanochannel assembly. Instead,

intermolecular orthogonal H-bonding interactions were found in the crystal structure of **1**. However, when **1** gets protonated in the presence of HCl, it participates in an antiparallel stacking interaction between the benzimidazole ring and the pyrrole ring. These antiparallely stacked moieties further connect, leading to a hollow nanotubular cavity filled with Cl^- ions and water molecules (Figure 5.2). Interestingly, the hydrophobic outer surface of the assembly faces outwards. In contrast, the polar hydrophilic recognition sites face inwards, making it ideal for the assembly to sit and span the lipid bilayer membrane to construct an ion channel.

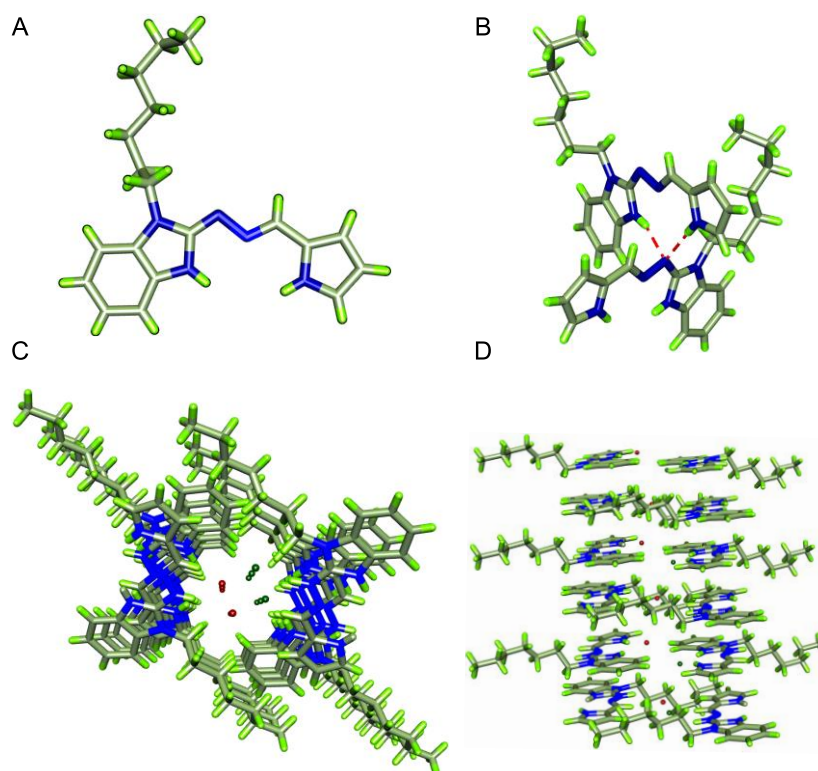


Figure 5.2 Crystal structure of compound **1** (A). Intermolecular hydrogen bonding among the monomer units of **1** showing orthogonal interactions (B). The top view (C) and the side view (D) of the self-assembled architecture of **1**·HCl show nanochannel assembly in the solid state.

5.2.3 Ion Transport Study

The ion transport activity of compound **1** was evaluated across large unilamellar egg yolk phosphatidylcholine vesicles (EYPC-LUVs) encapsulating a pH-sensitive probe 8-hydroxypyrene-1,3,6-trisulfonate (HPTS). During the experiment, a pH gradient was created across the bilayer membrane before the addition of transporter **1**. Compound **1** showed extremely high ion transport activity with a half-maximal effective concentration (EC_{50})

value of only 11 nM (Figure 5.3 B). The Hill analysis using the dose-dependent activity profile provided the Hill coefficient value of 1.01, indicating a thermodynamically stable ion channel formation.

To assess the selectivity of transporter **1** toward different anions, HPTS-based fluorimetric assays were conducted by changing both intravesicular and extravesicular buffers with sodium salts of various anions, such as Cl^- , Br^- , ClO_4^- , and NO_3^- . The pH was dissipated the most in the case of Cl^- as compared to the other anions, confirming the Cl^- selectivity of **1** (Figure 5.3 C).

To understand if cations participate in the transport process mediated by **1**, a lucigenin-based fluorimetric assay was conducted employing various salts of alkali metal cations. Lucigenin is a Cl^- ion-sensitive dye whose fluorescence gets quenched in the presence of Cl^- ions. During the experiment, a Cl^- ion gradient was created across the bilayer membrane before the addition of transporter **1**.²⁵ A significant quenching of the lucigenin fluorescence was observed when 2 μM of compound **1** was applied in NaCl buffer, re-confirming the Cl^- ion transport efficacy of compound **1** across the lipid bilayer membranes. However, no significant change in the quenching rate of lucigenin fluorescence was observed upon variation of alkali metal cations (i.e., Li^+ , Na^+ , K^+ , Rb^+ and Cs^+), ruling out the influence of cations in the overall ion transport activity (Figure 5.3 D).

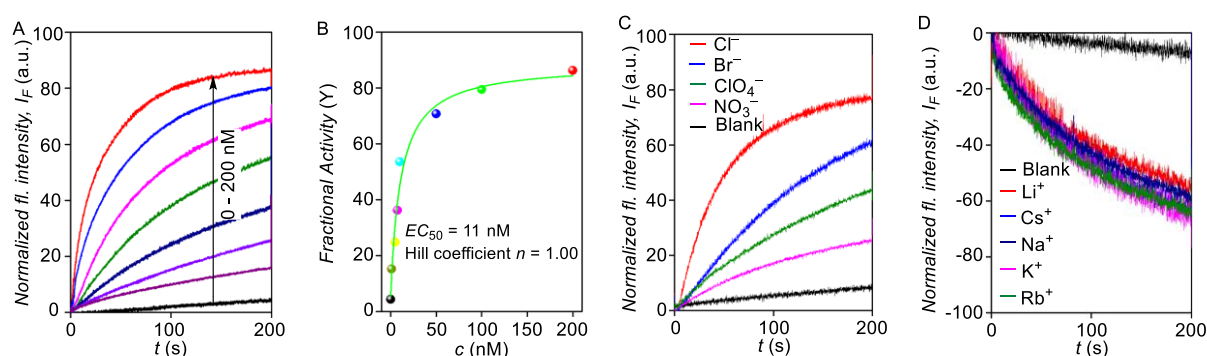


Figure 5.3 Dose-dependent ion transport activity of **1** in the HPTS assay (A). The Hill analysis of **1** (B). The ion transport activity of **1** in the HPTS assay by varying intravesicular and extravesicular anions, i.e., Cl^- , Br^- , ClO_4^- , and NO_3^- (C). The ion transport activity of **1** in the lucigenin assay by varying the extravesicular cations, i.e., Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+ (D).

We used a cationophore-coupled assay that Gale and colleagues recently reported investigating the underlying ion transport mechanism.²⁶⁻²⁹ For a transporter, the transport can

be electrogenic (i.e., the net charge would change across the membrane), electroneutral (i.e., the net charge would not change), or a combination of the two processes.³⁰ Valinomycin is an electrogenic K^+ cationophore that causes a change in net charge without affecting the transmembrane pH gradient.³⁰ Monensin, on the other hand, is an electroneutral cationophore that acts as an M^+/H^+ antiporter, dissipating the transmembrane pH gradient.²⁹ By using lucigenin-based cation selectivity assays, we have ruled out the involvement of various alkali metal cations in the transmembrane transport process, eliminating the possibility of M^+/Cl^- symport mechanism ($M^+ = Li^+, Na^+, K^+, Rb^+, \text{ and } Cs^+$). So, we tried to figure out whether the transport is electrogenic or electroneutral. A chloride ion-selective electrode was used to monitor the concentration of effluxed Cl^- during the valinomycin and monensin-coupled experiments across EYPC-LUVs. When **1** was looked at, we discovered that Cl^- efflux is only seen in the presence of monensin but not valinomycin (Figure 5.4). It means that K^+/H^+ antiport by monensin cooperatively couples to H^+/Cl^- co-transport, facilitated by **1**, resulting in overall KCl efflux from the liposomes. The lack of chloride efflux in the presence of valinomycin explains why H^+ and Cl^- transport by **1** is an integrated process, i.e., chloride cannot be transported without a proton, and protonated **1** cannot diffuse through the lipid bilayer without a counteranion.

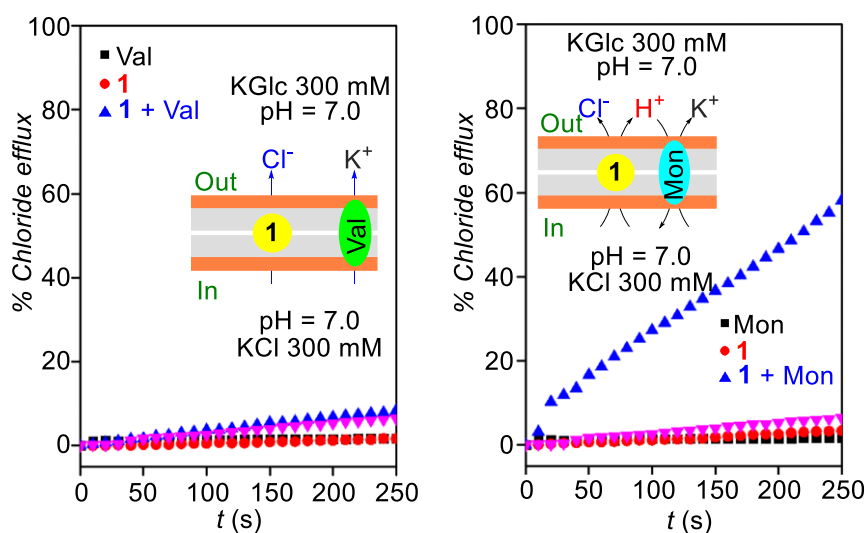


Figure 5.4 Chloride efflux activity of **1** from EYPC-LUV in the presence and absence of valinomycin (A) and monensin (B).

To confirm the formation of an ion channel, electrical conductance was assessed electrophysiologically³¹⁻³³ across a bilayer membrane made of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) lipid upon the addition of compound **1**. The experimental setup

includes two compartments, cis and trans, each filled with 1M KCl electrolyte. An Ag/AgCl electrode is connected to both chambers. A bilayer membrane was formed across a 150 μm diameter aperture in a polystyrene cup at the junction of the two compartments. The electrolyte solutions in both chambers are electrically isolated in this situation. When compound **1** (2 μM) was added to the trans chamber, and either positive or negative voltage was applied, distinct and periodic channel opening and closing events were observed (Figure 5.5 A), confirming the formation of an active ion channel at the black lipid membrane (BLM). Further, the current vs voltage plots was analyzed in symmetrical and asymmetrical electrolyte solutions, which revealed an ohmic behaviour, i.e., a linear relationship between current and voltage, indicating the non-dipolar nature of the ion channels. Using Hille's equation,¹ the diameter of the channels was found to be 5.32 ± 0.77 Å, suitable for recognising hydrated chloride anions. When the ion selectivity was tested using asymmetric KCl solutions in two compartments (0.5 M in trans and 1.0 M in cis), the reversal potential was found to be -23 mV (Figure 5.5 B), confirming that the conducting ion is Cl^- . According to the Goldman-Hodgkin-Katz equation¹, the permeability ratio $P_{\text{Cl}^-}/P_{\text{K}^+} = 3.60$ was calculated, supporting the anion selective nature of the channel created by **1**.³⁴

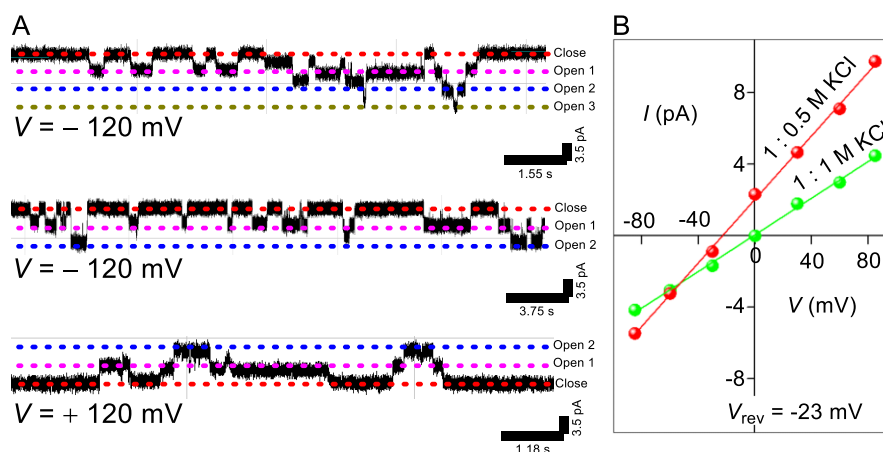


Figure 5.5 Current traces with **1** ($c = 2.0$ μM) at -120 mV and $+120$ mV in a symmetrical KCl solution (A). Current–voltage relationship of **1** in both symmetrical and asymmetrical KCl concentrations (B).

