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Review

P-type ATPases as drug targets: Tools for medicine and science

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ABSTRACT

P-type ATPases catalyze the selective active transport of ions like H^+, Na^+, K^+, Ca^{2+}, Zn^{2+}, and Cu^{2+} across diverse biological membrane systems. Many members of the P-type ATPase protein family, such as the Na^+,K^+-, H^+,K^+-, Ca^{2+}-, and H^+-ATPases, are involved in the development of pathophysiological conditions or provide critical function to pathogens. Therefore, they seem to be promising targets for future drugs and novel antifungal agents and herbicides. Here, we review the current knowledge about P-type ATPase inhibitors and their present use as tools in science, medicine, and biotechnology. Recent structural information on a variety of P-type ATPase family members signifies that all P-type ATPases can be expected to share a similar basic structure and a similar basic machinery of ion transport. The ion transport pathway crossing the membrane lipid bilayer is constructed of two access channels leading from either side of the membrane to the ion binding sites at a central cavity. The selective opening and closure of the access channels allows vectorial access/release of ions from the binding sites. Recent structural information along with new homology modeling of diverse P-type ATPases in complex with known ligands demonstrate that the most proficient way for the development of efficient and selective drugs is to target their ion transport pathway.

1. Introduction

Membrane proteins are in general regarded as extremely potent drug targets due to their role as transporters and mediators in the interaction of the cell with the surrounding environment, between cells, and between cellular compartments. This role, and their highly exposed position in the cell, renders them of utmost importance in cellular physiology. P-type ATPases form a superfamily of cation transporters of various biological membranes. These enzymes are found in all kingdoms of life and pump specific ions through the lipid bilayer at the expense of one ATP molecule per cycle [1–6]. Thus, P-type ATPases maintain steep electrochemical gradients and cell homeostasis [1–6]. Sequence motifs define five subfamilies, P_1 through P_5-type ATPases, with further subclassifications A, B, C, and D. These classifications also correlate with specific transport activities. P_{2B} pumps for example include the sarco(endo)plasmic reticulum Ca^{2+}-ATPases.

Maintenance of the proper gradients for essential ions across cellular membranes makes P-type ATPases crucial for cell survival. Indeed, Ca^{2+}, Na^+, K^+, and H^+ gradients control numerous secondary transport processes and impose a stringent regulation on diverse signaling pathways. Because of their pivotal role, these pumps have been linked to disorders displaying various degrees of severity. The Menkes and Wilson diseases arise from mutations in copper-transporting P_{1B}-type ATPases [7]. Neurodegenerative disorders are related to mutations in brain Na^+,K^+-ATPase, such as the migraine subtype familial hemiplegic migraine type 2 [8] and the movement disorder familial rapid-onset dystonia parkinsonism [9]. Aggravated parkinsonism is also linked to mutations within the lysosomal ATP13A2, a neuronal P_5-type ATPase [10]. Mutations in sarcoplasmic reticulum Ca^{2+}-ATPases (SERCA) isoforms can cause recessive Brody

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Abbreviations: ATP, Adenosine 5′-triphosphate; AMPPCP, Adenylyl 5′-(beta,gamma-methylene)diphosphonate; BHQ, 2,5-di-*tert*butyl-1,4-dihydroxybenzene; Br_2-TITU, 1,3-dibromo-2,4,6-tris(methylisothiouronium)benzene; CTS, Cardiotonic Steroids; CPA, Cyclopiazonic Acid; GERD, gastroesophageal reflux disease; IP3R, Inositol-3-Phosphate Receptor; PCAB, Potassium-Competitive Acid Blocker; PPI, Proton Pump Inhibitor; SERCA, Sarco(Endo)plasmic Reticulum Ca^{2+}-ATPase; TG, Thapsigargin; TM, transmembrane helix

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myopathy (SERCA 1) or Darier disease (SERCA 2) [11]. Abnormal cell proliferation resulting in malignant tumors can also be induced by a dysfunction of different pumps [12,13] and impairment of SERCA function has been linked to apoptotic events which are triggered by a rise in cytoplasmic calcium concentration [14]. P-type ATPases therefore appear as attractive drug targets in a broad range of pathologies and pharmacological settings. In this context, the cardiac Na^+,K^+-ATPase and the gastric H^+,K^+-ATPase already have a long history of therapeutic application. Inhibition of the Na^+,K^+-ATPase by cardiotonic steroids is used in the medical treatment of congestive heart failure [15], whereas inhibition of the gastric H^+,K^+-ATPase is part of the treatment of dyspeptic conditions [16]. Other members of the family are considered as prominent targets for future drug development. For instance, the inhibition of the human Ca^{2+}-ATPases by thapsigargin-derived prodrugs has been described as a promising tool in prostate cancer therapy [17] and fungal H^+-ATPases are regarded as potential targets for the development of novel antifungal medication and fungicides [18].

An abundant literature is available to describe the mode of action of each class of inhibitors on their respective target. However, a more global approach may be required if one aims at expanding the list of inhibitors to target new categories of pumps. A thorough analysis of the general mechanisms governing pump inhibition would therefore be particularly useful to define common principles applicable to the selection and design of second generation and new classes of inhibitors.

In the last couple of years, structural information on diverse P-type ATPases has emerged with an increasing pace. Crystal structures are now available for the Ca^{2+}-ATPase in nearly all conformations relating to the functional cycle [19–24] or as complexes with inhibitors [27–29], and the first structures of the Na^+,K^+-ATPase and the H^+-ATPase have been determined [25,26]. Most importantly, these structure determinations and insight on conformational changes together with a wealth of biochemical data have brought valuable information on how to address the future development of efficient and selective inhibitors leading the way to new drugs. In this context, and in light of the new structures available, the purpose of this review is to compare the mode of action of different classes of inhibitors and to draw general conclusions on how pumps are targeted and inhibited. Finally, perspectives in the application of these general principles to the design of new inhibitors – in particular for pumps with no selective inhibitors known yet such as the fungal H^+-ATPases – will be discussed.

2. General description of P-type ATPases

A hallmark of all P-type ATPases is the formation of a covalent aspartyl-phosphate intermediate by autophosphorylation of the pump catalytic subunit on a conserved aspartic residue — a mechanism which distinguishes them from other ATPases such as F- or V-type ATPases [2–4]. Many of the P-type ATPases perform two-way transport, such as the Na^+,K^+-ATPase and the Ca^{2+}-ATPase (in fact Ca^{2+},H^+-ATPase). The reaction cycle is traditionally described by the Post-Albers scheme (Fig. 1) in which the pump oscillates between two functional states, E1 and E2, related to the direction of the ion transport, i.e. from the cytosol and into the lumenal/extracellular environment, and counter-transport into the cytosol, respectively [5,6]. The ion translocation proceeds through a Ping-Pong mechanism, cation species being sequentially transferred in opposite directions across the membrane. The opening and closure of the access channels are tightly coupled to conformational changes within the cytoplasmic

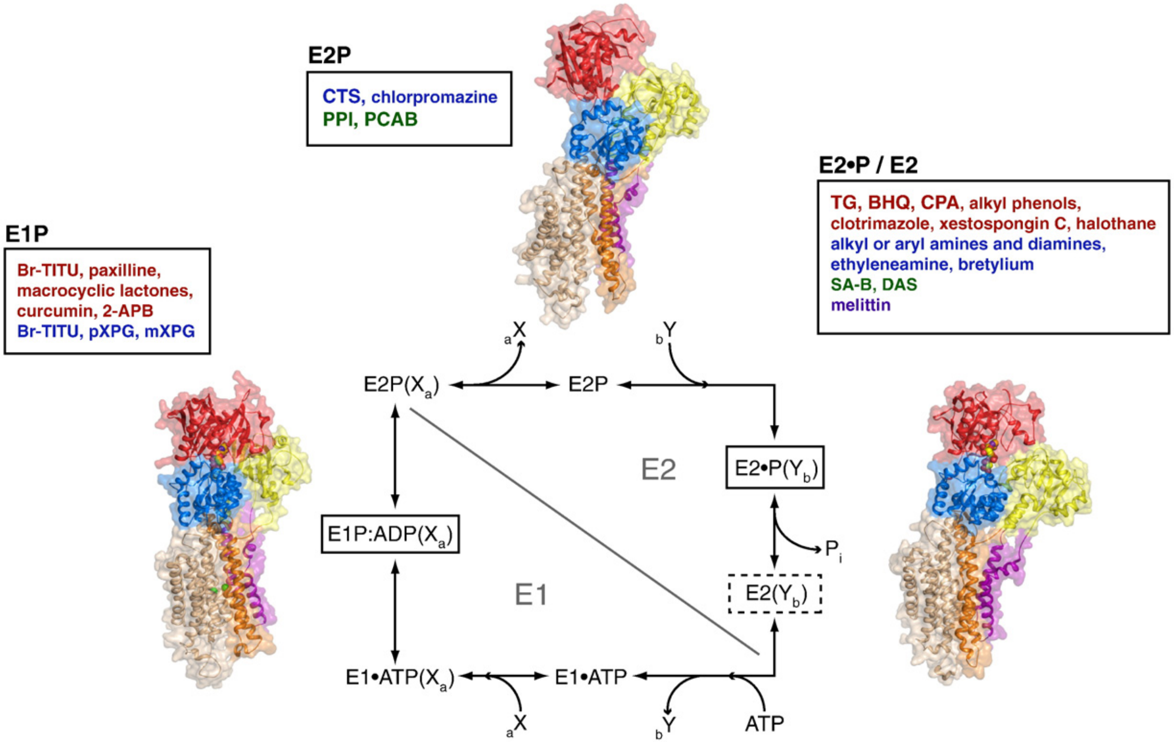


Fig. 1. Post-Albers scheme of the transport cycle for P-type ATPases. Following this scheme, a ions of species X and b ions of species Y are transported out of and into the cytoplasm of the cell, respectively. The two states represented in boxes correspond to the two cation occluded states where the access pathways to both sides of the membrane are closed. The dashed state corresponds to an occluded state for the Na+,K+-ATPase. Structures of SERCA in the (Ca_2)E1P [21], E2P [24] and E2 [78] states are included, representing pumps in general. Inhibitors that are known to target individual forms are indicated for each state (SERCA inhibitors in red; Na^+,K^+-ATPase inhibitors in dark blue; H^+,K^+-ATPase inhibitors in green; inhibitors common to the three pumps in light purple).

domains induced by ATP binding, phosphorylation, subsequent dephosphorylation, and phosphate release from the enzyme. Consequently, ions are successively taken up at ion binding sites from one side of the lipid bilayer, occluded within the transmembrane domain, and then released on the other side of the membrane, in exchange for the second ion species [2–6].

