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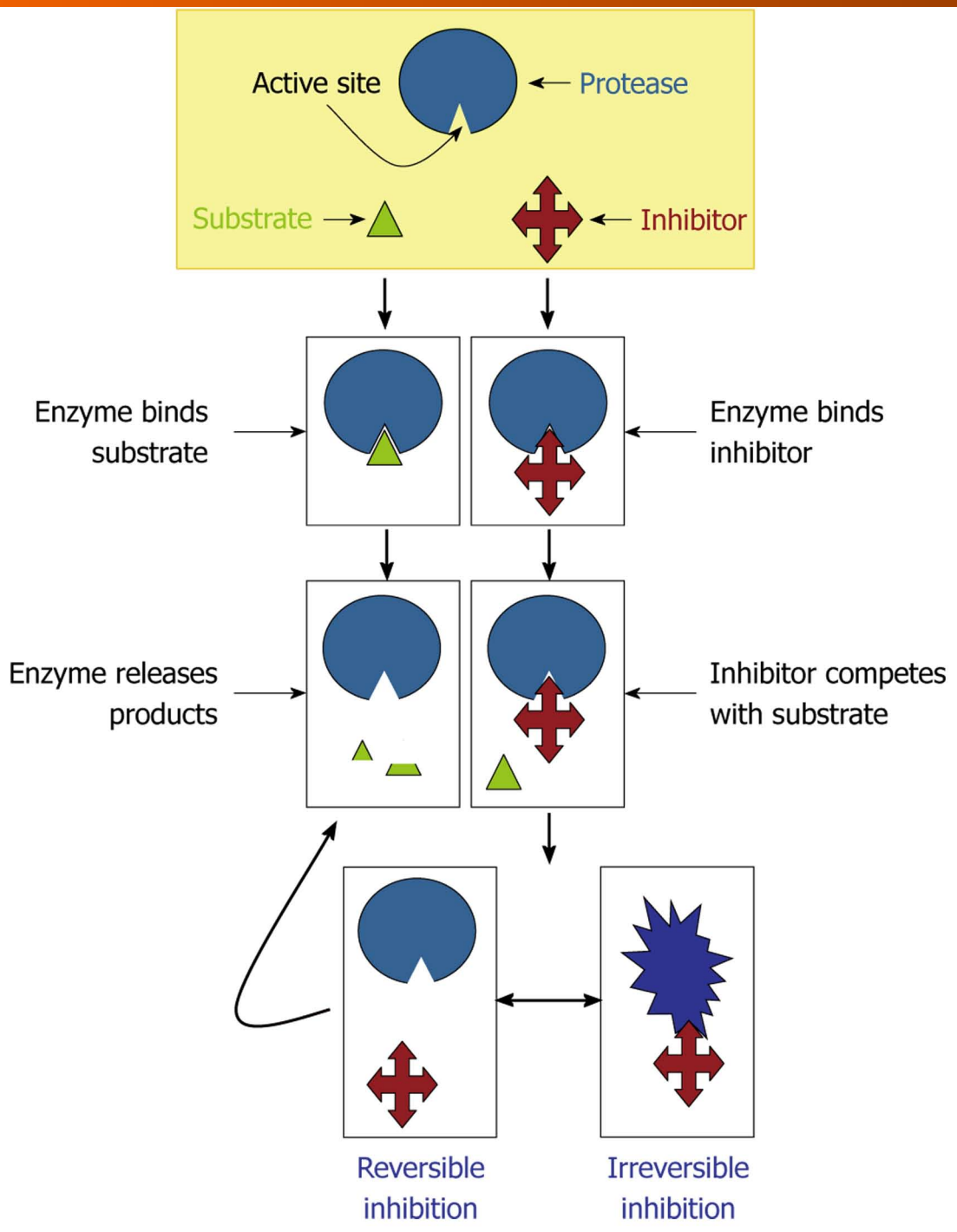

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TOPIC HIGHLIGHT

Emanuel E Strehler, PhD, Professor, *Series Editor*

Allosteric inhibitors of plasma membrane Ca^{2+} pumps: Invention and applications of caloxins

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Abstract

Plasma membrane Ca^{2+} pumps (PMCA) play a major role in Ca^{2+} homeostasis and signaling by extruding cellular Ca^{2+} with high affinity. PMCA isoforms are encoded by four genes which are expressed differentially in various cell types in normal and disease states. Therefore, PMCA isoform selective inhibitors would aid in delineating their role in physiology and pathophysiology. We are testing the hypothesis that extracellular domains of PMCA can be used as allosteric targets to obtain a novel class of PMCA-specific inhibitors termed caloxins. This review presents the concepts behind the invention of caloxins and our progress in this area. A section is also devoted to the applications of caloxins in literature. We anticipate that isoform-selective caloxins will aid in understanding PMCA physiology in health and disease. With strategies to develop therapeutics from bioactive peptides, caloxins may become clinically useful in cardiovascular diseases, neurological disorders, retinopathy, cancer and contraception.

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Key words: Phage display; Calcium signaling; Plasma membrane Ca^{2+} pumps; Hypertension; Neuronal disorders; Contraception; Cancer; Thrombosis; Lipid rafts

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INTRODUCTION

This review focuses on the concepts behind the invention of a novel class of plasma membrane (PM) Ca^{2+} pump (PMCA)-specific inhibitors called caloxins. PMCA is a high affinity Ca^{2+} removal system found in all mammalian cells. Regulation of Ca^{2+} concentration in the cytosol (Ca^{2+}i) is essential for cell survival and signal transduction. In this context, we provide an overview of membrane Ca^{2+} transport proteins responsible for control of Ca^{2+}i and the need for PMCA-specific inhibitors to delineate its role in Ca^{2+}i homeostasis and signaling. We introduce the extracellular domains (exdoms) of PMCA as allosteric targets to obtain prototype caloxins that have been used to study PMCA physiology. More recently, the importance of genetic diversity of PMCA isoforms is becoming obvious. Therefore, we have started to develop isoform-selective caloxins. We briefly outline the importance of various PMCA isoforms in health and disease and describe the isoform-selective caloxins ob-

tained to date. We conclude with the potential of caloxins as research and therapeutic tools in the field of PMCA physiology and pathophysiology.

