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Biomedical Potentials of Crown Ethers: Prospective Antitumor Agents

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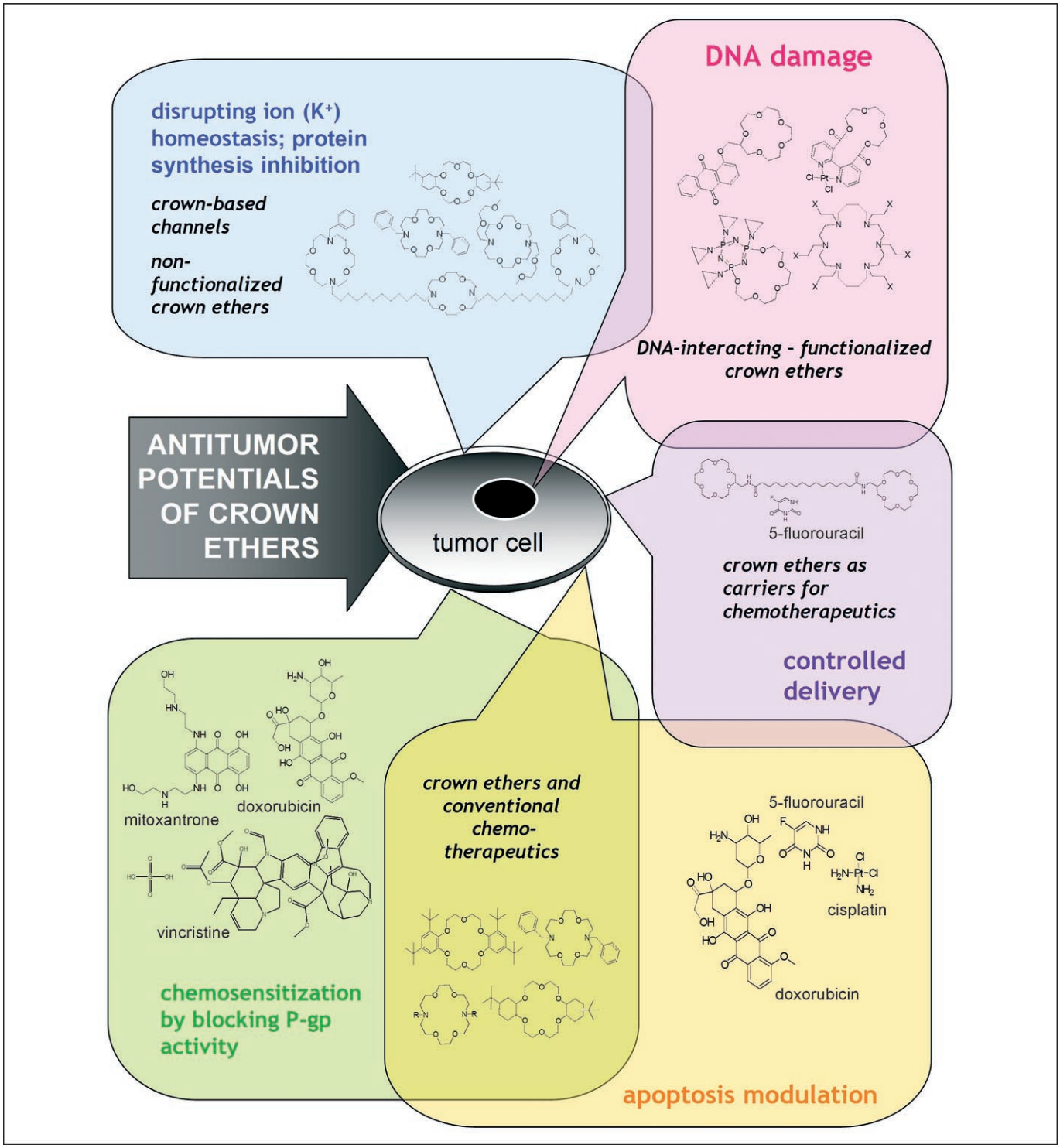

Crown ethers are of enormous interest and importance in chemistry, biochemistry, materials science, catalysis, separation, transport and encapsulated processes, as well as in the design and synthesis of various synthetic systems with specific properties, diverse capabilities, and programmable functions. Classical crown ethers are macrocyclic polyethers that contain 3–20 oxygen atoms separated from each other by two or more carbon atoms. They are exceptionally versatile in selectively binding a range of metal ions and a variety of organic neutral and ionic species. Crown ethers are currently being studied and used in a variety of applications beyond their traditional place in chemistry. This review presents additional applications and the ever-increasing biomedical potentials of these intriguing compounds, with particular emphasis on the prospects of their relevance as anticancer agents. We believe that further research in this direction should be encouraged, as crown compounds could either induce toxicities that are different from those of conventional antitumor drugs, or complement drugs in current use, thereby providing a valuable adjunct to therapy.

1. Introduction

Since Pedersen reported the synthesis and cation complexing properties of a new class of compounds termed crown ethers in 1967,[1,2] these first neutral synthetic heterocyclic compounds have attracted extensive and continuous attention through their unusual and powerful noncovalent cation binding properties. Classical crown ethers are macrocyclic polyethers that contain 3–20 oxygen atoms, each separated from the next by two or more carbon atoms. The most effective complexing agents, however, are macrocyclic oligomers of ethyleneoxy units, either substituted or unsubstituted, that contain 5–10 oxygen atoms. Selected examples are shown in (Figure 1). The common names for these macrocycles, which are generally used in favor of their systematic names, consist of the number and type of attached hydrocarbon rings (in the substituted derivatives), the number and type of atoms in the polyether ring, the class name "crown" that comes from their molecular shape and ability to "crown" a metal ion upon coordination, and the number of oxygen atoms in the polyether ring. For example: dibenzo-18-crown-6 (Figure 1), which represents the first crown compound isolated by Pedersen.

Crown ethers are exceptionally versatile in selectively binding a range of metal ions and a variety of neutral and ionic organic species,[3] providing development of the area of host–guest chemistry and in the construction of well-defined supramolecular assemblies.[4,5] The fundamental advances in studies of crown synthetic, structural, coordination, and solution chemistry have been summarized in a number of reviews and books.[6–10] The following sections are designed to introduce a wider readership to the ever-rising biomedical potentials of these intriguing compounds, with a particular emphasis on their potential application as promising anticancer compounds.

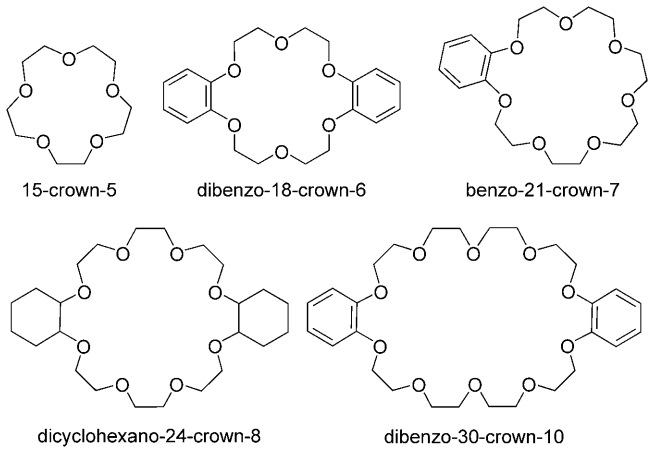

Figure 1. Examples of classical crown ethers.

2. An Overview of Crown Ether Chemistry

Crown ethers, which contain a hydrophobic ring of ethylenic groups surrounding a hydrophilic cavity of ether oxygen atoms, posses the greatest affinities for the alkali and alkaline earth cations. These small and hydrated metal ions become large and lipophilic as crown complexes; this allows the metal ions to be extracted into organic solvents, and this is widely used in a variety of organic reactions. The complexation process provides increased metal salt solubility and anion reactivity in nonaqueous solvents, enabling their widespread use in studies of mediated ion transport, solute separations, and anion-activated catalysis.[11] Due to their properties as ionophores, crown ethers have considerable biochemical relevance as models of naturally occurring ionophores (such as gramicidin and valinomycin) for the study of ion-transport processes in cell membranes, particularly of sodium and potassium ions.[12] Furthermore, crown ethers have been applied as complexing agents for primary and secondary alkylammonium ions,[10,13,14] some transition metal ions,[15] lanthanides and actinides,[16] and for some neutral molecules such as urea, thiourea, acetonitrile, and nitro compounds.[3,17] They may act also as encapsulating ligands for water molecules and oxonium

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Dr. Marijeta Kralj graduated with a degree in molecular biology from the Faculty of Natural Sciences, University of Zagreb (Croatia) in 1990. She received her PhD in biomedical sciences in 2001. From 2003 to 2008 she was Head of the Laboratory of Functional Genomics at the Division of Molecular Medicine, Ruđer Bošković Institute, Zagreb. She is currently Head of the Laboratory of Experimental Therapeutics at the same institute, where is in charge of organizing and managing the antitumor screening platform for the discovery of novel anticancer compounds. Her research includes cancer gene therapy and the search for novel potential antitumor compounds, with particular emphasis on the study of various cell death responses to tumor therapy.