Of the P-type ATPase family, some members have been characterized more extensively. The Na^+,K^+-ATPase, originally discovered in 1957 [30], was the first one to be identified. This house-keeping enzyme, found in various isoforms and in all animal cells, maintains proper K^+ and Na^+ concentrations in the cells. The gradients are vital for the processes of cell excitability and volume regulation and serve as a driving force for the secondary transport of ions, sugars, and amino acids [31,32]. The gastric H^+,K^+-ATPase is localized in the plasma membrane of the parietal cells of the gastric mucosa. It is responsible for the last step of acid secretion by actively exporting protons into the stomach lumen along with Cl^- anions extruded from conductance channels to form the gastric juice [33]. The Ca^{2+}-ATPase from sarco(endo)plasmic reticulum (SERCA) is an important regulator of intracellular calcium concentration. It enables muscle contraction/relaxation and exerts a general tight control on calcium signaling pathways whose abnormal regulation can lead to severe pathologies [34]. Finally, H^+-ATPases are responsible for the establishment and maintenance of the electrochemical gradient across fungal and plant plasma membranes, and thus play a vital role for secondary active transport in these organisms, such as the up-take of nutrients [35]. Though serving distinct cellular functions, all these enzymes share a high degree of resemblance, the Na^+,K^+-ATPase and the gastric H^+,K^+-ATPase being close relatives with a sequence identity of 62% [36].

A huge step in the understanding of the intricate mechanisms behind ion pumping was made when the first atomic structures of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase from rabbit skeletal muscle were determined [19–21]. These structures revealed the general architecture of the catalytic subunit of P-type ATPases which encompasses three cytoplasmic domains — the A (actuator), P (phosphorylation), and N (nucleotide binding) domains — and a transmembrane segment of 10 helices (TM1 to TM10) bearing the two embedded Ca^{2+} binding sites. At present, nearly all of the functional states of the SERCA pump (E1·ATP, E1P, E2P, E2·P_i, E2) have been structurally characterized [19–24] (Fig. 1). More recently, two other members of the P-type ATPase family have been structurally described: the pig renal Na^+,K^+-ATPase in the $[Rb_2]E2\cdot MgF_4^{2-}$ occluded state, representing the $[K_2]E2\cdot P_i$ form [25], and the *Arabidopsis thaliana* H^+-ATPase in an E1·AMPPCP partially occluded state, probably representing the protonated E1·ATP state [26]. These structures enabled a direct comparison between three members of the P-type ATPase family and shed new light on the general mechanism of cation transport as well as the specific properties of each transporter. Indeed, the H^+-ATPase belongs to the subgroup III and displays an autoinhibitory mechanism provided by an extended C-terminal regulatory (R) domain [35] while the Na^+,K^+-ATPase is one of the few oligomeric P-type ATPases and performs an electrogenic transport reaction with an additional Na^+ site in the cation binding pocket [37]. These new structures constitute a valuable source of information for the design of inhibitors targeting specific P-type ATPases.

3. Pumps as drug targets

Historically the discovery of drugs for the treatment of diseases has been based on trial and error principles. A vast number of plant, animal or mineral products were applied to prevent, diagnose, and cure illness. Some of them are still used by millions of people and a few of those ancient drugs are nowadays shown to be inhibitors of the ion transport systems, pumps included. In the past few decades, the critical role of P-type ATPases in the regulation of cellular metabolism attracted special attention to the design of new, highly specific inhibitors of these proteins. To date, several inhibitory compounds and families have been characterized for P-type ATPases. Table 1 presents a list of the most important known inhibitors for the Ca^{2+}-, H^+,K^+-, and Na^+,K^+-ATPases. These molecules display various degrees of potency and selectivity towards their cellular target. Thapsigargin, for example, is a highly specific inhibitor of SERCA Ca^{2+}-ATPase with an IC_{50} in the subnanomolar range [38]. On the other hand, Br_2-TITU has a lower potency and binds to both the Ca^{2+}-ATPase and the Na^+,K^+-ATPase [39]. Some compounds can also target other cellular proteins, such as celecoxib, a non-steroidal anti-inflammatory agent originally identified as a blocker of the cyclo-oxygenase-2 [40]. The principles of inhibition are quite well understood for the best characterized inhibitors, in particular for SERCA inhibitors thapsigargin (TG), cyclopiazonic acid (CPA), and 2,5-di-(*t*-butyl)-dihydroxybenzene (BHQ). Indeed, their binding sites on SERCA have been structurally determined [19–24,27–29]. The detailed mode of action of these compounds will be described later in this review. However, the precise blocking mechanism of many of the listed compounds remains elusive. More biochemical and structural data are needed to acknowledge them as inhibitors of potential therapeutic value.

3.1. Na^+,K^+-ATPase inhibitors: keeping the heart beat

Cardiac glycosides, also called cardiotonic steroids (CTS), are well-known inhibitors of the Na^+,K^+-ATPase — their only pharmacological receptor identified so far. They consist of a steroid core with a lactone moiety (five-membered, cardenolides, or six-membered, bufadienolides) attached at position 17 and sugars of different nature and length at position 3 [41] (see ouabain as an example in Fig. 3C). They are naturally found in many plants — the common foxglove (*Digitalis purpurea*) is, for example, a source of digoxin [42]. Bufadienolides are also secreted as toxins by a few animals including several species of toads (*Bufo marinus*), snakes (*Rhabdophis tigrinus*), and fireflies (*Photinus* species) [43]. The use of CTS in the treatment of cardiac insufficiency was first introduced by William Withering in 1785 [44], though some texts suggest that ancient Egyptians were aware of the curative properties of bufadienolide-containing plants such as squill and used them to treat heart diseases [45]. The generally accepted explanation for the positive effect of CTS on the patients heart (the so-called sodium pump lag hypothesis) suggests the following sequence of events: Inhibition of the Na^+,K^+-ATPase by CTS binding to its extracellular side provokes, at least locally, a raise in intracellular Na^+ concentration. As a result, the secondary active transport of Ca^{2+} out of the cell is slowed down and contractility of the cardiac muscle is increased (positive inotropic effect) [46]. This picture became more complicated a few decades ago when endogenous compounds exhibiting the same biological activity as exogenous CTS were identified in mammals [47]. So far, five different endogenous CTS have been chemically characterized and a more complex nature of their physiological function in mammals slowly emerges [48]. Indeed, it has recently been postulated that the Na^+,K^+-ATPase forms a compartment-specific receptor complex with the tyrosine kinase Src which, upon its activation induced by binding of CTS to the pump, initiates a cascade of phosphorylation events within the cell that regulate downstream effectors. This signaling pathway does not imply changes in the intracellular Na^+ concentration [49]. Therefore, even at concentrations far below those used in CTS medication, endogenous compounds can exert an effect on cell physiology and lead to a large spectrum of cellular responses including gene activation, motility, cell–cell contact, cell proliferation or apoptosis [48]. This new role of the Na^+,K^+-ATPase as a signal transducer has opened new perspectives for the development of anti-cancer drugs derived from CTS, notably for the treatment of breast cancer [13].