5.2.4 Molecular Modeling of the Ion Channel

Atomistic computer simulations were carried out to achieve mechanistic insight into ion transport and the state of channel integrity during the passage of ions through the channel. To precisely understand whether the studied channel is an HCl channel or chloride ion transporting channel, we have considered its protonated form and the non-protonated channel. At first, the coordinates of both the channel structures, the protonated and the non-protonated ones (composed of six consecutive layers), were minimised using MAESTRO.³⁵ Then, the semi-minimised structures were employed for the construction of the channel/membrane complex by utilizing the membrane builder protocol of CHARMM-GUI.^{36, 37}

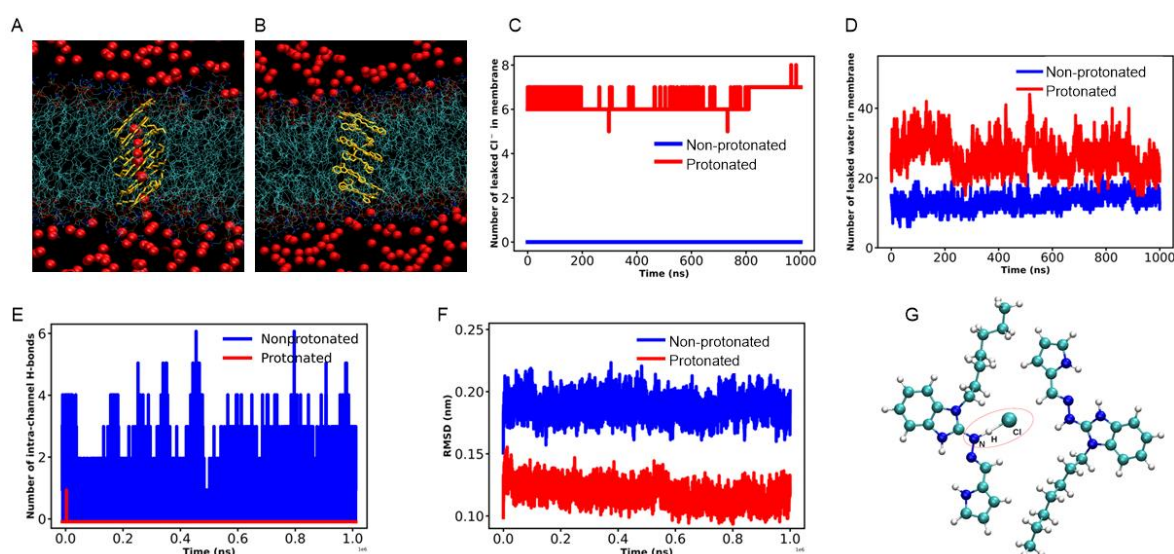


Figure 5.6. Snapshots obtained from equilibrium simulation of protonated channel (A) and non-protonated channel (B) representing chloride ion transport through only protonated form. The leakage profile of chloride ions through each of the channels (protonated and non-protonated) (C). The leakage profile of water molecules through each of the channels (D). The number of intra-channel hydrogen bonding calculated for each channel (E). The root mean square deviation (RMSD) profile was calculated for each channel (F). MD snapshot and a layer of monomers for the DFT calculations (G).

Upon analyzing the results of an equilibrium simulation of two channel structures, it was observed that only the protonated channel could transport Cl^- ions, whereas the non-protonated structure does not allow for the passage of Cl^- ions (Figure 5.6A, 5.6B). The channel consists of 12 monomers forming 6 layers, and Figure 2 shows that 6-7 Cl^- ions are constantly present inside the channel, allowing for the continuous transport of Cl^- ions from one side to the other. When a Cl^- ion enters the channel, it loses some water molecules from its solvation shell due to confinement and interaction with the ligand. Thus, it moves with

fewer water molecules, leading to water leakage in the channel, as shown in Figure 5.6D. The non-protonated channel, which forms multiple inter-channel hydrogen bonds (Figure 5.6E), interacts with itself rather than passing Cl^- ions and water. Therefore, our simulation suggests that the designed channel is involved in transporting HCl molecules, as chloride ions can only be transported through it when the channel is protonated.

To provide further evidence of our findings, we analyzed the leakage profile of chloride ions through both channels. Our observations revealed significant anionic leakage through the protonated channel, whereas the non-protonated channel did not allow for any leakage of Cl^- ions (as shown in Figure 5.6C). These findings indicate that the protonated channel efficiently transports hydrogen chloride through its lumen.

To better understand the transport mechanism through the channel, we analyzed the accessibility of water within the channel core. Figure 5.6D illustrates the count of water leakage through both channel structures. We found that water leakage through the protonated channel structure was comparatively higher than the non-protonated form, which can be correlated with the facile ion transport through the protonated channel. The presence of water in the channel core facilitates ion transport by keeping the ion hydrated within the channel and promoting ion passage.

We have calculated the number of hydrogen bonds for each channel structure (Figure 5.6E) to analyze their mechanistic details. We found that the non-protonated channel exhibits a significantly higher level of hydrogen bonding between the channel residues, which may impede ion transport by limiting the channel's core. In contrast, the protonated channel exhibits almost no significant hydrogen bonding among the channel residues, implying that the channel is better suited for transporting HCl.

Toward this end, we analyzed the channel structures' structural integrity to investigate the relationship between channel stability and the transport process. The root mean square deviation (RMSD) of each channel structure, both protonated and non-protonated, was measured (Figure 5.6F). While chloride ion transport was not observed through the non-protonated channel, we found that the non-protonated channel had a higher RMSD than the protonated channel. This suggests that the protonated form of the channel is more stable than the non-protonated form. Furthermore, the absence of abrupt fluctuations in the RMSD curve for the protonated channel structure during chloride transport indicates that the protonated

channel maintains structural integrity even after ion passage. This finding is consistent with the fact that the channel is formed via self-assembly only after protonation.

Classical molecular dynamics (MD) simulations reveal that Cl^- ions transport through the channel by hopping across the N-H bonds of the monomer. To understand this interaction better, we conducted density functional theory (DFT) calculations on a system consisting of a single Cl^- ion taken from an MD snapshot and a layer of monomers (Figure 5.6G). We performed energy minimization on this system to investigate N-H and Cl^- interaction. The resulting relaxed structure showed that the N-H bond elongated from 1.04 to 1.18 Å, and the H-Cl bond was 1.72 Å, which falls within the coordination range. The Mulliken overlaps populations of the H atom for H-N (0.34) and H-Cl (0.27) indicated an intermediate formation of the N-H-Cl bond. Additionally, the formation energy of -0.98 eV confirmed the stability of this intermediate bond. Our analysis supports the hopping of Cl^- ions along the monomers observed in the MD simulations.

5.2.5 Effect on Cellular Systems

We looked into the excellent Cl^- ion transport activity of channel **1** in terms of their biological applicability. Recent research has shown that altering the extracellular or intracellular pH level and affecting the homeostasis of Cl^- ions are linked to anticancer effects.³⁸⁻⁴² The cell viability of different cancerous and non-cancerous cell lines, including human lung adenocarcinoma epithelial cells-A549, human breast cancer cells-MCF7, triple-negative human breast cancer cells-MDA-MB-231, human osteosarcoma cells-U2OS, colon adenocarcinoma cells-SW480, human cervical cancer cells-HeLa; and the non-cancerous human embryonic kidney cells-HEK293T, and mouse embryonic fibroblasts (MEFs) was assessed by conducting the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.⁴³ Compound **1** exhibited high toxicity in all cancerous cell lines, as seen in Figures 5.7A and 5.16, with U2OS cells showing the highest toxicity ($IC_{50} = 0.1 \mu\text{M}$) followed by MCF-7 cells ($IC_{50} = 0.49 \mu\text{M}$). In contrast, the non-cancerous HEK293T cells and MEFs showed only marginal toxicity. Subsequent experiments were carried out using U2OS and MCF-7 cells, where the highest level of toxicity was seen.

Further investigations were carried out to comprehend how compound **1** mediates cytotoxicity in cancer cells. The viability of U2OS and MCF-7 cells was assessed using extracellular media containing or missing Cl^- ions in the presence of compound **1** for

determining the role of Cl^- ion transport in causing cytotoxicity. To accomplish this, Hank's balanced salt solution (HBSS)^{40, 44} with and without Cl^- ions were used, and viability of cells were evaluated upon incubation with channel **1** for 24 h following MTT assay. Significantly higher cytotoxicity was observed in both U2OS and MCF-7 cells treated with compound **1** in HBSS buffer containing Cl^- ions, as shown in Figure 5.7B and 5.7C, confirming the role of Cl^- ion transport in mediating cytotoxicity.

Previous research from our group¹⁵ and others^{9, 19} have shown that disrupting cellular Cl^- ion homeostasis is linked to activating apoptotic pathways in cells. To elucidate further, we

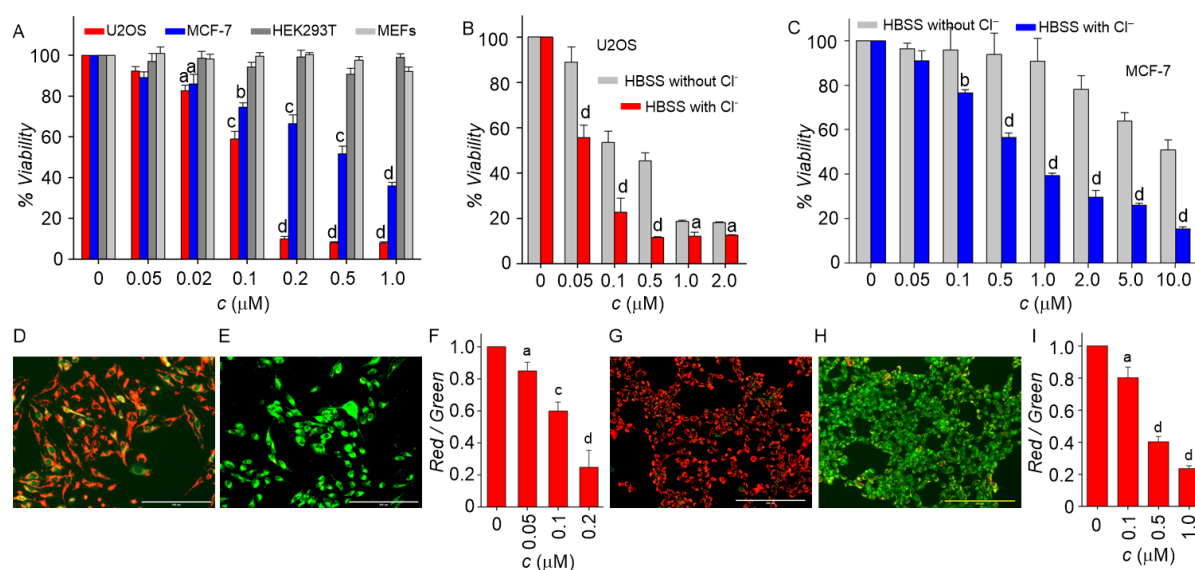


Figure 5.7 Assessment of cellular viability of cancer and non-cancerous cells in the presence of **1** by MTT assay (A). Viability of U2OS (B) and MCF-7 (C) cells upon treatment with **1** in the presence/absence of Cl^- ions in the extracellular medium. Representative live cell images of U2OS cells upon treatment with 0 μM (D) and 0.1 μM of **1** (E) followed by staining with JC-1 dye. Representative live cell images of MCF-7 cells upon treatment with 0 μM (G) and 0.5 μM of **1** (H) followed by staining with JC-1 dye. Red and green channel images were merged to generate the displayed image. Mitochondrial membrane potential as assessed by fluorescence intensity of JC-1 dye in the presence of **1** in U2OS (F) and MCF-7 cells (I). *a* represents $p < 0.05$; *b* represents $p < 0.01$; *c* represents $p < 0.001$; *d* represents $p < 0.0001$ using Tukey's HSD test when compared to the untreated control.

started with probing any change in the mitochondrial membrane potential (MMP) in the presence of channel **1**. U2OS and MCF-7 cells were treated with **1** in a dose-dependent manner, followed by staining with MMP-sensitive dye JC1. When JC1 accumulates in a

healthy mitochondrial membrane, it emits red fluorescence due to J aggregation; however, if the mitochondrial membrane is damaged, JC1 gets dispersed into the cytosol resulting in green fluorescence emission.^{45, 46} When U2OS and MCF-7 cells were preincubated with **1**, the ratio obtained from red/green fluorescence measurements for JC1 dye decreased dose-dependent (Figure 5.7F and 5.7I). Live cell images of JC-1-stained U2OS and MCF-7 cells that fluoresced red under control conditions support this data (Figure 5.7D and 5.7G). Treatment of these cells by **1** resulted in a sharp drop in the red fluorescence with a concomitant increase in the green fluorescence, suggesting that channel **1** is capable of rupturing MMP in these cells (Figure 5.7E and 5.7H).

