Recent Advances in Bioactive Artificial Ionophores

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Several life-threatening diseases, also known as 'Channelopathies' are linked to irregularities in ion transport proteins. Significant research efforts have fostered the development of artificial transport systems that facilitates to restore the functions of impaired natural transport proteins. Indeed, a few of these artificial ionophores demonstrate the rare combination of transmembrane ion transport and important biological activity, offering early promises of suitability in 'channel replacement therapy'. In this review, structural facets and functions of both cationophores and anionophores are discussed. Ionophores that are toxic to various bacteria and yeast, could be exploited as antimicrobial agent. Nevertheless, few non-toxic ionophores offer the likelihood of treating a wide range of genetic diseases caused by the gene mutations. In addition, their ability to disrupt cellular homeostasis and to alter lysosomal pH endow ionophores as promising candidates for cancer treatment. Overall, critically outlining the advances in artificial ionophores in terms of *in vitro* ion transport, possible modes of action and biological activities enables us to propose possible future roadmaps in this research area.

1. Introduction

Exchange of Na^+, K^+, Ca^{2+} and Cl^- ions across cell membranes is indispensable for sustaining cellular life as it controls diverse biological processes including metabolism, maintenance of osmotic pressure and cell volume, regulation of cellular pH and signalling pathways. In biological systems, the plasma membrane of a cell is a hydrophobic barrier, which separates the interior of the cells from the environment outside. It allows small neutral molecules such as CO_2 and H_2O to easily pass through the hydrophobic bilayer but exchange of charged solutes across the membrane needs assistance of natural channel proteins. These channel proteins are not only extraordinarily selective gatekeepers for specific ions but also maintain several physiological processes. For instance, Ca^{2+} channels are vital in regulating the cardiovascular system, Na^+ and K^+ channels form the basis of neuronal signal transduction. In addition, mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel is associated with cystic fibrosis disease. Consequently, dysfunction of these ion transport systems lead to 'Channelopathies' such as cystic fibrosis (CF), epilepsy, Long QT syndrome, Bartter syndrome, myotonia, nephrolithiasis, osteoporosis, and other ailments.[1]

Of late, development of synthetic ionophores has attracted considerable interest because they are structurally robust and offer adjustable ion transport behaviour relying upon simple structural fine-tuning as compared to the complex structured natural ion channels.[2] The transmembrane ion transport could be attained either via ion channels or ion carriers by exploiting various non-covalent interactions, *e.g.* electrostatic interactions, H-bonding, halogen bonding, chalcogen interactions, pnictogen interactions, cation/anion-π interactions, hydrophobic interactions, etc. Such transport machineries might potentially mimic the function of their natural congeners and have immense therapeutic potential in 'channel replacement therapies' that address 'Channelopathies'. Despite the current state of underexplored research knowledge, quite a few ion channels/carriers have delivered record-high biological activities.[3] For example, some artificial ion channels were identified with noteworthy antibacterial activity against Gram-negative and Gram-positive bacteria. A few studies also showed potential applications including DNA sequencing, sensors and delivery systems. In some cases, ion channels were found to kill cancer cells via the necrotic pathway.[4] Recent studies have established the advantages of apoptotic pathway over the necrotic cell pathway concerning cell death based therapeutic applications. This is linked to the latter's toxic nature, far from ideal considering the living organs.[5] In fact, of late, quite a few ion channels/carriers demonstrated the ability to alter the intracellular pH gradient in the cellular acidic components (*e.g.* lysosomes, endosomes and Golgi apparatus) or to destroy the ionic homeostasis of cells, thereby triggering the apoptosis-inducing pathway.[6] However, for the treatment of 'channelopathies' *e.g.* CF, the transport systems must be non-toxic so that they can be delivered in sufficient quantities (same order of magnitude as endogenous ion channels) to the targeted cell lines for producing considerable effects. Recently, a few exciting results have been published in this direction by developing anionophores with minimum or no toxicity towards epithelial cell lines.[7]

Simply put, focus of this review is to highlight the biological activities of artificial channels/carriers and recent advances towards achieving the diverse functions of natural ion channels. A comprehensive investigation of the structure-activity relation-

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ships, selectivity aspects and biological implications in terms of potential therapeutic applications of these ionophores will be summarized in this review.

2. Cationophores and Their Biological Studies

Since the seminal work on crown ether was reported by Pedersen,[8] research activities have spurred to afford the most popular receptors in the area of supramolecular chemistry and medicinal chemistry. Thanks to their strong binding affinities to various alkali and alkali earth metals, they were exploited widely to develop metal sensors, selective ion transport machineries and catalysts.[9] In the lipid bilayer, as a selectivity filter, the crown ether could serve as an entry gate for ions, qualifying them to be considered as essential building blocks in developing artificial channels. Recent studies reveal that ion transport activity of these systems are associated with numerous biological properties. For example, Gokel and co-workers reported a series of dialkyldiaza-18-crown-6 lariat ethers (**1**–**2**) with *n*-octyl, *n*-decyl, *n* -dodecyl, *n*-tetradecyl, *n*-hexadecyl, 1-oxodecyl and 1-oxododecyl side arms (Figure 1A).[10] The observed cation transport activities of these ether carriers could be directly correlated with toxicities towards the bacteria *E. coli*, *B. subtilis*, and the yeast *S. cerevisiae*. The authors also proposed that the variation of side chain length changes hydrophobicity, leading to different interactions and integration in the lipid bilayer. This subsequently impacted ion transport, membrane depolarization and toxicity.

The same group also developed closely related channels, named hydraphiles **3**–**4** (Figure 1B).[11] These channel molecules comprise of two crown ether moieties (head groups) at the entry and exit gate and are connected by hydrophobic spacer group with another one (compounds **3a**–**3c**)/two crown-ethers **4** in the middle, favouring cation transport through it. These hydraphiles offer easy access to modulate structural components such as number of diaza-18-crown-6 units, spacer chain length, and side arm to maximize toxicity. They demonstrated potent cytotoxicity against Gram-positive and Gram-negative bacteria, yeast, and mammalian cells (*e.g.* Human Embryonic Kidney (HEK 293) and colonic carcinoma (CaCo2) cells). Higher toxicity was observed for the distal diaza-18-crown-6 head group instead of aza-18-crown-6. The distal macrocycles with benzyl groups have increased activity (**3a**–**3c**) and tetramacrocycle hydraphile **4** further enhanced activity against *E. coli* by two-fold. Remarkably, maximum cytotoxicity was observed for the channels bearing C_{14}–C_{16} aliphatic spacers (**3b** and **3c**), perhaps because the channel length matches with the cell membrane. However, selectivity between bacteria and yeast was not observed in this case and the undesired cytotoxic side effects limit their use in practical applications. To overcome this limitation, the same research group reported a possible solution by directly injecting the toxic hydraphiles to the target cells.[4b] This approach proposed possible application of hydraphiles as chemotherapeutic agents by destroying cancer cells locally.

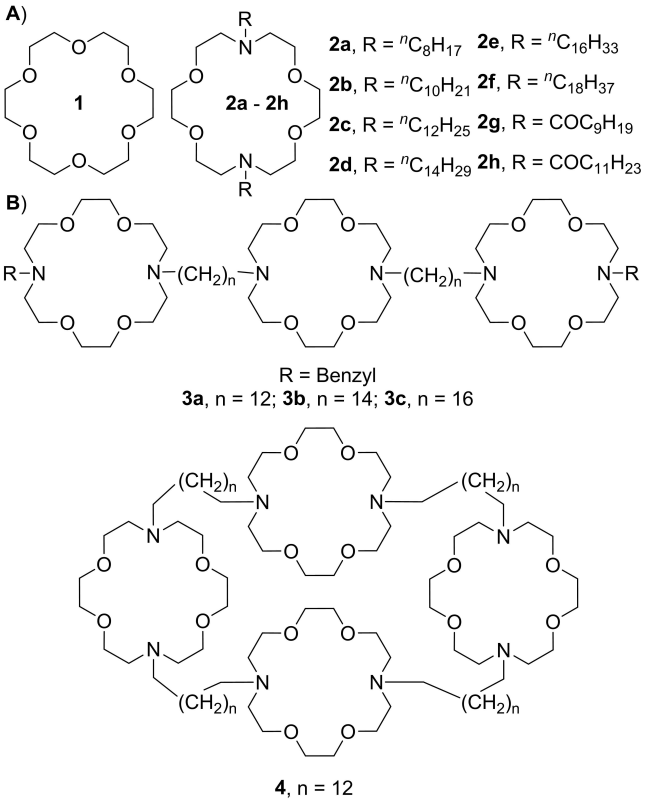

Figure 1. A) Structures of a select few crown ethers, **1**–**2**. B) Structures of hydraphiles, **3**–**4**.

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Prof. Pinaki Talukdar was born in 1975 in India. He obtained his PhD in 2005 from University of Geneva, under the guidance of Prof. Stefan Matile. He completed his postdoctoral research at the University of Illinois at Urbana Champaign, USA with Dr. Mary S. Gin. After working in Industry for three years, he joined IISER Pune in 2009 as Assistant Professor of Chemistry and now is a Professor. His research covers synthetic chemistry, supramolecular chemistry as well as the development of fluorescent probes.

The potential cation selectivity and biological activities of crown ethers have also been further exploited by conjugating of oligopeptide sequence bearing crown ethers in such a way that the macrocycles are positioned on top of each other. For instance, Voyer and co-workers strategically designed a series of crown α-helical peptides **5a**–**5d** that adopts an active channel conformation in the lipid membrane (Figure 2A).[12] The cavity diameter could be controlled by the size of the ether which subsequently resulted cation selectivity, while the length of the oligopeptide sequence would define the channel length, allowing the systems to facilitate cation transport across bilayer membrane. No significant antimicrobial activity was found in these channels with several bacteria including Gram positive and Gram negative.

However, they displayed promising cytotoxicity towards breast cancer cells (MDA) and mouse leukaemia cells (P388). The results further emphasised the importance of channel length (3–4 nm) to span the cell membrane and to observe cytotoxic property. Later, the same authors proposed a strategy to selectively destroy cancerous cells by disrupting their membranes after selective enzymatic activation.[4a] The N- or C-termini of the crown-peptide nanostructure **5d** could be attached with negatively charged dipeptide chains but that caused loss of activity. Nevertheless, the dipeptide chains could be explicitly cleaved by the proteolytic activity of prostate-specific membrane antigen (PSMA), a type II membrane protein selectively over-expressed in LNCaP prostate cancer cell line. Hence the inactive peptide-crown ether conjugates converted into their active forms *in vivo* by desired metabolic processes, killing the cancer cells selectively.

Recently Zeng and co-workers prepared a series of small molecule cation transporters (**8a**–**8c**) comprising of three modular components: a head group, a flexible alkyl chain-derived body, and a crown ether-derived foot for cation transport.[13] The cell viability assay demonstrated that the most selective cation transporter **8a** revealed anticancer activities with very low IC_{50}, 4.35 μM and 6.00 μM towards HeLa and PC3 cells, respectively. Thanks to their modular composition, this new series of transporters could find use in therapeutic applications, particularly deemed suitable as anticancer agents of future (Figure 2B).