MAJOR PLAYERS OF CALCIUM DYNAMICS

Ca^{2+} is a key intracellular signaling molecule which controls different cellular processes in various cells. In a resting cell, $Ca^{2+}i$ is kept low at around 0.1 μmol/L, which is 10,000-fold lower than its concentration outside the cell. Since the membrane potential is negative inside the cell, the large electrochemical gradient allows Ca^{2+} entry through pathways in the PM that open during cell excitation[1-4]. The Ca^{2+}entry upon cell stimulation involves a number of Ca^{2+} channels and reverse mode Na^{+}-Ca^{2+}-exchanger (NCX) in the PM. Ca^{2+} is also stored at high concentration in the sarco/endoplasmic reticulum from where it can be released by Ca^{2+} channels. In addition, other organelles, like mitochondria and Golgi bodies, may also store and release Ca^{2+} into the cytosol. Following the completion of the signaling event, $Ca^{2+}i$ has to be lowered to the resting levels. The lowering of $Ca^{2+}i$ in the cytosol occurs mainly by its sequestration into the sarco/endoplasmic reticulum or its extrusion from the cell. Ca^{2+} sequestration into the sarco/endoplasmic reticulum is carried out by the sarco/endoplasmic reticulum Ca^{2+} pumps (SERCA). Ca^{2+} extrusion from the cells can be carried out by PMCA and forward mode NCX. NCX uses the electrochemical gradient of Na^{+} to extrude Ca^{2+} from the cells with a low affinity (Km for Ca^{2+} = 1-10 μmol/L). PMCA uses the energy derived from ATP hydrolysis to expel Ca^{2+} from cells with a high affinity (Km for Ca^{2+} = 0.2-0.5 μmol/L). Thus, PMCA is the only high affinity Ca^{2+} extrusion mechanism found in eukaryotic cells which may play a key role in long-term regulation of $Ca^{2+}i$. PMCA act as dynamic regulators of $Ca^{2+}i$ involved in both short-term signaling events as well as long-term cellular processes like cell growth and differentiation. PMCA are crucial to cell survival and signaling. However, it is difficult to study the exact role of PMCA in a cell due to the presence of other $Ca^{2+}i$ lowering systems like NCX and SERCA. Specific inhibitors are needed to understand the role of PMCA in various cell types in health and disease.

CALOXINS - A NOVEL CLASS OF ALLOSTERIC INHIBITORS OF PMCA

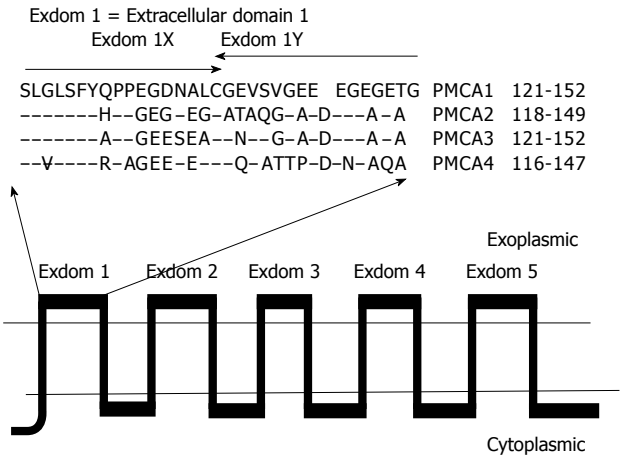
Allosteric sites have been exploited as pharmacological targets because of their greater specificity than the active sites of enzymes and receptors[5-7]. Digoxin and ouabain are allosteric inhibitors of Na^{+}-pump which have proven useful as research and therapeutic tools[8,9]. Similarly, SERCA pump inhibitors, such as thapsigargin, have led to the exploration of the role of this protein in signal transduction[10,11]. In contrast, inhibitors based on regulatory and active sites with consensus sequences conserved among various proteins may not be specific. For example, vanadate and eosin are currently used as inhibitors to study PMCA[12-15]. Vanadate is a phosphate analog that competes for ATP binding in the catalytic domain of all ATPases. It has much higher affinity for the Na^{+}-pump than for PMCA. Decavanadate also inhibits a variety of ATPases and other proteins[16]. Similarly, eosin is also not PMCA specific as it acts by interfering with the binding of ATP to a conserved site found in all ATPases.

Therefore, PMCA specific inhibitors are required to understand the role of PMCA in Ca^{2+} homeostasis and cell signaling.

We pioneered the concept of caloxins: short peptides that specifically inhibit PMCA by binding to the allosteric sites on the protein. The exdoms, which are short loops connecting the transmembrane helices of PMCA on the extracellular surface, were chosen as allosteric targets to obtain caloxins. PMCA have 5 exdoms and the regulatory and active sites of PMCA are all cytosolic. The first reason for choosing exdoms was that the exdom sequences of PMCA do not have significant homology with other proteins[2,17,18]. The second reason was that thapsigargin inhibits SERCA by binding to its luminal loops which have been shown to undergo conformational changes during its reaction cycle and the exdoms of PMCA would be similar in function to luminal loops of SERCA[10,11,19]. Also, exdom 1 of Na^{+}-K^{+}-ATPase is the allosteric site involved in its inhibition by ouabain[20]. Hence, exdoms of PMCA were chosen as the allosteric sites to which caloxins would bind and specifically inhibit PMCA when added extracellularly.

INVENTION OF CALOXIN 2A1

The first caloxin to be invented was caloxin 2a1[21]. A phage library displaying 12-amino acid random peptides fused to its coat protein was screened by a process called panning for binding to a target[17]. The target was a synthetic peptide corresponding to the exdom 2 sequence (Figure 1) of PMCA1 conjugated to carrier protein keyhole limpet hemocyanin or ovalbumin. The target peptide conjugated to keyhole limpet hemocyanin was immobilized by passive adsorption to the wells of a plastic microtiter plate. The phage library in solution was allowed to bind to the target in the well. The wells were washed extensively to remove the non-specific phage. This was followed by elution of the target-bound phage with a solution of target peptide conjugated to ovalbumin. The eluted phage was amplified and the above screening process repeated for 8 cycles. Following the last round of phage screening, the consensus peptide sequence VSNSNWPSFPSS was selected. This peptide was chemically synthesized with addition of GGG sequence at its C-terminus to obtain caloxin 2a1 (VSNSNWPSFPSSGGG-amide). GGG sequence is found as a short spacer between the variable peptide sequence and the coat pro-



Caloxin nomenclature
The first number is the targeted exdom
The letter is based on how it was obtained:
a: Panning
b: Panning and PMCA affinity chromatography
c: Mutagenesis of b
d: Bidentate caloxin
The last number shows the chronological order of the invention

Figure 1 Extracellular domains (exdoms) in human plasma membrane Ca^{2+} pump protein. Sequences of exdom 1 of human plasma membrane Ca^{2+} pump protein (PMCA)1 to 4 are also compared (Swiss protein bank accession #P20020, Q01814, Q16720, P23634). Exdom 1 is long and contains a C in the middle. It was divided into two segments: exdoms 1X and 1Y around the C.