Changes in mitochondrial membrane potential and dynamics have been linked to increased oxidative stress and the accumulation of reactive oxygen species (ROS).^{47, 48} In fact, treatment of **1** in U2OS and MCF-7 cells resulted in elevated levels of both mitochondrial and cellular ROS as measured by MitoSox red (Figure 5.8C and 5.8F) and shown by increased green fluorescence using the H₂DCFDA probe (Figures 5.8B and 5.8E), respectively.

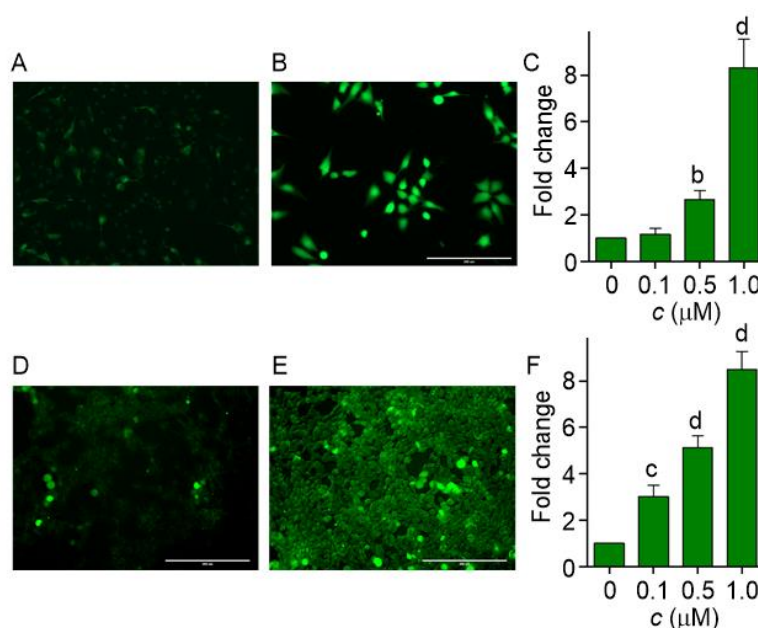


Figure 5.8 The representative live cell images of H₂DCFDA dye-stained U2OS cells upon treatment with 0 μ M (A), 0.1 μ M (B) of **1**. The representative live cell images of H₂DCFDA dye-stained MCF-7 cells upon treatment with 0 μ M (D), 0.5 μ M (E) of **1**. Mitochondrial ROS levels, as estimated by MitoSox red in the presence of **1** in U2OS cells (C) and MCF-7 cells (F). *a* represents $p < 0.05$; *b* represents $p < 0.01$; *c* represents $p < 0.001$; *d* represents $p < 0.0001$ using Tukey's HSD test when compared to the untreated control.

Increased levels of ROS in the cells trigger the release of apoptotic factors, which ultimately cause the mitochondrial respiratory cycle's electron transport chain to break.^{49, 48, 50} In keeping with this, U2OS and MCF-7 cells were immunostained with a primary antibody specific to cytochrome c to examine the release of cytochrome c from the mitochondria into the cytosol. As evident from Figure 5.9B and 5.9C, channel **1** mediated the release of cytochrome c from the mitochondria into the cytosol in a dose-dependent manner when compared with the untreated cells (Figure 5.9A). Furthermore, a dose-dependent increase in the cleaved poly(ADP-ribose) polymerase (PARP) (86 kDa), a marker of apoptosis,⁵¹ was observed in immunoblot assays upon incubation of channel **1** in MCF-7 cells in a dose-dependent manner (Figure 5.9D and 5.9E). These findings collectively demonstrated that channel **1** induces apoptosis in cancer cells.

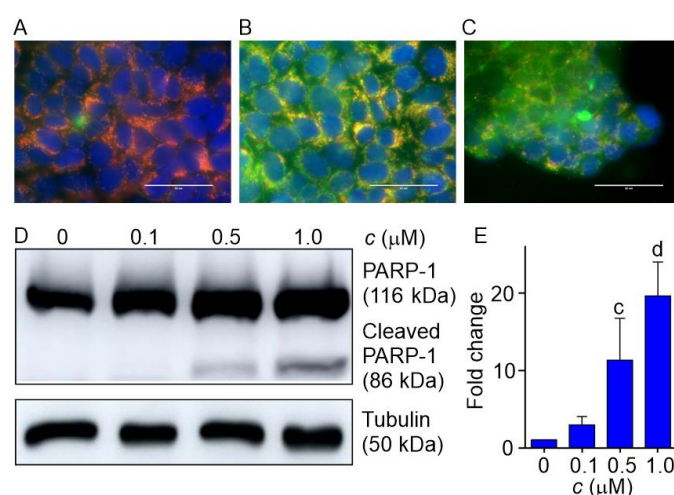


Figure 5.9 Representative live-cell images of MCF-7 cells treated with 0.1 μ M (B) and 0.5 μ M (C) of **1** for 24 h, and untreated cells (A) analyzed for cytochrome c release by immunostaining with cytochrome c specific primary antibody (green). Cells were counterstained with Phalloidin (red) and DAPI (blue). Immunoblot assay for PARP-1 in MDA-MB-231 cells after 24 h incubation with **1** in a dose-dependent manner (D) concentrations. Densitometric analysis (N = 3) of the expression of cleaved PARP-1 with respect to Tubulin (used as reference gene) using ImageJ (E). *a* represents $p < 0.05$; *b* represents $p < 0.01$; *c* represents $p < 0.001$; *d* represents $p < 0.0001$ using Tukey's HSD test when compared to the untreated control.

Doxorubicin (DOX) has previously been shown to be a potent inhibitor of MCF-7 cells in 2D and 3D culture models.⁵² It has been shown to significantly affect cell proliferation at concentrations ranging from 0.8 to 1.0 μ M. The excellent Cl^- ion-mediated cytotoxicity of channel **1** in 2D cultures prompted us to investigate its effect on MCF-7 3D spheroid cultures.

Using a seven-day protocol, the growing 3D cultures were treated with DOX on days 4, 5, and 6 at a concentration of 0.8 μM . Whereas channel 1 was treated on the growing 3D cultures only on day 4 at a concentration of 0.5 μM . Immunofluorescence staining with Phalloidin and Hoechst 33258 (Figure 5.10A) clearly shows that channel 1 significantly reduces surface area (Figure 5.10B), surface volume (Figure 5.10C), and the number of cells per spheroid (Figure 5.10D) when compared to the control. In fact, channel 1 was more effective at reducing the growth and spread of MCF-7 3D spheroids than the widely used anticancer drug DOX, suggesting that channel 1 may very well be able to reduce tumour growth *in vivo*.

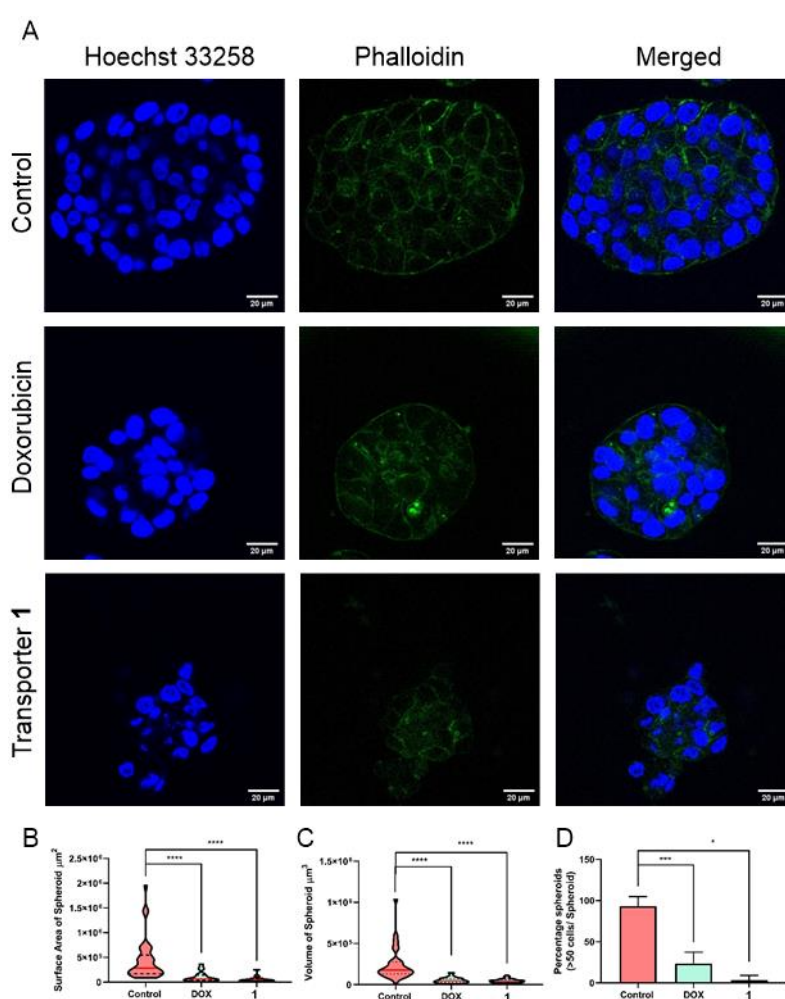


Figure 5.10 The immunofluorescence technique was employed to visualize 3D cultures, and two staining agents, Phalloidin and Hoechst 33258 (A), were used to label actin filaments and cellular nuclei, respectively. The ImageJ software was used to quantify various parameters, such as surface area (μm^2) (B), spheroid volume (μm^3) (C), and the number of cells present in each spheroid (D),

under different experimental conditions. DOX = doxorubicin. *Represents $p < 0.05$, **, $p < 0.01$ and **** for $p < 0.001$ using Kruskal-Wallis test followed by Dunn's multiple comparison test.

5.3 Conclusion

In conclusion, we introduced a pyrrole-linked benzimidazolyl hydrazone **1** that self-assembles to form an oligomeric supramolecular nanochannel assembly capable of effective and selective transmembrane transport of H^+/Cl^- . In-depth ion transport studies revealed that the operative ion transport mechanism is a non-electrogenic H^+/Cl^- symport. Detailed MD simulations, quantum mechanical calculations, and electrophysiological experiments confirmed the formation of a stable HCl channel across the membranes at ambient temperatures and pressures.

Studies on cells using cell lines from cancerous and non-cancerous origins demonstrated that the Cl^- ion transport mediated by compound **1** imparts toxicity to cancerous cells but remains relatively nontoxic to cell lines from non-cancerous origins. The increased level of intracellular Cl^- ion concentration disrupts the mitochondrial membrane potential (MMP) and builds up reactive oxygen species (ROS). This elevated production of ROS eventually dysfunctions mitochondrial transition pores leading to the release of cytochrome c into the cytosol from the mitochondria, commencing the apoptotic process. Furthermore, channel **1** inhibits the growth and spread of MCF-7 3D spheroids more effectively than the widely used anticancer drug DOX, implying the potential of channel **1** to inhibit tumour growth *in vivo*.