In addition to CTS, the pharmacological effects of several other compounds have been related to their interaction with the Na^+,K^+-ATPase. The antipsychotic drug chlorpromazine and the anti-malarial

Table 1

List of known inhibitors of therapeutic interest for the Ca^{2+}-, Na^{+},K^{+}-, H^{+},K^{+}-, and H^{+}-ATPases

Class of inhibitor	Compounds	Type and origin	Therapeutical applications
SERCA inhibitors			
Thapsigargin and derivatives [38,84]	Thapsigargin (TG), Boc12-ADT	Sesquiterpene lactones from the plant *Thapsia garganica*	Prodrugs for prostate cancer therapy
2,5-di-(*t*-butyl)-dihydroxybenzene [27]	BHQ	Synthetic compound	Anti-oxidant agent
Cyclopiazonic acid [28,29,127]	CPA	Mycotoxin originally isolated from *Penicillium* and *Aspergillus* species	
Estrogenic alkylphenols [71,73,88]	Bisphenol A, Diethylstilbestrol (DES), Tetrabromobisphenol A (TBBPA), Nonylphenol, Octylphenol, 3,5-dibutyl-4-hydroxytoluene (BHT)	Estrogenic alkylphenols	Toxic effect on testis development
		Bisphenol A: plasticizing agent	
		DES: Artificial estrogen	DES: Therapeutic agent to treat different gynecological problems and growth promotant factor in livestock
		TBBPA: Brominated Flame Retardant	
Paxilline [128]	Paxilline	Alkaloid mycotoxin from *P. paxilli*	Tremorgenic effect
Macrocyclic lactones [70]	Ivremectin, Cyclosporin A, Rapamycin	Macrocyclic lactones	Immunosuppressant agent, antihelminthic agent (Ivermectin)
Curcuminoids [74]	Curcumin	Component of the Indian curry spice turmeric	Antitumoral, antioxidant, antiarthritic, anti-amyloid and anti-inflammatory agents
Aromatic isothiouronium derivatives [39]	Br_2-TITU		
2-aminoethoxy-diphenyl borate [86]	2-APB		
Clotrimazole [129]	Clotrimazole	Antifungal	Antimycotic agent
Celecoxib [71]	Celecoxib	Non-steroidal drug (Celebrex®)	Anti-inflammatory drug used in the treatment of arthritis and acute pain
Chlorpromazine [50]	Chlorpromazine (CPZ)		Phototoxic antipsychotic drug
Chloroquine [50]	Chloroquine (CLQ)		Anti-malarial drug
Xestospongin C [87]	Xestospongin C	Marine alkaloid from the Okinawan sponge *Xestospongia Sp.* I	
Organo-chlorine pesticides [130]	Chlordecone, Toxaphene	Organo-chlorine pesticides	Pesticide
Artemisinin and derivatives [75]	Artemisinin	Sesquiterpene lactones from sweet wormwood (*Artemisia annua*)	Anti-malarial drug
Halothane [131]	Halothane		Volatile anesthetic
Melittin [132]	Melittin	Amphipathic peptide from bee venom	
Na,K-ATPase inhibitors			
Cardiotonic Steroids [41,48]	Ouabain, Digoxin, Digitoxin, Digoxigenin, Bufalin, Bufagenin, Strophanthidin, Proscillaridin A	Cardiotonic Steroids	Treatment of congestive heart failure and cardiac arrhythmia
Coumestans [133]	PCALC36	Wedelolactone from *Eclipta prostrata*	
Palytoxin [58]	Palytoxin (PTX)	Marine toxin from *Palythoa* polyps	Tumor promoter
Aromatic isothiouronium derivatives [55]	Br-TITU, Br_2-TITU	Aromatic isothiouronium derivatives	
Guanidium derivatives [55]	pXBG, mXBG	Guanidium derivatives	
Tetrapropylammonium [56]	TPA		
Amines [54,57]	Ethylenediamine, Bretylium	Alkyl or aryl, secondary, tertiary and quaternary amines	Class III antiarrhythmic drug (Bretylium)
Oligomycin [134]	Oligomycin	Macrolides from *Streptomyces*	Antibiotic
Chlorpromazine [50]	Chlorpromazine (CPZ)		Phototoxic antipsychotic drug
Chloroquine [50]	Chloroquine (CLQ)		Anti-malarial drug
Procaine, Tetracaine and Dibucaine [51]	Procaine, Tetracaine, Dibucaine	Amino-esters	Local anesthetics
Gramidicin A [52]	Gramidicin A		Antibiotic
Melittin [135]	Melittin	Amphipathic peptide from bee venom	
Gastric H,K-ATPase inhibitors			
PPIs (Proton Pump Inhibitors) [59,60]	Omeprazole, Tenatoprazole, CMN 131, Lansoprazole, Timoprazole, Rabeprazole, Pantoprazole, Esomeprazole, …	Synthetic compounds	Anti-ulcer (duodenal and gastric) agents
PCABs (Potassium-competitive acid pump antagonists, formerly APA) [59–62]	SCH28080, BYK 99, Imidazopyridines, YH1885, MDPQ 2,4-Diaminoquinazoline, soraprazan	Synthetic compounds	Anti-ulcer agents
Diterpenoids [63]	Scopadulcic Acid B (SA-B), Diacetyl Scopadol (DAS)	From the Paraguayan traditional medicinal herb *Scoparia dulcis*	Antiviral agent
Cibenzoline [64]	Cibenzoline		Class I antiarrhythmic agent
Prodigiosins [65]	Prodigiosins	Antibiotic red pigments produced by microorganisms including *Streptomyces* and *Serratia*	Antibiotic
Melittin [136]	Melittin	Amphipathic peptide from bee venom	
H-ATPase inhibitors			
NEM [137]	NEM		
Dio-9 [138]	Dio-9		Antibiotic
Ebselen [120]	Ebselen		

agent chloroquine were shown to inhibit different transport ATPases, including Ca^{2+}- and Na^+,K^+-ATPases [50]. Local anesthetics (procaine, tetracaine, dubucaine) are also affecting the Na^+,K^+-ATPase pump activity in a cation-dependent manner [51]. The antibiotic gramicidin A, known to form a membrane-spanning channel that selectively transports monovalent ions such as Na^+ and K^+ through the lipid bilayer, has recently been described as a mixed-type inhibitor of the Na^+,K^+-ATPase [52]. The antibiotic oligomycin has also been shown to stabilize E1-occluded states of the pump [53]. The fact that either these inhibitors are known to interact with other proteins or their inhibitory effects are observed at high concentrations makes them however irrelevant from a practical point of view.

Cationic compounds including isothiouronium and guanidinium derivatives, and different types of amines are also known to block the Na^+,K^+-ATPase [54–56]. In particular bretylium, an antiarrhythmic drug used to treat ventricular tachycardia and fibrillation, is acting as a K^+-antagonist on the Na^+,K^+-ATPase [57]. Finally, palytoxin constitutes a unique type of inhibitor of the Na^+,K^+-ATPase. This marine toxin produced by coral polyps binds to the extracellular side of the ATPase and transforms the pump into an ion channel. The fact that palytoxin, being a specific inhibitor of the Na^+,K^+-ATPase, at the same time acts as a skin tumor promoter, suggests (as in the case of ouabain) involvement of the ATPase in the process of signal transduction [58].

3.2. Gastric H^+,K^+-ATPase inhibitors: controlling acid secretion

Gastric diseases are generally not life-threatening but can be quite painful. The two major pathological disorders associated with acidification of the digestive tract are peptic ulcers, which in 80% of the cases are caused by *Helicobacter pylori* infection of the stomach, and gastroesophageal reflux disease (GERD). Since the export of protons into the stomach lumen by the H^+,K^+-ATPase constitutes the last step of acid secretion, inhibition of this gastric pump has now supplanted all other therapeutic strategies in the treatment of gastric complications, including the targeting of histamine H2 receptors [59].

The first generation of H^+,K^+-ATPase inhibitors, simply called the Proton Pump Inhibitors (PPIs), was introduced in the 1970s when derivatives of a compound termed CMN 131 were shown to have antisecretory activity. Further development of these compounds led to the synthesis of omeprazole, first commercialized in the late 1980s, and being now the most commonly used drug to treat peptic ulcers and GERD [60]. The binding of these benzimidazole derivatives to the extracellular side of the H^+,K^+-ATPase is irreversible and requires the acidification of the surrounding environment by active H^+-transport to be activated. Indeed, PPIs must be converted into an active form by gastric acid before they can react with the cysteines located on the lumenal surface of the pump. Because of this activating mechanism, there is a lag time in the cellular response to PPI absorption and the pain relief is consequently delayed. For this reason, new types of inhibitors which would be fast-acting and acid-independent have been sought. In the 1980s, the imidazopyridine SCH28080 was found to display the same antisecretory properties as PPIs even at higher pH values [61]. Several tertiary amines derived from SCH28080 were subsequently synthesized to gain in activity and bypass the side effects observed with this first compound. A new class of inhibitors was born: the Potassium-Competitive Acid Blockers (PCABs). PCABs act as K^+ antagonists on the H^+,K^+-ATPase and their binding to the pump from the lumenal side of the membrane is reversible. PCABs act faster than PPIs as they do not require any activation. However, because of their non-covalent binding to the H^+,K^+-ATPase, higher doses are needed to produce an effect comparable to what is observed with PPIs [59,60]. Nevertheless, if PCABs succeed in fulfilling the requirements of clinical trials, they could constitute a new promising class of pharmacological agents to treat gastric diseases. The last PCAB to date, soraprazan, has already proved to be superior to PPIs in terms of onset of action and complete control of acid secretion [62].

Other inhibitors of the gastric H^+,K^+-ATPase include scopadulcic acid B and its derivatives, which are antiviral agents against herpes simplex virus type 1, and cibenzoline, a class I antiarrhytmic drug [63,64]. Finally, prodigiosins are potent inhibitors of acid secretion and they inhibit several types of proton-transporting pumps, including the H^+,K^+-ATPase, through a chloride-dependent uncoupling mechanism [65]. These antibiotics have also been shown to inhibit *H. pylori* growth and could therefore constitute promising agents in the treatment of gastroesophageal diseases.