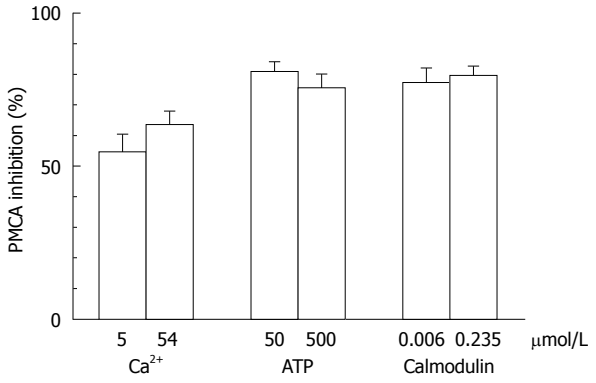


Figure 2 Caloxin 2A1 inhibition of plasma membrane Ca^{2+} pump ATPase in the erythrocyte ghosts is non-competitive with respect to Ca^{2+}, ATP and calmodulin[22]. The percent inhibition with caloxin 2a1 (1.8 mmol/L) did not differ significantly between low or high concentration of Ca^{2+}. Similarly, concentrations of ATP and calmodulin did not influence the percent inhibition with caloxin 2a1. The low and high concentrations compared for each ligand are shown. Concentrations of all the other ligands were saturating in each experiment. Details of the experiments are available in[22].

tein of the phage. Caloxin 2a1, at a concentration of 0.4 ± 0.1 mmol/L, produced 50% inhibition of Ca^{2+}-Mg^{2+}-ATPase activity of PMCA in the human erythrocyte ghosts. It also inhibited the formation of Ca^{2+}-dependent acylphosphate in a partial reaction of PMCA catalytic cycle. Caloxin 2a1 did not inhibit any other ATPases tested. Consistent with being an allosteric inhibitor, caloxin 2a1 inhibited the PMCA ATPase in a manner non-competitive with respect to Ca^{2+}, ATP and calmodulin (Figure 2)[22]. Screening protocol similar to that used for caloxin 2a1 resulted in affinity selection of caloxins 1a1 and 3a1 for binding to synthetic exdom 1 and 3 respectively[23,24]. The caloxins invented by screening phage display libraries using synthetic exdoms as targets were termed series A caloxins (see Figure 1 for nomenclature).

APPLICATIONS OF CALOXIN 2A1

Caloxin 2a1 has been used to answer a number of questions concerning PMCA action. It was used to investigate a long standing hypothesis about the Ca^{2+} transport mechanism of PMCA. PMCA had been suggested to exchange Ca^{2+} for proton(s) so as to remain electroneutral[18,25]. Increase in Ca^{2+}i in cultured mouse cerebellar granule cells induced by phytotoxin (Palytoxin) or glutamate receptor activation was shown to be accompanied by intracellular acidification. This acidification was blocked with caloxin 2a1, suggesting that it was related to proton influx that accompanied Ca^{2+} removal by PMCA[12,13]. The synchronous activation of CA1 pyramidal neurons of hippocampus was associated with a rapid increase in extracellular pH. The pH transients at the extracellular surface were measured using a single neuron or neuronal population. Caloxin 2a1 inhibited the extracellular alkaline transient observed in single neurons or neuronal population. This provided direct evidence that the countertransport of proton(s) by PMCA generates extracellular alkaline shifts observed upon synchronous activation of a neuronal population[26].

Caloxin 2a1 has helped in determining the role of PMCA in Ca^{2+}i oscillations which are a mode of signaling in both excitable and non-excitable cells. The extracellular Ca^{2+} sensing receptor translates the changes in extracellular Ca^{2+} to the cell interior *via* oscillatory Ca^{2+}changes. HEK293 cells expressing the extracellular Ca^{2+} sensing receptor elicited Ca^{2+}i oscillations upon receptor activation. PMCA-mediated Ca^{2+} extrusion was essential in reinforcing the receptor stimulation to maintain and modulate the periodicity of Ca^{2+} oscillations which were eliminated in the presence of caloxin 2a1[27]. The role of PMCA in generation of spontaneous Ca^{2+}i oscillations was also shown in human bone marrow-derived mesenchymal stem cells. Caloxin 2a1 inhibited the oscillations as did the non-selective inhibitor, carboxyeosin. However, caloxin 2a1 induced a Ca^{2+}i transient followed by the return of Ca^{2+}i to basal levels. In contrast, carboxyeosin markedly increased the basal Ca^{2+}i before stopping the oscillations. The difference in the action of the two agents is consistent with PMCA-specific inhibition by caloxin 2a1 leading to an initial increase in Ca^{2+}i which is then lowered by other Ca^{2+}i removing systems. In contrast, the increase in basal Ca^{2+}i by carboxyeosin can be explained by its non-specific effects on sodium pump or SERCA, that in turn can affect Ca^{2+} influx or uptake into the intracellular Ca^{2+} pool[14]. Similar differences in the effects of caloxin 2a1 and carboxyeosin have been observed in other cell types like mouse embryonic stem cells[28].

Caloxin 2a1 has also been used to study the effect of PMCA inhibition in vascular tissues. Consistent with the inhibition of PMCA in vascular endothelium, caloxin 2a1 produced an endothelium-dependent relaxation that was reversed by N(G)-nitro-L-arginine methyl ester[21]. Thus caloxin 2a1 is the first known PMCA selective inhibitor. Despite its low affinity for PMCA, it has been used to study PMCA physiology in various tissues and cell types.

GENETIC DIVERSITY IN PMCA

PMCA are encoded by four genes: PMCA 1-4. Alternative splicing of the primary gene transcripts results in a large number of PMCA variants which differ in their regulatory and kinetic properties, as reviewed elsewhere[1,2,29-32]. Here, we focus on the diversity observed in the expression of the PMCA isoforms 1-4 in various tissues and cell types and the differences in their subcellular localization. PMCA1 and 4 are most widely expressed while PMCA2 and 3 have tissue-specific distribution.