5.4 Experimental Section

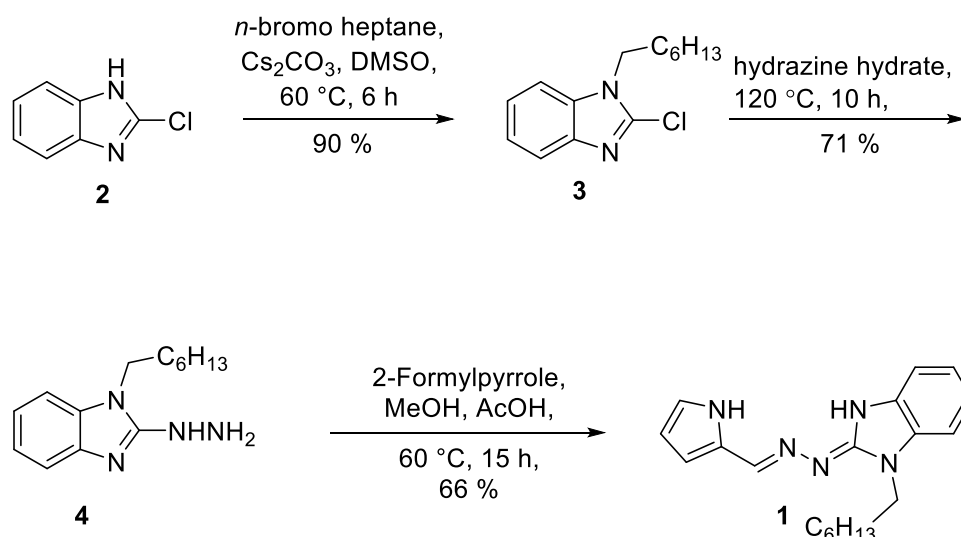
5.4.1 General Methods

Reagents for synthesis were purchased from commercial sources (Sigma Aldrich, and Spectrochem) and used as it is without further purification. HPTS, HEPES buffer, Triton X-100, NaOH and inorganic salts were of molecular biology grade obtained from Sigma. Egg-yolk phosphatidylcholine (EYPC) lipid as a solution in $CHCl_3$ (25 mg/mL), polycarbonate membrane (100 nm and 200 nm pore size), and mini-extruder used for vesicle preparation was obtained from Avanti Polar Lipids. All the Stock solutions were prepared in either HPLC grade DMSO or Methanol.

5.4.2 Physical Measurements

^1H NMR spectra were recorded at 400 MHz, and ^{13}C NMR spectra at 101 MHz. All the spectra were calibrated with respect to the residual solvent peak. The following abbreviations were used to describe peak patterns wherever appropriate: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants are reported in hertz (Hz). High-resolution mass spectra (HRMS) were performed on an electron spray ionization time-of-flight (ESI-TOF). Melting points were measured with a micro melting point apparatus. Single Crystal X-Ray Diffraction (SCXRD) measurements were done on a Bruker-KAPPA APEX II CCD diffractometer. Fluorescence spectra were recorded from Fluoromax-4 from JobinYvon Edison equipped with an injector port and a magnetic stirrer. Measurements of pH were done using a Helmer pH meter. All data from fluorescence studies were processed by either KaleidaGraph or Origin 8.0 software.

5.4.3 Synthesis Procedures



Scheme 5.2 Synthesis of compounds **1**.

Synthesis of compound 2, 3, and 4: Synthesis of these compounds were done according to known protocol.²⁵

Procedure for the synthesis 1: In a 50 mL round-bottomed flask, a mixture of pyrrole-2-carbaldehyde (270 mg, 2.84 mmol), hydrazine **4** (700 mg, 2.84 mmol) and MgSO_4 (1700 mg, 14.2 mmol) in methanol (10 mL) containing a catalytic amount of glacial acetic acid (3 drops) was heated to reflux using an oil bath until thin layer chromatography monitoring indicated no further conversion (approximately 15 h). The resulting hydrazone was then extracted in EtOAc. The organic extract was dried over Na_2SO_4 and evaporated in a vacuum

to obtain a crude residue which was then subjected to purifying by column chromatography using EtOAc/Hexane to get the pure solid compounds **1** (66%).

Characterization data of 1: Purified using EtOAc/Hexane (15:85 (v/v)); grey solid, Yield 66%, 179 mg, **¹H-NMR (400 MHz, CDCl₃):** δ 9.69 (s, 1H), 8.37 (s, 1H), 7.03 – 6.80 (m, 4H), 6.74 (d, J = 1.8 Hz, 1H), 6.41 (dd, J = 3.6, 1.7 Hz, 1H), 6.18 (dt, J = 3.6, 2.2 Hz, 1H), 3.91 (t, J = 7.5 Hz, 2H), 1.76 (t, J = 7.4 Hz, 2H), 1.27 (dddd, J = 24.4, 9.3, 7.6, 3.7 Hz, 8H), 0.86 (t, J = 6.9 Hz, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 155.9, 138.6, 133.0, 131.1, 130.5, 120.8, 120.1, 109.5, 109.1, 108.2, 107.6, 41.2, 31.6, 28.8, 27.8, 26.6, 22.5, 14.4. **IR (Neat, v_{cm}⁻¹):** 3729, 2922, 1743, 1615, 1571, 1481, 1416, 1370, 1284, 1232, 1155, 1124, 1092, 1027, 1012, 967, 933, 884, 788, 732, 719, 604. **HRMS (ESI):** m/z : [M + H]⁺ calculated for C₁₉H₂₅N₅ 324.2183; found: 324.2188.

5.4.4 Single Crystal X-Ray Diffraction Studies

Crystals of **1** suitable for single crystal X-ray diffraction analysis was obtained by the slow evaporation of a solution of **1** in CH₂Cl₂. Compound **1**·HCl was crystallized by a slow diffusion of HCl into the solution of **1** in CH₂Cl₂. Bruker KAPPA APEX II CCD Duo diffractometer (operated at 1500 W power: 50 kV, 30 mA) using graphite-monochromated Mo K α radiation (λ = 0.71073 Å) was used to collect single-crystal X-ray data at 100 K. SAINT software was used to integrate and reduce the data. SHELXTL was used to solve all the structures and refined on F^2 by a full-matrix least-squares technique using the SHELXL-97 program built in the Apex-3 package⁵³. Hydrogen atoms were constructed in geometric positions to their parent atoms.⁵⁴ Adsym subroutine of PLATON was used to confirm that there is no additional symmetry possible.

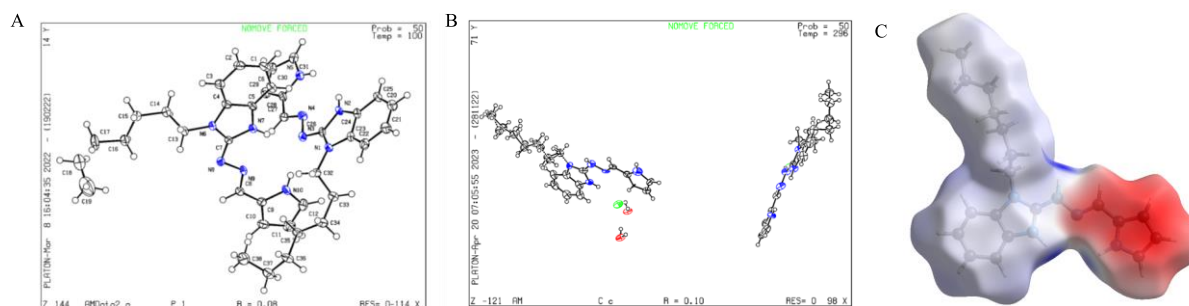


Figure 5.11 ORTEP diagrams of the structure **1** (A) and **1**·HCl (B) with 50% probability ellipsoids established by single X-ray crystallography. The electrostatic potential on [1·H]⁺ surface generated in crystalExplorer21 software using the HF/3-21G level of theory (C). In electrostatic potential surface the blue and red colors indicate the electron deficient and electron rich areas on a molecule.

Hirshfeld surface analysis:

We generated the electrostatic potential of $[1\cdot\text{H}]^+$ using the crystal data. The electrostatic potential on receptor surface generated in crystalExplorer21 program⁵⁵ using the default program “Tonto”, method “HF” and basis set “3-21G”. The blue and red colors indicate the electron deficient and electron rich areas on a molecule (Figure S2). The analysis clearly indicates that the pyrrole ring is electron rich and the protonated benzimidazole is a electron deficient center, promoting antiparallel stacking to form the ion channel.

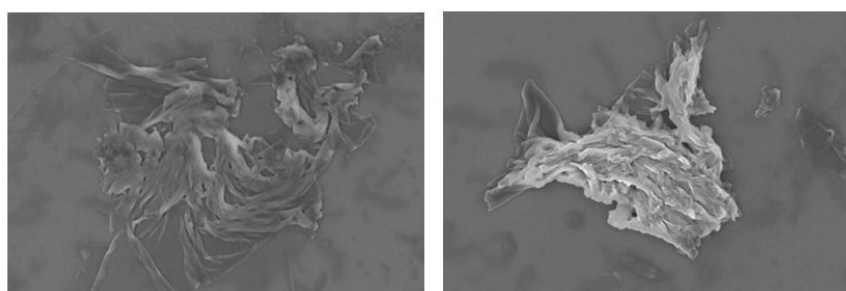
Field Emission Scanning Electron Microscopy (FESEM) Images:

Figure 5.12: FESEM images of **1** in methanol (with a drop of 35 % HCl) showing distinct aggregation patterns.

5.4.5 Ion Transport Experiments**Ion transport across EYPC-LUVs $\Rightarrow$ HPTS:**

Preparation of EYPC-LUVs $\Rightarrow$ HPTS: Vesicles were prepared following a reported protocol.⁵⁶

Final conditions: EYPC $\sim$ 5 mM. **Inside:** 10 mM HEPES, 100 mM NaCl, 1 mM HPTS, pH 7.0; **Outside:** 10 mM HEPES, 100 mM NaCl, pH 7.0.

Ion transport activity by HPTS assay: In a clean and dry fluorescence cuvette, 1975 μL of buffer (10 mM HEPES, 100 mM NaCl, pH 7.0) was added, followed by the addition of 25 μL EYPC-LUV $\Rightarrow$ HPTS vesicles. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The change in HPTS fluorescence intensity at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$) was monitored as a function of time. During the experiment, base (20 μL , 0.5 M NaOH to create pH gradient) was added at $t = 20 \text{ s}$, and transporter **1** in

DMSO was added at $t = 100$ s. Finally, 10% Triton X-100 (25 μ L) was added at $t = 300$ s to lyse the vesicles to achieve complete destruction of the pH gradient. The fluorescence intensity was monitored up to 350 s.⁵⁶

The time data were normalized according to Equation 1 and then the fluorescence intensity data was normalized to the percentage change in fluorescence intensity as a course of time using Equation 2:

$$t = t - 100 \quad (\text{Equation 1})$$

$$I_F = [(I_t - I_0) / (I_\infty - I_0)] \times 100 \quad (\text{Equation 2})$$

where I_F is the normalized fluorescence intensity in percentage, I_0 is the initial intensity, I_t is the intensity at time t , and I_∞ is the final intensity after adding a Triton X-100.

For concentration-dependent ion transport data of each compound, the I_F values at 150 s after the addition of the transporter were plotted against respect concentration values. Subsequently, the plot was fitted with the Hill equation (Equation S3):

$$Y = Y_\infty + (Y_0 - Y_\infty) / [1 + (c / EC_{50})^n] \quad (\text{Equation 3})$$

where, Y_0 is the fluorescence intensity just before the transporter addition (at $t = 0$ s), Y_∞ is the fluorescence intensity with excess transporter concentration, c is the concentration of transporter compound, and EC_{50} is the effective concentration required to reach the 50% of the maximum activity.

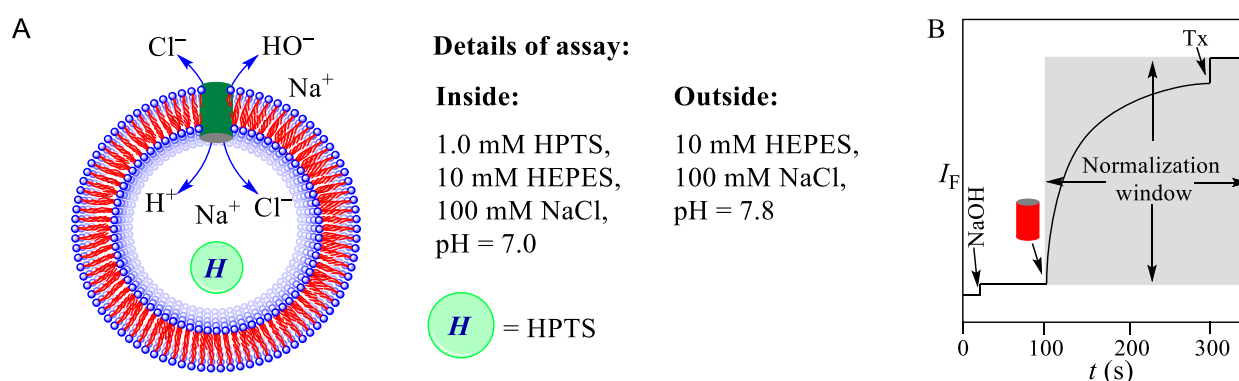


Figure 5.13 Representations of fluorescence-based ion transport activity assay using EYPC-LUVs (A) and representative ion transport kinetics showing normalization window (B).