Dr. Ljerka Tušek-Božić graduated with a degree in chemical technology from the Faculty of Technology, University of Zagreb (Croatia), and received her BSc and PhD in chemistry from the Faculty of Natural Science, University of Zagreb in 1975. After graduation she started work at the Ruđer Bošković Institute, where she is currently research adviser in the Division of Physical Chemistry. She worked for a year under an IAEA fellowship at the Centro di Studi Nucleari in Rome (Italy). Her current research interests are focused on the chemistry of complex compounds, including the design, synthesis, and characterization of novel organic ligands and their metal complexes with specific structural and biological properties.

Dr. Leo Frkanec studied biology and chemistry at the Faculty of Sciences, University of Zagreb (Croatia). He received his PhD in 2000 for work in supramolecular chemistry on amino acid derivatives of crown ethers and calixarenes. After a postdoctoral fellowship in the research group of Professor Raphael Darcy at University College Dublin (Ireland), where he worked on the synthesis of amphiphilic cyclodextrins with particular emphasis on their development as gene delivery vectors, he returned to the group of Professor M. Žinić at the Ruđer Bošković Institute, Zagreb in 2004. His scientific interest is focused on the field of supramolecular chemistry, particularly on molecular recognition, self assembly, and artificial ionophores.

ions,[18] and as proton-solvating agents, they enable the solubilization and ionization of acids in nonpolar solvents with low dielectric constants. This is of considerable interest for acid–base catalysis, electrochemical processes, and various separation techniques such as solvent extraction, isotope isolation, and selective membrane permeation.[19] A number of metal aquo species readily form hydrogen bonded complexes with crown ethers, acting as second-sphere ligands.[20] Hydrogen bonding is very important for determining the physical and chemical characteristics of compounds and influences various fundamental chemical functions such as molecular conformation, species recognition, and selectivity.

The main purpose in the design of crown ethers is to synthesize macrocycles that can discriminate between different chemical species. There has been enormous productivity in this field, and an impressive number of synthetic procedures have been developed. Variations in structure and binding selectivity can be achieved by adjustments in the dimensions of the macrocyclic cavity, variation in shape and topology, changing the number and nature of substituted groups on the macrocyclic ring (such as benzene, cyclohexane, and heterocyclic subunits such as tetrahydrofuran or pyridine) and tailoring the number, type, and arrangement of donor atoms (oxygen, nitrogen, sulfur, phosphorus) within the specific ligand frame employed. In the chemistry of metal ion–crown ether complexes, the relative dimensions of the crown ether cavity and of the linked cation primarily control the coordination behavior of small-ring crowns with a planar conformation and limited flexibility (crowns up to 18-membered rings). Therefore, 18-crown-6 has high affinity for potassium, and 15-crown-5, for sodium cations.[10] High complex stability is generally associated with greater penetration of the metal cation into the polyether hole. In contrast, a given cation's large diameter, strong solvation by the solvent, or effects of steric hindrance can prevent significant penetration into the polyether hole, resulting in the formation of weaker complexes; in such cases, greater interaction between the cation and solvent molecules and counterions is possible.[8,21] On the other hand, cavity size effects are not of major importance for the larger crowns (24- and 30-membered polyether rings), which have high conformational mobility that allows a wide variety of coordination environments in the complexes formed. Such macrocycles may complex cations by partially wrapping around them with a change in conformation either by expelling the conjugate anion and solvent molecules from the cation coordination sphere, or by leaving space for coordination with the anion. These crowns can also accommodate two cations if repulsive forces are not too great.

If a given metal ion is too large to fit inside the available crown cavity, formation of so called "sandwich" complexes with metal/crown ratios at 1:2 and 2:3 can occur.[22] For example, the complexation modes of crown ethers with various ring sizes toward sodium and potassium cations are illustrated in Figure 2. Notably, complexation characteristics also strongly depend on the charge density of the cation as well as the nature of the applied solvent and nucleophilicity of the counterion, because complex formation is based on weak noncova-

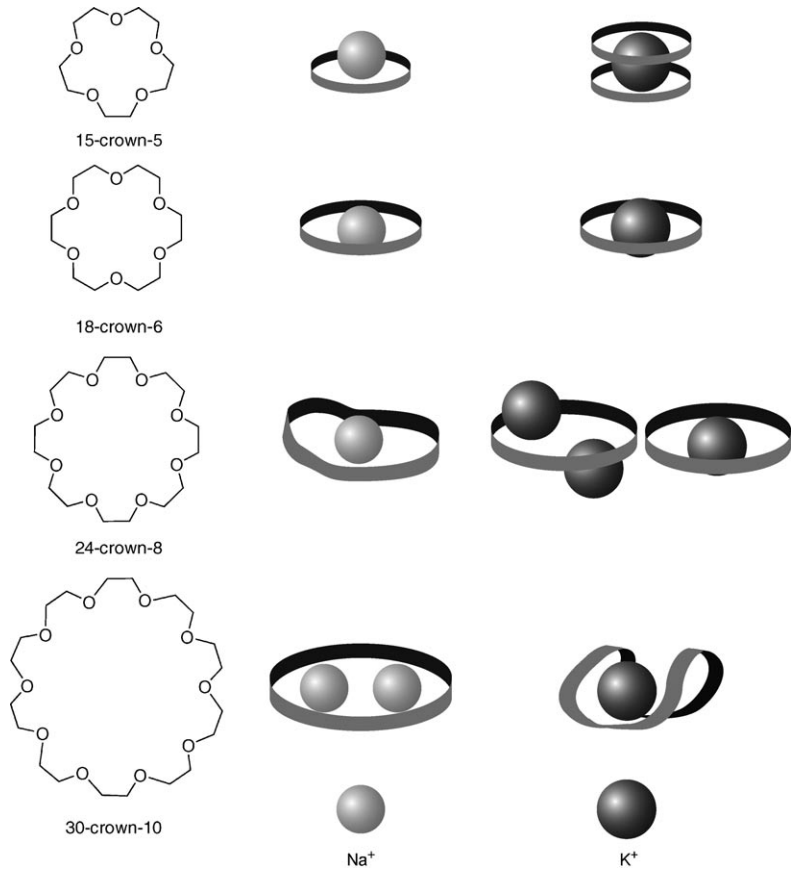

Figure 2. Complexation modes of crown ethers of various ring size toward sodium and potassium cations.

lent interactions. Moreover, the relative strain energies of the crown in various conformations may also contribute.

The identity and placement of donor atoms play important roles in macrocyclic selectivity. Thus, in the aza-crowns obtained by substitution of nitrogen in place of oxygen in the classical crown ethers, the size and shape of the macrocycle remain similar, although the conformations may change; their acid–base properties are substantially altered. The presence of basic amines in the macrocycle enables the diffusion of a proton inside the molecular cavity, followed by ligand protonation and formation of the protonated derivatives,[23,24] which can interact with simple as well as more complex inorganic and organic anions. The main driving forces in the formation of these complexes are electrostatic and hydrogen bonding interactions between the protonated moiety and the accompanying anion. In general, proton transfer reactions play an important role in chemistry and biology. They are fundamental to numerous processes such as acid–base neutralization and electrophilic addition, and are involved in transport phenomena, photosynthesis, enzyme reactions, and more.[25] As nitrogen and oxygen have inherently different binding selectivity, the oxygen–nitrogen mixed-donor macrocycles are able to bind a wider variety of both cations and anions than either the purely oxygen- or nitrogen-containing macrocycles.[3,4,8] Although these compounds prefer transition and post-transition metal ions,[26] the selected derivatives also bind alkali, alkaline earth, and lanthanide metal ions,[27] as well as ammonium and oxonium cations.[4] By reactions with some anionic[28] and neutral organic and biological substrates, supramolecular compounds with specific properties and applications can be formed.[29] This principle of receptor–ligand noncovalent binding is one of the foundations of biological chemistry. It is also important to note that the amine groups in the aza-crowns could be used as reactant sites for building more complex structures such as three-dimensional cage ligands (cryptands), which are able to completely encapsulate guest moieties to form inclusion complexes, and lariat ethers, which contain side arms attached to the macro-ring at nitrogen atoms (Figure 3). Dipeptide-derived lariat ethers with chiral side arms enable the enantioselective transport of amino acid and dipeptide K^+ carboxylates through bulky membranes.[30]

The arrangement of the side arms containing donor atoms may alter the host–guest selectivity pattern, as the macro-ring and side arm could cooperate in binding the guest moiety, leading to novel possibilities for controlling the properties of complex formation.[4,10] Pendant arms that bear additional potential ligation groups can be attached at both the nitrogen