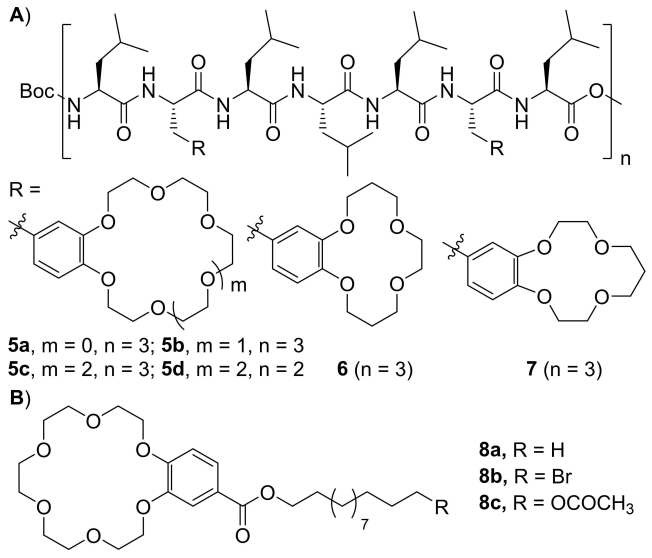

Figure 2. Structures of A) crown α-helical peptides channels (**5**–**7**) and B) small molecule-based transporters (**8a**–**8c**).

Crown ether appended ionophores were further exploited to perturb K^+ ion homeostasis which can profoundly impact various cellular functions, leading to the onset of programmed cell death such as apoptosis. Despite several ionophores revealing potential therapeutic applications as apoptosis inducers, the mechanism of cell death remains elusive.[14] Recently, Kim and co-workers reported the first evidence of synthetic helical polypeptides which can induce endoplasmic reticulum (ER) stress-mediated apoptosis (Figure 3A).[15] Introduction of tetramethylammonium group and hydrocarbon chain enhanced interactions between plasma membranes and the polypeptide (Figure 3B). These polypeptides (**AIP1**–**AIP4**) disturbed K^+ ion homeostasis and enhanced Ca^{2+} influx in the cytosol. As a result, the ER stress-related proteins could be detected followed by onset initiation of apoptosis. The ER pathways were not affected by caspase signalling which is indicative of the generation of ER stress prior to the apoptosis activation. They further demonstrated the generation of ER stress-mediated apoptosis by oxidative stress and provided complete mechanistic pathways by various apoptosis-related assays including immunoblot (Figure 3D). To verify *in vivo* applicability of AIPs as potential anti-cancer drugs, they further revealed that AIP could suppress tumour proliferation by ER stress-mediated apoptosis in a tumour-bearing mouse model without any side effects (Figures 3C and 3E).

Yamamura *et al.* studied simple amino group-containing cyclodextrin (CD) derivatives **9a**–**9c** to mimic membrane-active polycationic antibacterial peptide polymyxin B (Figure 4A).[16] These CDs could efflux K^+ ion from both *S. aureus* FDA 209P (Gram-positive bacteria) and *E. coli* K12 strain W3110 (Gram-negative bacteria). Addition of hydrophobic benzyl groups in these amino CDs created appropriate amphiphilicity, thereby increasing antimicrobial activity. In fact, these modified CDs caused higher disruptions in the bacterial membrane *versus* the unmodified CDs without amino groups. Potential antimicrobial activities were observed by Xin *et al.*, in a new series of unimolecular channels based on dual helical peptide structure modified pillar[5]arene **10a**, (Figure 4B).[17] Length of these channels were found to significantly impact both activities: membrane-incorporation and K^+ ion transport. Interestingly, their transport properties could be directly correlated with their antimicrobial activities (for *S. aureus* $IC_{50} = 4.34$ μM) and inversely correlated with their haemolytic toxicity (< 5.5%). They further showed that introduction of positively charged groups in the peptide domain had noteworthy impact on the membrane insertion ability, ion transport and biological activity.[18] The modified compound **10b** displayed a higher antimicrobial activity towards *S. aureus* ($IC_{50} = 0.55$ μM) and negligible hemolytic toxicity. These data validated their potential to serve as systemic antibiotics in future.

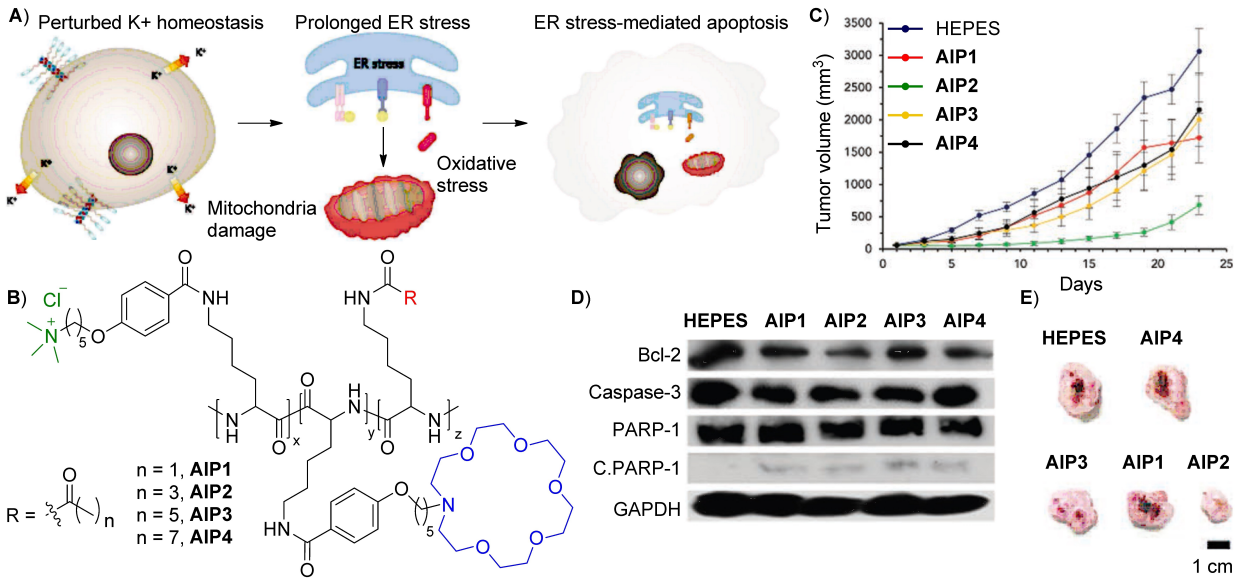

Figure 3. A) AIPs induce ER-mediated apoptosis by disturbing potassium homoeostasis. B) Representative structures of AIPs, AIPi, (i = 1–4). C) Changes in tumour volume after treatment with HEPES and AIPs. D) Immunoblot assays of the harvested tumour tissues for apoptosis-related proteins. E) Optical images of the excised tumour tissues after all mice were sacrificed on day 23.

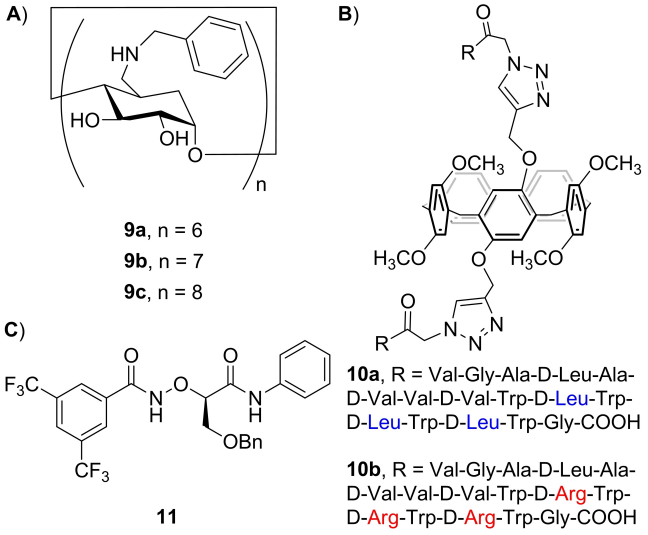

Figure 4. A) Structures of cyclodextrins, **9a**–**9c**. B) Structures of peptide modified pillar[5]arene channels **10a** and **10b**. C) Structure of the aminoxy acid monomer **11**.

In 2019, Barboiu and co-workers reported a new series of synthetically substituted cereulide K^+ ionophores (Figure 5A).[19] Interestingly, substitution of one hydroxy acid *i.e.* the (S)-2-hydroxy-3-methylbutanoic acid, by (*S*)-2-hydroxypentanedioic acid in the backbone structure of the synthetic cereulide **12** altered biological property significantly. Besides, this modified compound exhibited K^+ transport activity with a different electroneutral mechanism and could elicit highly selective activation of glucose-induced insulin secretion in a constitutive manner in the β-cells of the rat pancreas.

Disruption of ion homeostasis by a synthetic cation transporter based on α-aminoxy acid monomer scaffold **11** has been recently reported by Yang and co-workers (Figure 4C).[20] These transporters displayed potential capability to kill cancer stem cells selectively over healthy cells. By exploiting endogenous subcellular proton gradients and membrane potential in cells, this molecule displayed exceptional selectivity in killing drug-resistant ovarian cancer stem cells (CSCs) via apoptosis induction and autophagy suppression with significant selectivity (up to 47-fold). Recently, composed of a human telomeric G-quadruplex DNA (*h*-TELO; PDB: 1KF1) and a lipophilic monoguanosine derivative (MG) **13** (Figure 5B), Dash and co-workers presented an excellent stimuli responsive K^+ ionophore.[21] In this approach, MG stabilized h-TELO by non-covalent interactions and promoted the insertion of *h*-TELO within the hydrophobic lipid bilayer membrane by taking advantage of its lipophilic side chain. Authors demonstrated the transport of K^+ ions across Chinese hamster ovary (CHO) and human erythroleukemia (K-562) cell membranes using Voltage clamp experiments and these transporters might offer potential therapeutic applications by targeting specific cellular receptors. Seipelt and colleagues demonstrated the activity of zinc ionophores Pyrithione (PT) and Hinokitiol (HK) against multiplication of human rhinoviruses, coxsackievirus, and mengovirus.[22] These compounds inhibited the replication of picornaviruses considerably by impairing viral polyprotein processing.

Izzo and co-workers developed new cyclic peptoids containing (2S,4R)-4-hydroxyproline (Hyp) residues via "submonomer/monomer" approach (Figure 6A).[23] These compounds displayed significant ionophoric activities across liposomal membrane and transported alkali metal ions via carrier mechanism. MTT assay for the most active compounds **14a** and **14c** were conducted on A375 (human melanoma) and A549 (human lung carcinoma)

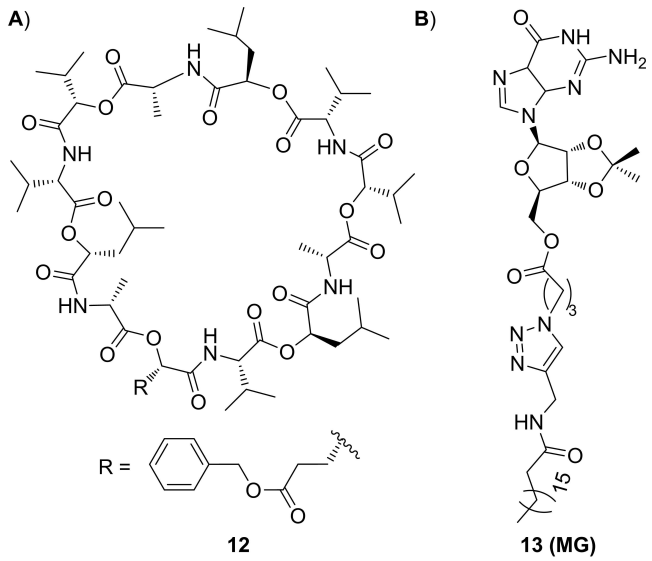

Figure 5. A) Structure of the synthetically substituted cereulide ionophore **12**. B) Structure of the MG derivative **13**.