Anion selectivity by HPTS assay:

Preparation of EYPC-LUVs $\supset$ HPTS: Vesicles were prepared following a reported protocol.⁵⁶

Final conditions: EYPC $\sim$ 5 mM. **Inside:** 10 mM HEPES, 100 mM NaX, (where, $X^- = F^-$, Cl^- , Br^- , I^- , OAc^- , and NO_3^-), 1 mM HPTS, pH 7.0; **Outside:** 10 mM HEPES, 100 mM NaX, (where, $X^- = F^-$, Cl^- , Br^- , I^- , OAc^- , and NO_3^-), pH 7.0.

Anion selectivity experiments: In a clean and dry fluorescence cuvette 1975 μ L of buffer (10 mM HEPES, 100 mM NaX, pH 7.0; where, $X^- = F^-$, Cl^- , Br^- , I^- , OAc^- , and NO_3^-) was added followed by the addition of 25 μ L EYPC-LUV $\supset$ HPTS vesicles. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The change in HPTS fluorescence intensity at $\lambda_{em} = 510$ nm ($\lambda_{ex} = 450$ nm) was monitored as a function of time. During the experiment, base pulse (20 μ L, 0.5 M NaOH to create pH gradient of 0.8) was given at $t = 20$ s, and transporter **1** in DMSO was added at $t = 100$ s. Finally, 10% Triton X-100 (25 μ L) was added at $t = 300$ s to lyse the vesicles to achieve complete destruction of the pH gradient. The fluorescence intensity was monitored up to 350 s. The collected data was then normalized according to Equations 1 and 2 to get the percentage change in fluorescence intensity as a function of time.⁵⁶

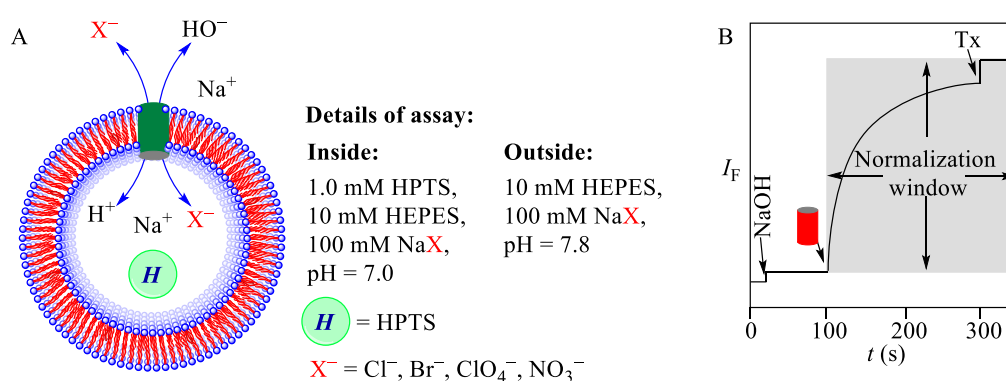


Figure 5.14 Representations of fluorescence-based ion transport activity assay using EYPC-LUVs (A) and representative ion transport kinetics showing normalization window (B).

Effect of extravesicular cations in chloride influx:

Preparation of EYPC-LUVs $\supset$ lucigenin: Vesicles were prepared following a reported protocol.⁵⁶ Final conditions: $\sim$ 3.3 mM EYPC; *inside*: 200 mM $NaNO_3$, 1 mM lucigenin; *outside*: 200 mM $NaNO_3$.

Cation selectivity using lucigenin assay: In a clean and dry fluorescence cuvette, 1975 μL of NaNO_3 solution (200 mM) was added, followed by the addition of 25 μL of EYPC-LUVs $\supset$ lucigenin. The cuvette was placed in the fluorescence instrument in a slow stirring condition by a magnetic stirrer equipped with the instrument (at $t = 0$ s). The time course of lucigenin fluorescence emission intensity, F_t was observed at $\lambda_{\text{em}} = 535$ nm ($\lambda_{\text{ex}} = 455$ nm). 33.3 μL of 2 M MCl ($\text{M}^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+$ and Cs^+) was added to the cuvette at $t = 25$ s to create the chloride gradient between the intra and extra vesicular system. Transporter **1** was added at $t = 100$ s, and finally, 25 μL of 10% Triton X-100 was added at $t = 300$ s to lyse those vesicles resulting destruction of the chloride gradient. The collected data was then normalized according to Equations 1 and 2 to get the percentage change in fluorescence intensity as a function of time.⁵⁶

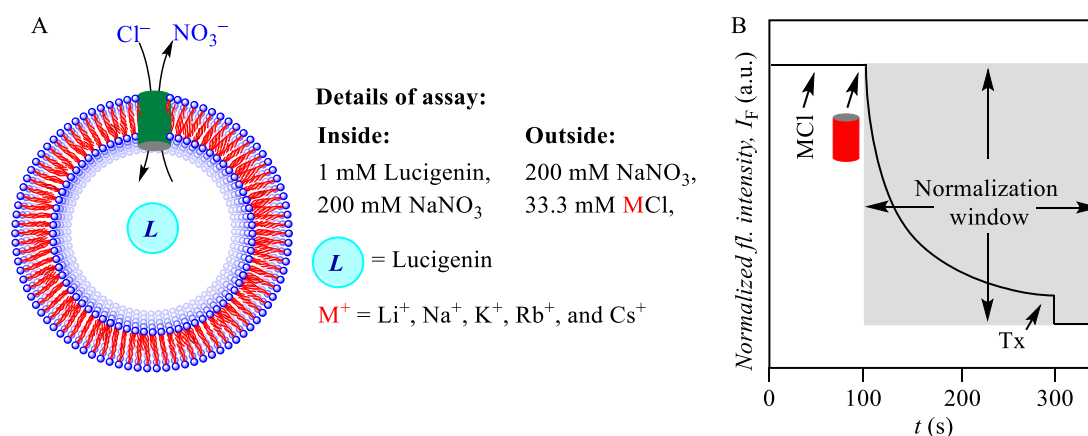


Figure 5.15 Representations of chloride influx activity across EYPC-LUVs $\supset$ lucigenin in the presence of different extravesicular cations (A). Illustration of chloride influx kinetics showing normalization window (B).

Ion transport activity in the presence of valinomycin:

Preparation of EYPC-LUVs $\supset$ lucigenin: The aforesaid lucigenin vesicles were used for the experiments.

Description of the assay:

In a clean and dry fluorescence cuvette, 1975 μL of NaNO_3 solution was added, followed by the addition of 25 μL of EYPC-LUVs $\supset$ lucigenin. The cuvette was placed in a fluorescence instrument in a slow stirring condition by a magnetic stirrer equipped with the instrument (at $t = 0$ s). The time course of lucigenin fluorescence emission intensity, F_t was observed at $\lambda_{\text{em}} =$

535 nm ($\lambda_{\text{ex}} = 450$ nm). 33.3 μL of 2 M KCl was added to the cuvette at $t = 25$ s to create the chloride gradient between the intra and extra vesicular system. Valinomycin (1.0 μM) was added to the cuvette at 50 s. After that **1** was added at $t = 100$ s, and finally, at $t = 300$ s, 25 μL of 10% Triton X-100 was added to lyse those vesicles resulting destruction of the chloride gradient. The collected data was then normalized according to Equations 1 and 2 to get the percentage change in fluorescence intensity as a function of time.

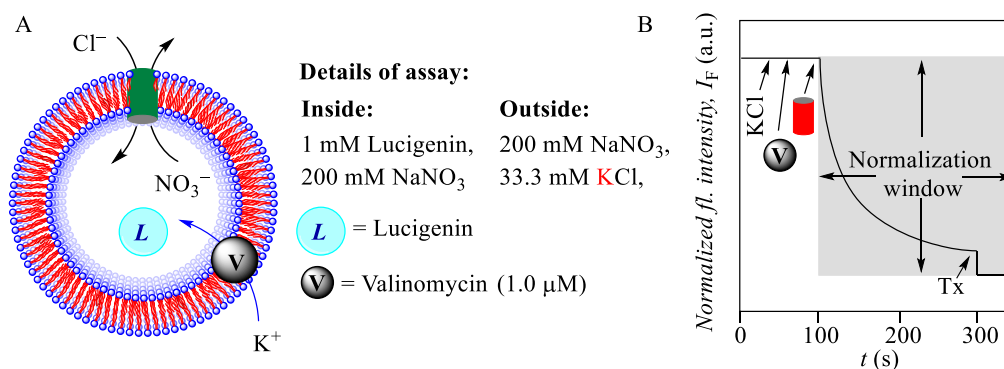


Figure 5.16 Representations chloride influx activity across EYPC-LUVs $\supset$ lucigenin in the presence and absence of valinomycin (A). Illustration of chloride influx kinetics showing normalization window (B).

Ion transport across EYPC-LUVs by chloride ion selective electrode:

Transport mechanism by valinomycin and monensin coupled chloride screen:

Preparation of EYPC-LUVs:

A thin film of egg yolk phosphatidylcholine (EYPC) was formed from a chloroform solution (25mg/mL) with purging nitrogen and dried under a vacuum for at least 6 hours. The lipid film was rehydrated by vortexing with a metal chloride (KCl) salt solution and then buffered to pH 7.0 with sodium phosphate salts. The lipid suspension was then subjected to 10 freeze-thaw cycles, where the suspension was alternatively allowed to freeze in a liquid nitrogen bath, followed by thawing in a water bath (55 $^{\circ}\text{C}$). The lipid suspension was allowed to age for 30 min at room temperature and was subsequently extruded 19 times through a 200 nm polycarbonate membrane. The resulting unilamellar vesicles were dialyzed (using membrane MWCO 1 kD) against KGlc (300 mM) in potassium phosphate buffer of pH 7.0 to remove unencapsulated Cl^- ions.⁵⁷

Internal solution: KCl 300 mM, KH_2PO_4 10 mM, pH 7.0

External solution: KGlc 300 mM, KH₂PO₄ 10 mM, pH 7.0

Description of the experiments:

Chloride efflux during transport experiments was measured using a chloride-selective electrode (*Accumet*). The electrode was calibrated against potassium chloride solutions of known concentrations before the experiments. The lipid concentration in the cuvette was 1.0 mM. A DMSO solution of the anionophore (0.1 μ M) was added to start the experiment and the chloride efflux was monitored using the chloride-sensitive electrode. When a cationophore (valinomycin or monensin) was used, a DMSO solution of the cationophore (0.5 μ M) was added to the liposome solution prior to the addition of the anionophore at 60 s. At 300 s, the vesicles were lysed with 25 μ L of Triton X-100 (10% solution in water), and the total chloride reading was taken at 360 s. The value at 60 s was set at 0% chloride efflux, and the final chloride reading (at 360 s) was set as 100% chloride efflux to normalize the collected data from each measurement.⁵⁷

The time data were normalized according to Equation 4, and then the fluorescence intensity data was normalized to the percentage change in fluorescence intensity as a course of time using Equation 5:

$$t = t - 60 \quad \text{(Equation 4)}$$

$$E_F = [(E_t - E_0) / (E_\infty - E_0)] \times 100 \quad \text{(Equation 5)}$$

where E_F is the normalized chloride efflux, E_0 is the initial chloride reading, E_t is the chloride reading at time t , and E_∞ is the final chloride reading after the addition of a Triton X-100.

5.4.6 Planner bilayer conductance measurement

The conductance measurement across the bilayer lipid membrane was done with the help of Warner instrument (USA). For that, a solution of diphytanoylphosphatidylcholine lipid (DPhPC; Avanti Polar Lipids) was prepared by dissolving it in *n*-decane of concentration 20 mg/mL. Then a bilayer membrane was formed by using this solution at the 150 μ m diameter aperture within a cup made of polystyrene (Warner Instrument, USA). In both chambers (*cis* and *trans*) of the instrument, 1 M KCl was symmetrically injected. The setup was made in such a way that the *trans* compartment was held at virtual ground. The BC 535 head stage (Warner Instrument, USA) connects the *cis* compartment through Ag-AgCl electrodes. Then

the channel-forming molecule **1** (2 μM) was added to the *trans* chamber. An inbuilt magnetic stirrer was used to stir the solution for 20 min. Channel formation through the lipid bilayer was confirmed from channel opening and closing events upon application of different voltages using pClamp11 software (Molecular probes, USA). An analogue-to-digital converter (Digidata 1440A, Molecular Devices) and pClamp11 software (Molecular Probes, USA) were used to low-pass filter the currents at 1 kHz.