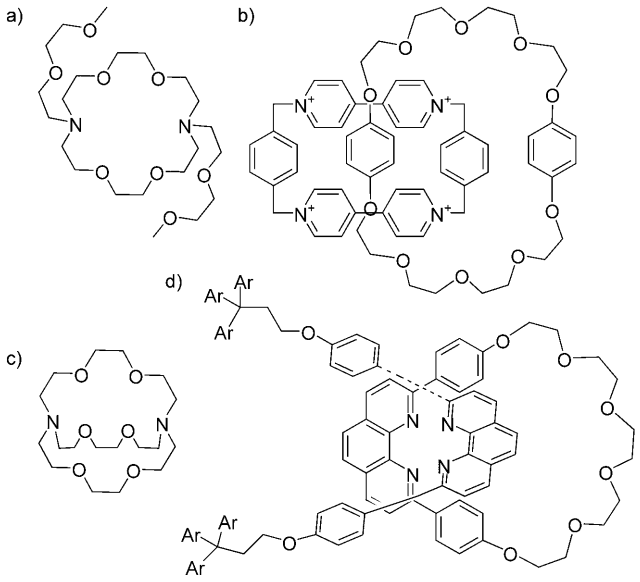

Figure 3. Structures of: a) lariat ether; b) [2]catenane; c) [2.2.2]cryptand; d) [2]rotaxane.

and carbon atoms at appropriate positions on a macrocyclic framework. The structural modifications of crown receptors, which allow the control of complexation strength and selectivity, are mainly based on concepts of molecular switching and sensing. The response of host molecules to cations and amines may be detected by changes in their electrochemical, optical, or chemical properties; thus, the molecular switching is connected with a change either in the charge state, conformation, or in the structure of host molecules that enable or prevent cation complexation. Photochemical, redox, and electrochemical switching are among the most common.[10,31]

Crown-based sensors designed for cation recognition with high selectivity and a variety of responses can be developed by the incorporation of a chromophore, fluorophore, or luminophore into the crown macrocyclic framework.[32] These types of synthetic host ionophores have been developed into molecular sensors for application in areas as diverse as chemistry, biochemistry, medicine, cell biology, and environmental detection. In supramolecular chemistry, crown ethers can serve not only as substrate binding sites but also as function-tuning sites. With the combination of synthetic versatility and well-tailored design, diverse capabilities such as molecular recognition, chirality, and catalytic properties become possible at the molecular or mesoscopic level.[33] Crown ethers integrated with other structural units can also provide various polymeric systems such as nanotubes[34] or light-emitting devices (LEDs),[35] which are important in nanoscience and materials science.

Indeed, crown-based macrocyclic compounds have proven to be of interest and importance in chemistry, biochemistry, materials science, catalysis, separation, transport and encapsulated processes, as well as in the design and synthesis of various synthetic systems with specific properties, diverse capabilities, and programmable functions.

3. Crown Ethers as Biological Model Systems

Essential biological processes such as recognition, membrane transport, signal transduction, biocatalysis, information storage, processing, and reproduction are based on supramolecular interactions between molecular components. Enzymes, viruses, membranes, and many other complex structures with biologically relevant functions are mainly built up through simple self-assembly processes.[36] These processes can be mimicked in small artificial supramolecular derivatives such as crown ethers. The knowledge obtained by investigating the interactions between host and guest species (reaction partners) that are thought to be important in biology, such as hydrogen bonding, π-stacking, ion–dipole interactions, dipole–dipole interactions, charge transfer phenomena (electron donor–acceptor interactions), and the influence of solvent, provides a reliable basis for the design of chemical structures that function in the same way as complex chemical structures in biological systems.[37] In these studies the "structurally developed" macrocyclic systems are most useful, such as those which are able to incorporate more than one metal ion and nonmetallic species, crown-appended and interlocking macrocyclic systems (catenanes), cryptand- and cavitand-type structures, as well as those with rings threaded by molecular string-like components (rotaxanes and pseudorotaxanes) (Figure 3).[3,5,38]

Indeed, macrocyclic crown ether compounds have been used as model systems for imitating biological processes involving enzymes, antibodies, receptors, membranes, carriers, and channels that are based on molecular recognition. For example, carrier-assisted transport through liquid membranes is one of the most important applications of supramolecular chemistry.[5a] Biological systems have evolved two general strategies for the selective transport of metal ions across cellular membranes: ion carriers (ionophores) and ion channels (Figure 4).

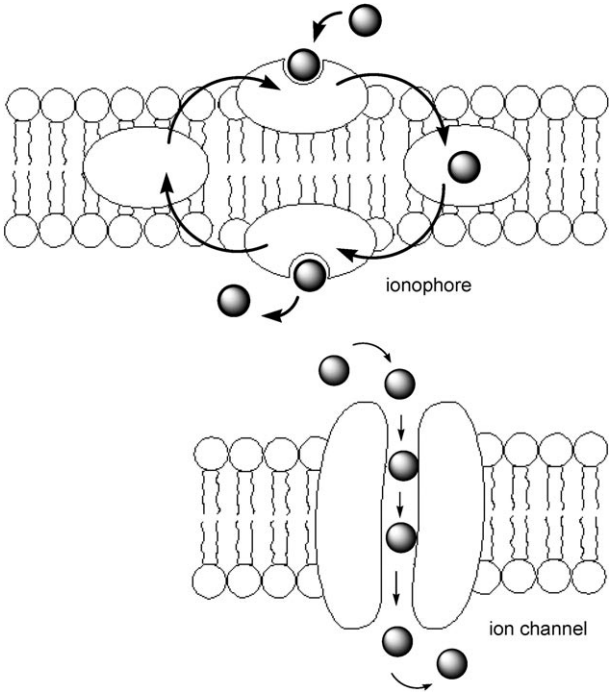

Figure 4. A schematic representation of ionophore- and ion-channel-mediated ion transport across a cell membrane. An ionophore binds an ion on one side of a lipid bilayer, where the concentration is high, and releases it on the other side, where the concentration is low. Ion channels are membrane-spanning proteins that directly mediate transmembrane ionic flux, acting as pathways for ions down their transmembrane gradients.

During the past few years a number of artificial ionophores and non-natural channel models have been developed that show some but not all characteristics of ionophores and protein natural channels. Since the discovery of crown ethers, a number have been recognized as potential artificial models of natural ionophores, for example, valinomycin (Figure 5), which is probably the best-known example of a natural ion carrier with high selectivity for potassium ions.[39] Ionophores in general can be regarded as molecules with backbones of diverse structures that contain strategically spaced oxygen atoms. They are compounds of moderate molecular weight (~200–2000 Da) that form lipid-soluble complexes with polar cations, of which K^+, Na^+, Ca^{2+}, Mg^{2+}, and the biogenic amines are the most biologically significant. The ion selectivity of iono-

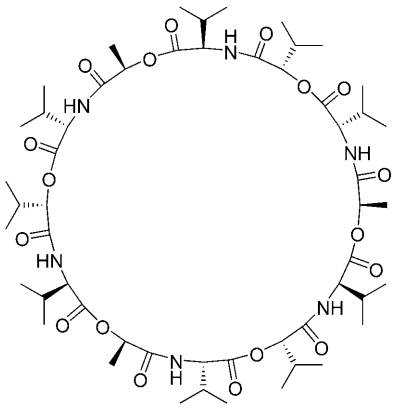

Figure 5. Structure of valinomycin.

phores is a combined function of the energy required for desolvation of the ion and the liganding energy obtained upon complexation.[39] A wide variety of naturally occurring macrocyclic antibiotics have been shown to exhibit differing degrees of ion selectivity in the processes of active ion transport, photosynthesis, oxidative phosphorylation, and metal binding. There are numerous reports of cation transport through bulk liquid membranes mediated by crowns of widely varying structures;[40] however, remarkable effects on ion selectivity have not yet been reported.[8]

Ion channels and pores, on the other hand, are integral membrane proteins that regulate the fast and selective movement of ions across the cell membrane. In general, protein ion channels are exceptionally complex and have been studied for decades because of their tremendously important functions in various cellular processes. However, their detailed mechanisms are still unclear.[41] Furthermore, they are difficult to isolate in their pure and functional form and they tend to denature easily.[42] Synthetic channels should therefore be more appropriate alternatives, as they mimic natural systems. From the first synthetic ion channel based on amphiphilic cyclodextrin,[43] numerous examples of synthetic channels structurally based on different building blocks have been reported.[44] Owing to the properties of crown ethers discussed above, they were, soon after their discovery, considered as essential building blocks for channels that would function in bilayers. For example, crown ethers were intended to serve both as headgroups in the amphiphilic sense and as entry portals for ions, whereby the crown would impose selectivity on the ion-transport process.[45,46]

Synthetic ion channel models include, among other things, the peptide nanotubes described by Ghadiri and co-workers[47] and the peptide-linked crown ethers described by the Voyer research group,[48] in which the general concept is to use α-helical peptidic structures as a scaffold to support a chain of crown ether compounds (Figure 6a). When the peptidic framework adopts an α-helical conformation, the crown rings are proposed to align to form a polar pore long enough to allow the passage of ions across a membrane lipid bilayer.[42]