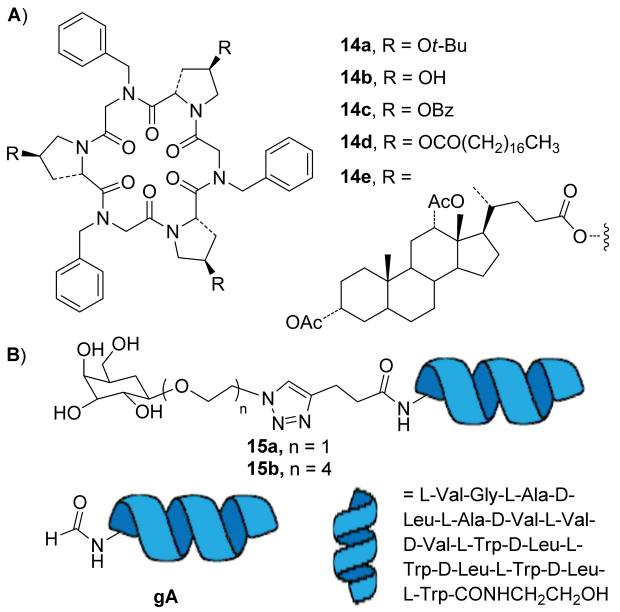

Figure 6. A) Structures of (2*S*,4*R*)-4-hydroxyprolinated cyclic peptoids, **14a**–**14e**. B) Structure of glycoside-gA conjugates, **15a**–**15b** and gA.

cancer cell lines. Experimental data showed that both ionophores inhibited the proliferation of the two cancer cell lines. The lowest IC_{50} values of 6.2 ± 0.9 μM (in A375 cells) and 4.1 ± 0.5 μM (in A549 cells) were found for **14c**.

N-terminal of the natural peptide Gramicidin A (gA) was functionalised with a galactose (Gal) moiety and the ion transport property of these Gal-gA conjugates (**15a**–**15b**) were recently reported by Haoyang *et al.* (Figure 6B).[24] Planar lipid bilayer measurements revealed that these conjugates could form unimolecular ion channels whereas the conductance (γ) and dwell time ($\tau_{1/2}$) of compound **15a** were found be 17.1 ± 2.1 pS and 0.7 ± 0.08 s, respectively. These values were significantly different from that of gA, 23.0 ± 1.6 pS and 0.4 ± 0.04 s, respectively. **15a** displayed the highest cytotoxicity and anticancer activity towards HepG2 liver cancer cells with overexpressed ASGPR (asialoglycoprotein receptor) among other Gal-gA conjugates whereas **15a** exhibited lower cytotoxicity towards HeLa (cervical cancer) cells, human pulmonary carcinoma cells (A549), human breast cancer cell lines (MCF-7), and healthy liver cells (LX-2). In addition, western blot analysis confirmed that selective death in liver cancer cells was induced by apoptotic pathway.

3. Anionophores and Their Biological Studies

Unlike cation transport machineries, anion selective transport systems remained an uncharted territory only until recently, thanks to the recent upsurge primarily driven by supramolecular chemists. Although the reports of quite a few synthetic anion transport carriers/channels are known, only a few of them are associated with exhibiting potential biological activity, suitable to mimic the functions of natural anion channels.[3d,25]

3.1. Natural products derived ionophores: synthetic prodiginine, obatoclax, tambjamines, and perenosins

Prodigiosin belongs to the family of prodiginines and is the first example of alkaloid based transmembrane anion transporter. The antimalarial, antimicrobial, immunosuppressive, and cytotoxic activities of these compounds were well studied and recently they were recognized as a novel group of agents with pro-apoptotic anticancer properties.[26] In a seminal work by J. L. Sessler's group, the biological activities of the prodigiosin analogues, also known as prodigiosenes (**16**–**19**) were introduced (Figure 7A–D).[27] The rate of transport could be correlated with the anticancer activity which was confirmed by cell proliferation assays with A549 human lung and PC3 human prostate cancer cells. The electronic nature of the O-aryl substituent on the B-ring of the tripyrrolic skeleton influenced pK_a values of the prodigiosene derivatives (**20a**–**20h**) which could control Cl^-/NO_3^- antiport rates (Figure 7E).[28] The anticancer activity of four of these synthetic prodigiosenes (**20a**, **20b**, **20d** and **20h**) (NCI60 human tumour panel) were found to be powerful with mean GI_{50} values ranging from 18 to 74 nM range. Another series of synthetic analogue of prodigiosin, known as obatoclax derivatives, were developed by Quesada and colleagues (Figure 7F).[29] The active anionophores (**21a**, **21b**) induced an increase in the lysosomal pH possibly due to influx of HCO_3^-, deacidifying the acidic organelles to induce cell death. This consequently resulted in complete disappearance of orange colour from the cytoplasm granules of GLC-4 cell line. Further studies showed that *in vitro* cytotoxic activities of these compounds correlates well with the ionophoric activity.

The tambjamines are a class of natural products having similar structural motif as prodiginines. The anticancer and

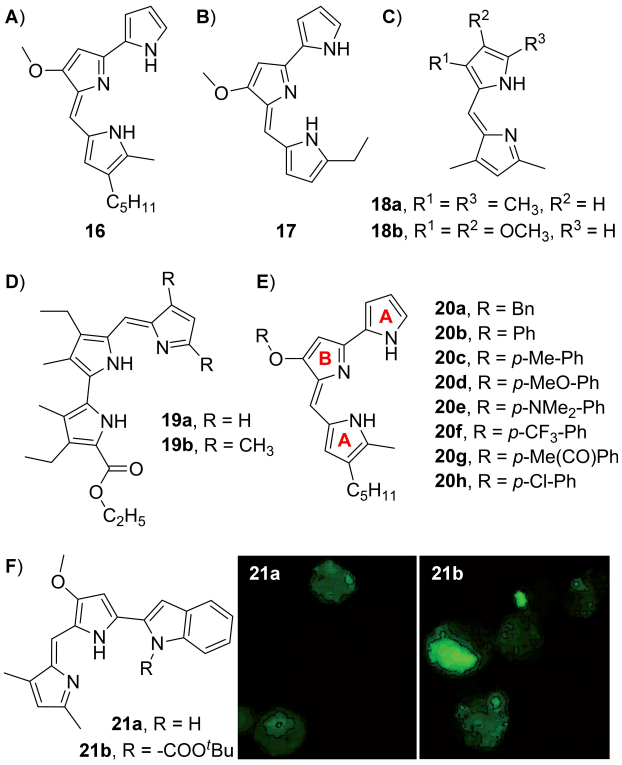

Figure 7. A)–D) Structures of prodigiosin analogues, **16**–**19**. E) Structure of prodigiosenes, **20a**–**20h**. F) Structure of obatoclax, **21a**–**21b** and AO staining of the GLC4 cell line after 1 h exposure to the indicate compounds (800 nM). Adapted with permission from Ref. [29] Copyright 2011, Wiley.

antimicrobial properties of these natural products were reported by several research groups.[30] Quesada and co-workers studied anion transport by **22**, **23a** and **23b** across liposomal membrane and on small cell lung cancer line (GLC4) cells using acridine orange (AO) dye based vital staining (Figures 8A and 8B).[31] The active transporters (**22d**, **23a**–**23b**) increased the intracellular pH via possible influx of HCO_3^- to cause disappearance of orange emission of the dye. Authors further studied *in vitro* activity of new tambjamine derivatives (**23c**, **23d**, **24**–**25**), bearing aromatic enamine moieties.[32] These anionophores altered intracellular pH, induced basification of acidic organelles and triggered apoptosis that could be observed by Hoechst 33342 staining on melanoma (A375) human cancer cell line. Later, the same group reported another series of tambjamine-inspired anionophores (**26a**–**26c**) which were capable of Cl^- and HCO_3^- transport across lipid bilayer (Figure 8D).[6b] This further induced acidification of cytosol and hyperpolarization leading to differentiation of the cancer stem cells (CSCs) and selective elimination of cancer cell subpopulation. Authors later analysed the cellular and molecular mechanisms of killing the lung cancer cells and cancer stem cells by these tambjamine anionophores (**23b**–**23d**, **24a**–**24d**, and **25b**).[33] The transmembrane transport caused imbalance in cell homeostasis activated mitochondrial dysfunction and lysosomal deacidification. These events consequently generated potential cytotoxic effects through necrosis in cancer cell lines. Moran and co-workers studied anion transport activity of prodigiosin, a triazole derivative of prodigiosin, obatoclax and two synthetic analogues (**27**–**29**) of the tambjamines.[34] These molecules were found to be efficient Cl^- and HCO_3^- transporters across cell membrane with a higher transport efficiency at acidic pH without affecting the cystic fibrosis transmembrane conductance regulator (CFTR) function. Therefore, these could be used as potential therapeutic agents to replace the defective or missing CFTR. Quesada and colleagues reported a new series of anionophores, called 'click tambjamines' (**29a**–**29i**).[35] In this design, one of the pyrrole groups of the 4-methoxy-2,2′-bipyrrole core of the natural tambjamines was replaced by a 1,2,3- triazole ring. Few of these molecules showed significant anion transport across the liposomal membrane and displayed moderate IC_{50} values (3.5 to 10.7 μM), determined on human lung (A549), breast adenocarcinoma (MCF7) and mammary epithelial (MCF10 A) cell lines. The research groups of Quesada and Caci recently developed anionophores (**28** and **29b**), inspired by the structure of tambjamine as potential future CF drugs which were independent of the CFTR function and the genotype of the CF patient.[36] These compounds enhanced the periciliary fluid composition, decreasing the fluid reabsorption, improving the abnormal ASL pH, could reduce the viscosity of the mucus and all these parameters are linked to CF pulmonary disease (Figure 8G). Inspired by prodigiosin structure, Cho and colleagues rationally designed a series of ionophores that feature a terminal ethynyl group, comprising non-pyrrolic H-bond motifs such as phenolic OH, amide NH, and triazole CH.[37] These Cl^- transporters (Figure 8H) were found non-toxic even at 500 μM concentration in various carcinoma cell lines, including HT-29 and DLD-1. In essence, this could overcome the cytotoxicity of synthetic prodigiosins (IC_{50} = 2-3 μM in A549 human lung cancer cells) and could find potential use for Cl^- malfunction caused diseases such as CF.