3.3. SERCA inhibitors: controlling intracellular calcium concentration

In contrary to the Na^+,K^+- and the H^+,K^+-ATPases, no large family of inhibitors has been defined for SERCA. However, several compounds and derivatives of high potency and selectivity have been identified including thapsigargin, cyclopiazonic acid, and 2,5-di-(*t*-butyl)-dihydroxybenzene. Thapsigargin is the most potent inhibitor of SERCA described so far [38]. The natural product was originally extracted from the plant *Thapsia garganica* which has been used in Arabian medicine for more than 2000 years [66]. Several studies demonstrated that thapsigargin can induce apoptosis in different types of cancer cells [67,68]. Later it was shown that programmed cell death is a direct consequence of an increase of the intracellular calcium concentration due to the blocking of the SR/ER Ca^{2+}-ATPase. The steps following Ca^{2+}-ATPase shut down by thapsigargin have been further characterized by several groups (see [17] for a review). First, a primary elevation of intracellular calcium concentration is observed as a result of Ca^{2+} leakage by passive diffusion from the ER stores. This primary raise stimulates the translocation of several proteins to the plasma membrane, among them the IP3R receptor and the annexin-V complex. This allows increased influx of calcium into the cytoplasm and leads to a second elevation of the intracellular calcium that eventually activates apoptotic factors and finally engages the cell death machinery [17]. Because thapsigargin will target all sarcoplasmic reticulum Ca^{2+}-ATPase pumps regardless of the cell type, its cytoxicity is too high to consider it as a general antitumoral agent. However, a derivative that selectively targets cancer cells would constitute a promising alternative to existing antitumoral drugs (such as paclitaxel, docetaxel, doxorubicin), in particular for certain types of cancers which are resistant to current therapies. Prostate cancer cells, contrary to most cancer cells, are slowly growing and therefore hard to target by conventional chemotherapy. Treatment of this type of cancer is a typical example where synthesis of new drugs would be beneficial. The use of thapsigargin derivatives was therefore introduced as a potential therapeutic tool to induce death of metastatic cells [17]. This new approach is based on the synthesis of inactive prodrugs which contain a thapsigargin molecule coupled to a small peptide carrier. Activation of the thapsigargin molecule requires the cleavage of the peptide moiety by specific proteases. Therefore, one can synthesize a tissue-specific drug by selecting a peptide which will only be recognized by proteases of this cell type. Prostate cancer cells produce such a specific protease and secrete it into the extracellular fluid. A thapsigargin-derived prodrug being a substrate for the protease should consequently be cleaved in the vicinity of the malignant cells. Complete validation of this strategy is still needed, but it seems clear that more efforts will be put in this direction in the future and this appealing approach could be extended to other inhibitors of the Ca^{2+}-ATPase [17].

Indeed, other inhibitors of SERCA have been proposed to have therapeutic properties. Cyclopiazonic acid may have a cardioprotective action on myocardial ischemia [69]. How CPA inhibition of SERCA could attenuate damages caused by oxygen deprivation is not fully understood. But it is clear that modulation of the pump activity is affecting the relaxing/contractile cycle of the heart muscle through calcium flux regulation.

A number of molecules used as therapeutic agents have also been shown to inhibit SERCA to different degrees. The list includes macrocyclin lactones such as cyclosporin A or rapamycin, which are used as immunosuppressant agents, the anti-inflammatory drug celecoxib, the antihelminthic agent ivermectin, the artificial estrogen diethylstilbestrol, and curcumin derivatives [70–74]. Whether their inhibition of the Ca^{2+}-ATPase is part of their therapeutic response or is just a secondary effect is however still unclear.

Finally, it was proposed that artemisinins (the most important class of antimalarial drugs used today, which are sesquiterpene lactones isolated from sweet wormwood *Artemisia annua*), could inhibit the SERCA orthologue PfATP6 of *Plasmodium falciparum*, the parasite causing malaria [75]. Surprisingly, although these compounds structurally resemble thapsigargin, they seem to have no effect on mammalian SERCA pumps. It has been postulated that a single residue in transmembrane helix TM3 near the thapsigargin pocket is governing the selectivity of the pump for artemisinins [76]. Artemisinins mode of action is however still controversial and some studies suggested that they could act by haem-dependent activation of an endoperoxide bridge occurring within the parasite's food vacuole rather than by inhibition of PfATP6 [77].

4. The molecular mechanisms behind pump inhibition

Biochemical and structural studies on P-type ATPases inhibition have so far been centred on a limited set of inhibitors with the highest affinity and the most immediate pharmacological interest. Intensive mutagenesis studies have enabled to define more precisely the binding pockets of several families of inhibitors, including the CTS binding site on the extracellular side of the Na^+,K^+-ATPase and the PPI and PCAB binding sites on the lumenal side of the H^+,K^+-ATPase. In the case of SERCA, several crystallographic structures of the enzyme–inhibitor complexes are now available and have enabled a precise description of the mechanism by which these compounds affect the enzyme's function. The new structures together have opened up for possibilities of molecular modeling of inhibitor binding and for further rational drug design attempts.

4.1. SERCA inhibitors: blocking the cytoplasmic pathway

In the absence of calcium, the detergent solubilized Ca^{2+}-ATPase enzyme from SR membranes is quite unstable and starts denaturing rapidly [20]. Therefore, the E2 state of the pump has been a poor target for crystallographic studies and attempts made so far to trap this conformational state have largely failed although poorly diffracting crystals have been reported [78]. However, it was shown that inhibitors of SERCA favor an E2 conformation of the pump. They were consequently used to stabilize this state and several E2 structures of SERCA have been obtained in the presence of TG, CPA, BHQ, or different combinations of these three inhibitors [20,22,23,27–29].

TG, CPA and BHQ all affect the Ca^{2+}-ATPase pump cycle in a similar way. They bind to the ATPase in a calcium-free state (referred to as E2–P transition state, $E2{\cdot}P_i$ or E2) and act as Ca^{2+}-antagonists on the enzyme, preventing the binding of calcium ions from the cytoplasmic side by blocking the pump in a conformational state which has very low affinity for calcium [79–81]. Due to these similarities in their mode of action, these compounds were thought to share a common mechanism of inhibition and consequently a common binding site on the pump. However, structures of SERCA obtained in the presence of these inhibitors revealed two distinct binding pockets, one pocket for TG and one pocket for CPA and BHQ [27–29] (Fig. 2A). Moreover, both binding pockets can be occupied by inhibitors at the same time without affecting the respective affinities for each compound and this concomitant binding has even proved to further stabilize the E2 state in crystallization experiments [27–28].

Several functional states have been crystallized in the presence of these three inhibitors. Individual complexes of the Ca^{2+}-ATPase with CPA, BHQ, and TG have been obtained both in the $E2{\cdot}P_i$ ($E2{\cdot}MgF_4^{2-}$) and E2 states [20,23,27–29], and in the case of TG, the complex has also been described in the $E2{\cdot}AlF_4^-$ state [22] mimicking the E2–P transition state of

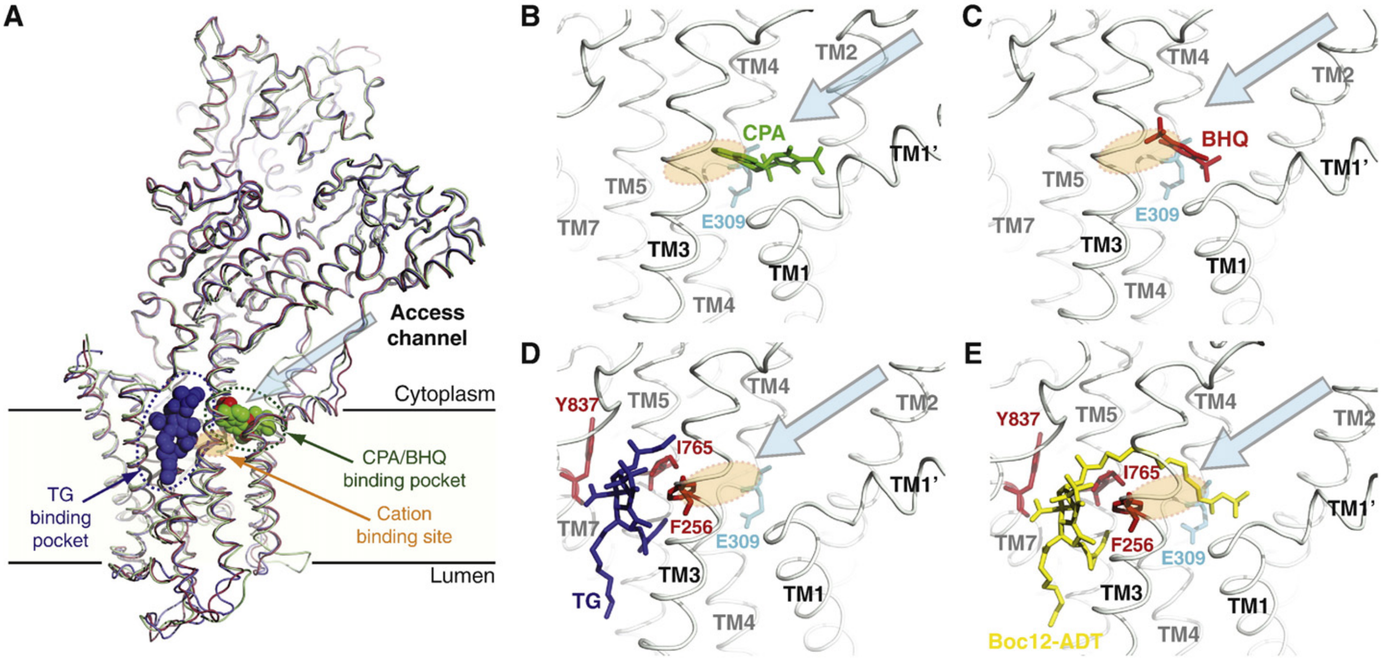


Fig. 2. Visualization of the TG, CPA, BHQ and Boc12-ADT binding sites on SERCA. (A) General view of the inhibitors position in the transmembrane domain of SERCA. The structures of the $E2{\cdot}AlF_4^-$–TG complex [22] (SERCA backbone and TG molecule in blue), the $E2{\cdot}MgF_4^{2-}$–CPA complex [29] (SERCA backbone and CPA molecule in green) and the E2–BHQ complex [27] (SERCA backbone and BHQ molecule in red) have been superimposed in O [140]. The gating residue Glu309 is shown in cyan as a stick representation. The cation binding site (represented in orange) is derived from the $(Ca_2)E1{\cdot}AMPPCP$ state [21] whose transmembrane region has been superimposed to the transmembrane region of the 3 other E2 structures. (B) Binding pocket of CPA within the $E2{\cdot}MgF_4^{2-}$–CPA complex [29]. (C) Binding pocket of BHQ within the E2-BHQ complex [27]. (D) Binding pocket of TG within the $E2{\cdot}AlF_4^-$–TG complex [22]. The three most important residues for TG binding (Phe256, Ile765 and Tyr837) are shown in red. (E) Binding pocket of Boc12-ADT within the $E2{\cdot}AlF_4^-$–TG complex [84].