PMCA isoform expression varies in a cell-type dependent manner in various tissues, e.g. in coronary arteries, the endothelial cells express mainly PMCA1 whereas the smooth muscle cells express more PMCA1 than PMCA4[33] (unpublished). Similarly, various regions of the brain differ in their expression of the four PMCA genes[34,35]. The frontal cortex expresses high levels of all four PMCA genes; the hippocampus expresses PMCA genes 1, 3 and 4 and the cerebellum has higher levels of PMCA2 and 3[34-36]. Cell-type specific expression of PMCA isoforms has also been observed in different classes of retinal neurons that differ in their signaling. The neurons can signal with graded potentials involving sustained elevation of Ca^{2+}i (photoreceptors, horizontal and bipolar cells), action potentials involving Ca^{2+} transients (ganglion cells) or both (amacrine cells). PMCA1 is expressed in photoreceptors, horizontal cells and cone bipolar cells. PMCA2 is found in rod bipolar cells and it is coexpressed with PMCA3 in amacrine and ganglion cells. In stratified corneal epithelium, there are differences in the PMCA isoforms expressed in the different layers of cells[37,38]. Thus, the PMCA isoforms exhibit tissue and cell-specific expression that may reflect differences in their Ca^{2+}i handling requirements.

The PMCA isoforms may exhibit differential PM localization within a single cell type. The spatially distinct demands of Ca^{2+} influx and efflux observed especially in polarized cells like epithelial and neuronal cells may determine the differential distribution of PMCA isoforms in the PM. Duodenal mucosa cells express PMCA1, which is localized in the basolateral membrane. This allows the transcellular transport of luminally absorbed Ca^{2+}, which is pumped out of the basolateral membrane into the interstitial space[39]. In some instances, PMCA may be localized in lipid rafts which are cholesterol/sphingolipid-rich microdomains of PM or in a specialized subset of lipid rafts called caveolae[40,41]. Caveolae are small (50-100 nm) invaginations of PM that are enriched in the protein caveolin. They are rich in receptors, channels, signal transducers, effectors and structural proteins and may act as Ca^{2+} signaling microdomains[42]. It is not known if the PMCA isoforms localized in these specialized PM domains are different from those present in non-caveolar PM. PMCA4b has been shown to localize in caveolae where it can interact with neuronal NO synthase and regulate its activity[43]. The functional significance of differences in the distribution of PMCA isoforms observed at tissue, cell or subcellular levels needs to be determined and would require the isoform specific inhibitors of PMCA.

PMCA ISOFORMS AND DISEASE

Changes in the levels of expression or activity of various PMCA isoforms have been associated with several pathologies including heart disease, hypertension, carcinogenesis, cataract formation, diabetes and neurodegenerative diseases[44-47]. Gene targeting studies have shown that PMCA1 null mice are embryolethal suggesting an essential housekeeping role for PMCA1[44,45]. However, mice with heterologous PMCA1 ablation are normal. Studies on oral squamous cell carcinoma have suggested an epigenetic inactivation of PMCA1 gene as a frequent and early event during oral carcinogenesis[46]. Altered PMCA1 expression has also been observed in breast cancer[47]. In vascular smooth muscle cells, the regulation of PMCA1 expression by the transcription factor c-Myb may control cell proliferation[48]. An increase in the level of PMCA1 expression is associated with the loss of Ca^{2+}i homeostasis observed in human cataract lenses[49,50]. Studies using cultured human lens epithelial cell line have shown that oxidative stress, a major contributor to cataract development, also induces changes to the level of expression of PMCA1.

The changes in the levels of PMCA2 have been reported in several diseases including hearing defects, multiple sclerosis, spinal cord injury, cancer and cataract. PMCA2 null mice exhibit deafness and ataxia, whereas the heterozygous mice are predisposed to age or noise related hearing loss[45]. The role of PMCA2 in hearing loss has also been verified in humans. The hearing loss in a family, caused by homozygous mutations in cadherin 23, was exacerbated in individuals heterozygous for a mutation in the PMCA2 gene[51]. Besides being a regulator of Ca^{2+} homeostasis, PMCA2 has been shown to be essential for Ca^{2+} secretion in milk[44]. An increase in levels of PMCA2 is seen during lactation, which decreases again upon weaning. An increase in levels of PMCA2 is also observed in breast cancer cell lines[47,52-54]. Its inhibitory interaction with calcineurin, and therefore of calcineurin-mediated apoptosis, suggests a regulatory role for PMCA2 in breast tumorigenesis. PMCA2 expression is also altered in cataract lenses as compared to age-matched clear lenses[55].

PMCA3 is expressed at high levels in choroid plexus and may thus play a role in regulating ionic composition

of the cerebrospinal fluid which is essential in brain function and development[31]. PMCA3 mRNA levels in the placenta correlate with neonatal bone mineral content, bone area, placental weight and birth weight suggesting that it may be crucial in calcium absorption by fetal bones[56]. Lack of availability of PMCA3 knockout animals has limited studies on PMCA3 physiology.

Despite the ubiquitous distribution of PMCA4, the major phenotype observed in null mice is male infertility due to loss of sperm hypermotility[44]. The role of PMCA in sperm motility has also been confirmed by carboxyeosin-mediated inhibition of PMCA in wild type mice[57]. Although PMCA is thought to play a less important role than NCX in excitable cells, a loss of PMCA4 impaired phasic contractions and caused apoptosis in the portal vein smooth muscle studied *in vitro* from some strains of mice[44]. PMCA4 may also play a more direct role as modulator of Ca^{2+} signaling pathways. PMCA4b overexpression in mice gave unexpected increase in arterial reactivity and increased blood pressure[58,59]. In cardiomyocytes, the overexpression attenuated the β-adrenergic inotropic response[60]. The observed results were due to a direct interaction of PMCA4 with the neuronal NO synthase leading to impairment of its NO synthase activity. PMCA4 may also regulate hypertrophy and heart failure. In human failing hearts, PMCA4 protein expression decreased by 60% as compared to the normal hearts. PMCA4 was also downregulated by 25% in hypertrophic mouse hearts following transverse aortic constriction[61]. Gene targeting of PMCA4 increased the susceptibility of hearts to hypertrophy, whereas its cardiac-specific inducible expression rendered it anti-hypertrophic in response to pressure overload. Association of PMCA4 with calcineurin and alteration of calcineurin-mediated hypertrophic response may be the underlying mechanism of PMCA4 regulated hypertrophy. Abnormal platelet Ca^{2+} homeostasis in diabetes mellitus is also associated with increase in the level of PMCA4 expression[62]. PMCA4 expression levels may change during cancerogenesis. Differentiation of HT-29 colon cancer cells was associated with an upregulation of PMCA4, whereas breast cancer cell lines show decrease in levels of PMCA4[47,54,63].