A new class of prodigiosin-inspired anion transporter (**30a**–**30e**), also known as 'perenosins' was studied by Gale and co-workers (Figure 9).[38] The indole based perenosins affected the viability of two breast cancer cell lines such as breast carcinoma MDA-MB-231 (invasive) and MCF-7 (non-invasive) with ~5.5-fold selectivity over normal breast cells (MCF-10 A). They introduced a new class of perenosin derivatives with improved lipophilicity (Figure 9).[39] Variation of the alkyl substituent at the R^2 position enabled fine-tuning of cellular cytotoxicity. These ionophores (**31**–**32**) triggered death of cancer cell lines *e.g.* MCF7, MDA-MB-231, A549 and SW620 and non-cancerous human cell line MCF-10 A. These transporters decreased cell viability of MDA-MB-231 over other normal cells and cancer cells. The most potent perenosin, **32b** induced cell death via a combination of apoptotic pathway and the cell cycle arrest mechanism.

3.2. Acyclic anionophores based on urea and thiourea

Urea or thiourea scaffolds are often incorporated into receptors to promote anion binding and transport across the lipid bilayer. Gale and others explored anion transport activity of urea/thiourea containing transporters extensively.[40] For example, a

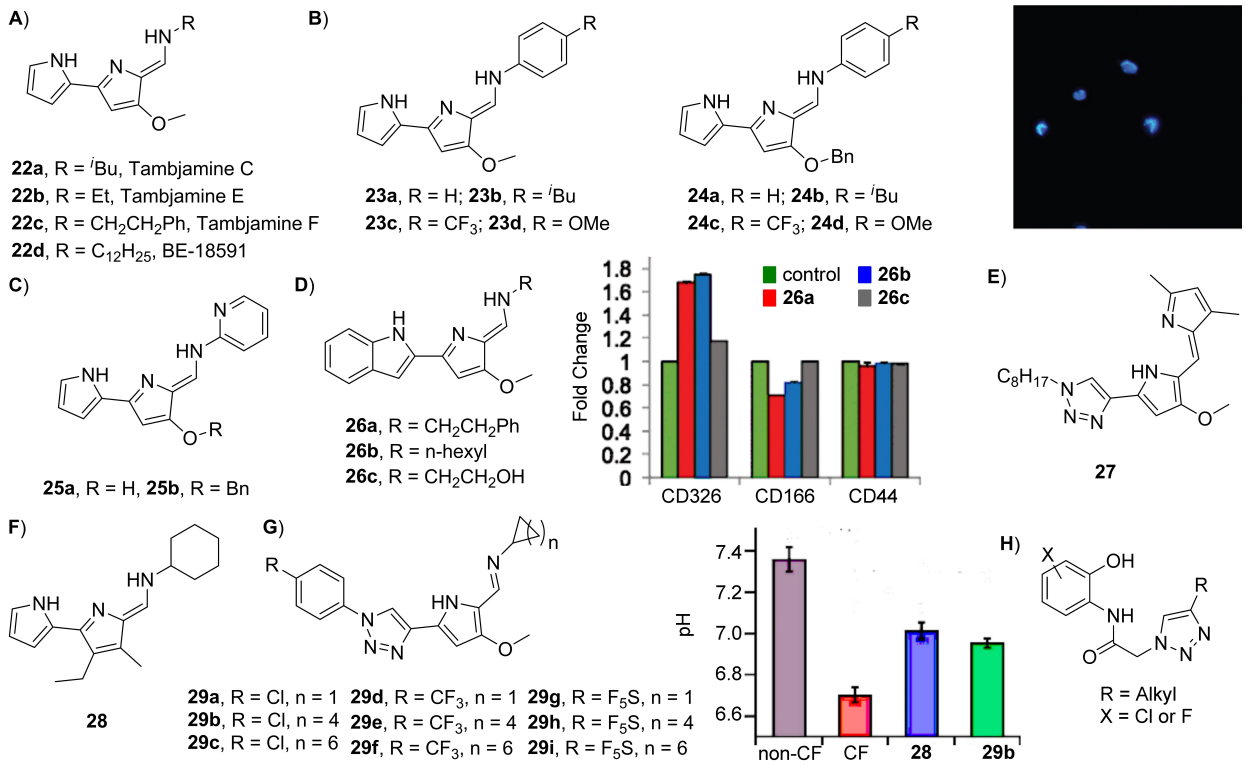

Figure 8. A) Tambjamine derivatives **22a**–**22d**. B) and C) Synthetic tambjamine analogues (**23**–**25**) and Hoechst 33342 staining on melanoma (A375) human cancer cells treated with **23c**. D) Chemical formula of tambjamine-inspired anionophores (**26a**–**26c**) and data showed that **26a** (2.5 μM), **26b** (2.5 μM) and **26c** (2.5 μM) affect expression of cancer stem cell surface markers in A549 cells and the expression of CD326 (EpCAM), CD166, and CD44 was analysed by flow cytometry. E–G) Chemical structures of the synthetic analogues (**27**–**29**). G) Evaluation of the apical fluid pH. Untreated CF and non-CF epithelia were incubated with 0.1% DMSO as control. The CF epithelia were incubated with 0.25 μM of **28** or **29b**. H) Prodigiosin inspired non-pyrrolic anion transporters. Adapted with permission from Ref. [32] Copyright 2014, Royal Society of Chemistry. Ref. [6b]. Copyright 2015, American Chemical Society.

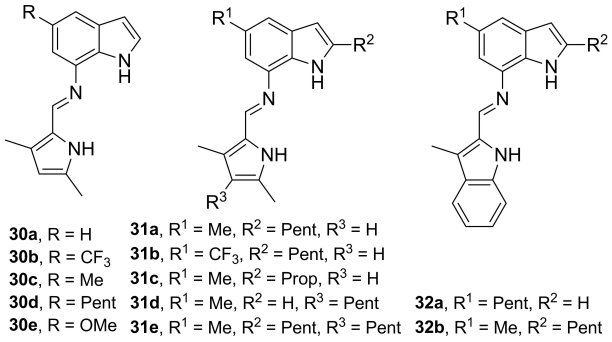

Figure 9. A) Structures of the perenosins, **30a**–**30e.** B) Structure of new analogues **31a**–**31e** based on the same indole-pyrrole scaffold and on a modified bis-indole scaffold, **32a**–**32b.**

series of tripodal receptors (**33a**–**33h**) based on urea/thiourea scaffold were reported by Gale and co-workers in 2011 (Figure 10A).[40a] Anion transport abilities of these compounds were found to be directly proportional to the degree of fluorination which in turn increased their lipophilicity. The transporters (**33c**–**33h**) changed the internal pH regulation which subsequently resulted in apoptosis in GLC4 cancer cell line. Inspired by the anticancer activities revealed by these transporters, the authors developed a series of "drug-like" indole based anion transporters (**35**–**37**), whose lipophilicity and transport activity were dependent on the trifluoromethyl functionalisation (Figure 10C).[41] The *In vitro* fluorescence and viability assay displayed that the most active transporter can be utilized as an anticancer agent by killing human melanoma A375 cancer cell line via apoptosis. The authors further developed a series of anion transporters (**34a**–**34f**) consisting of an urea/thiourea scaffold and a naphthalimide fluorophore (Figure 10B).[42] These transporters were found to be cytotoxic towards cancer cells and the most lipophilic, fluorinated thiourea based fluorescent transporter **34f** was found to be localised homogeneously throughout the cell. Moreover, this transporter induced killing of A549 cells via apoptotic pathway and might function as antineoplastic agents.

Davis and co-workers proposed that anion transporters could find potential use to treat cystic fibrosis when they were not toxic towards cells.[7] A much convenient fluorescence based protocol on Fischer rat thyroid (FRT) cells expressing YFP-H148Q/I152 L (YFP-FRT cells) was used to test anionophoric activity. Among 15 transporters, bis-(*p*-nitrophenyl) ureidodecalin **38a** was identified with the most promising activity including deliverability, potency and persistence (Figure 11A). In Epithelia, the activity of the compound was confirmed by electrophysiological studies and the observed current was similar to those obtained for naturally expressed

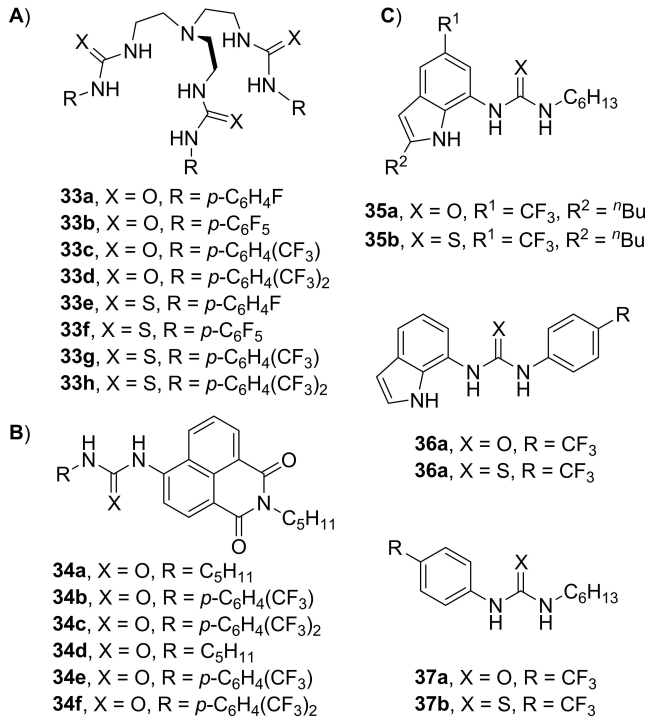

Figure 10. A) Chemical structures of tripodal transporters, **33a**–**33h**. B) Chemical structures of fluorescent transporters, **34a**–**34f** and C) "drug-like" transporters, **35**–**37**.

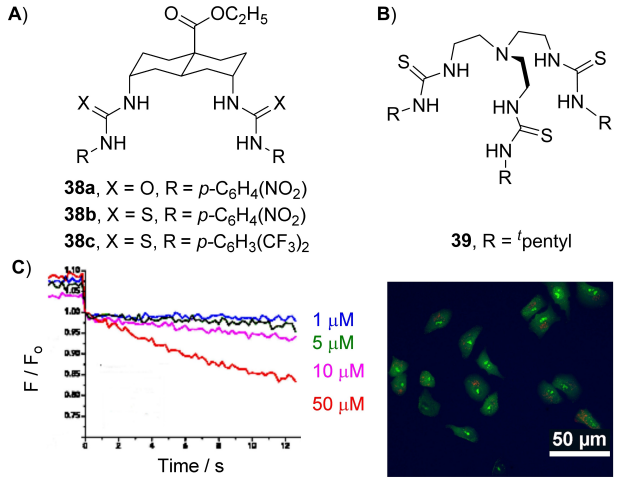

Figure 11. A) Chemical formulae of Decalin bisureas, **38a**–**38c**. B) Chemical formula of tripodal thiourea **39**. C) Facilitated Cl^- transport through the plasma membrane was measured indirectly by I^- entry into the cells coupled with the exit of intracellular Cl^-, leading to the quenching of YFP fluorescence by **39** (left). AO staining of human lung adenocarcinoma (A549) cells treated with compound **39** (50 μM) (right).