the dephosphorylation reaction. No major differences are observed in the positioning of each inhibitor within the so-far crystallized states, and we will for this reason mainly refer to only one state for each inhibitor. Fig. 2A shows a general view of the binding sites of TG, CPA, and BHQ on the Ca^{2+}-ATPase molecule in the calcium-free E2 (CPA [29] and BHQ [27]) or E2–P (TG) state [22]. The two distinct cavities are clearly visible on the structure. The TG binding cavity lies between transmembrane helices TM3, TM4, TM5, and TM7, whereas the CPA/BHQ binding cavity is formed by TM1, TM2, TM3, and TM4. CPA and BHQ binding sites are only partially overlapping and the two molecules make specific and distinct interactions with the surrounding residues [27–29]. Two different orientations of the CPA molecule have been described within the binding pocket, one with the tetramic acid buried in a hydrophobic pocket [28] and another with the tetramic acid facing the surface [29] — clearly a matter of diverging interpretations of the same structure that needs to be resolved. Still, the global occupancy of the cavity remains the same and the overall conformation of the protein is not affected within the two models. Therefore the conclusions concerning the mechanism of inhibition that can be drawn from both models have remained the same, although at least in one case they have been based on incorrect arguments.

Fig. 2B and C shows a close-up view of the binding pockets for both CPA and BHQ at the cytoplasmic cation access channel. In the background, the cation binding sites from the $(Ca_2)E1\cdot AMPPCP$ state [21] are indicated in orange. Both inhibitors are clearly bound right in the middle of the putative entry pathway for cytoplasmic calcium and stabilize the E2 conformation of the transmembrane domain. Their interactions with the surrounding residues also help pushing TM1 and TM2 towards TM4, therefore slightly closing the access channel. Moreover, the proposed gating residue Glu309 (shown in cyan in FIG. 2) is blocked by the inhibitory molecule. Both CPA and BHQ are located at sites close to TM4 and prevent the Glu309 side chain from extending towards the entrance channel. The Glu309 residue can therefore no longer act as a gating residue [27–29,82]. Thus, in short: structures clearly show that the inhibitory effect of both CPA and BHQ is based on the obstruction of the cytoplasmic access channel, which consequently prevents calcium binding and the E2 to E1 transition.

While CPA and BHQ create a physical hindrance on the way of Ca^{2+} ions, TG has a distinct binding site (Fig. 2D) and therefore a unique mode of inhibition. Its position in a cleft between TM3, TM5, and TM7 does not interfere directly with the cation access pathway. Rather, TG immobilizes TM3, TM5, and TM7 in positions that are incompatible with calcium binding. Indeed, mutagenesis studies have shown that the main residues involved in TG binding are Phe256 (TM3), Ile765 (TM5), and Tyr837 (TM7) (Fig. 2D) [83]. These residues, and in particular Phe256, undergo a large shift within the structure during the E2 to $(Ca_2)E1$ transition, due to the repositioning of the transmembrane helices to accommodate the two calcium ions [21,83]. Such a large movement is incompatible with the presence of TG and the TG-bound protein is consequently trapped in a dead-end complex which is almost irreversible due to the very high affinity binding of TG to the enzyme. Therefore, TG acts through a unique mechanism on the Ca^{2+}-ATPase by trapping it in a conformation that prevents calcium re-entry without physically blocking the access pathway.

In the search for TG analogs applicable in prostate cancer therapy, several molecules were synthesized and tested for their ability to inhibit SERCA [84]. In particular, a 12-Boc-aminododecaonoyl derivative (Boc12-ADT) was synthesized and co-crystallized with SERCA [84]. Fig. 2E shows its position on the Ca^{2+}-ATPase. The TG main core of the compound occupies the exact same pocket as the TG molecule alone whereas the long 12-Boc-aminododecaonoyl tail extends right through the protein and ends up at the cavity where CPA and BHQ binding sites are located. This compound displays the same potency as TG, probably because the network of interactions within the CPA/BHQ cavity is not sufficient to make new stabilizing contacts. It would seem possible to conjugate both modes of inhibition within a single molecule to strengthen the inhibitory effect.

All three compounds (CPA, BHQ, and TG) have also been shown to block the opening of the lumenal gate, hereby preventing the reaction cycle from going backwards. Thus, even though they are positioned deep within the membrane and near the cytoplasmic access channel, these inhibitors also influence the lumenal exit pathway [85].

It is not known if other inhibitors of SERCA act according to one of the mechanisms described above. Nevertheless, several compounds have been shown to stabilize E2 states and act as Ca^{2+}-antagonists on the Ca^{2+}-ATPase (Fig. 1). 2-aminoethoxydiphenyl borate (2-APB), which inhibits SERCA with an IC_{50} of 720 ± 45 μM, binds to the pump in a Ca-competitive manner, reducing the affinity of the pump for calcium by more than 20-fold [86]. It has been proposed that this inhibitor could act in a way similar to TG and modeling studies have identified the TG binding pocket as a putative site for 2-ABP interaction with SERCA [84]. Xestospongin C, a SERCA inhibitor at the vertebrate synapse, has been shown to compete with TG for the binding to SERCA, suggesting that they could share a partially overlapping binding site [87]. Finally, tetrabromobisphenol A, a potent inhibitor of SERCA, yet used as a brominated flame retardant, also favours an E2 state of the enzyme and reduces its affinity for calcium. Competition experiments have suggested that it could share a common binding site with BHQ [88].

4.2. Na^+,K^+-ATPase and cardiotonic steroid inhibitors: blocking the extracellular pathway

Cardiotonic inhibitors (CTS) interact with the extracellular part of the Na^+,K^+-ATPase. Despite their long tradition of use in cardiology, the structural basis for their high affinity binding towards the enzyme remains elusive. The consequences of CTS binding for the binding properties of other substrates of the Na^+,K^+-ATPase have been subjected to kinetic investigations for many years [89–91]. However, a complete understanding of the CTS mode of inhibition has up to now been prevented by the absence of high-resolution 3D structure of the Na^+,K^+-ATPase in complex with CTS — a structure which would allow a description of the CTS–pump interaction at the atomic level. The definition of the CTS binding pocket presents not only an academic interest, it would be highly appreciated in drug design, advancing synthesis of new highly potent and tissue-specific inhibitors.

Mutagenesis studies have implicated several residues in the potential binding pocket for CTS on the Na^+,K^+-ATPase. Gln111 and Asn122 in transmembrane helices TM1 and TM2 (residue numbering corresponds to the pig renal α1 Na^+,K^+-ATPase sequence) were identified as key residues for the interaction of the Na^+,K^+-ATPase with CTS. Their change into respectively Arg and Asp is responsible for the insensitivity of the rat and mouse Na^+,K^+-ATPase α1 isoforms towards ouabain and analogs [92]. Subsequently, other residues within the extracellular loops of the protein were involved in CTS binding: Cys104, Tyr108, Pro118, Asp121, and Tyr124 in TM1 and TM2; Tyr308, Leu330, Ala331, Thr338, and Cys367 in TM3 and TM4; Phe783, Phe786, Leu793, and Thr797 in the TM5–TM6 loop; Phe863 and Arg880 in the TM7–TM8 loop; and Phe982 in TM10 [93–97]. Finally, using chimeras between the Na^+,K^+-ATPase and the gastric H^+,K^+-ATPase, a subset of seven residues was found sufficient to convert the low-affinity ouabain binding site of the gastric H^+,K^+-ATPase into a binding site possessing the same high affinity as native Na^+,K^+-ATPase: Glu312, Val314, Ile315, Gly319, Phe783, Thr797, and Asp804 [98].