PMCA ISOFORM SELECTIVE CALOXINS: NEEDS AND CHALLENGES

The genetic diversity that exists in PMCA emphasizes the need for isoform-selective caloxins to understand PMCA physiology and pathophysiology. The invention of isoform-specific inhibitors, however, is an unprecedented task. The challenges in the invention of isoform-selective caloxins are discussed below.

The first challenge in the invention of isoform-selective caloxins is the identification of allosteric target sites in PMCA where ligand binding can produce inhibition and which are sufficiently diverse among the four PMCA gene products to confer isoform selectivity to the ligand. Our initial work shows that exdoms 1, 2 or 3 can be used as targets to obtain caloxins[21,23,24]. Of these, only the amino acid sequence of exdom 1 differs significantly among the proteins encoded by the four PMCA genes (Figure 1). Therefore, exdom 1 was chosen as a target to invent isoform-selective caloxins[33]. In addition, the alternative splicing of the four primary gene transcripts does not affect the exdom 1 sequence in the splice variants. Therefore, exdom 1 based isoform-selective caloxin would be expected to inhibit all the splice variants of the gene. However, exdom 1 is very long and has a cysteine residue in the middle. It is not known if the cysteine participates in any disulfide bonding affecting the conformation of the exdom. Therefore, exdom 1 has been arbitrarily divided into exdom 1X and 1Y around the cysteine. Synthetic peptides corresponding to each half are used as targets to screen for isoform-selective caloxins.

The second challenge is the development of appropriate protocols to screen the phage display random peptide libraries for binding to the target[19,33]. Series A caloxins have been obtained using only the synthetic exdom peptides as targets. In nature, the conformation of the exdom 1 in PMCA protein may be different from that in the synthetic peptides and hence the resulting caloxins would have low affinities and may not distinguish between different isoforms. This problem may be overcome by screening phage libraries using purified PMCA as the target. However, a major disadvantage of this method is the loss of specificity due to exdom recognition. Therefore, a two-step screening procedure was developed to affinity select phage-encoded peptides that retain exdom specificity and can bind the exdom in its native conformation in PMCA with high affinity. In the first step, synthetic exdom peptide is used as a target to screen the phage library by biopanning for 3-4 rounds to obtain a sub-library of phage clones showing some preference for binding to the synthetic exdom. In the second step, the sub-library is screened for binding to PMCA protein by affinity chromatography. This takes advantage of the ability of PMCA to bind to calmodulin only in the presence of Ca^{2+}. The phage is allowed to bind to PMCA immobilized on a calmodulin resin. PMCA-phage complex is then eluted in a solution containing Ca^{2+} chelator. The method can be further refined by introducing negative chromatography to eliminate selection of non-specific phage. The phage pool is pre-adsorbed with calmodulin resin alone or with immobilized PMCA that differs in its isoform type as compared to the target before use in screening by affinity chromatography. Phage copy number bias after screening can arise from methods used in library construction or by preferential amplification of certain phage clones in between the screening rounds. Therefore, following the two-step screening, the enriched phage pool was subjected to competitive screening by affinity chromatography. In competitive screening, equal plaque forming units of each type of phage clone in the enriched pool was allowed to compete for binding to PMCA to select a dominant clone based on its

affinity for the target. The resulting caloxins are termed series B caloxins and exhibit higher affinity with PMCA-isoform preference as compared to series A caloxins. The affinity and isoform selectivity of series B caloxins can be improved further by limited mutagenesis of series B caloxin to create a library and then screen it by affinity chromatography to obtain series C caloxins. The next major concept is to take advantage of the information that exdom 1 has been arbitrarily divided into exdom 1X and 1Y to be used as targets for screening as shown in Figure 1. The series C caloxins directed against exdom 1X and 1Y can be optimally linked to obtain series D bidentate caloxin. The bidentate caloxin is expected to have affinity and PMCA-isoform selectivity much higher than the either of the two partner caloxins.

The third challenge is to obtain PM source that is relatively rich in only one of the four PMCA isoforms. Human erythrocyte PM expresses mainly PMCA4 and pure PM can be obtained as erythrocyte ghosts[29]. Several tissues were tested to discover that PM of rabbit duodenal mucosa is rich in PMCA1 (unpublished). We have not yet tested tissues to identify a rich source for PMCA2 or 3. However, microsomes prepared from the insect cells overexpressing these isoforms have been used in biochemical assays for measuring the activity of PMCA2 and 3[64].

PMCA4 SELECTIVE CALOXINS

Caloxin 1b1 is a series B caloxin that was obtained by two-step screening using synthetic exdom 1X of PMCA4 and PMCA protein purified from erythrocyte ghosts as a target[33,64]. It inhibited the Ca^{2+}-Mg^{2+}-ATPase activity of PMCA in leaky erythrocyte ghosts that express mainly PMCA4 isoform with a Ki (inhibition constant) value of 46 ± 5 μmol/L - an affinity which was 10 × higher than that of the first reported series A caloxin 2a1. It was selective for PMCA4 as it inhibited the other PMCA isoforms with lower affinity: PMCA1 (105 ± 11 μmol/L), PMCA2 (167 ± 67 μmol/L), PMCA3 (274 ± 40 μmol/L). It did not inhibit any other ATPases tested. It increased the force of contraction produced by a submaximum concentration of phenylephrine in de-endothelialized rat aortic rings. In cells cultured from pig coronary artery, it caused a greater increase in Ca^{2+}i in the arterial smooth muscle cells (expressing PMCA4 and PMCA1) than in endothelial cells (expressing mainly PMCA1).

Caloxin 1c2 is a series C caloxin that was obtained by limited mutagenesis of caloxin 1b1[64]. Caloxin 1c2 inhibited the PMCA activity in erythrocyte ghosts with a Ki value of 2-5 μmol/L. This affinity is 10-20x higher than that of the parent caloxin 1b1, and 100-200x higher than that of caloxin 2a1. The structure activity relationship of various residues in caloxin 1c2 is illustrated in Table 1. Caloxin 1c2 has the 1c2 specific sequence TAWSEVLDLLRR and the conserved domain GGGSK. A study of the mutants of 1b1 that were selected for the selection of 1c2 showed that the residue W was crucial. Substitution of W with benzoylphenylalanine decreased the affinity while substitution of K with the same residue had very little effect. Based on mutagenesis and the modification studies, the moiety WSEV(L/V) was key to this inhibition. Caloxin 1c2 was PMCA4 selective in that it had greater than 10-fold higher affinity for PMCA4 than for PMCA1, 2 or 3.