CFTR channels. Later, Davis and Gale developed the first example of anionophores on tripodal thiourea with bulky *tert*-pentyl group **39** that facilitated $Cl^- > H^+/OH^-$ selectivity (Figure 11B).[43] This compound mediated effective anion transport by FRT cells expressing the halide sensor YFP-H148Q/I152 L and exhibited modest toxicity to human lung adenocarcinoma (A549) cells with a half-maximum inhibitory concentration (IC_{50}, determined after treatment for 24 h) of 43 ± 4 μM. However, only a slight disappearance of AO fluorescence in the same cell line was observed for **39** (at 50 μM concentration) when compared with DMSO control (Figure 11C). Hence, **39** transported anions without neutralizing lysosomal pH in cells, essentially mimicking the electrogenic behaviour of valinomycin. In 2019, by monitoring fluorescence emission from the halide-sensitive YFP, Davis and colleagues screened 22 anionophores, searching for biological activity.[44] Four of these carriers (**33h**, **38a**–**38c**) were found to be effective in YFP-modified CF airway epithelial cells without any cytotoxic effects. When tested together with the clinically licensed CFTR modulators such as lumacaftor and ivacaftor, activities were found to be additive to rescue the predominant CF-causing variant F508del-CFTR. Hence these anionophores, either alone or together with CaCC potentiator (or CFTR modulator), as a part of combination therapy might find potential use in therapeutic applications that address CF.

Moore *et al.* studied simple orthophenylenediamine based bisurea scaffolds which facilitated Chloride, carboxylate and carbonate transport across the liposomal membrane (Figure 12).[45] Few of these transporters showed cytotoxicity in various human cancer cell lines of diverse origin (melanoma A375, small-cell lung carcinoma GLC4, colon adenocarcinoma SW480, alveolar adenocarcinoma A549, and oral adenosquamos carcinoma CAL27). The ability to induce apoptosis in A375 cells could be observed for the two most cytotoxic transporters (**40a**–**40b**). Félix and colleagues reported thiophene appended transporters (**41**–**43**) on an *ortho*-phenylenediamine central core which bind with anions by urea and C–H binding groups in a cooperative fashion (Figure 12B–C).[46] The fluorinated analogues were found to be more active towards anions over non-fluorinated derivatives and display cytotoxicity against tumour cell lines such as HeLa, MCF-7 and A549. Recently Manna and co-workers described conformationally controlled bis(thiourea) derivatives (**44a**–**44f**) based on 1,2-diphenylethylenediamin scaffold. These molecules carried anions with significant transport efficiency *versus* other biologically relevant cations and the antiport mechanism could be identified (Figure 12D).[47] Here, anion carriers induced perturbation of Cl^- ion homeostasis and led to the death of HeLa cell line by promoting the caspase-mediated intrinsic pathway of apoptosis. Last year, Rawling *et al.* reported a series of fatty acid substituted urea-based (also termed as, aryl-urea) anion transporters (**45a**–**45d**) and their function as mitochondrial uncouplers.[48] The carboxylic acid group and a π-conjugated motif were separated by a long alkyl chain and the aryl-urea group engaged in forming intermolecular H-bonds with the fatty acid carboxylate, facilitating permeation across mitochondrial inner membrane (MIM) alongside repetitive cycling. These compounds induced mitochondrial dysfunction in breast cancer cells and damaged ATP production at concentrations exceeding their JC-1 IC_{50} (at 10 and 40 μM). Using Seahorse Mito Stress test, the cellular oxygen consumption rates (OCR) were determined in presence of the ATP synthase inhibitor oligomycin. The active ionophores and the known protonophore FCCP

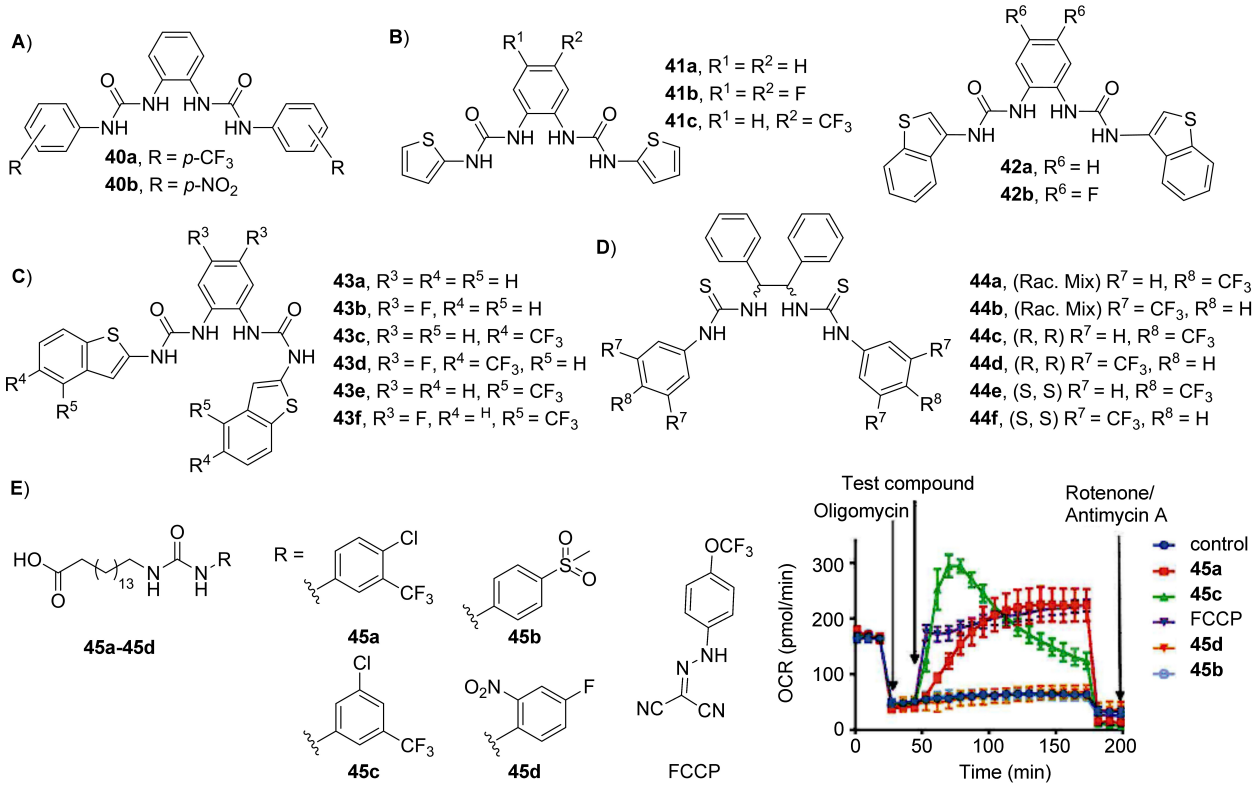

Figure 12. Chemical structures of A) *ortho*-phenylenediamine based bisureas, **40a**–**40b**; B) and C) thiophene appended transporters, **41**–**43**; D) Diphenylethylenediamine based transporters, **44a**–**44f** and E) aryl urea substituted fatty acids based protonophoric mitochondrial uncoupler as anion transporters (**45a**–**45d**) and Oxygen consumption rates (OCR) in MDA-MB-231 cells after sequential addition of the ATP-synthase inhibitor oligomycin (1 μM), protonophore FCCP (1 μM) or transporters (20 μM).

increased the OCR *ca.* 5-fold *versus* the control probe and subsequently introduced the first biological example of a mitochondrial uncoupler.

A new class of low-toxic, glutathione mediated sulfonium-based proanionophore was developed by Manna and co-workers (Figure 13A).[49] These stimuli-responsive carriers could be potentially used to treat ion transport related diseases such as CF. Nevertheless, the apical fluid of CF patients contains relatively less glutathione (GSH); additional GSH would be needed to generate the active anionophore from the proanionophore **46** for both pulmonary and oral drug delivery methods. The transport and biological activities of the acylthiourea based phosphatidylinositol-3,4,5-trisphosphate (PIP3)

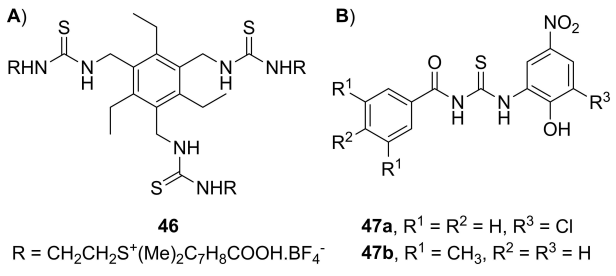

Figure 13. A) Chemical structures of glutathione mediated proanionophore **46**. B) Chemical structures of PIT-1, **47a** and DM-PIT-1, **47b.**

inhibitors (PITENINs) were reported by the same research group recently (Figure 13B).[50] Among these PITENINs, **47a** (PIT-1) and **47b** (DM-PIT-1) displayed significant Cl^- transport ability. The cell viability test on HeLa cell suggested that compounds **47a** and **47b** disrupted the Cl^- ion homeostasis and promoted cell death via apoptotic pathway.

3.3. Squaramide based anion transporters

Squaramide based receptors have been exploited for developing anion receptors and transmembrane carriers as these conformationally rigid square-shaped compounds feature stronger ability as a H-bond donor than the urea counterpart. In 2017, Sessler, Gale, Shin and co-workers reported the activities of Squaramide based anionophores (**48**, **49a**–**49b**) which affected the function of a subcellular organelle (Figure 14A).[51] The caspase-dependent apoptosis activities of the active transporters were in correlation with the liposomal and cellular transport activities. The squaramide derivative **48** increased liposomal pH that reduced the activity of lysosomal cathepsin B and L, each. This affected the normal function of liposome and therefore inhibited autophagy. Conversely, regardless of autophagic processes, the transporter promoted the release of cytochrome c from mitochondria, which led to the caspase-dependent apoptotic cell death (Figure 14B–D). Hence, this