Though CTS can bind to different functional states of the pump, they seem to favour the E2P conformation of the Na^+,K^+-ATPase [89–91]. Some of the mutations mentioned above therefore exert their effect indirectly through a shift of a conformational equilibrium, e.g. Phe786Leu, which increases the stability of the K^+ bound form and, thereby, the K^+-ouabain antagonism [99]. By contrast, Phe783 seems to be directly involved, as a Phe783Leu substituted enzyme showed 60-fold reduction in ouabain affinity without any associated effect on K^+ binding or conformational equilibria [97]. Application of solid state NMR techniques for structural examination of the ouabain-binding site of pig kidney Na^+,K^+-ATPase revealed that the steroid core of the ouabain derivatives was more constrained than the sugar group [100]. It suggests that the steroid part establishes hydrogen bonds with the amino acid residues, while the sugar faces away from the surface of the protein. With the first high-resolution structures of a P-type ATPase, namely the Ca^{2+}-ATPase [19–22], several attempts were made to dock CTS on the extracellular side of a Na^+,K^+-ATPase model derived from the SERCA structures [96,101]. Though bringing some useful information, these models are lacking a high accuracy in their description of the extracellular region because of the low level of sequence identity between SERCA and the Na^+,K^+-ATPase in this region. Furthermore these models were restricted by the use of occluded forms of SERCA as starting models, possibly of small relevance to the conformational state of CTS-bound forms. The recent first structure of the Na^+,K^+-ATPase, the pig renal $\alpha1\beta1\gamma$ complex in the $[Rb_2]E2{\cdot}MgF_4^{2-}$ occluded form [25], is therefore a critical new source of information to create an accurate model for the CTS binding on the Na^+,K^+-ATPase. Based on the conformational changes of SERCA observed between the calcium-free E2P state, obtained by an $E2{\cdot}BeF_3^-$ complex [24], and the occluded E2P state, obtained by the $E2{\cdot}AlF_4^-$ [22] or $E2{\cdot}MgF_4^{2-}$ form [28], we have constructed an E2P model of the Na^+,K^+-ATPase and attempted by a simple approach to manually place ouabain at its extracellular site according to mutagenesis data (Fig. 3 — see legend for the description of the model building and docking). As shown in Fig. 3A and B, in the E2P conformation, the cation exit pathway is open towards the extracellular side of the membrane. The cation binding site (visible at the bottom of the entrance channel in orange) is now accessible to extracellular K^+. Because of this open conformation, a large cavity is available at the top of the transmembrane region to accommodate ouabain or other CTS with bulky glycosylations (Fig. 3B). The proposed model of ouabain (Fig. 3C) binding positions the CTS molecule within this cavity (Fig. 3D), in close proximity to residues that are shown to be involved in ouabain binding (Fig. 3E), notably Gln111, Asn122, Tyr308, and Glu312. The steroid core and the lactone moiety have been positioned near TM1–TM2 and TM3–TM4, i.e. the residues affecting CTS binding according to mutagenesis studies. However, a different orientation of the ouabain molecule within this cavity can not be ruled out. Some of the residues in TM5–TM6, namely Phe783, Leu793, and Thr797, shown to be very important for CTS binding in mutagenesis studies, are a little too distant from the CTS molecule (approx. 5 Å) for direct involvement in binding with in this model. It is however likely that while the initial binding occurs in this open E2P configuration a further "induced fit" rearrangement leads to a dead-end inhibited complex in which TM5–TM6 has approached TM3–TM4, hereby narrowing the cleft in which the CTS binds. It is also possible that the large loop between TM7 and TM8 is rearranged upon ouabain binding so that some residues, such as Arg880, may come in close contact with the CTS molecule. The model suggests that the CTS molecule obstructs the extracellular cation exit pathway, preventing K^+ re-entry and occlusion. Since CTS display a large variety in size, it is not certain that all compounds would block the cation exit tunnel with the same efficacy. However, even a partial overlap could be sufficient to block the enzymatic cycle. Leu311 has for example been proposed to participate in the cation gating mechanism [102] and the position of ouabain in the present model clearly interferes with this residue.

4.3. Gastric H^+,K^+-ATPase inhibitors: blocking the extracellular pathway

Omeprazole and derivatives (PPIs) are the main class of agents in clinical use to treat peptic ulcers and GERD. Like CTS on the Na^+,K^+-ATPase, they bind to the gastric H^+,K^+-ATPase in an E2P conformation and act as cation antagonists. PPIs are sulphur-containing compounds that need to be converted into a reactive species, namely a cyclic sulphenamide or a sulphenic acid, to be able to react with thiol groups on the surface of their protein target [60]. This conversion is insured by successive protonations of the PPI molecule which can only occur in the very acidic environment of the lumen of the parietal cells (Fig. 4A). Once activated, PPIs can form disulphide bonds with thiol groups on the H^+,K^+-ATPase, forming a covalent complex with the pump which in turn is irreversibly inhibited, forming a so-called suicide complex [60]. Cross-linking and mutagenesis studies identified several cysteines in the transmembrane region of the H^+,K^+-ATPase potentially involved in PPI binding: Cys321, Cys813, Cys822, and Cys892 (residue numbering according to the human H^+,K^+-ATPase sequence) [103,104]. Attempts to use molecular docking of the PPI molecule on the H^+,K^+-ATPase lumenal surface have previously been presented based on homology models of the H^+,K^+-ATPase using SERCA structures as templates, however using other conformations than the E2P-form and manual manipulations [105].

Here we propose a new homology model of the H^+,K^+-ATPase in the E2P state based on the newly available structure of the Na^+,K^+-ATPase [25] (Fig. 4B). Because of the high sequence identity between the gastric H^+,K^+-ATPase and the Na^+,K^+-ATPase, this new model enables a more precise localization of the cysteines possibly involved in PPI binding (Fig. 4B and C). As shown in Fig. 4C, Cys813 appears as the best candidate for PPI binding since it is lying at the end of helix TM6, right in the middle of the lumenal cavity, at the exit of the cation pathway. Mutagenesis studies also indicated that Cys813 could be the predominant residue involved in PPI interaction since it binds to all PPIs, whereas some other cysteines are PPI-specific (such as Cys822 which can crosslink with pantoprazole [60]). Mutagenesis studies have been mainly focused on cysteine residues directly participating in the covalent linking of PPIs and no other residues possibly stabilizing the PPI molecule within its binding pocket have been identified so far. Due to the formation of a covalently bound complex, a docking model of PPIs on the H^+,K^+-ATPase is a quite challenging task, and several different approaches have been investigated here in the attempt to model the binding of omeprazole to the pump molecule. Each of the activated forms of omeprazole, the sulphenic acid and the cyclic sulphenamide, have been docked into the homology model of the H^+,K^+-ATPase using the two isomeric structures shown in Fig. 4A, reflecting the possible different placement of the methoxy group due to stereochemical scrambling in the spiro-intermediate. Omeprazole sulphide, a metabolite of omeprazole, has been shown to reduce the inhibition of the H^+,K^+-ATPase by omeprazole and reduce the omeprazole labelling of the pump [103]. However, the sulphide does not inhibit the pump itself. It has therefore been suggested that this compound competes with the activated form(s) of omeprazole for a binding site on the pump prior to formation of the covalent bond between the activated form of omeprazole and the protein [103]. To investigate if a binding site for the protonated sulphide could give some hints on the binding site for activated forms of omeprazole, this compound has also been docked. The molecular docking attempts resulted in a clear picture: all poses placing the sulphur atom of the ligand in close proximity to the nucleophilic Cys813 have the omeprazole-fragment binding behind helices TM1 and TM2. Fig. 4D and E shows the two possible orientations of omeprazole in the identified binding site within the lumenal side of the ATPase based on the covalent docking calculations. The PPI molecule is located in the groove that is just on top of the cation exit pathway towards the lumenal side of the membrane. In this configuration, PPIs would act through the exact same mechanism as CTS on the Na^+,K^+-ATPase by directly blocking the access of K^+ ions to the cation binding site, therefore trapping the H^+,K^+-ATPase in an inactive E2P state. A clear preference in the docking poses generated is found for the model displayed in Fig. 4E. In this model, omeprazole is covalently bound to the Cys813 side chain placing the benzimidazole ring system behind helices TM1 and TM2. The other identified binding mode for the