Furthermore, extensive biophysical studies have characterized hydraphiles as ion channels. Hydraphiles are synthetic ion channels that mimic the structure of known protein channels by having two distal crown ethers (headgroups) extend to

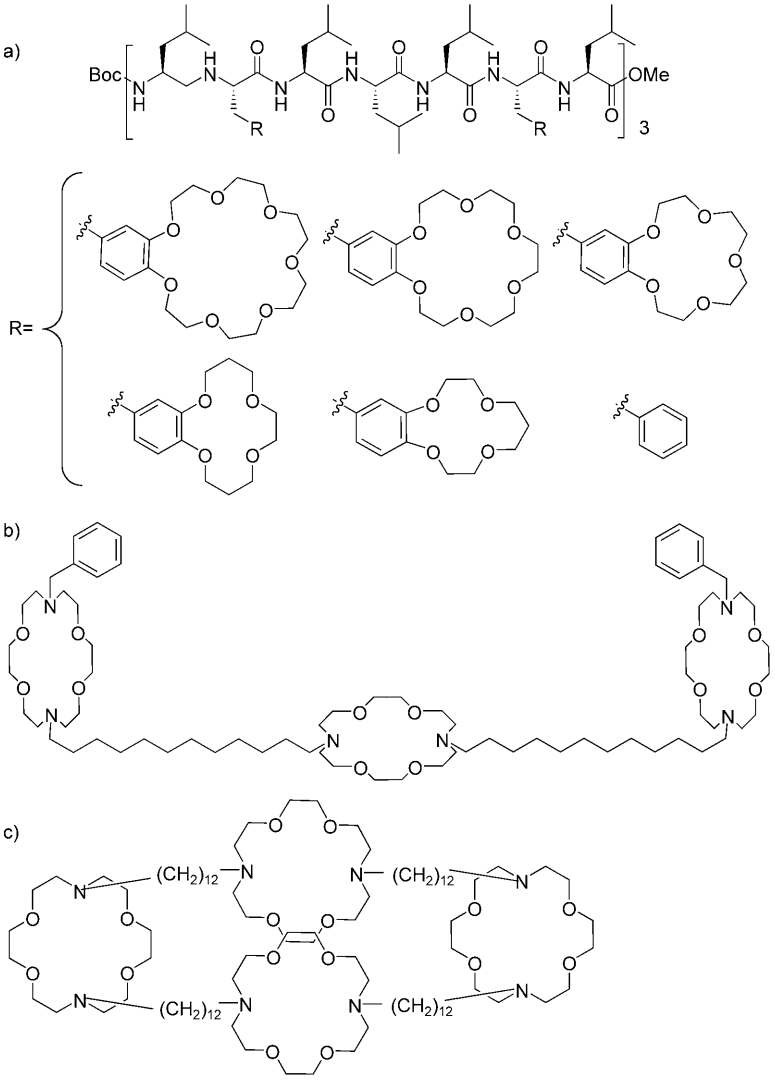

Figure 6. An illustration of crown-ether-based synthetic ion channels: a) crown peptide nanostructures;[42] b) hydraphile, benzyl channel;[49] c) tetramacrocycle hydraphile.[45]

form the entry and exit portals of the pore (Figure 6 b,c).[49] Each headgroup is connected to a hydrophobic spacer chain that is, in turn, linked to a central crown ether. This middle unit was specifically designed to stabilize a cation in transit through the membrane. Hydraphiles arrange in the bilayer with the distal macrocycles at opposite ends of the membrane, while the hydrocarbon chains align with the fatty acid chains.[49] These channel model compounds have demonstrated the open/closed channel properties in planar bilayer conductance experiments[50] and have shown significant activity in synthetic liposomes and other biological systems, as discussed bellow.[51]

In summary, numerous synthetic approaches have been developed for different supramolecular models of ion channels, and many of them have been successful in mimicking the characteristics of native protein ion channels. Crown ethers promise to be useful templates for the synthesis of supramolecular devices in the study of biological processes.

4. Biological Activity of Crown Ethers

4.1. Antimicrobial activity

Although research on the potential biological activity of crown compounds is still in its early stages, their potential impact remains large. From the biological or biomedical point of view, one of the most interesting features of crown ethers is the fact that they behave very similarly to the natural ionophores, such as gramicidin, valinomycin (Figure 5), and nonactin owing to their ionophoric properties in membranes.

Naturally occurring ionophores as metabolites of microorganisms (e.g. *Streptomyces* spp.) were first recognized through their effect of stimulating energy-linked transport in mitochondria. They disrupt the flow of ions either into or out of the cells, thus dissipating cellular ion gradients and leading to physiological and osmotic stress. Bacteria (particularly Gram-positive bacteria) are very sensitive to this effect. Because cyclic polyethers clearly discriminate among different ions, they can serve as convenient synthetic model compounds for their biological counterparts and have similar functions.[10,52] Indeed, crown ethers were found to be toxic in prokaryotes and eukaryotic cellular systems, and this led to further studies on their potential for development into pharmacological agents.[53] It was shown that certain ionophores have antiparasitic (e.g. antimalarial or anticoccidial) activity;[54,55] therefore, attempts were made to prepare efficient crown compounds as potential antiparasitic drugs. For example, Brown and Foubister synthesized crown compounds with ring sizes from 14 to 30 atoms that showed anticoccidial activity in vitro against *Eimeria tenella*, but unfortunately no activity in vivo.[55] In addition, certain crown ethers were found to show significant antifungal activity against some wood-decay fungi, phytopathogenic fungi and eumycetes, and *Trichophyton* spp. for dermatomycosis. Yagi et al. showed that among the 26 crown ethers tested, 3,5-di-*tert*-butylbenzo-15-crown-5 showed relatively high activity, while unsubstituted crown compounds, or those with a polar substituent were inactive.[56]

Tso and co-workers found that substituted 18-crown-6 ethers show different inhibitory effects on the growth of *E. coli*, and this effect is influenced by the presence of potassium and sodium ions in the nutrient medium.[53,57] Thereafter, various approaches were developed to prepare crown-based antimicrobial agents. Leevy et al. determined minimum inhibitory concentrations of several alkyl-substituted lariat ethers on *E. coli*, *B. subtilis*, and yeast (Figure 7 a).[58]

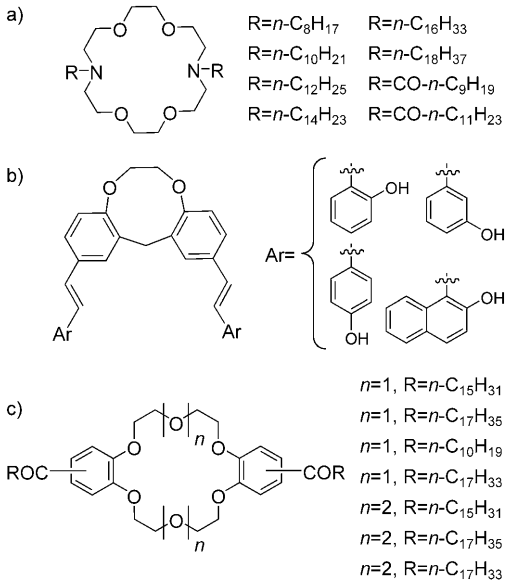

Figure 7. Examples of various crown-ether-based potential antimicrobial agents: a) alkyl-substituted lariat ethers;[58] b) crown ether ligands of the Schiff base type;[59] c) various acyl-substituted benzo-18-crown-6, dibenzo-18-crown-6, and dibenzo-24-crown-8 compounds.[60]

The authors proposed a mechanism for toxicity, which depends on the ability of these compounds to transport ions, most probably by inserting and integrating into membrane bilayers and conducting cations as expected for carriers, whereby the side chain length and hydrophobicity play essential roles. Depending on the membrane structures, the sensitivity to various compounds differed among the organisms tested; *B. subtilis* and yeast were the most sensitive, as they lack the second membrane. Similarly, Yildiz et al. prepared a group of new crown ether ligands of the Schiff base type that showed prominent activity against various microorganisms.[59] Indeed, some of them were more susceptible to the novel macrocyclic compounds, than to standard antibiotics (Figure 7 b). The compounds differ significantly in their activity against the microorganisms tested, pointing again to the differences of the cell walls between Gram-positive and Gram-negative bacteria. The former have single layered structures, whereas the cell walls of the latter are composed of a multilayered structure; the yeast cell wall is also quite complex.[59] Uğraş et al. described the synthesis and complexation properties of palmityl-, stearyl-, oleyl-, and undecenoyl-substituted benzo-18-crown-6, dibenzo-18-crown-6, and dibenzo-24-crown-8 and tested them for antimicrobial activity (Figure 7 c). However, no activities were observed against eight standard bacterial and fungal strains.[60]