The channel opening and closing events at positive, as well as negative potentials, were analyzed by pClamp 11 software. A complete trace comprised of a series of opening and closing events for the time period of 2 h from where a small fraction is presented in the manuscript.

The channel diameter was calculated using the equation (Hille equation) mentioned below.¹

$$1/g = (l + \pi d/4) (4\rho/\pi d^2) \quad (\text{Equation 6})$$

where, g is the corrected conductance, calculated by multiplying measured single-channel conductance with the Sansom's correction factor⁵⁸ 5.61; l is the length of the ion channel (34 Å), and ρ signifies resistivity of the KCl solution ($\rho = 10.1 \Omega \text{ cm}$).

Determination of anion selectivity permeability ratio by Planar Bilayer Conductance

Measurements: The *cis* and *trans* chambers were filled with unsymmetric solutions of KCl. The *cis* chamber was filled with 1.0 M KCl solution and the *trans* chamber was filled with 0.5 M KCl. The compounds **1** (2 μM) were added to the *trans* chamber and stirred for 5 minutes. The permeability ratio ($P_{\text{Cl}^-}/P_{\text{K}^+}$) was calculated by using the Goldman-Hodgkin-Katz equation (Equation S5).¹

$$\frac{P_{\text{Cl}^-}}{P_{\text{K}^+}} = \frac{a_{\text{K}^+}^{\text{cis}} - a_{\text{K}^+}^{\text{trans}} \times \exp\left(-\frac{zV_{\text{rev}} \times F}{R \times T}\right)}{a_{\text{Cl}^-}^{\text{cis}} \times \exp\left(-\frac{zV_{\text{rev}} \times F}{R \times T}\right) - a_{\text{Cl}^-}^{\text{trans}}} \quad (\text{Equation 7})$$

where, $P_{\text{Cl}^-}/P_{\text{K}^+}$ = anion/cation permeability ratio; $a_{\text{K}^+}^{\text{cis}}$ = K^+ activity in the *cis* chamber; $a_{\text{K}^+}^{\text{trans}}$ = K^+ activity in the *trans* chamber;⁵⁹ $Z_{\text{K}^+} = +1$, $Z_{\text{Cl}^-} = -1$ $a_{\text{Cl}^-}^{\text{cis}}$ = Cl^- activity in the *cis* chamber; $a_{\text{Cl}^-}^{\text{trans}}$ = Cl^- activity in the *trans* chamber; V_{rev} = reversal potential; F = Faraday constant; R = gas constant; T = temperature (K).

5.4.7 MD Simulations

Simulation Model and Methods:

System setup and simulation model: First, the coordinates of both the channel structures, the hydrogenated and the non-protonated (composed of six consecutive layers), were minimised using MAESTRO.³⁵ Then, the semi-minimised structures were employed for the construction of the channel/membrane complex by utilizing the membrane builder protocol of CHARMM-GUI^{36, 37} The protocol comprises six consecutive steps as described by Jo et al⁶⁰: reading of the object and its suitable orientation, determination of system size, then the lipid bilayer constitution followed by its assembly, and finally equilibrium of the system. The channel/membrane complex involves the self-assembled superstructures (either hydrogenated or non-hydrogenated structure) accompanying 12 monomer molecules arranged in six repetitive layers of the corresponding monomeric units at the centre of the lipid bilayer membrane. The bilayer membrane constitutes 144 units of 1,2- diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) lipids (each membrane leaflet contains 72 lipid molecules). The channel-embedded membrane was finally packed in a rectangular box of dimension $7.7 \times 7.7 \times 9.0 \text{ nm}^3$. Towards the end, the whole system was solvated with water on both the compartments of the system separated by the lipid bilayer, and 1 M chloride ion concentration (1 M KCl) was maintained in the aqueous compartments throughout the simulation. The constitution of each of the simulation system corresponding to each of the channel structures are provided in Table I. The channels were modeled based on the general AMBER forcefield (GAFF) parameters.^{61, 62} The membrane is modeled with the CHARMM36m forcefield parameters⁶²⁻⁶⁴ and the CHARMM-TIP3P⁶⁵ water model is employed in our simulation.

Table 5.1: Details of the simulation systems.

System	Number of Cl ⁻ ions	Number of K ⁺ ions	Number of water molecules
Non-protonated	166	166	9340
Protonated	178	166	9339

Simulation method: For all the simulations, periodic boundary conditions (PBC) was set in all three dimensions. Electrostatic long-range interactions were maintained using the particle mesh Ewald (PME) method⁶⁶ with cubic interpolation. For short-range electrostatic interactions, the cut-off of 1.2 nm was practiced. The neighbor lists were updated every 20 steps during the simulation. For constraining each of the bonds involving hydrogen atoms of the lipid molecules and the channel monomers, the LINCS algorithm⁶⁷ was utilized. To maintain the rigidity of the water molecules, the SETTLE⁶⁸ algorithm was applied. For our simulation the Verlet cut-off scheme⁶⁹ accompanying Lenard Jones interaction extending to 1.2 nm involving dispersion corrections was utilized. At first, the system with restraints was energy minimized with the steepest-descent algorithm followed by equilibration in six successive steps of 10 ns each. Throughout the equilibration process, the simulation systems were restrained, but the restrain value gradually lowered in each consecutive step. During equilibration, the average temperature was kept fixed at 298 K using a Berendsen thermostat⁷⁰ and a 1.0 bar of average pressure was maintained employing a Berendsen barostat. Finally, each equilibrated system was subjected to an NPT production run for 1.0 microseconds under slight restrain applied to each channel structure. During production simulation, the 298 K average temperature was maintained with the help of the Nose-Hoover thermostat⁷¹ (relaxation constant of 1.0 ps) via individual coupling of the channel structure, membrane bilayer, and solvent. The pressure was kept constant at 1.0 bar utilizing Parrinello-Rahman barostat⁷² (with 5 ps coupling regular and the compressibility factor of 4.5×10^{-5} bar) under semi-isotropic coupling (coupled XY and Z directions separately) of the system to retain bilayer tensionless during simulation. The 2 fs simulation (production run) time step was chosen by employing leapfrog integrator. Simulation of both the channel (protonated and non-protonated) systems were carried out following similar protocol of simulation and replicated twice. To execute all simulations GROMACS software of version 20x were utilized.

Density functional theory: DFT calculations were performed using the SIESTA 4.0 package,⁷³ considering a single monomer layer and Cl^- , with Na^+ for charge compensation. All pseudo potentials are generated using the generalized gradient approximation (GGA) Perdew–Burke–Ernzerhof (PBE) exchange-correlation functional.⁷⁴ The energy cut-off for the real space grid is set at 500 Ry, and the Broyden method in a variable-cell-relaxation scheme is employed for geometry relaxation. We use a $4 \times 4 \times 1$ k-point sampling of the Brillouin zone using the Monkhorst–Pack scheme.⁷⁵

5.4.7 Biological Studies:

Cell lines used: Different human and non-human cell lines of cancerous and non-cancerous origin were used for the study. The human cancerous cell lines included: lung adenocarcinoma – A549 (basal epithelial cell); breast cancer – MCF-7; triple-negative breast cancer – MDA-MB-231; osteosarcoma – U2OS; colon adenocarcinoma – SW480; cervical cancer – HeLa; and the non-cancerous human line: HEK293T. In addition, mouse embryonic fibroblasts (MEFs) were also used as a control.

Cell culture: All cell lines used for the study were grown in High Glucose containing Dulbecco's Modified Eagle Medium (DMEM; Lonza) containing 10% fetal bovine serum (FBS; Invitrogen), 2.0 mM L-glutamine (Invitrogen) and 100 units/mL penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were maintained in tissue culture-treated T25 flasks (Corning) at 37 °C in a humidified 5% CO₂ incubator (Thermo Scientific).³¹ Cells were subcultured every 2 days once the flask showed 80% confluency.

MTT-based cytotoxicity assay: To assess cellular viability under the influence of **1**, MTT assays were performed. Briefly, cells were dispersed in 96-well flat-bottom tissue culture-treated plates (Corning) at the density of 8×10^4 cells/well (per 100 µL) in complete DMEM media and were incubated at 37 °C in a 5% CO₂ incubator overnight for recovery. Post-recovery, the cells were treated with increasing concentrations of the compound **1** (0 to 1.0 µM) by maintaining a maximum amount of DMSO at 2 µL/well and were further incubated for 24 h. Post incubation, the media containing the compounds was replaced with 100 µL of MTT solution (0.5 mg MTT/mL of PBS) and incubated 37 °C for 4 h. After 4 h, 100 µL of solubilization buffer (10% SDS containing 0.01 N HCl in PBS) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a multi-mode plate reader (HiDex). All experiments were performed in triplicates, and the relative cell viability (%) was expressed as a percentage of untreated cells (set as 100%).³¹ The cellular viability has been depicted in Figure S7. Since maximum cytotoxicity was observed in U2OS followed by MCF-7 cells, all further experiments were carried out using these two cells.

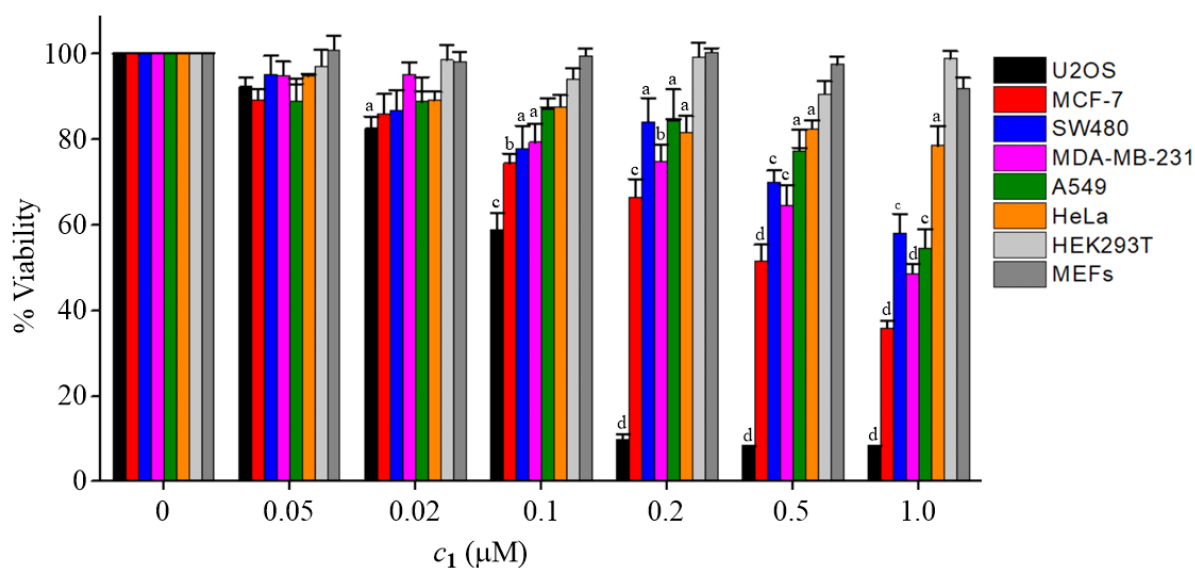


Figure 5.17. Cellular viability (as assessed by MTT assays) of U2OS (Black), MCF-7 (Red), SW 480 (Blue), MDA-MB-231 (Pink), A549 (Green), HeLa (orange), HEK293T (light grey), MEFs (dark grey) in response to compound **1**. *a* represents $p < 0.05 = *$, *b* represents $p < 0.01 = **$, *c* represents $p < 0.001 = ***$, and *d* represents $p < 0.0001 = ****$ (* represents $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$).