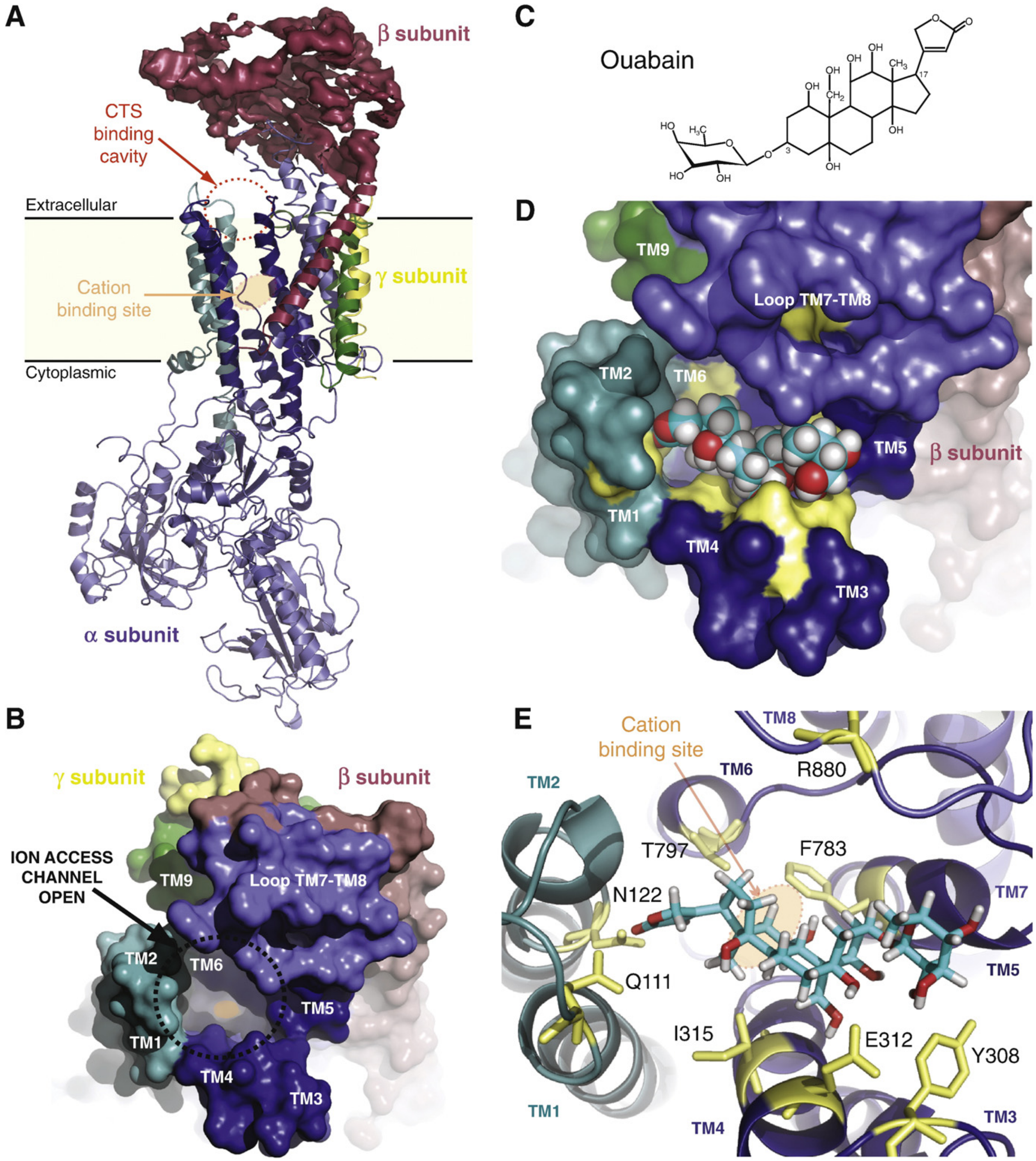
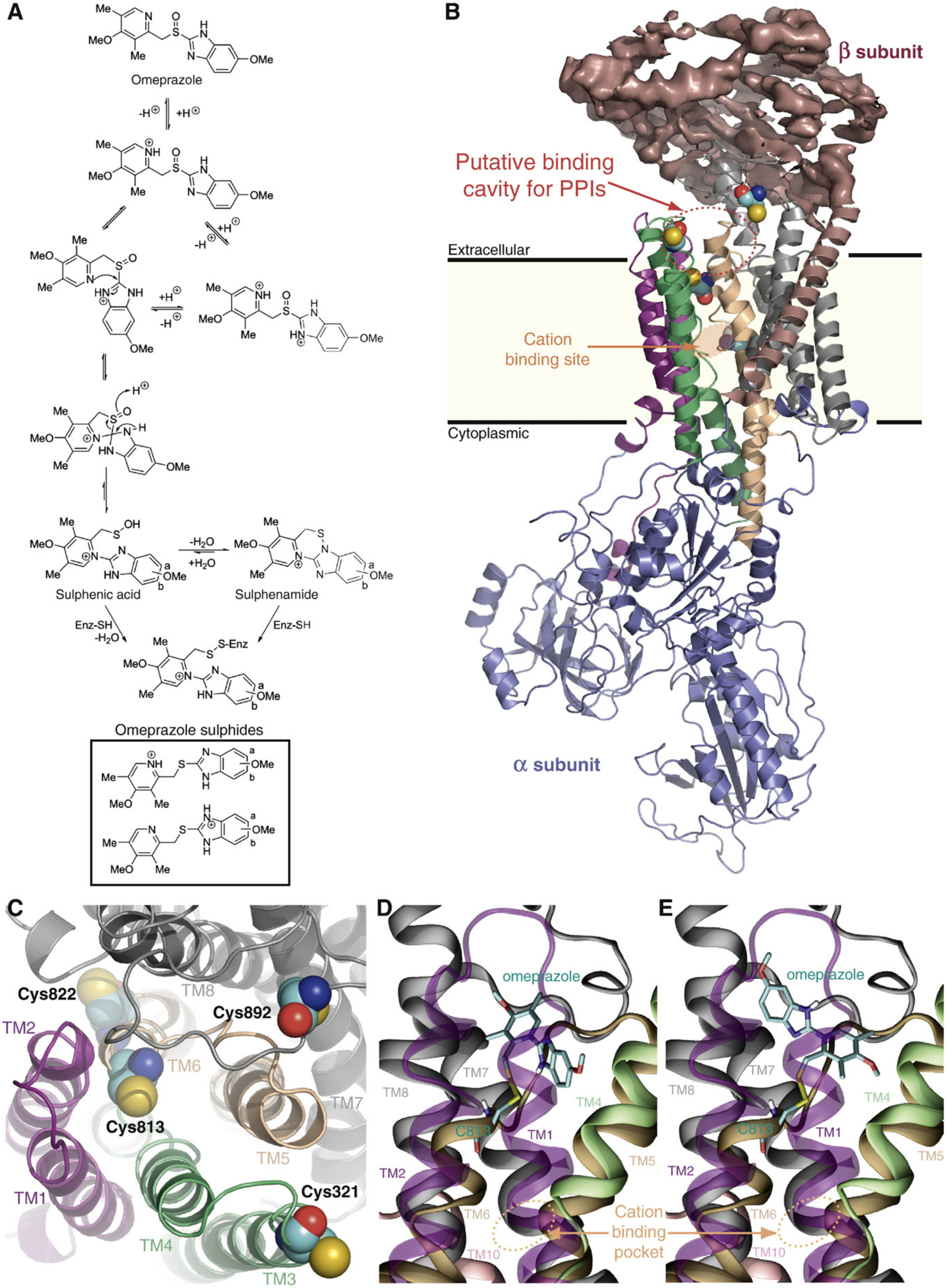


Fig. 3. Binding model of ouabain into an homology model of the E2P state of the pig renal Na$^+$,K$^+$-ATPase. To build the E2P homology model of the Na$^+$,K$^+$-ATPase, the structure of the [Rb]$_2$E2·MgF$_4^{2-}$ form of the \raster(100%,p)="figure3"pig renal Na$^+$,K$^+$ ATPase [25] was manually combined with the observed conformation of the Ca^{2+}-ATPase in the open E2·BeF$_3^-$ form [24]. The invariant regions between these two E2 states were initially deduced by comparison of the Ca^{2+}-ATPase structure in the E2·BeF$_3^-$ and in the [H$_{2-3}$]E2·AlF$_4^-$ forms using the program ESCET [139]. Of particular importance, the region consisting of the transmembrane helices TM6 to TM10 was found to be significantly invariant. The E2·BeF$_3^-$ structure of the Ca^{2+}-ATPase (representing the genuine E2P state) was then superimposed with the [Rb]$_2$E2·MgF$_4^{2-}$ form of the Na$^+$,K$^+$-ATPase on the TM6 to TM10 invariant region. The transmembrane helices TM1–TM2 and TM3–TM4 and the A domain from the Na$^+$,K$^+$-ATPase were then superimposed on the Ca^{2+}-ATPase E2·BeF$_3^-$ structure to impose the E2P state of the Na$^+$,K$^+$-ATPase (assuming a conserved mechanism of lumenal/extracellular opening in the E2P state). The connecting loop regions were manually aligned in O [140]. (A) General view of the E2P model of the Na$^+$,K$^+$-ATPase. The approximate location of the membrane is indicated. (B) View of the transmembrane region of the pig renal Na$^+$,K$^+$-ATPase in the E2P state from the extracellular side of the membrane. The cation binding site (positioned according to the [Rb]$_2$E2·MgF$_4^{2-}$ structure [25]) is visualized in orange down the open cation exit pathway. (C) Ouabain molecule. (D) Binding model of ouabain within the extracellular cavity on the surface of the Na$^+$,K$^+$-ATPase E2P homology model. The ouabain molecule has been docked manually according to mutagenesis studies. The residues described as important for CTS binding are shown in light yellow on the surface of the protein. (E) Same as D but with cartoons and sticks representations to visualize the possible interactions of residue side chains with the ouabain molecule.


A
Omeprazole
-H⊕
+H⊕
Sulphenic acid
-H₂O
+H₂O
Sulphenamide
Enz-SH
S-Enz
Omeprazole sulphides
B
β subunit
Putative binding cavity for PPIs
Extracellular
Cation binding site
Cytoplasmic
α subunit
C
Cys822
TM8
Cys892
TM2
TM6
TM7
Cys813
TM5
TM1
Cys321
TM4
TM3
D
omeprazole
TM7
TM8
TM4
C813
TM1
TM5
TM2
TM6
TM10
Cation binding pocket
E
omeprazole
TM7
TM8
TM4
C813
TM1
TM5
TM2
TM6
TM10

covalently linked omeprazole-fragment has an almost 180° rotation of the two aromatic ring systems, placing the benzimidazole ring near helix TM4. The inhibition mechanism of the two binding modes will be the same, as the omeprazole-derivative sits in the same binding pocket, only interchanging the two aromatic groups, and effectively blocking the ion-transfer pathway. The inhibitory mechanism may also be due to the possibility that omeprazole bound in this cavity can obstruct the conformational changes needed when going from E2P to E2·P_i. It is interesting to note that a partly overlapping cavity has been proposed as the binding site for PCABs, the other class of H^+,K^+-ATPase inhibitors [106].