Numerous approaches have been developed to build more complex synthetic ion transporters with potential biological activity: channels. Namely, as discussed above, ion transporters are present in the form of either carriers or channels.[10,45] Biron et al. designed and prepared multiple crown ether peptide nanostructures having ion channel activity (Figure 6a), as mentioned in Section 3. No antimicrobial activity was detected for the peptide nanostructure. However, accentuated cytotoxicity was observed with various crown peptides against breast tumor and mouse leukemia cells, underscoring the necessity of having nanostructures of appropriate length (3–4 nm) in order to efficiently form a membrane pore and thus effect cytotoxicity.[42]

Hydraphiles, on the other hand, are potent cytotoxic compounds against Gram-positive and Gram-negative bacteria, yeast, and mammalian cells. They have the ability to transport ions in both directions, disrupting the organism's osmotic balance, and leading to death. However, possible alternative mechanisms cannot be excluded, such as: 1) disruption of membrane structure and 2) organization and interaction with membrane enzymes in a deleterious way.[60] The side arms of hydraphiles anchor the crown headgroup in the membrane and are crucial for their ion-conducting activity.[10] In comparing the biological activity (toxicity) of various hydraphiles, it can be concluded that: 1) dramatically higher toxicity is observed with the distal macrocycles as diaza-18-crown-6 instead of aza-18-crown-6; 2) increased activity is observed with benzyl groups attached to the distal macrocycles; 3) in linking the dodecyl side chains through an additional macrocycle (tetramacrocycle hydraphile (Figure 6c) leads to another twofold enhancement in activity against *E. coli* and, interestingly and encouragingly, significantly lower activity against *S. cerevisiae*), maximal toxicity is reached in the C_{14}–C_{16} channels, but with no observed selectivity between bacteria and yeast.[45,49]

Interestingly, several hydraphiles are substantially more active against all microbes tested than valinomycin. Moreover, maximal toxicity values for the C_{14}–C_{16} channels is similar to the toxic concentration for penicillin. In summary, hydraphile channels are promising biologically active compounds for several reasons: they have an abiotic structure that should prevent microbial resistance, they possess unparalleled synthetic flexibility for channel compounds, and all major components of the hydraphile can be synthetically tailored to fit the various unique properties of the target organism in question.[10]

4.2. Biological activity in mammalian cells

Immediately after the crown ethers were discovered, their toxic effects in higher organisms were observed. Certainly, targeting the cell membrane and disrupting the homeostasis of important physiological cations should have toxic consequences to normal tissue. This advanced further toxicological research on more detailed mechanisms. More than 20 years ago various studies were performed, showing the toxicity of various cationic ionophores (including crown ethers) in multiple species such as mice, rats, and dogs.[61–64] These studies clearly showed that the majority of the ionophores induced mainly neurobehavioral toxic effects. For example, tremulous motion, salivation, and paralysis of the hind legs were observed after the administration of 18-crown-6 to beagle dogs.[61] Acute oral toxicity studies of 12-crown-4, 15-crown-5, 18-crown-6, and 21-crown-7 have also shown neurological and behavioral effects in rats, mice, and rabbits (tremor, aggressive behavior, muscle contractions), as well as eye and skin irritations, and testicular atrophy.[62,63,65] Interestingly, the relative lethality of cyclic polyethers increases with both ring size and hydrophilicity, a trend quite opposite to the onset and extent of neurobehavioral symptoms. Moreover, the attached substituent groups (such as dicyclohexano or dibenzo groups) significantly augment or decrease (respectively) the lethal potency of 18-crown-6, while having no influence on the neurological effects.[64] Importantly, the acclimation to effects at successively higher doses and the complete disappearance after discontinuation of dosing support the view that the effects observed are of a reversible pharmacological nature. Moreover, it was shown that the lethal concentration (LD_{50}) of several crown ethers in mice is about the same as that found for aspirin.[63]

It was also found that dicyclohexyl-18-crown-6 and valinomycin inhibited protein synthesis in reticulocytes, and this effect was not observed in a cell-free system. The inhibition was reversible at low concentration, and the ribosomes isolated from treated cells were normal in their structural and functional properties. However, inhibition was irreversible at high concentrations of valinomycin, whereby the isolated ribosomes were completely inactive.[66]

The aforementioned studies stimulated further research on the potential genotoxicity of crown compounds. It was shown that various tested crown ethers are neither genotoxic nor do they exhibit co-mutagenic properties.[67] This supported the concept that their toxicity is due to their interaction with membranes, and not with nucleic acids; they do not possess DNA-damaging activity.[68,69] Moreover, it was shown that dicyclohexyl-21-crown-7 has antimutagenic effects on heavy-metal-induced sister chromatid exchanges.[70] More recently, Zasukhina et al. showed that several *N*-carboxyalkyl derivatives of aza- and benzoaza-crown compounds have antimutagenic and protective effects toward human cells according to the criteria of primary DNA injury after exposure to γ irradiation and $CdCl_2$. The antimutagenic effect was similar to that of garlic extract. However, the protective effect of garlic extract is associated with its antioxidant properties, whereas crown compounds show non-antioxidant activity.[71]

In contrast, Boojar and Goodarzi showed that 18-crown-6 and 15-crown-5 markedly inhibit the viability of, and enhanced the oxidative damage toward normal human fibroblasts WI 38 and rat lung tissue cells.[72,73] Additionally, both compounds induced the production of reactive oxygen species (ROS) along with activation of antioxidant enzyme activities, such as that of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). Similarly, Boojar and Shockravi reported that two novel tri-aza macrocyclic diamides also produce oxidative stress in the V79 cell line, and that their cytotoxicity effects are due to the oxidative damage of proteins, lipids, and DNA.[74] However, it should be noted that the concentrations of crown

ethers sufficient for these effects were extremely high: up to 2 mmol L^{-1}.

4.2.1. Antitumor potential of crown ethers

As mentioned above, crown ethers are being studied and used in a variety of applications beyond their traditional place in chemistry. Although their cytotoxic effects toward mammalian cells (including tumor cells) were recognized early, no systematic study had been performed on the potential antitumor activity of crown ethers. The exceptions are functionalized crown ethers, which, for example, have been designed to interact with, alkylate, and/or cleave DNA in order to effect their antitumor activity.[75] The emphasis was put on the mutual effect of two functionally different parts: one part carries a DNA-intercalating function, and the other binds metal ions. Thus, the DNA binding capacity of such compounds should be influenced or regulated by the complexation of metal ions, as metal complexation should lead to a change in net electronic charge, along with a global conformational change in the ligands.[76] DNA binding and intercalation studies have been carried out with various crown compounds possessing various side arms (Figure 8a,b).[77] Crown-ether-linked DNA intercalators such as

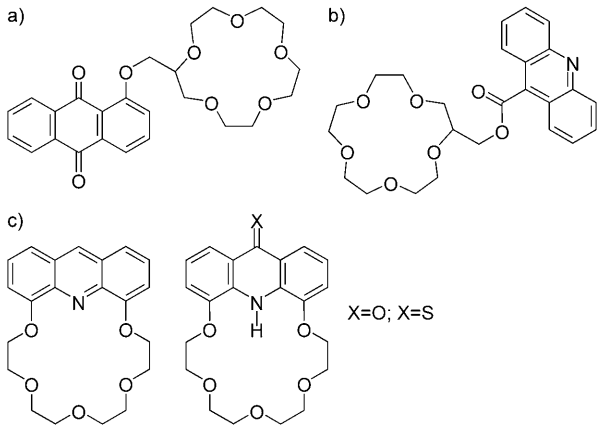

Figure 8. Crown ether compounds synthesized to interact with DNA: the a) anthraquinone[76] or b) acridine[77] subunit intercalates into DNA while the crown binds cations that interact with the phosphate backbone; c) fluorescent acridino- and acridono-18-crown-6 ligands.[80]

acridine and anthraquinone derivatives have indeed shown enhanced binding to DNA in the presence of certain metal ions due to the cationic property given by the coordination of the crown ether to the metal ions. These studies revealed that the acridine subunit binds DNA while the crown binds cations which interact with the phosphate backbone, thus stabilizing the complex.[77] It was clear that the activity of a given metal ion for DNA binding and cleavage is highly dependent on the nature of metal chelating moiety of the DNA ligand; a crown-based metal binding moiety significantly enhanced DNA cleavage activity, whereas an iminodiacetic acid chelator diminished it.[76]