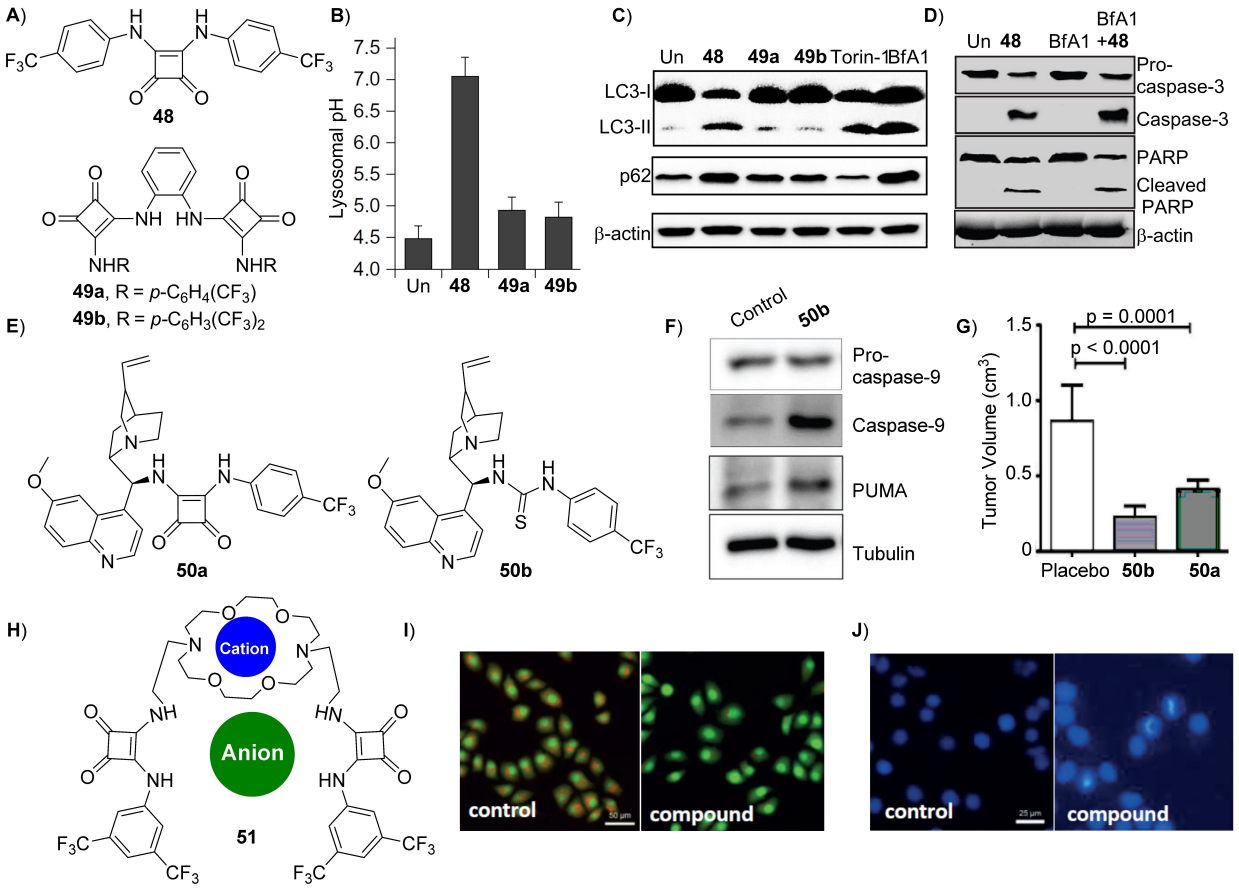

Figure 14. A) Structures of squaramide based transporters **48**, **49a**–**49b**. B) The effect of transporters (4 μM) on the lysosomal pH. C) Western blot analysis of HeLa cells to evaluate their effect on autophagy. Torin-1 (1 μM) and BfA1 (5 μM) were used as controls for autophagy induction and inhibition, respectively. D) Effect of apoptosis induction promoted by **48**. E) Structure of squaramide and thiourea derivatives of quinine (**50a**–**50b**). F) Whole-cell protein extracts of MCF-7 cells grown in the absence and presence of **50b** (10 μM) for 24 h were immunoblotted for the indicated proteins. G) The extent of tumour growth inhibition by **50a** and **50b**. H) Structures of aza-crown ether-squaramide conjugate transporter **51**, I) AO was used to stain HeLa cells and the characteristic pH-sensitive fluorescence colour changes from orange to green was observed for **51**, and J) changes in the nuclear morphology by fluorescence microscopy due to apoptosis by compound **51**. Reprinted by permission from the Springer Nature [Nature Chemistry] Ref. [51], Copyright 2017. Ref. [52], Copyright 2020, American Chemical Society. Ref. [53], Copyright 2019, Future Science Ltd.

transporter promoted apoptotic cell death as well as disrupted autophagy, thereby validated early signatures of a potential anticancer agent of the future. Recently Manna and co-workers developed quinine derivatives with squaramide (**50a**) and thiourea (**50b**) scaffolds and investigated their ion transport and biological activities.[52] The anionophores caused death of MCF7 cells through caspase dependent apoptosis; in turn, reducing the tumour volume efficiently with slight immunotoxicity to other organs (Figure 14E–G). Recently anion/cation symporters consisting of aza-crown ether-squaramide conjugates were reported by Chen and co-workers.[53] The most efficient transporter bearing 3,5-bis(trifluoromethyl)phenyl substituents **51** exhibited moderate cytotoxicity and could basify the acidic organelles, unsettling ionic homeostasis towards the HeLa cells and triggered cancer cell death via apoptosis (Figure 14I–J).

3.4. Indole, benzimidazole/imidazole based anion transporters

Chen and colleagues developed a series of 1,3-bis (benzimidazol-2-yl)benzene (m-Bimbe) derivatives (**52**–**53**) as transmembrane anionophores which exhibit anticancer activity (Figure 15A).[54] The 5-nitrated derivatives bearing trifluoromethyl (**53b**) and the ones bearing nitro (**53c**) at the benzimidazoloyl subunits were the most cytotoxic carriers among other derivatives tested against a few select cancerous and normal cells. The cellular mechanistic study by using Hoechst 33342 and JC-1 staining suggested that these compounds triggered cell death possibly via apoptotic pathway. Fluorinating the same class of molecules (**54**–**55**), the same research group later demonstrated the importance of lipophilicity on anion transport and biological activity (Figure 15B).[55] Improvements in anion transport ability and cytotoxicity were detected for these fluorinated bisbenzimidazoles, which too induced apoptosis in the cancer cell. Of late, Manna and co-workers reported a new class of bis(iminourea) derivatives (**56a**–**56b**) as Cl^- ion

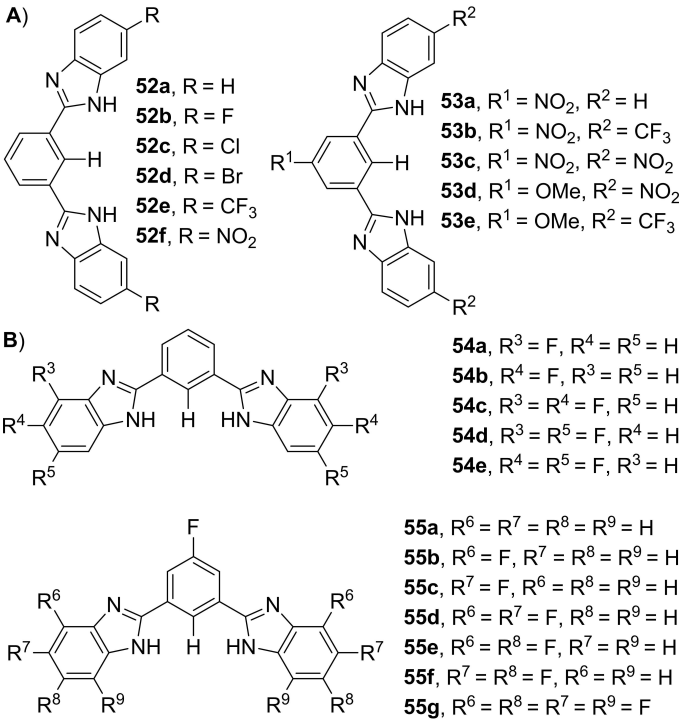

Figure 15. Chemical structures of A) 1,3-bis(benzimidazol-2-yl)benzene (m-Bimbe) derivatives (**52**–**53**) and B) fluorinated bisbenzimidazoles (**54**–**55**).

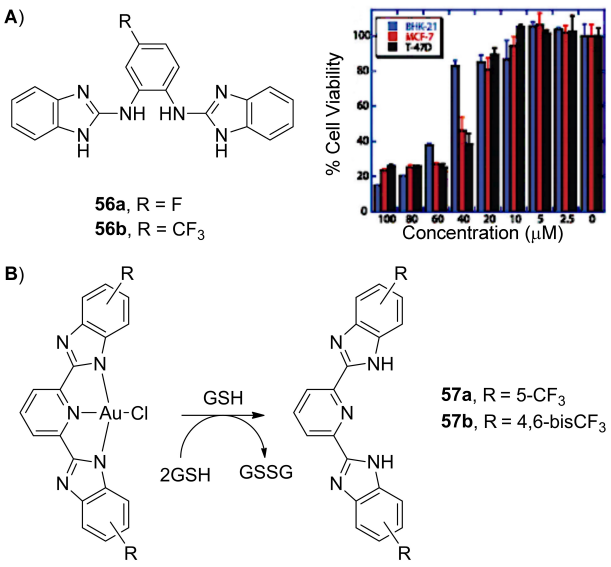

Figure 16. A) Chemical structures of bis(iminourea) carrier (**56a**–**56b**) (left side) and cell viability of the BHK-21, MCF-7 and T-47D cell lines in the presence of **56b**, measured at different concentrations (right). B) Chemical structures of bisimidazole derivatives (**57a**–**57b**). Adapted with permission from Ref. [56] Copyright 2019, Royal Society of Chemistry.

carriers.[56] These compounds showed pH dependent switching of Cl^- transport (9-fold) within a narrow window which could be due to their pK_a values (6.2–6.7) within the lipid bilayer. MTT assay revealed that these compounds brought about 2-fold higher cytotoxicity for the cancerous cells (MCF-7 and T-47D) over the studied normal cell lines (Baby Hamster Kidney fibroblasts BHK-21) (Figure 16A).

Recently, Gale and co-workers reported stimuli-responsive complexes which was developed by complexing bisimidazole anionophores with Au(III).[57] Au(III) blocked the binding site and could be decomplexed in presence of the reducing agent such as GSH to release active transporters (**57a** and **57b**) (Figure 16B). Since tumour cells contain higher amounts of GSH than the healthy cells, these compounds could be targeted for cancer cells selectively. In fact, the cell viability test showed significant cytotoxic effect for cancerous cells (SW620) and less toxicity for non-cancerous cell lines (HEK293 and MCF10 A).

3.5. Other anion transporters

Gale, Roelens, Sessler and co-workers developed a family of amino pyrrolic anionophores (**58**–**59**) which inhibited the growth of Gram positive bacteria (*S. aureus*) *in vitro* (Figure 17A).[58] The most active carriers **59a** and **59b** were found to be effective against the methicillin resistant *S. aureus* strains (MRSA) *e.g.* HP1173 and Mu50, as well as the non-methicillin resistant UAMS1 strain. Recently, Kulsi *et al.* synthesised a hybrid triazole/aminoxy amide macrocycle **60** which was found to bind Cl^- via aminoxy amide N–H and triazole C–H interactions, while transporting Cl^- ions across the lipid bilayer following antiport mechanism (Figure 17B).[59] The macrocycle imposed cytochrome c leakage and altered mitochondrial membrane potential along with activation of a family of caspases. These data further suggested that cellular apoptosis occurred through a caspase-dependent intrinsic pathway and this macrocycle could be used as a potential biological tool for treating defective chloride transporter related diseases.