Table 1 Structure activity relationship of caloxin 1c2 like peptides[64]

Caloxin	Peptide sequence	Ki (μmol/L)
1b1	TAWSEVLHLLSR GGGSK	45 ± 4
1c1	TTWSEVVHRLSR GGGSK	20 ± 3
1c3	ASWSEVLHLLSR GGGSK	18 ± 3
1c2	TAWSEVLDLLRR GGGSK	2.3 ± 0.3
3B1c2biotin	TABSEVLDLLRR GGGSK(biotin)-amide	50 ± 6
16B1c2biotin	TAWSEVLDLLRR GGGBK(biotin)-amide	5.1 ± 0.8

Caloxins 1c1, 1c2 and 1c3 were obtained by random limited mutagenesis of caloxin 1b1. The values of inhibition constants (Ki) are for plasma membrane Ca^{2+} pump (PMCA) ATPase in the human erythrocyte ghosts, which contain mostly PMCA4. B: Benzoylphenylalanine.

Caloxin 1c2 increased the basal tone of the de-endothelialized arteries and increased the Ca^{2+} sensitivity of the tissue to produce greater force of contraction at low extracellular Ca^{2+} when NCX and SERCA were inhibited[64]. In another study, the effect of caloxin 1c2 on smooth muscle contractility was examined in intestine of caveolin-1 knockout mice[40]. Caloxin 1c2 increased the carbachol-induced contractions in the intestinal longitudinal smooth muscle from the control mice. However, a similar effect of caloxin 1c2 was not observed in the tissues from caveolin-1 knockout mice. Immunohistochemistry and immunoblot studies suggested caveolar localization of PMCA4 and its role in Ca^{2+} extrusion from a privileged cytosolic space formed by close spatial arrangement between caveolae and sarcoplasmic reticulum. Thus, caloxin 1c2 is being used in studies to provide insight into the physiological function of PMCA4 in tissues.

We have also obtained caloxin 1b2 based on exdom 1Y of PMCA4 (unpublished). In a preliminary experiment, caloxins 1c2 and 1b2 were linked to obtain a bidentate caloxin which inhibited PMCA activity in erythrocyte ghosts with a Ki of 500 nmol/L, indicating higher affinity compared to either caloxin 1c2 or 1b2. This preliminary experiment shows the feasibility of linking the exdom 1X- and exdom 1Y-based caloxins to obtain the series D bidentate caloxins with nanomolar affinity and very high selectivity for PMCA4.

PMCA1 SELECTIVE CALOXINS

Our interest in coronary artery endothelium led us to identify PMCA1 as the major isoform expressed in this tissue. Hence, a PMCA1-specific caloxin is needed to elucidate its role in coronary artery function. We carried out a two-step screening of the phage library using synthetic exdom 1X of PMCA1 and PMCA protein purified

from rabbit duodenal mucosa as target. This led to the invention of caloxin 1b3 which inhibited the Ca^{2+}- Mg^{2+}-ATPase activity of PMCA in the PM preparation from the rabbit duodenal mucosa (PMCA1) with a Ki of 17 ± 2 μmol/L (unpublished). It exhibited isoform selectivity due to its higher affinity for PMCA1 as compared to PMCA2, 3 or 4. Adding caloxin 1b3 to the medium increased Ca^{2+}i in the endothelial cells. The work on the limited mutagenesis of caloxin 1b3, to further improve its affinity and isoform selectivity, is in progress. Recently, we have also obtained a caloxin based on exdom 1Y of PMCA1 (unpublished). Experiments will be carried out to improve its affinity by limited mutagenesis, followed by its linking to exdom 1X based caloxin to obtain a PMCA1-selective high affinity bidentate caloxin.

FUTURE OF CALOXIN RESEARCH

The existence of gene diversity and differential tissue and cell-specific distribution of PMCA has turned the initial concept of developing PMCA inhibitors into a more daunting challenge of obtaining isoform-selective inhibitors. Significant progress has been made in this area with the invention of prototypes of PMCA4 and PMCA1 selective caloxins (unpublished)[64]. Caloxin research may unfold in several directions: study of PMCA-isoform specific physiology and pathophysiology, developing PMCA2 and 3 selective caloxins, designing targets based on PMCA protein structure with higher modulatory potential, tissue targeted expression of caloxins, obtaining non-peptide inhibitors by caloxin displacement.

In the invention of isoform-selective caloxins, exdom 1 has been divided arbitrarily into 1X and 1Y, due to the presence of a cysteine residue in the middle (Figure 1). The proximity of exdom 1X and 1Y can allow for the development of a bidentate caloxin. However, optimization of the orientation of exdom 1X and 1Y based caloxins and the length of the linker between them is essential in obtaining a high affinity bidentate caloxin. Synthetic peptide based on the complete sequence of exdom 1 was not used as a target due to the presence of cysteine whose participation in disulfide bond is not known at present. Examination of the primary structure of PMCA protein shows the presence of a cysteine residue is in the middle of the exdom 2 and exdom 5. There is a subsurface cysteine in transmembrane loop 5 of PMCA. Taken together, these cysteines may be involved in disulfide bridge formation. In homology model based on X-ray structure of SERCA, exdom 1 is in proximity to exdom 5. Therefore designing a target where synthetic peptides based on exdom 1 and 5 are joined by a disulfide bridge may result in higher affinity caloxins. A layer of selectivity may also be added by targeted expression of secretable caloxins. Regardless of these approaches to obtain high selectivity of inhibition by caloxins, the problem will remain that peptides will be susceptible to proteolytic attack and hence short-lived. In this context, one can select non-peptide inhibitors by high-throughput screening. Thus, there are several ways to improve the affinity and isoform-selectivity of current caloxins with the potential to improve their bioavailability.