Several studies were carried out using crown ether derivatives of actionomycin D (AMD) containing benzo-15-crown-5 and benzo-18-crown-6 groups attached by amide bonds.[78,79] The rationale for these studies is based on the characteristic of AMD to act as an ionophore antibiotic consisting of a phenoxazone chromophore substituted with two cyclic pentapeptide lactone rings. The biological activity involves intercalation of the planar phenoxazone chromophore into GC-rich sequences of DNA, while the pentapeptide rings lie in the minor groove. AMD forms complexes with sodium ions, but not potassium ions. This, in turn, suggests that the activity of AMD may only be manifested when the pentapeptide rings form complexes with sodium ions. Combining the AMD chromophore with crown groups in the side chains should give different specificities of metal cation binding and possibly to different antitumor activities. It was found that the activity of compounds with five-membered crown ether side chains showed antitumor activity similar to that of AMD, whereas derivatives with side chains containing six-membered crown ethers abolished all cytotoxic effects in two model systems: MOLT-3 leukemia cells[78] and in mice bearing Ca755 adenocarcinoma.[79]

Cationic recognition studies using crown-type fluorophore macrocycles have also been carried out. Huszthy et al. synthesized fluorescent acridono- and thioacridono-18-crown-6 ligands and their precursors, which could be useful building blocks for acridine, acridone, and thioacridone derivatives of chemotherapeutic importance (Figure 8c).[80] Whereas the above examples present DNA binding and intercalating compounds, some functionalized crown compounds have been designed to covalently modify (alkylate) and cleave DNA in an ion-regulated manner. Two research groups have developed compounds that alkylate and cleave DNA and also halt the growth of cancer cells (Figure 9a,b).[81–83]

The aziridine-derived DNA alkylators are powerful antitumor agents, but suffer from undesirable side effects, such as bone marrow toxicity. Therefore, a great deal of research has been carried out to minimize these drawbacks by the incorporation of various substituents as tumor-targeting moieties. Brandt et al. developed compounds that have aziridinyl groups attached to a crown-bearing cyclotriphosphazene in order to improve the therapeutic properties of aziridinylcyclophosphazenes.[81] They synthesized a tetraaziridinyl lariat ether and tested it for in vitro antitumor activity as well as in an investigational AIDS-related lymphoma screen. This compound showed remarkable cytostatic activity as a result of interacting with DNA through the synergistic effect of the interacting metal center and the (di)alkylating capacity of the aziridinyl group. The resulting DNA damage halts cell proliferation, making this compound a cytostatic drug.[81]

Propargylic sulfone-containing molecules also show DNA-cleavage activity. They isomerize in mildly basic medium to allenic sulfones and consequently may serve as reactive electrophiles that alkylate DNA at sites such as N7 of guanine residues.[82] Propargylic sulfone-armed lariat crown ethers and bis(-propargylic) sulfone crown ethers have been prepared to couple the molecular recognition of specific alkali metal ions with DNA damage under the conditions of increased alkali

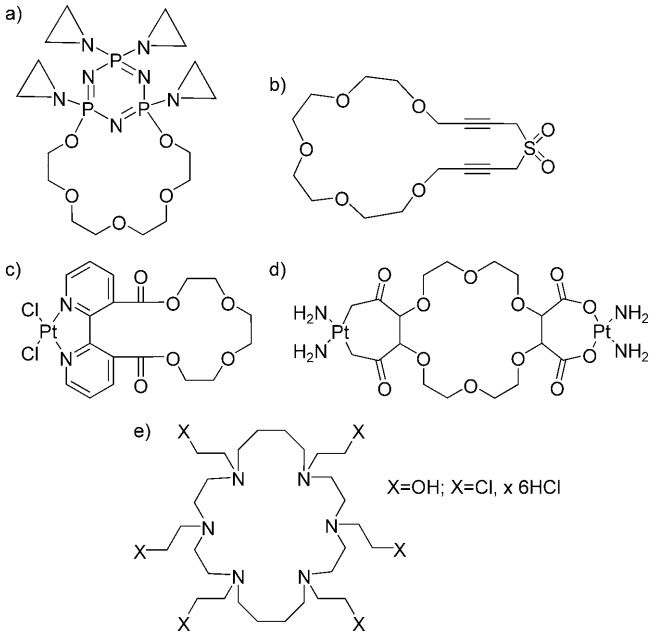

Figure 9. Crown ether compounds designed to covalently modify (alkylate) and cleave DNA: a) tetraaziridinyl lariat ether;[81] b) bis(propargylic)sulfone crown ether;[82] c) bipyridyl platinum complex;[84] d) 18-crown-6-tetracarboxybis-diammineplatinum(II);[85] e) hexaazamacrocyclic mustards.[87]

metal ion levels present in tumor cells. These compounds have been tested against cancer cell lines at the National Cancer Institute (US National Institutes of Health) to assess their ability to inhibit growth in culture. Some of them showed significantly more pronounced DNA cleavage and cytotoxic activity than the non-crown ether analogues.[82,83] Platinum-based DNA binding/alkylating agents containing crown ether moieties have also been prepared and tested for their potential antitumor activity. The antitumor effect of platinum compounds is ascribed to a reaction between the platinum center and nucleophilic sites on the DNA; in the case of cisplatin, for example, major adducts are generated by intrastrand cross-links formed by the binding of cisplatin to two neighboring guanines. Yoo et al. synthesized new crown-ester-linked bipyridine platinum homologs with three to five ethylene glycol units that showed moderate cytotoxic effects in murine and leukemia cells (Figure 9c).[84] Another example of potential platinum-based anticancer compounds with the platinum centers linked through a spacer or to pendant coordinating groups is 18-crown-6-tetracarboxybis-diammineplatinum(II) (Figure 9d). Its antitumor activity has been tested in various tumor models and, in general, is equal to cisplatin in cisplatin-sensitive as well as cisplatin-resistant cells. Moreover, its toxicity in vivo is considerably lower.[85] Jansen and co-workers prepared cisplatin derivatives in which the platinum center is coordinated directly to the nitrogen atom in an aza crown ether. Although these compounds have higher DNA binding capacity, their biological activity is either negligible or lower than that of cisplatin.[86]

The synthesis of a new class of mustard drugs as potentially more effective alkylating agents was recently reported. These compounds are tri-, tetra-, and hexaazamacrocyclic compounds that contain two or more potential alkylating sites, which effect highly efficient DNA cross-linking and allow the development of alternative strategies for prodrug formation (Figure 9e). Indeed, their antiproliferative activity against human leukemia cell line K562 was shown to be similar to the clinically relevant drugs melphalan and chlorambucil.[87] Conversely, a series of naphthoquinone thiol crown ethers was investigated as biologically active compounds with a completely different mode of action.[88] The quinone structure is widespread in natural products that are associated with antitumor, antibacterial, antimalarial, and antifungal activities. Its mode of action is ascribed to the ability to accept electrons to form the corresponding radical anion species. Various substituents at the quinone moiety modulate the redox properties responsible for the resulting oxidative stress. Indeed, quinones substituted with a metal cation-binding crown ether have modulated redox properties, and this type of molecule is classified as a redox-switched crown ether. All of the compounds in this quinone series displayed a variety of biological activities, and the bis-naphthoquinone thiol crown ether was observed to be the most potent inhibitor of methicillin-resistant *Staphylococcus aureus*.[88]

In summary, all the examples discussed above show attempts to prepare potential antitumor or other biologically active compounds in which crown ethers, as a part of the molecule, facilitate or enhance the inherent mode of action of the other part(s) of the same compound. However, as previously mentioned, no systematic study has been performed on the potential antitumor activity of non-functionalized crown compounds. Interestingly, in all experiments described above, the crown ether moieties alone were not tested in parallel with the crown-ether-substituted derivatives in order to assess their independent activity. In general, there are limited reports on the antiproliferative activity of crown ethers in mammalian cells,[70,89] although it has been known for more than 20 years that ionophores such as valinomycin, an antibiotic with potassium-selective ionophoric activity, have been reported to display strong antitumor effects.[90] Its use has been limited by its extreme toxicity, yet it was shown that this toxicity could be decreased by incorporating valinomycin in liposomes, while maintaining or even enhancing its antitumor activity.[91] Therefore, recent studies on the potential cytotoxicity caused by disrupted ion transport by the Gokel research group[49] inspired us[68] to check the possible antiproliferative/antitumor activity of conventional crown ethers and their derivatives in vitro and to compare this activity with valinomycin. We chose various derivatives of 18-crown-6 as the most frequently studied crown ether compounds, along with one derivative with 15 ring atoms and two derivatives with larger macrocyclic rings: dibenzo-24-crown-8 and dibenzo-30-crown-10. All of these, except that with the smallest ring size (compound **9**) are preferentially selective for complexation of potassium over sodium (Figure 10). The results clearly reveal that crown ethers possess marked tumor cell growth inhibitory activity and that this activity strongly correlates with both the type of hydrophilic cavity (the size and the nature of donor atoms) and the characteristics of the surrounding hydrophobic ring. Thus, the most