Table 5.2: IC_{50} values for compound **1** against cell lines of cancerous and noncancerous origins

Cell lines	A549	MCF-7	MDA-MB-231	U2OS	SW480	HeLa	MEFs	HEK293T
IC_{50} (μM)	1.86	0.49	0.96	0.10	1.98	>20	>20	> 20

Chloride-mediated cytotoxicity assay

HBSS buffer solution. Hank's balanced salt solution (HBSS with Cl^-) was prepared with the following compositions: 136.9 mM NaCl, 5.5 mM KCl, 0.34 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 0.81 mM MgSO_4 , 1.25 mM CaCl_2 , 5.5 mM D-glucose, 4.2 mM NaHCO_3 and 10 mM HEPES (pH 7.4). Chloride-free HBSS was prepared by mixing 136.9 mM Na-gluconate, 5.5 mM K-gluconate, 0.34 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 0.81 mM MgSO_4 , 1.25 mM Ca-gluconate, 5.5 mM D-glucose, 4.2 mM NaHCO_3 and 10 mM HEPES (pH 7.4).⁴¹

U2OS and MCF-7 cells were dispersed separately in 96-well flat bottom tissue culture-treated plates (Corning) at a density of 8×10^4 cells/well (per 100 μL) in complete DMEM media

and were incubated at 37 °C in a 5% CO₂ incubator overnight for recovery. Post-recovery, media was replaced by HBSS buffer (either with Cl⁻ or without Cl⁻) containing 10% FBS and 100 units/mL penicillin and 100 µg/ml streptomycin (Invitrogen). Compound **1** was added to each well in increasing concentrations (0 – 100 µM) by maintaining the maximum amount of DMSO at 2 µL/well and incubated for 24 h. Post incubation, 10 µL of MTT (5 mg MTT/mL of PBS) was added to each well and the plates were incubated at 37 °C for 4 h. After 4h, 100 µL of solubilization buffer (10% SDS containing 0.01 N HCl in PBS) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a multi-mode plate reader (HiDex).

Measurement of Reactive oxygen species (ROS) levels:

U2OS and MCF-7 cells were dispersed separately in 96-well flat bottom tissue culture-treated plates (Corning) at a density of 8×10^4 cells/well (per 100 µL) in complete DMEM media and were incubated at 37 °C in a 5% CO₂ incubator overnight for recovery. Compound **1** was added to each well in increasing concentrations (0, 0.05, 0.1 and 0.2 µM for U2OS and 0, 0.1, 0.5, 1.0 µM for MCF-7) by maintaining the maximum amount of DMSO at 2 µL and incubated for 24 h. In separate experiments, H₂DCFDA (cellular ROS probe) or MitoSox Red (mitochondrial superoxide indicator) was added to each well at a final concentration of 5 µM. The plates were incubated further for 20 min in the dark. Two washes with 1X ice-cold PBS followed this. For quantitation of MitoSox, fluorescence intensities were read using a multi-mode plate reader (Hidex) at (excitation 510 nm/emission 580 nm). for mitochondrial ROS measurements. All experiments were performed in triplicate, and the relative emission intensity was normalized with respect to the untreated cells. For H₂DCFDA assay, fluorescence imaging was performed by acquiring images in the green channel using EVOS FL auto imaging system (Life Technologies).³¹

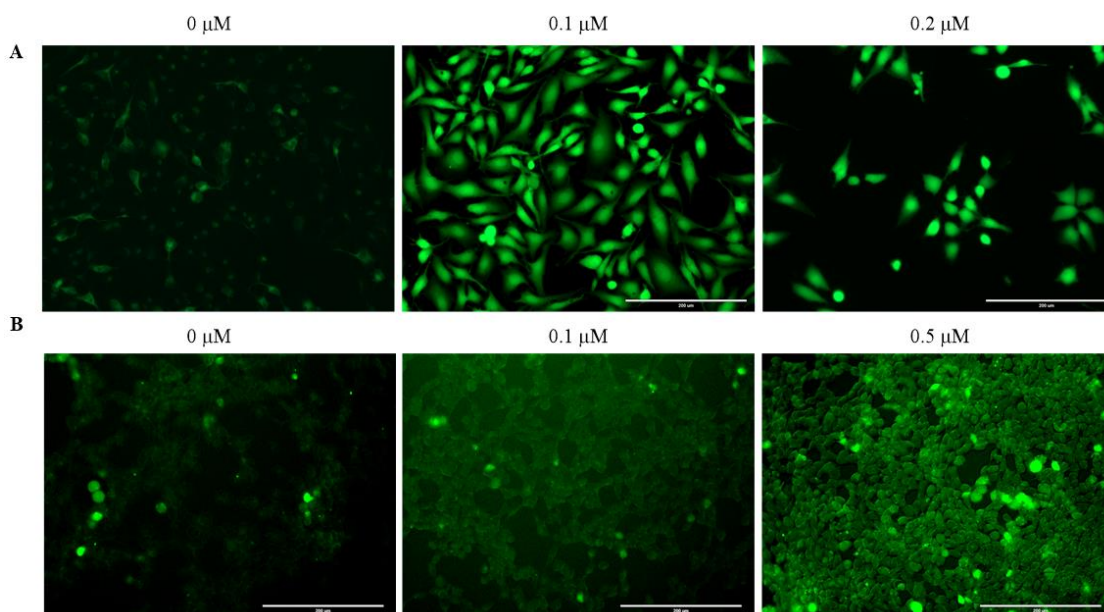


Figure 5.18 Representative live cell images of H₂DCFDA stained U2OS (A) and MCF-7 cells (B) under control conditions and after treatment with **1**.

Mitochondrial membrane potential measurement

U2OS and MCF-7 cells were dispersed separately in 96-well flat bottom tissue culture-treated plates (Corning) at a density of 8×10^4 cells/well (per 100 μL) in complete DMEM media and were incubated at 37 °C in a 5% CO₂ incubator overnight for recovery. After recovery, cells were incubated with compound **1** in increasing concentrations (0, 0.05, 0.1 and 0.2 μM for U2OS and 0, 0.1, 0.5, 1.0 μM for MCF-7) by maintaining the maximum amount of DMSO at 2 μL and incubated for 24 h. The cells were incubated with JC-1 (Invitrogen), a dye used to assess mitochondrial membrane potential, at the final concentration of 2 μg/mL for 30 min. After incubation, the cells were washed with ice-cold 1X PBS, and fluorescence was measured at 485 nm/530 nm (green) and 510 nm/580 nm (red) using a multi-mode plate reader (HiDex) for the quantitation. For qualitative analysis, fluorescent images were acquired after PBS washes in red and green channels using EVOS FL auto imaging system (Life Technologies).

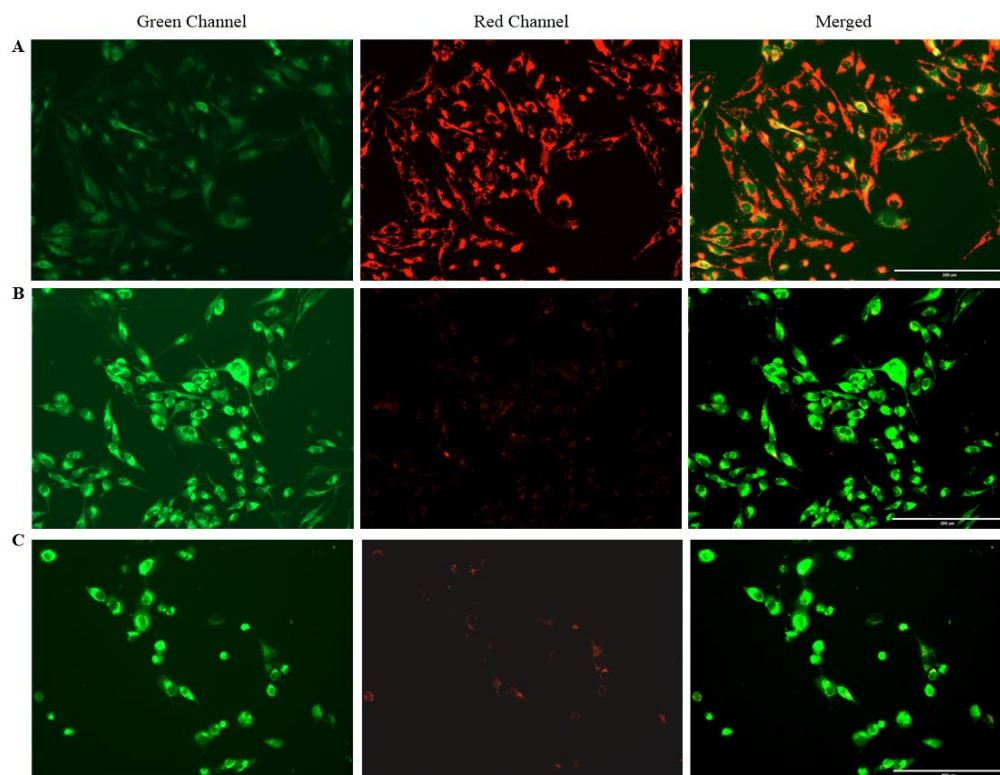


Figure 5.19 Representative live cell images of U2OS cells stained with JC1 upon treatment with **1** at 0.1 μM (B) and 0.5 μM (C) and in untreated cells (A).

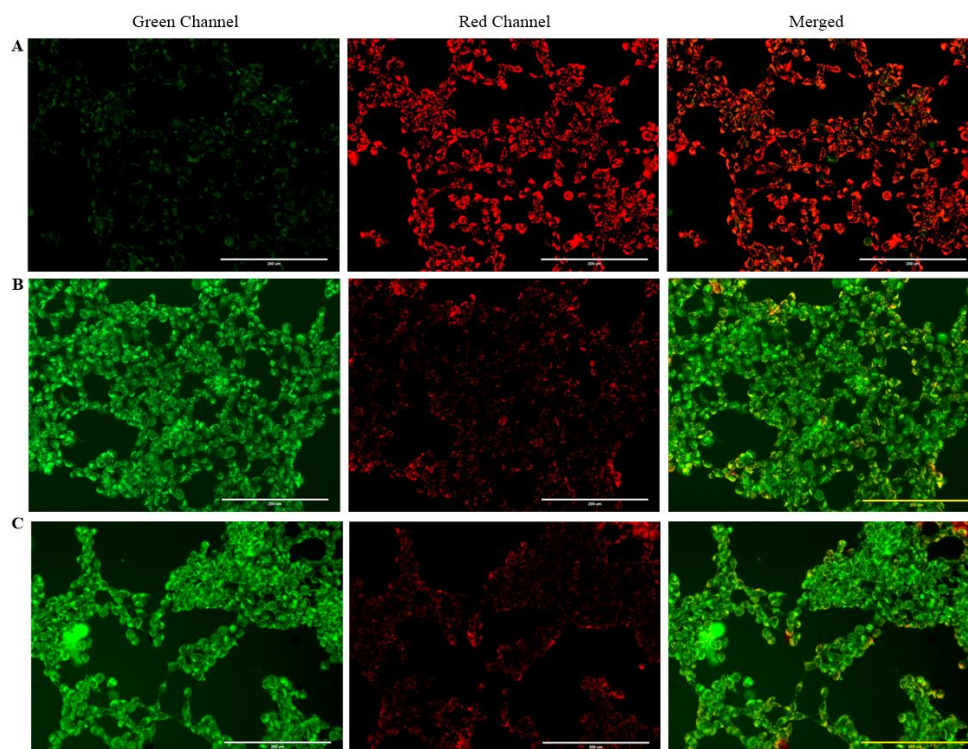


Figure 5.20 Representative live cell images of MCF-7 cells stained with JC1 upon treatment with **1** at 0.1 μM (B) and 0.5 μM (C) and in untreated cells (A).