A few examples are provided where caloxins may also aid in understanding the basis of various disorders and become clinically useful. Hypertension is associated with defects in PMCA[65]. Current antihypertensive therapies involve decreasing arterial excitability with Ca^{2+} channel blockers or angiotensin converting enzyme inhibitors. Caloxins will help us explain how the increased artery tone in hypertension can result from PMCA inhibition in smooth muscle. PMCA1 selective caloxins may be preferentially used to increase endothelial NO production, resulting in a decrease in artery tone. PMCA4 is required for sperm motility[44,57]. Thus, PMCA4 selective caloxins may form a new class of contraceptive agents. The reduction in sperm motility may also aid in storing sperm for future use. Ca^{2+}i regulation plays a key role in cell cycle and changes in PMCA expression have been reported in several forms of cancer[46-48,52-54]. The effects of caloxins on cancer are being realized and isoform-specific caloxins may aid in developing therapies for cancer. PMCA isoforms play specific roles in neuronal signaling that may change in various neuronal pathologies[65]. PMCA isoform-selective caloxins will aid in delineating the underlying mechanisms. Isoform specific caloxins may help in understanding the basis of retinopathies as expression and distribution of the PMCA isoforms in retina and lens may alter during disease. We anticipate that isoform-selective caloxins will become useful tools in understanding and/or managing some of the disorders discussed above.

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Protease expression by microorganisms and its relevance to crucial physiological/pathological events

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Abstract

The treatment of infections caused by fungi and trypanosomatids is difficult due to the eukaryotic nature of these microbial cells, which are similar in several biochemical and genetic aspects to host cells. Aggravating this scenario, very few antifungal and anti-trypanosomatidal agents are in clinical use and, therefore, therapy is limited by drug safety considerations and their narrow spectrum of activity, efficacy and resistance. The search for new bioactive agents against fungi and trypanosomatids has been expanded because progress in biochemistry and molecular biology has led to a better understanding of important and essential pathways in these microorganisms including nutrition, growth, proliferation, signaling, differentiation and death. In this context, proteolytic enzymes produced by these eukaryotic microorganisms are appointed and, in some cases, proven to be excellent targets for searching novel natural and/or synthetic pharmacological compounds, in order to cure or prevent invasive fungal/trypanosomatid diseases. With this task in mind, our research group and others have focused on aspartic-type proteases, since the activity of this class of hydrolytic enzymes is directly implicated in several facets of basic biological processes of both fungal and trypanosomatid cells as well as due to the participation in numerous events of interaction between these microorganisms and host structures. In the present paper, a concise revision of the beneficial effects of aspartic protease inhibitors, with emphasis on the aspartic protease inhibitors used in the anti-human immunodeficiency virus therapy, will be presented and discussed using our experience with the following microbial models: the yeast *Candida albicans*, the filamentous fungus *Fonsecaea pedrosoi* and the protozoan trypanosomatid *Leishmania amazonensis*.

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Key words: Protease; Aspartic protease inhibitors; Trypanosomatids; Fungi; Cell biology; Virulence; Chemotherapy

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INTRODUCTION AND EDUCATIONAL EXPERIENCE

Since I was young, I (Figure 1) have been interested in being a teacher, and that feeling grew and consolidated along with my professional journey. The scientific world was introduced to me during high school. From 1990 to 1994, I studied the Biotechnology course in a reputable Federal Institution from Rio de Janeiro State, Brazil, called Escola Técnica Federal de Química - ETFQ (currently CEFETEQ), an excellent technical school. Over those years, the disciplines related to the Microbiology area (Bacteriology, Mycology, Virology, Protozoology and Immunology) and the laboratory classes produced a great curiosity, motivation and stimulation of scientific thought, which ignited my desire to be a scientist. With this proposal in mind, in 1994, I started my bachelor degree in the Microbiology and Immunology course at the Federal University of Rio de Janeiro (UFRJ), being one of the 35 students approved to constitute the first class of that novel graduation course. In parallel, I worked as a Biotechnology technician at the Biochemistry Department of the State University of Rio de Janeiro (UERJ) under the supervision of Dr. Claudia Vitória de Moura Gallo, an exemplar professional and an excellent person, who contributed notably to turn my dream into reality. In early 1999, I finished the undergraduate program and started a Master's degree at the Institute of Microbiology Prof. Paulo de Góes (IMPPG)-UFRJ. During the period from mid 2000 until early 2002, I developed my doctoral thesis at the IMPPG-UFRJ under the supervision of Dr. Rosangela Maria de Araújo Soares. Since August 2002, I have been Professor at the Department of General Microbiology of the IMPPG-UFRJ and, since then, I have been teaching lessons to several undergraduate courses including Microbiology and Immunology, Nursing, Biology and Pharmacy. Still, I effectively participate in two postgraduate courses at UFRJ: Microbiology from IMPPG and Biochemistry from Chemistry Institute.

Indubitably, my professional work has only been fully developed because I have a research group consisting of competent professionals, including technicians and undergraduate, masters, doctoral and postdoctoral students, who are extremely dedicated and committed to scientific thinking. I would like take this opportunity to express and reiterate my full admiration and gratitude to all my students. I would also like to thank to the several Brazilian researchers who have contributed immensely to my work, in particular Dr. Marta Helena Branquinha (IMPPG-UFRJ), Dr. Eliana Barreto-Bergter (IMPPG-UFRJ), Dr. Lucy Seldin (IMPPG-UFRJ), Dr. Celuta Sales Alviano (IMPPG-UFRJ), Dr. Claudia Masini d'Avila-Levy (Fundação Oswaldo Cruz-FIOCRUZ) and Dr. Lucimar Ferreira Kneipp (FIOCRUZ). My research has been supported by the Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Conselho de Ensino para Graduados e Pesquisas (CEPG/UFRJ), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação Universitária José Bonifácio (FUJB) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). I have also been supported by a CNPq fellowship since 2005 and by a FAPERJ fellowship since 2007.

Over the past 10 years: (1) I supervised 16 monographs of graduate students, 10 master theses and 4 doctoral theses; (2) I published 79 papers in the field of Bacteriology (n = 5), Mycology (n = 22) and Protozoology (n = 52) (Figure 2); and (3) I was invited to participate as a speaker at national and international meetings. I am a peer reviewer for international scientific journals, as well as career and research grant committees. In addition, I have accepted invitations to write reviews and book chapters on the themes: (1) relevance of proteolytic enzymes produced by microorganisms; and (2) antimicrobial properties of protease inhibitors[1-11].