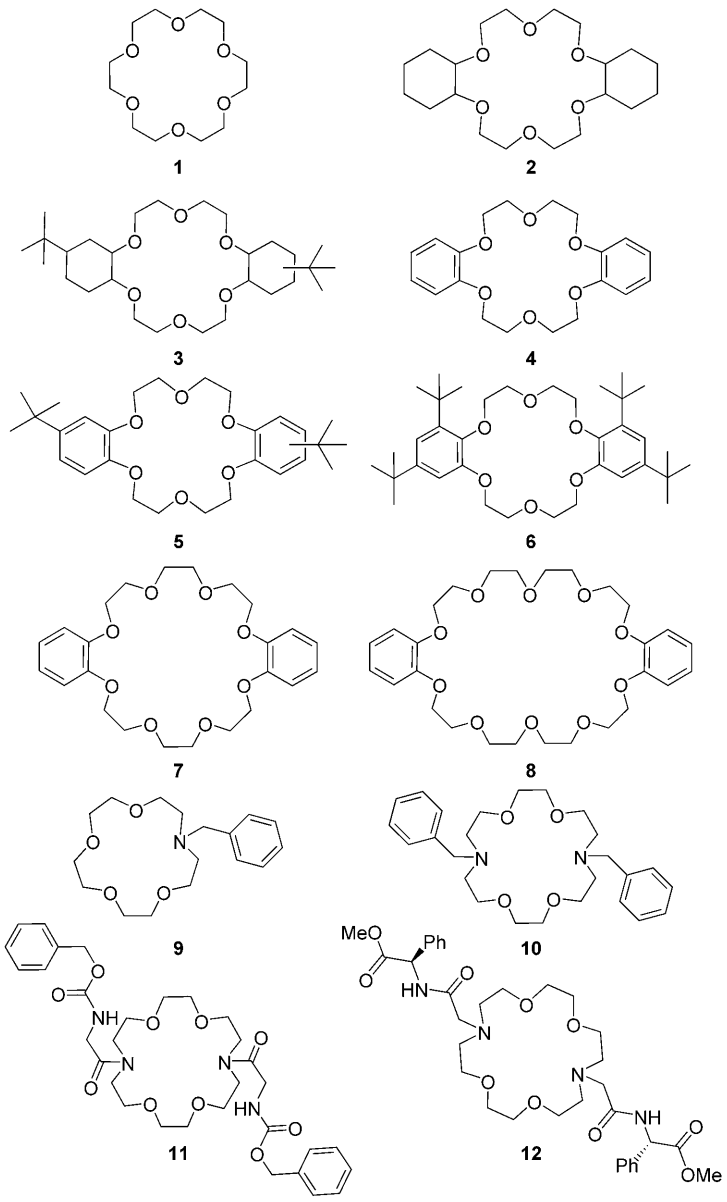

Figure 10. Structures of various crown ether compounds tested for their antiproliferative activity, as described by Marjanović et al.[68]

active compounds were di-*tert*-butyldicyclohexano-18-crown-6 **3**, which exhibited cytotoxicity in the sub-micromolar range (still having lower activity than valinomycin) and di-*tert*-butyldibenzo-18-crown-6 **5** (IC_{50} values of ~2 μM) (Figure 11).

This clearly demonstrates that the substituents are of great importance for this effect, which is enhanced by increasing the hydrophobicity, possibly due to requirements for membrane insertion. Even so, neither the lipophilicity nor the K^+ binding constants exhibit a linear relationship with antiproliferative activity, indicating that a combination of various molecular properties determine their biological activity. Therefore, we attempted to computationally model the structure–activity relationships of crown ethers using the support vector machines (SVM) algorithm for regression. The SVM algorithm takes many descriptors into account simultaneously, along with their possible interactions, and allows for nonlinear dependencies. A reasonably high estimate of predictive ability obtained in cross-validation (Pearson's $r = 0.77$ between predicted and actual log IC_{50} concentrations) indicates the SVM model may be useful in predicting the activity of molecules that are similar in structure and in their mechanism of action. Applying the Relief-F algorithm[92] for descriptor relevance evaluation, we have shown that the two main groups of attributes have consistently shown a very high rank and are therefore quite likely to be related to the antiproliferative activity of the crown ethers tested, representing versions of the BCUT and d COMMA2 descriptors.[93] In other words, the orientation and asymmetry of hydrophobic groups and distribution of polarizable elements are of utmost importance for the activity of tested crown ether compounds. This activity is connected with the interaction between the compounds and the receptor/target, which is most likely the membrane. The distributions of polarizable elements also influence their ability to complex metal ions. This is in agreement with previous investigations which found that crown ethers can only transport metal ions through membranes if both prerequisites for ion complexation (polarizable atoms) and hydrophobicity (membrane penetration) are met.[68] We are currently working on measuring the antiproliferative activity of a series of novel crown ether derivatives; these data will be used to refine our SVM regression model with the long-term goal of obtaining accurate in silico predictions of activity for any 18-crown-6 molecule.

Compounds **3** and **5** had a marked influence on cell-cycle phase distribution: they induced strong G1 arrest followed by induction of apoptosis. Similar results were obtained using valinomycin. Indeed, it has been recognized

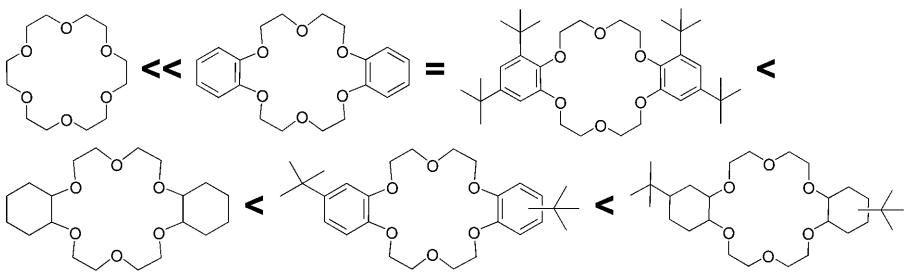

Figure 11. Comparison of inhibitory activity of the crown ethers shown toward tumor cell growth based on their measured IC_{50} values.[68]

that potassium currents play a role in cell proliferation, specifically in the regulation of progression through the G1 phase.[94] These results again support the hypothesis that crown ether compounds can inhibit tumor cell growth by disrupting potassium ion homeostasis, which in turn leads to cell-cycle perturbations and apoptosis. Further in vivo studies are undoubtedly very important.

Mirkhodjaev and co-workers studied the mechanism of antitumor action in vivo of diacetyl-, divaleryl-, and dinonanoyldibenzo-18-crown-6. They concluded that diacetyldibenzo-18-crown-6 inhibited tumor growth by blocking the Ca^{2+} channels of tumor cells, whereas divalerildibenzo-18-crown-6, acting as a Ca^{2+} ionophore, even stimulated the growth of rat sarcoma tumors.[89]

Another described, but still underexplored approach of using crown ether compounds in antitumor therapy is the combination treatment of tumor cells with conventional chemotherapeutics along with crown compounds. Specifically, it was shown that various ionophores such as valinomycin, nonactin, and nigericin inhibit the P-gp-mediated efflux of a variety of drugs, which causes resistance in treated tumor cells, that is, the multidrug-resistance (MDR) phenotype. Thus, blocking the activity of P-gp could overcome the MDR phenotype and lead to chemosensitization.[95] Among other compounds, some crown ethers were demonstrated to inhibit the P-gp-mediated efflux of antitumor drugs such as anthracyclines. Moreover, the potassium ion modulators amphotericin B and bumetanide could significantly influence tumor cell apoptosis induced by cisplatin or other chemotherapeutics.[96] This supports the need for further evaluation of other ion-modulating compounds, such as crown ethers, for their utility in modulating apoptosis in tumor cells.

Finally, it was recently shown that crown ethers can be used as vesicular systems for the delivery of 5-fluorouracil (5-FU), a common and effective chemotherapeutic agent against various tumor types. Specifically, the addition of a lipophilic long-chain alkyl group to a hydrophilic crown ether results in the formation of a crown-ether-based surfactant known as bolaform (Figure 12). This can form micelles or more complex supramolecular structures in water similarly to the common nonionic surfactants, niosomes. The formulation of 5-FU in niosomes could optimize the oral absorption or increase the biological half-life in the case of parenteral administration, thus decreasing toxic side effects.[97]

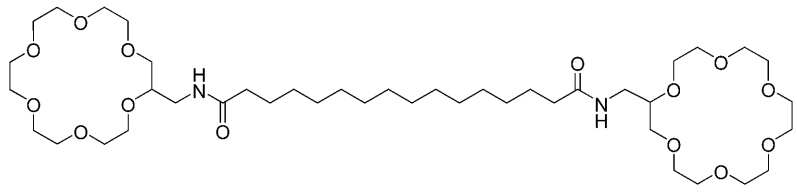

Figure 12. Chemical structure of a bolaform surfactant, prepared as a vesicular carrier for 5-fluorouracil.[97]