The biological activity of pH dependent ionophores based on macrocycles and cages have been explored lately by quite a few research groups. Shin and colleagues reported pyridine diamide-strapped calix[4]pyrrole **62b** as Na^+/Cl^- symporter which could promote apoptosis via a caspase-dependent pathway (Figure 17D).[6a] The influx of NaCl via these macrocycles caused an increase in the reactive oxygen species (ROS), released cytochrome c from mitochondria and directed apoptosis via caspase activation. Alfonso and co-workers developed a family of pseudopeptidic cage-like ionophores **61a**–**61b** displaying H^+/Cl^- symport activity.[60] The side chain dependent activity of these compounds was further exploited to induce significant enhancement of cytotoxicity towards lung adenocarcinoma cells in the presence of pH gradients that mimic the tumour microenvironments (Figure 17C). Talukdar group reported a series of Bis(sulfonamide) ionophores (**64a**–**64f**) that exhibited lipophilicity-induced anion binding and transport activity.[61] Upon conducting experiments in HeLa cell line, the most active anionophore **64f** induced caspase-dependent intrinsic pathway of apoptosis (Figure 17F). Later Talukdar and co-workers developed a series of procarriers based on indole-2-carboxamide receptors, sustained by a photo-sensitive *o*-nitrobenzyl-linked derivative.[62] Upon photoirradiation, 90% trans-

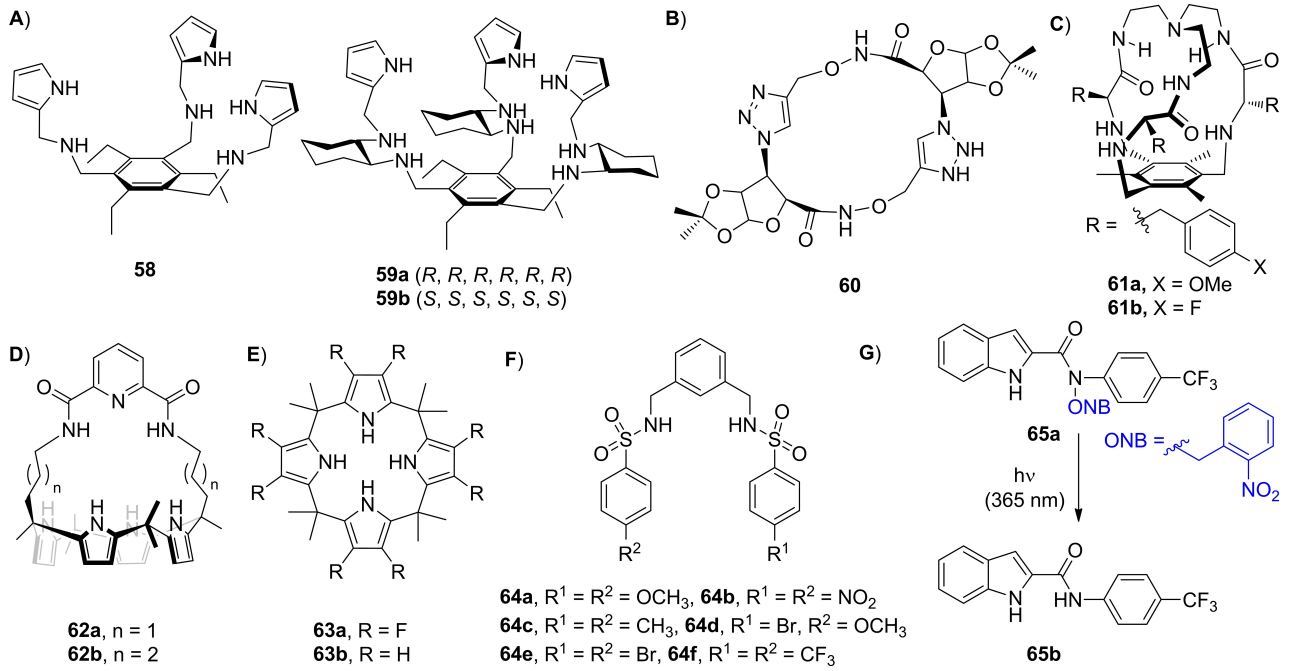

Figure 17. A) Structures of aminopyrrolic receptors **58**–**59**. B) Chemical structure of hybrid triazole/aminoxy amide macrocycle **60**. C) Structures of pseudopeptidic cages **61a**–**61b**. D) Structures of pyridine diamide-strapped calix[4]pyrroles **62a**–**62b**. E) Macrocyclic transporters (**63a**–**63b**). F) Chemical structure of bis(sulfonamide) anionophores **64a**–**64f**. G) Structures of phototriggered ONB-protected procarriers **65a** and active carrier **65b**.

port efficiency of the active transporter **65b** could be recovered within just 3 minutes (Figure 17G). Such efficiency was observed even in the cancer cell line, causing active transporter **65b** to trigger cancer cell death (MCF 7) efficiently, shedding light upon the potential scopes of these compounds in cancer treatment.

In order to understand the cellular mechanisms for cancer cell death, Shin and co-workers studied transport behaviour and bioactivity of the compounds **48**, **62a**, **63a** and **63b**.[63] Except **63b**, the other compounds increased intracellular Cl^- and Na^+ concentrations to cause (hypo)osmotic stress in cells, thus affording ROS via sequential processes. This further induced caspase-dependent apoptosis. Transporters **48** and **63a** increased the concentration of cytosolic Ca^{2+} ion, while activating AMPK and beclin-1 both to induce autophagy. However, these reduced lysosomal Cl^- concentrations and elevated lysosomal pH inhibited lysosomal function, consequently blocking autophagy. Overall, **62a** promoted the death of cancer cells by inducing apoptosis whereas the compounds **48** and **63a** triggered cancer cell death via apoptosis as well as by suppressing autophagy (Figure 17D–E).

3.6. Ion channels

In 2012, a small molecule channel **66** was reported by Shen *et al.* that mediate Cl^- ion across the lipid bilayer.[64] This channel **66** demonstrated potential ability to restore Cl^- permeability in CF airway epithelial cells with defects in their native CFTR chloride channels (Figure 18A). A new series of Cl^- ion channels based on triazole-capped octameric α-aminoisobutyric acid (Aib) foldamers (**67a**–**67c**) was reported by Webb's group (Figure 18B).[65] These channels could 'switch on' the transmembrane ion transport activity in presence of $CuCl_2$ which was "switched off" upon extracting Cu^{2+}. The channels formed by $CuCl_2$/Aib octamer complexes exhibited capability of transporting both cations and anions, especially Cl^-. The channels exhibited significantly reduced minimum inhibitory concentrations (MICs) against B. megaterium stain DSM319 *versus* the uncomplexed foldamer. However, perhaps due to lower hydrophobicity, their haemolytic activities were found 90% lower as compared to the antibiotic Alamethicin. Kimoon Kim's group reported a shape-persistent porphyrinic covalent organic cage **68** as I^- channel, exemplifying the dehydration-driven channel mechanism.[66] The outer maximum diameter of **68** was 3.64 nm that could span the bilayer lipid membrane. PB-1 A could be inserted into the YFPHEK- 293T cell membrane, mediating I^- transport across the cell membrane, thus indicating the likelihood of devising a biological tool for replacing defective I^- channels in living organisms (Figure 18C).

In 2016, Talukdar and co-workers described a self-assembled barrel-rosette Cl^- transporting ion channels (**69a**–**69d**) constructed by vicinal bis(diol) moieties. **69c** forms a H-bonded network at two termini of each monomer (Figure 19A).[67] Cl^- transport across the bilayer membrane caused disruption of cellular ionic homeostasis which triggered cell death by inducing the caspase dependent intrinsic pathway of apoptosis. Importantly, the mechanism of apoptosis was proved from the expression of proteins in the caspase family, namely, caspase 3 and caspase 9.

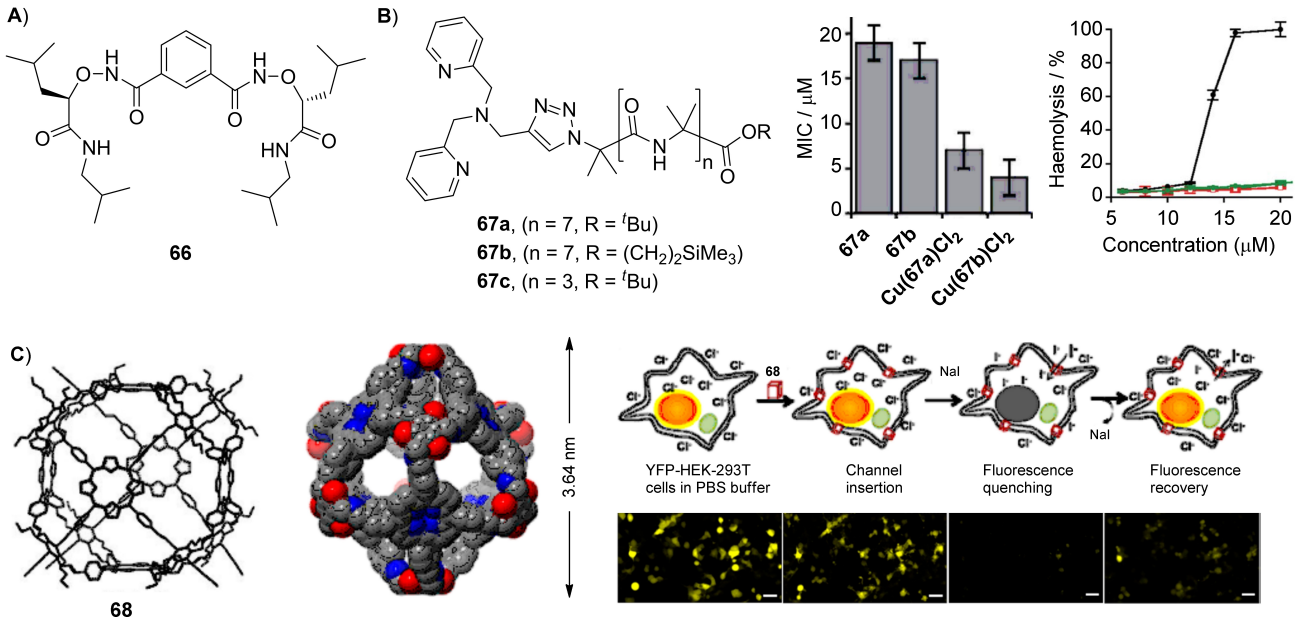

Figure 18. A) Chemical structure of small molecule ion channel **66**. B) structure of Aib foldamers ion channels **67a**–**67c**, MIC against B. megaterium strain DSM319. Haemolysis of human erythrocytes caused by alamethicin (●) and **67a** (□), Cu(II)[**67a**]Cl_2 (□), **67b** (●) and Cu(II)[**67b**]Cl_2 (●). C) Structure of **68** showing an average diameter ~3.7 Å, (including alkyl chains) estimated from the X-ray crystal structure of a closely related compound. Schematic representation of iodide transport in YFPHEK- 293T cells, and YFP-HEK-293T cells images (from left to right). Untreated cells in PBS buffer, after 1 h incubation with **68**, after 10 min treatment with NaI, and after the removal of extracellular NaI. Scale bar is 50 μm. Adapted with permission from Ref. [66a] Copyright 2015, Wiley. Ref. [66b] Copyright 2017, American Chemical Society.