More certain understanding of the mechanism of PPI inhibition will require an atomic description of the inhibitor–pump complex through high-resolution 3D structures combined with studies of the dynamics and function of the protein. Nevertheless, all studies and models proposed so far tend to conclude that PPIs, and possibly PCABs, inhibit the H^+,K^+-ATPase by blocking the re-entry of K^+ ions from the lumenal side of the membrane, either by a direct obstruction of the channel or by inducing conformational changes within the pump that close the extracellular gate towards the stomach lumen.

4.4. Plasma membrane Ca^{2+}- and H^+-ATPases: autoinhibited pumps at the cell surface

In addition to the above discussed Na^+,K^+- and H^+,K^+-ATPases, two other P-type ATPases are localized in the plasma membrane, namely the plasma membrane Ca^{2+}-ATPase (P_{2B}-type) and the plasma membrane H^+-ATPase (P_3-type). Plasma membrane Ca^{2+}-ATPases are found in most eukaryotic cells where they serve important roles in intracellular calcium regulation by being responsible for extrusion of calcium from cells [107]. Plasma membrane H^+-ATPases are restricted to fungi and plants, where they are essential and function as physiological analogs to the animal Na^+,K^+-ATPase by generating the electrochemical gradients required for nutrient uptake through secondary active transport systems [35].

Interestingly, P_{2B} and P_3 pumps share the same principle of regulation. Typically, their cytoplasmically located carboxy-termini are extended (although plant P_{2B}-type ATPases have extended amino-termini) and interact with the catalytic machinery of the pump to down-regulate its activity in this way serving as built-in inhibitors. Peptides have been designed that mimic the action of the autoinhibitory domains [108,109], but obviously such peptides have to act from the cytoplasmic side of the membrane obscuring their therapeutic potential. Peptides that bind to the autoinhibitory domain have not been described yet, but it can be predicted that at least some of these would act as pump activators.

A number of chemicals modulate the activity of P_{2B} [110] and P_3 [111–113] pumps, but in most cases their mechanism of action is from the cytoplasmic side (e.g. eosin) or uncertain (e.g. juglone). Other low molecular weight compounds target P_{2B} and P_3 pumps indirectly by perturbing the lipid bilayer [114,115] or by interfering with the regulatory machinery that controls auto-inhibition [116–118]. In addition, fungal P_3 pumps are targeted by compounds such as omeprazole [119] and ebselen [120], which are unspecific in that they also affect the gastric H^+,K^+-ATPase.

Screening of peptide libraries has become an interesting new approach to design inhibitors that target P_{2B} [121] and P_3 [122] ATPases from the cell surface. Cysteine bridge-constrained random peptide libraries have been designed for isolation of peptides that bind specifically to e.g. extracellular portions of plasma membrane Ca^{2+}-ATPase [123]. Peptide libraries with carboxy-terminal triarginine motifs to concentrate library members at the fungal cell exterior have been employed in case of the plasma membrane H^+-ATPase [124]. Although the results of peptide-based approaches are still preliminary, promising new drug leads have been discovered this way and await further studies.

5. Concluding remarks

The acknowledgement of P-type ATPases as versatile proteins has increased the interest in their specific inhibitors in the last few years. Regular papers on this subject were supplemented by reviews, but each publication was devoted to one particular enzyme. Tadini-Buoninsegni et al. [81] for example discussed in detail different mechanisms of inhibition of Ca^{2+}-ATPase, while Mijatovic et al. [125] explored the applicability of cardiotonic steroids in cancer therapy. The aim of the current publication is to summarize information about the interactions between several representatives of the P-type ATPase family with their respective inhibitors, deduced from the crystallographic data, and to present some guidelines for the development of the new generations of drugs, able to modulate (or shut down) exclusively their target.

The high selectivity of the drug, i.e. its ability to bind to a particular protein in a particular tissue, is crucial for its pharmacological applicability. The problem with CTS medication is a perfect illustration of this issue: despite their curative properties, their overall toxic effects may lead to severe poisoning and eventual death of the patients [126], limiting pharmacological use of cardiotonic steroids.

Thus, the major strategies for the design of inhibitors of such important and wide-spread enzymes are: 1) synthesis of tissue (or organ)-specific drugs; 2) development of isoform-specific drugs.

In the first case a pro-drug is converted into an active form either by specific environment or tissue-specific enzymes. This approach has proven successful and resulted in the targeted delivery of omeprazole and thapsigargin towards restricted pools of H^+,K^+-ATPase and Ca^{2+}-ATPase, respectively. Here the knowledge of physiology and metabolism of the particular cells is decisive. In addition, the structural data on the enzyme–inhibitor complex are

Fig. 4. Binding model of omeprazole into a homology model of the E2P state of the gastric H^+,K^+-ATPase. The above E2P model of the Na^+,K^+ ATPase was used as template structure in MODELLER [141] to generate a model of the E2P state of the human gastric H^+,K^+ ATPase. Out of 100 models generated, the one with the lowest Discrete Optimized Protein Energy (DOPE) score [142] was used for docking simulations with omeprazole. (A) Biochemical activation and inhibition mechanism of omeprazole. The first step, which is the cause of accumulation of omeprazole in the lumen of the parietal cell, is protonation of the nitrogen atom in the pyridine moiety. Subsequently follows a protonation of the benzimidazole moiety which increases the electrophilicity of the C-2 carbon. A spiro intermediate is then formed that collapses into the sulphenic acid. The cyclic sulphenamide is formed from the sulphenic acid by dehydration. Both the sulphenic acid and the sulphenamide can react with accessible thiols on the luminal surface of the H^+,K^+-ATPase [143]. The structure of omeprazole sulphide is also indicated. (B) General view of the E2P model of the H^+,K^+-ATPase. The approximate location of the membrane is indicated. (C) View of the transmembrane region of the human gastric H^+,K^+-ATPase in the E2P state from the lumenal side of the membrane. The four cysteines possibly involved in the binding of PPIs according to crosslinking studies are shown as van der Waals representation. (D) and (E) Tentative binding models of omeprazole within the extracellular cavity on the surface of the H^+,K^+-ATPase E2P homology model created by covalent docking. The putative position of the cation binding site (shown in orange) is inferred from the position of the two K^+ ions within the Na^+,K^+-ATPase $[Rb_2]E2·MgF_4^{2-}$ state [25]. The homology model was prepared for docking calculations using the Protein Preparation Wizard in the Schrödinger 2007 Suite (Schrödinger, LLC, New York; NY, 2007) and the ligands were drawn in Maestro 8.0 (Schrödinger, LLC, New York; NY, 2007) with charges as indicated in panel A. The structures were minimized with the MMFFs force field as implemented in MacroModel 9.5 (Schrödinger, LLC, New York; NY, 2007). To localize the global minimum, a conformation search was conducted using the Torsional Sampling, MCMM method in MacroModel 9.5. Molecular docking simulations were performed using Glide version 4.5 from Schrodinger Inc (Schrödinger, LLC, New York; NY, 2007) using the SP scoring function [144]. The possibility of performing covalent docking in Prime 2.0 (Schrödinger, LLC, New York, NY, 2008) was also attempted. After covalent docking, a minimization (PRCG algorithm, 15,000 steps) was conducted using the OPLS2005 force field [145,146] and an implicit water model. The majority of the generated poses for all compounds binds as shown in panel E. Structures shown in D and E are after minimization of the covalently docked poses. The orientation of the protein is rotated approximately 90° compared to B. TM1 and TM2 are shown in purple; TM4 in green; TM5 and TM6 in brown; TM7 and TM8 in grey; and TM10 in pink. For clarity, TM1 and TM2 are transparent. Only non-polar hydrogen atoms are shown. All structures representations in this review have been drawn in PYMOL [147].

extremely useful since they reveal the sites where derivatization induces minimal disturbances in the affinity. The potentials of such strategies in cancer treatment are evident.

The second approach is even more challenging. It implies that the inhibitor interacts selectively with one isoform of the enzyme, leaving the other molecules intact. No examples are known so far, because the design of such an inhibitor demands a detailed knowledge of the minute structural differences between isoforms.

The above strategies are applicable when the targeted enzyme has a specific inhibitor. However, some of the P-type ATPases, being potentially attractive drug targets, have no specific inhibitors or modulators so far. In these cases, the homology between the enzymes may be advantageous. It is obvious that the common transport mechanism stipulates similar scaffolding of the enzymes. The recent determination of the three-dimensional structures of the Na$^+$,K$^+$- and H$^+$-ATPases exhibited overall resemblance with the Ca^{2+}-ATPase and suggested that all P-type ATPases share a similar domain organization. It is most likely that also the spatial position of the ion access channels is conserved throughout the family. Binding of a substance within these channels or in their vicinity will disturb ion binding (or the conformational transitions following that binding) and, therefore, interfere with the pump cycle. The crystallographic structures of the enzyme–inhibitor complexes, as well as docking studies described in this review, show that this is apparently a common mechanism of inhibition of P-type ATPases. It seems also to be a rather efficient way of blocking the enzymes, specially for plasma membrane embedded proteins, since the “pharmacological activity” of the inhibiting substance does not depend on its ability to permeate the membrane. Additionally, multidrug transporters, that often abolish the effect of drugs by effectively extruding them from cells, cannot circumvent the action of compounds that act from the outside of cells.

Crystallization of the enzyme of interest and detailed mapping of its extracellular surface in order to find a site for specific “wedging” may be the first step in the design of a new synthetic highly specific inhibitor. This structure-based drug design strategy will compete with the traditional drug screening schemes in terms of time and economic costs. Constant development and standardization of the membrane protein crystallization methods will turn the scale in favor of the structural approach.

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