5. Biomedical Perspectives

All the studies mentioned above form the foundation for more detailed research on either biological mechanisms or novel synthetic approaches focused on variations of substituent groups that should augment or modify the activity of crown ethers as potential novel membrane-active drugs. It has been well established that cellular ionic homeostasis, fundamentally for K^+, Cl^-, and Ca^{2+}, is indispensable for cell proliferation and death. Membrane ion channels are thus basic equipment for all living cells, and they are essential for cell proliferation and are sometimes critical regulators of apoptosis.[98] For example, the activation of K^+ channels is required for proliferation, primarily for progression through the G1 phase of the cell cycle, whereas changes in ionic strength due to K^+ efflux, Cl^- efflux, and Ca^{2+} influx have been proposed as important inducers of apoptotic processes. Most reported studies support the notion that K^+ efflux induces cell shrinkage and apoptosis by disrupting either the mitochondrial or plasma membrane potential. Other studies claim that K^+ channel blockers inhibit proliferation by arresting the cells in the G1 phase, either without the activation of apoptosis, or even by its inhibition.[99,100]

In spite of the growing body of evidence that points to the importance and potential of ion transport regulation (primarily K^+) in the treatment of cancer, there is conflicting evidence for the role of ion transport in apoptosis and inhibition of cancer cell growth, and this requires more attention. Such discrepancies result from the fact that these studies mostly concern the activity or expression of K^+ channels, which are known to be among the most widespread and diverse family of plasma membrane ion channels; they play a wide variety of essential roles in different cell types.[98,101] Ion channel blockers represent a potential class of tool to determine the impact of ion channels on cell proliferation and cancer growth, but their lack of specificity makes it difficult to determine precisely which of the many channels is important for a given activity. Moreover, the effect of potassium concentration on apoptosis and proliferation is paradoxical: K^+ efflux plays a necessary and probably pivotal role in programmed cell death and may be triggered by various K^+ channel types. However, the same type of Ca^{2+}-activated K^+ channel that is activated during apoptotic cell shrinkage also promotes cell proliferation. Still, the activation of K^+ channels during apoptosis is much more pronounced than during proliferation, and the magnitude of the activated conductance along with environmental conditions essentially determine whether the channel supports proliferation or apoptosis.[98]

6. Summary and Outlook

In conclusion, many discrepancies in our awareness of the relationship of ion transport and cancer, along with the fact that

most of the ion channels claimed to promote cancer are also expressed and have a clear function in many other unaffected tissues, still hinder clinical applications of manipulating ion permeability in treating cancer. Nevertheless, a few studies are focused on K^+ ionophores and their potential in antitumor treatment, with the exception of valinomycin, which was shown to be too toxic. We therefore believe that further research on other ionophores, including crown ethers, as potential activators or regulators of transport of K^+ and other ions should enhance the scope therapeutic opportunities for combating cancer. The focus should be put on the preparation of novel crown compounds with various side arms or other functional moieties that allow their ion transport capacities to be adjusted, with consequent fine-tuning of their activity in suppressing tumor growth. Research on novel crown-based channel structures with cytotoxic activities should also be encouraged.

Although no anticancer drug is likely to be altogether free of toxic side effects, the potential toxicity of such compounds should be minimized or avoided if possible. Indeed, there are several lines of evidence that indicate substantial differences between normal and tumor cells with respect to membrane potentials, potassium and other ion currents, and ion concentrations[102–104] that could be exploited for therapy by membrane-active ionophores. For example, the concentration of potassium ions in cancer cells has been found to be twice that in normal cells.[105] Moreover, differentiated neuronal cells have been shown to be more resistant to the toxic effects of potassium ionophores than non-differentiated cells.[106] This phenomenon should be studied further and demonstrated with tumor cells of various differentiation status and compared with non-tumor cells. Alternatively, new means of imparting selectivity between tumor and normal cells should also be considered, such as cell targeting. This can be achieved either by "passive" methods, in which ionophores are encapsulated or attached to a lipid- or polymer-based carrier system, or by "active" targeting, in which the attachment of a homing moiety, such as monoclonal antibodies or ligands in the form of peptides, sugars, or lectins is used to deliver the drug to the

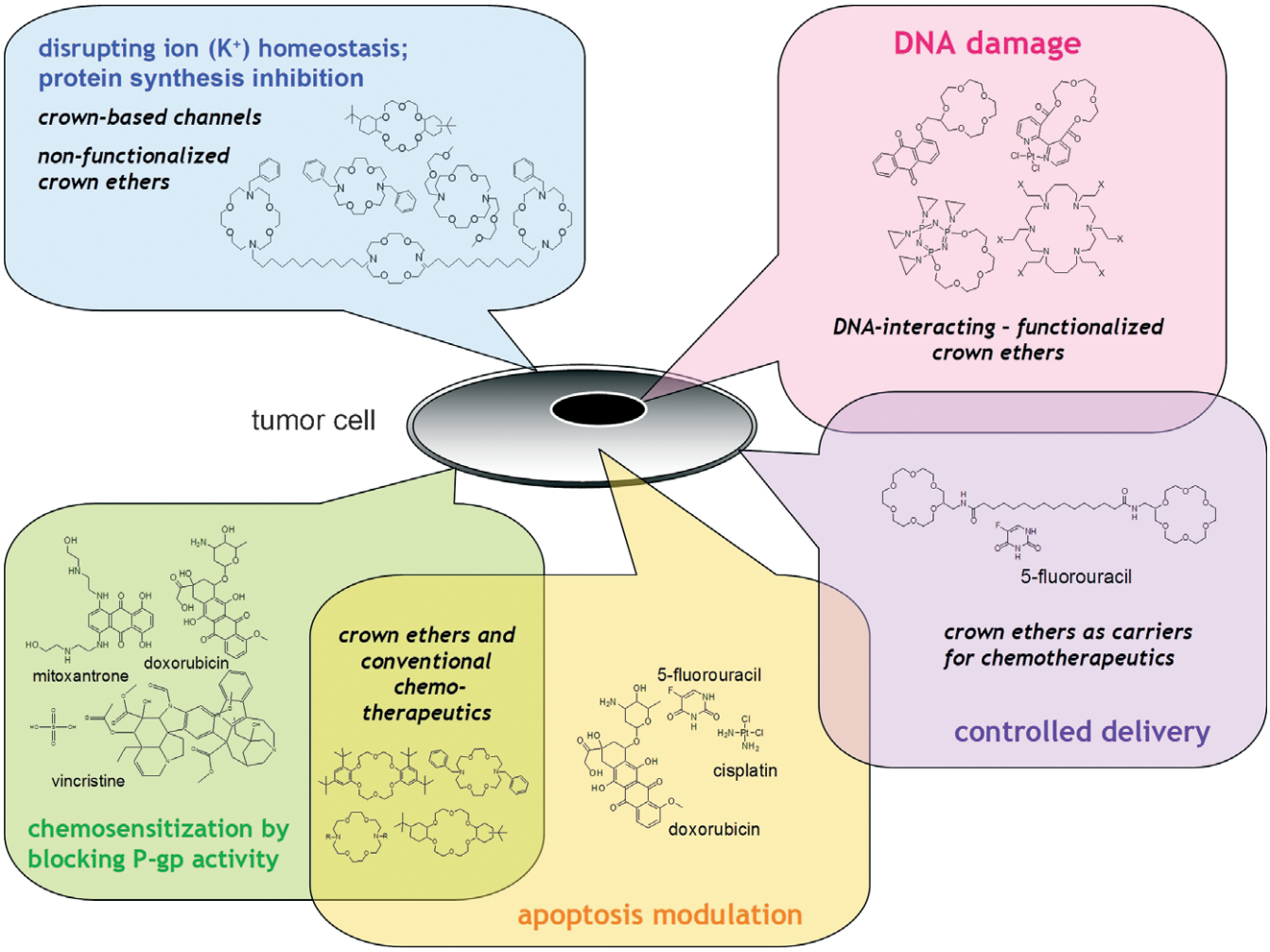

Figure 13. Various overlapping areas of potential application of crown ether compounds in antitumor therapy. Non-functionalized crown ethers and crown-based channels could be used for antitumor treatment, inducing the disruption of potassium transport (or that of other cations) and/or inhibition of protein synthesis, whereas DNA-interacting functionalized crown complexes should induce DNA damage. The combination of crown compounds and conventional chemotherapeutics have been shown to induce chemosensitization in multidrug-resistant cells, and to modulate (accentuate) apoptosis in tumor cells. Crown-ether-based surfactants could be efficient vehicles for controlled drug delivery.

intended target by attaching it to specific receptors on the appropriate cell surfaces.[68]

In conclusion, crown ethers are a promising and emerging group of compounds which, in addition to their enormous versatility and broad use in chemistry and industry, could have a firm footing in the biomedical sciences as well. We are sure that they will generate new and innovative applications with future generations of compounds, hopefully as potential novel anticancer drugs (Figure 13). They should either induce toxicities that differ from those of conventional antitumor drugs and could be used as such, or should complement drugs in current use and provide a valuable adjunct to therapy. We believe that further research in this direction should be encouraged.

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Keywords: antitumor agents · biological activity · crown compounds · ion channels · ionophores

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