Immunofluorescence analysis for cytochrome *c* release:

MDA-MB-231 cells were dispersed at 2×10^4 cells/ml density on glass coverslips (Micro-Aid, India) in 24 well plates (Corning) and recovered overnight at 37 °C in a 5% CO₂ incubator. Post recovery, the cells were allowed to incubate in the presence of **1c** (0, 5 and 10 μ M) for 24 h. Post-incubation, the cells were washed with ice-cold 1X PBS, fixed using 4% p-formaldehyde (Sigma) for 30 min, and permeabilised using 0.25% Triton X-100 (Sigma) for 10 min at RT. Following this, the cells were blocked with 10% (v/v) FBS (Invitrogen) for 1 h, stained with primary antibody (cytochrome *c* antibody; sc13561, 1:250 dilution in blocking buffer) overnight and then incubated with secondary antibody (goat anti-rabbit AlexaFluor-488) for 1 h. Cells were counterstained with FITC-conjugated Phalloidin (1:300; P5282, Sigma) and mounted onto glass slides (Micro-Aid, India) using Vectashield mounting medium with DAPI (Vector). Cell images were taken on EVOS FL auto imaging system (Life Technologies) using a 60X oil-immersion objective.⁴¹

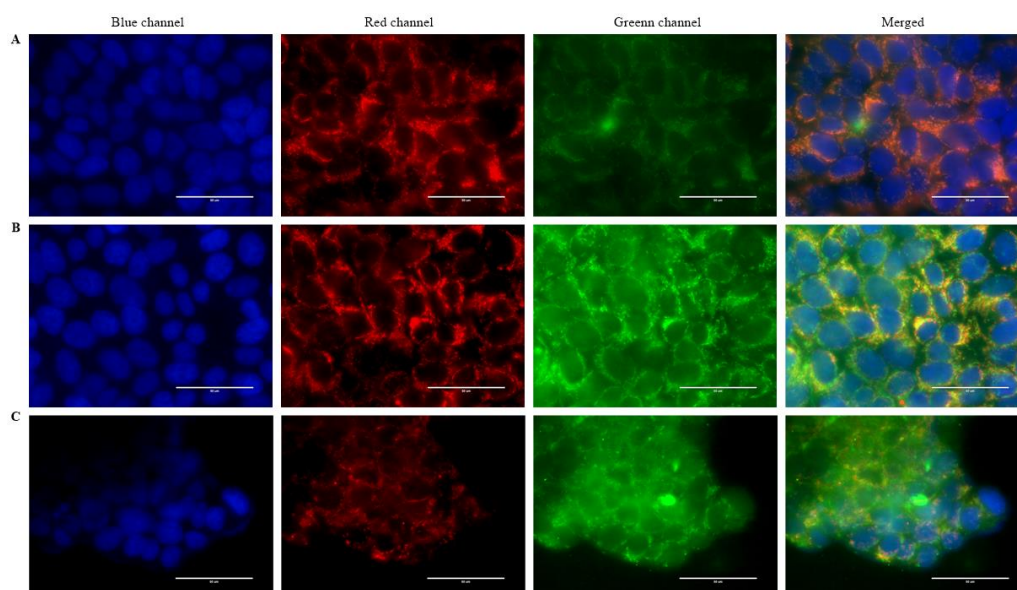


Figure 5.21 Representative live-cell images of MCF-7 cells treated with 0.1 μ M (B) and 0.5 μ M (C) of **1** for 24 h and untreated cells (A). Immunostaining for cytochrome *c* release was performed using a cytochrome *c*-specific antibody (green). Cells were counterstained with Phalloidin (red) and DAPI (blue).

Immunoblot analysis:

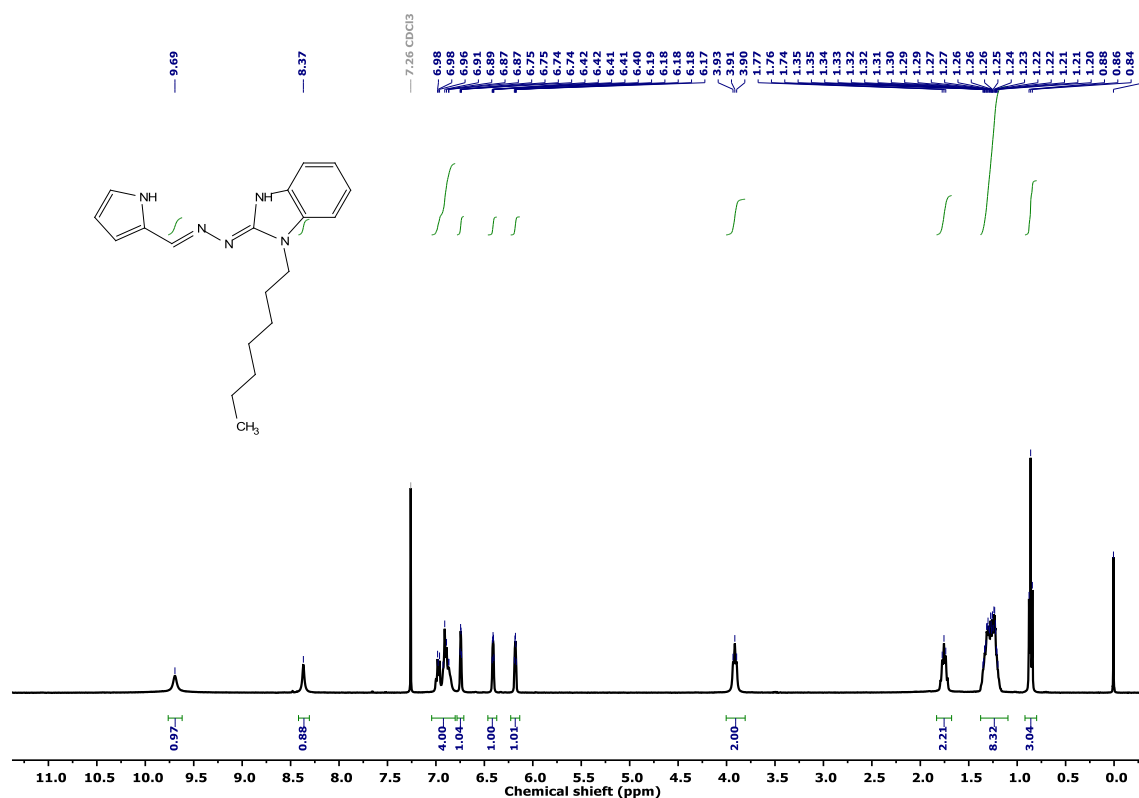
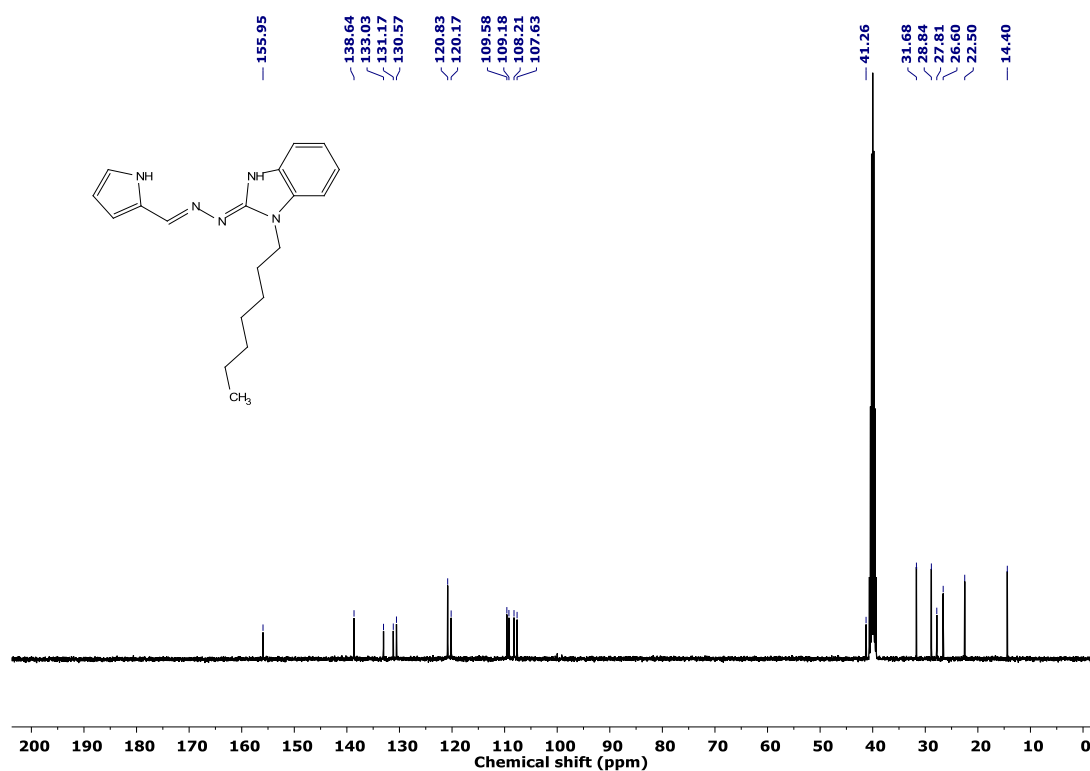
MDA-MB-231 cells were dispersed at a density of 8×10^4 cells/ ml media in 60 mm tissue culture-treated dishes (Corning) in complete DMEM media and maintained at 37 °C for 24 h.

Cells were then treated with **1** for 24 h at different concentrations (0, 10, 20, and 40 μ M). Control cells were treated with an equivalent volume of DMSO. After 24 h treatment, media was aspirated, and cells were washed twice with ice-cold 1X PBS and lysed in RIPA lysis buffer (Himedia) containing protease inhibitors (Roche). Cell lysates were resolved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Blocking was performed in 5% (w/v) skimmed milk (SACO Foods, USA) prepared in 1X Tris-buffered saline (TBS) containing 0.1% Tween 20 (1X TBS-T) for 1 h at room temperature. Blots were incubated overnight at 4 °C in primary antibodies (PARP; (46D11), and Tubulin; (T9026), Sigma) diluted in blocking buffer. Following washes, blots were incubated with peroxidase-conjugated secondary antibody (Sigma) prepared in 5% (w/v) skimmed milk in 1X TBS-T for 1 h at room temperature, following which blots were developed using the WesternBright ECL kit (Advansta) and visualized using ImageQuant LAS 4000 (GE Healthcare).⁴¹ Quantitation of the intensities for the bands was measured using densitometric scanning (ImageJ; NCBI).

Statistical Analysis:

All cellular experiments were performed in triplicate unless stated otherwise. Data has been represented as Mean $\pm$ SEM, where SEM represents the standard error of mean and is expressed as the standard deviation divided by the square root of sample size. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey HSD posthoc test. $p \leq 0.05$ was considered to be statistically different when comparing different groups.

5.4.8 NMR Spectra:

Figure 5.22 400 MHz ¹H NMR of compound 1 in CDCl₃.Figure 5.22 101 MHz ¹³C NMR of compound 1 in DMSO-*d*₆.

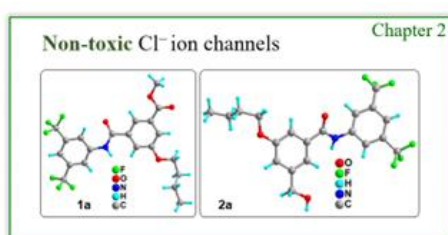
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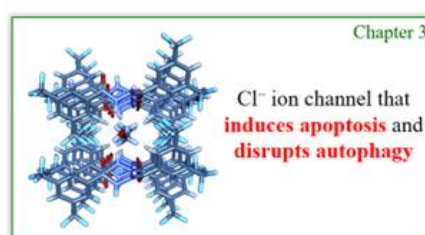
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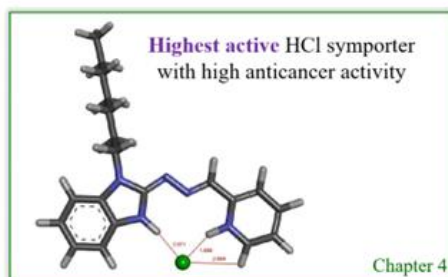
Long story short, the main objective of this thesis was to develop artificial ion transport systems and explore their potential applications in the field of biomedicine. Particularly, I focused on designing, synthesizing, and characterizing biomimetic artificial ion channels and ion carriers that have shown promising biological applications for future therapeutics. This thesis primarily focuses on the supramolecular architecture of artificial ion channels that can selectively transport chloride ions across cell membranes. Chapter 2 introduces isophthalic acid-based small molecules that self-assemble to form chloride ion channels, which are non-toxic to cells even at elevated concentrations. Such non-toxic chloride channels hold realistic potential for their application in the treatment of “channelopathies”. In Chapter 3, we present a benzohydrazide-based artificial chloride channel, which is capable of inducing apoptosis as well as disrupting autophagy, a combination seldom seen in cancer-targeting drugs. As an important contribution to the field, chapter 4 of the thesis reports the highest active artificial HCl symporter hitherto reported in the literature. Chapter 5 presents a rare example of an HCl channel, which is highly toxic to cancer cells while remaining relatively non-toxic to healthy cells. The compound also shows better efficacy when compared to the commercially available anticancer drug, Doxorubicin, in inhibiting the growth of MCF-7 3D spheroid cultures, indicating that it may be a promising candidate for combating cancer in due course.



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