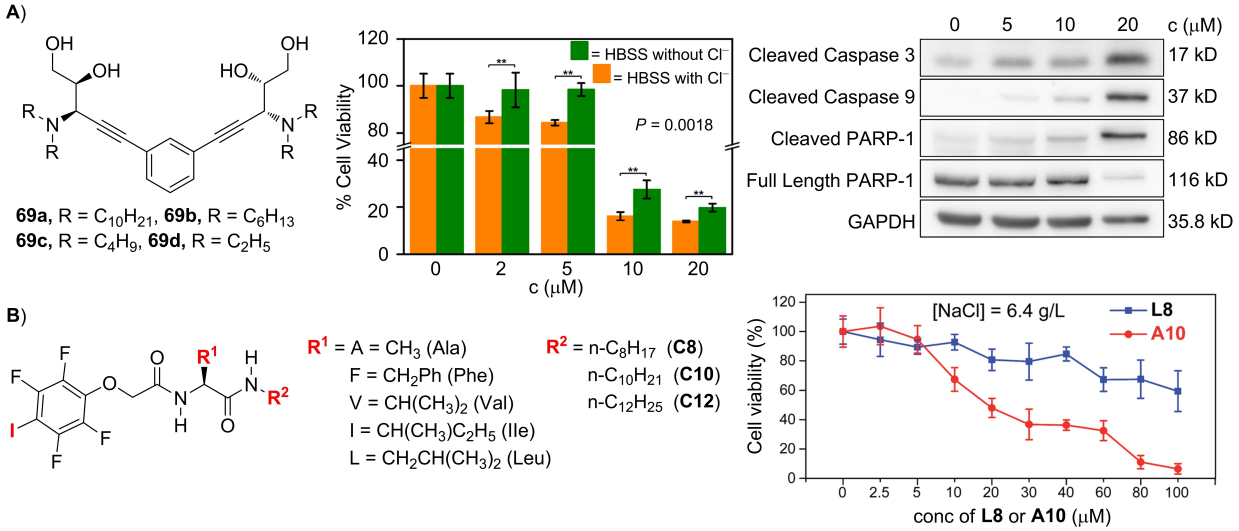

Figure 19. A) Structure of barrel-rosette ion channels based on vicinal bis(diol) moieties (**69a**–**69d**), MTT assay comparing cell viability of HeLa cells in the presence and the absence of Cl^- ions in extracellular media (HBSS buffer) and Immunoblot assay for cleaved caspase 9, caspase 3, and PARP-1 in HeLa cells, after 24 h incubation with various concentrations of **69c**. B) The structure of halogen bond-mediated artificial anion channels based on monopeptide scaffold and viabilities of BT-474 cells in the presence of various concentrations of **L8** and **A10**. Adapted with permission from Ref. [67] Copyright 2016, American Chemical Society.

Zeng and colleagues developed halogen bond-mediated artificial anion channels based on a monopeptide scaffold. These ion channels presented a transmembrane pathway for selective anion transport over a few alkali metal ions (e.g. Li^+, Na^+, K^+, Rb^+ and Cs^+) via the directional assembly of electron-deficient iodine atoms (Figure 19B).[68] These also facilitated the passage of anions across two types of membranes: cholesterol-containing and the free bilayer type. Excellent anion transport capabilities of **L8** and **A10** further inhibited the growth of human breast cancer cells (BT-474) with an IC_{50} of 20 μM, suggesting potential uses as anticancer agents in the future.

Later Talukdar's group developed a series of 2-hydroxy-N^1,N^3-diarylisophthalamide based fluorescent ion channel-forming compounds that facilitate ion transport via M^+/Cl^- symport mechanism **70** (Figure 20A).[69] Cell viability tests using MCF7 cells for the most active channel showed that the cell death is facilitated by both Cl^- and M^+ ions. The apoptosis mechanism was established by monitoring mitochondrial membrane depolarization, generation of ROS, release of cytochrome c, followed by activation of the caspase 9 pathway, PARP cleavage, and lastly the staining of nuclear contents by propidium iodide dye in the treated MCF7 cell line. Shortly after, the same group has published a new series of esterase enzyme mediated self-assembled rosette ion channel based on N^1,N^3-dihexyl-2-hydroxyisophthalamide scaffold. This ion channel **71** triggered ion transport across lipid membrane by M^+/Cl^- symport mechanism.[70] Interestingly, this system induced cancer cell death not only by apoptosis (following an intrinsic pathway), but also by autophagy disruption in MCF 7 cells (Figure 20B).

Glutathione (GSH) is a well-known antioxidant in the epithelial lung lining fluid, with potential resistance against chronic lung inflammation, a major cause of death in CF. The amounts of GSH in plasma, bronchoalveolar lavage fluid and CF apical fluid collected from CF patients is low because of impaired secretion of GSH. Gao *et al.* proposed that an increase in Cl^- ion transport could stimulate GSH efflux in CF patients.[71] A synthetic peptide channel (NK4-M2GlyR) was used to evaluate the dependency of GSH efflux on Cl^- transport. Authors noticed a significant increase in the GSH efflux by restoration of apical Cl^- secretion, not with the CFTR per se. However, this might eventually inhibit oxidative stress and mend the quality of life among CF patients. Recently, Talukdar's group reported 2,4-nitrobenzenesulfonyl (DNS) protected protransporter **72** based on 2-hydroxyisophthalamide which could be activated in presence of GSH and subsequently formed supramolecular M^+/Cl^- channels across lipid bilayer (Figure 20C).[6c] The active ionophore then increased ROS levels that subsequently reduced the intracellular GSH levels and altered mitochondrial membrane permeability (MMP). This further facilitated cytochrome c release which is associated with the activation of caspase 9 and PARP cleavage and finally this event promoted the intrinsic apoptosis pathway. The active transporter **72** hampered the growth and proliferation of 3D spheroids of MCF-7 cells with an efficiency same as that of the anticancer drug doxorubicin

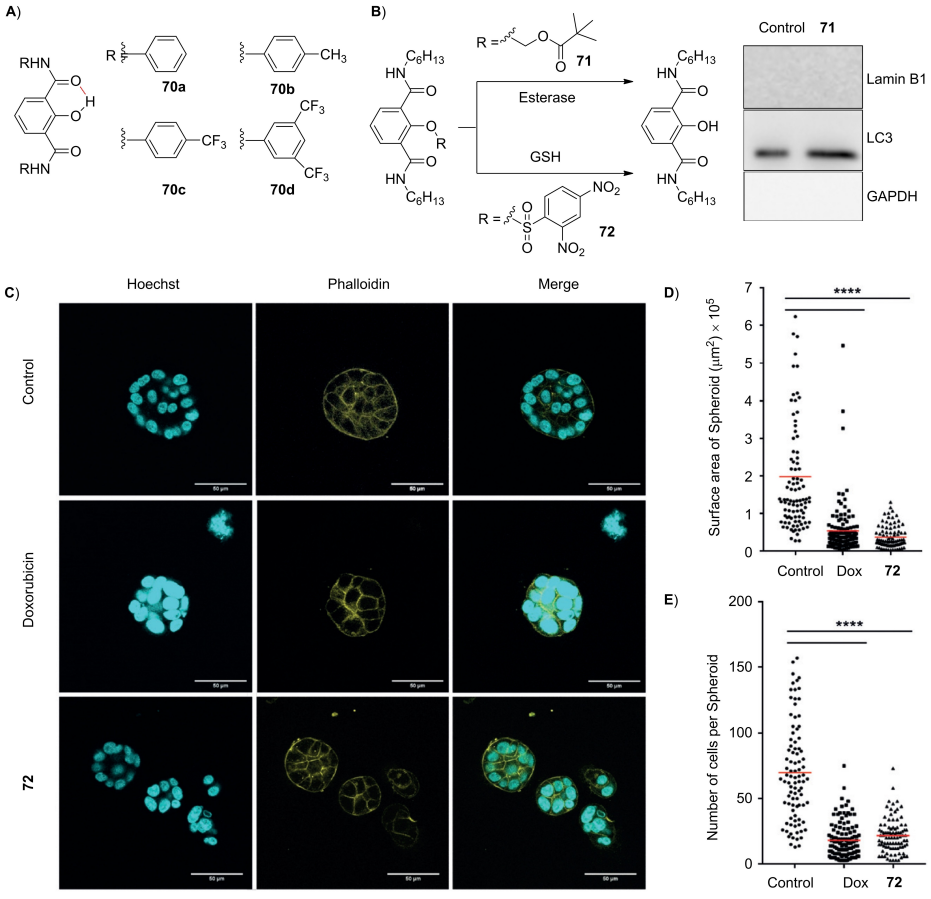

Figure 20. A) Structure of 2-hydroxy-N^1,N^3-diarylisophthalamide-based fluorescent ion channels (**70a**–**70d**). B) Structures of protransporters **71** and **72**. Immunoblot assay of compound **71** for a decrease in L amin B1 expression and an increase in LC3 expression in MCF 7 cells, with GAPDH as a loading control. C) Immunofluorescence for 3D cultures in MCF-7 cells using Hoechst 33258 and Phalloidin (stains actin filaments) D) Quantitation of surface area (μm^2) and E) quantitation of number of cells per spheroid. Con = control (DMSO was used in this experiment), DOX = doxorubicin (positive control, 0.8 μM) and compound **72** (2 μM). **** represents $p < 0.001$ using Tukey HSD tests. Adapted with permission from Ref. [70] Copyright 2020, Wiley. Ref. [6c] Copyright 2020, Wiley.

(Figure 20C–E). As a result, immunofluorescence staining experiment in MCF-7 cells using Hoechst 33258 and Phalloidin showed that the compound **72** reduced the surface area (Figure 20D), and the number of cells per spheroid (Figure 20E) significantly as compared to the control (here DMSO) while the observed data was comparable to doxorubicin.

4. Concluding Remarks

In the past two decades, arguably there has been a rapid development in the area of synthetic ionophores. These structurally simple and robust molecules offer remarkable ion transport activity, selectivity and promising bioactivity. In the context of transporting molecules of biological relevance, quite a few ionophores have been recently discovered with potential biological activities.[72] In fact, few of these membrane-active synthetic molecules are taking the leap from model bilayer membrane to cells. In this review, we have comprehensively summarised the progress on biological activity of the synthetic ionophores. A few studies have demonstrated their potential applications as antimicrobial agent. However, some of the non-toxic ionophores promoted Cl^- transport in epithelial cells and they could indeed be promising candidates for 'Channel replacement Therapy'.

Another significant therapeutic application of these systems could be observed in antitumour treatment. Recent reports show that the active transport systems trigger cell death by apoptosis in a wide range of human cancer cell lines and often they are identified with selective toxicity towards specific cell lines. Also another possible mechanism for cell death could be by the disruption of autophagy process and this function might be entirely independent of apoptosis. We hope that the examples discussed herein will stimulate future research across the existing and hitherto unreported generations of new ionophore families. Such intensive fundamental research in this area is highly likely to catalyse the discovery of novel modes of functions. Potential biological properties will evolve in the synthetic ionophores of the future, to mimic the natural ion transport protein, in essence.

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Conflict of Interest

The authors declare no conflict of interest.

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