ACADEMIC STRATEGIES AND GOALS

Our work group is distinguished by its multidisciplinary nature, with direct involvement of different research institutions from Brazil (other Departments and Institutes from UFRJ, UERJ, FIOCRUZ, Universidade Federal Fluminense (UFF), Universidade Federal do Estado do Rio de Janeiro (UNIRIO), Universidade do Estado de São Paulo (USP), Universidade Federal de São Paulo (UNIFESP), Universidade Federal do Espírito Santo (UFES)) and from other countries, generating productive and effective collaborations. Several publications in high-ranked journals, e.g. *FEMS Microbiology Reviews*, *PLoS One*, *Archives of Biochemistry and Biophysics*, *Journal of Antimicrobial Chemotherapy*, *Journal of Clinical Microbiology*, *International Journal of Antimicrobial Agents*, *Microbes and Infection*, *International Journal for Parasitology*, *Protist*, *Parasitology* and *Medical Mycology*, were produced in collaboration with these partners.

Over the last years, my group has focused on the identification, biochemical characterization and discovery of biological functions of proteases produced by microorganisms, with emphasis in trypanosomatids and fungi (Figure 3). More recently, we have started to study protease inhibitors in an attempt to use these bioactive compounds as a new therapeutic proposal against eukaryotic pathogenic microorganisms (Figure 3).

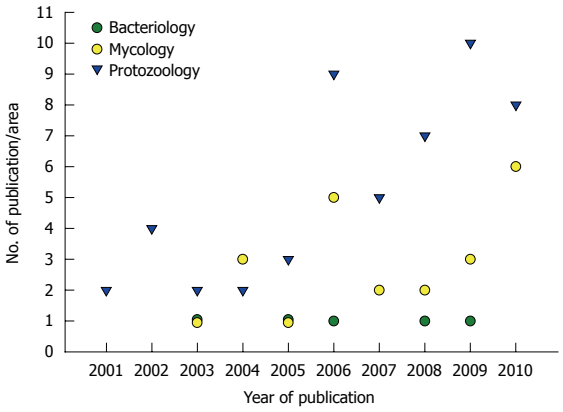

Figure 2 Publication of scientific papers by the research group led by André Santos. The graphic summarizes the numbers and specific areas of Microbiology in relation to papers published during the past ten years.

RESEARCH ACHIEVEMENTS

Proteolytic enzymes and their inhibitors: an overview

Proteolytic enzymes catalyze the cleavage of peptide bonds, which link amino acid residues in proteins and peptides. A redundant set of terms is used by the scientific community to refer to proteolytic enzymes, including: peptide hydrolase, peptidase and protease. All proteases bind their substrates in a groove or cleft, where peptide bond hydrolysis occurs (Figure 4). Amino acid side chains of substrates occupy proteolytic enzyme sub-sites in the groove, designated as S_3, S_2, S_1, S_1', S_2', S_3', that bind to corresponding substrate/inhibitor residues P_3, P_2, P_1, P_1', P_2', P_3' with respect to the cleavable peptide bond (Figure 4). After the proteinaceous substrate cleavage, at least two smaller peptides can be generated (Figure 4)[12-15].

Proteases are subdivided into two major groups depending on their site of action: exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino (NH_2) or carboxy (COOH) termini of the proteinaceous substrate, whereas endopeptidases cleave peptide bonds within a polypeptide chain. Based on their site of action at the NH_2 terminal, the exopeptidases are classified as aminopeptidases, dipeptidyl peptidases or tripeptidyl peptidases that act at a free NH_2 terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide or a tripeptide, respectively. Carboxypeptidases or peptidyl peptidases act at the COOH terminal of the polypeptide chain and liberate a single amino acid or a dipeptide (which can be hydrolyzed by the action of a dipeptidase). Carboxypeptidases can be further divided into three major groups: serine, metallo and cysteine carboxypeptidases, based on the functional group present at the active site of the enzymes. Similarly, endopeptidases are classified according to essential catalytic residues at their active sites in: serine, metallo, glutamic, threonine, cysteine and aspartic endopeptidases (Figure 5). Conversely, there are a few miscellaneous proteases that do not precisely fit into the standard classification[12-15].

The class of a protease is characteristically determined according to the effects of proteolytic inhibitors on the enzymatic activity[16,17]. Protease inhibitors enter or block a protease active site to prevent substrate access. In competitive inhibition, the inhibitor binds to the active site, thus preventing enzyme-substrate interaction. In non-competitive inhibition, the inhibitor binds to an allosteric site, which alters the active site and makes it inaccessible to the substrate[16,17]. The proteolytic inhibitors can be divided into two functional classes on the basis of their interaction with the target protease: (1) irreversible trapping reactions and (2) reversible tight-binding reactions (Figure 6). Inhibitors which bind through a trapping mechanism change conformation after cleaving an internal peptide bond and "trap" the enzyme molecule covalently; neither the inhibitor nor protease can participate in further reactions. In tight-binding reactions, the inhibitor binds directly to the active site of the protease; these reactions are reversible and the inhibitor can dissociate from the proteolytic enzyme in either the virgin state, or after modification by the protease. Based on their structural dichotomy, proteolytic inhibitors can be generally classified into two large groups: low molecular mass peptidomimetic inhibitors and protein protease inhibitors composed of one or more peptide chains. Proteolytic inhibitors can be further classified into five groups (metallo, serine, threonine, cysteine and aspartic protease inhibitors) according to the mechanism employed at the active site of proteolytic enzymes they inhibit. Some proteolytic inhibitors interfere with more than one type of protease[16,17].

Proteases produced by microorganisms: global functions

Proteases are essential for all life forms. They are involved in a multitude of physiological reactions from simple digestion of proteins for nutrition purposes to highly-regulated metabolic cascades (e.g. proliferation and growth, differentiation, signaling and death pathways), being essential factors for homeostatic control in both prokaryote and eukaryote cells (Figure 7)[12]. Proteases are also essential molecules in viruses, bacteria, fungi and protozoa for their colonization, invasion, dissemination and evasion of host immune responses, mediating and sustaining the infectious disease process (Figure 7). Collectively, proteases participate in different steps of the multifaceted interaction events between microorganism and host structures, being considered as virulent attributes. Consequently, the biochemical characterization of these proteolytic enzymes is of interest not only for understanding proteases in general but also for understanding their roles in microbial infections and thus their exploitation as targets for rational chemotherapy of microbial diseases[3,6,10,18-24].

Antimicrobial properties of proteolytic inhibitors

Current therapy for both fungal and trypanosomatid infections is suboptimal due to toxicity of the available therapeutic agents and the emergence of drug resistance[25-28]. Compounding these problems is the fact that many endemic countries and regions are economically poor. For that reason, the development of novel antifungal and/or

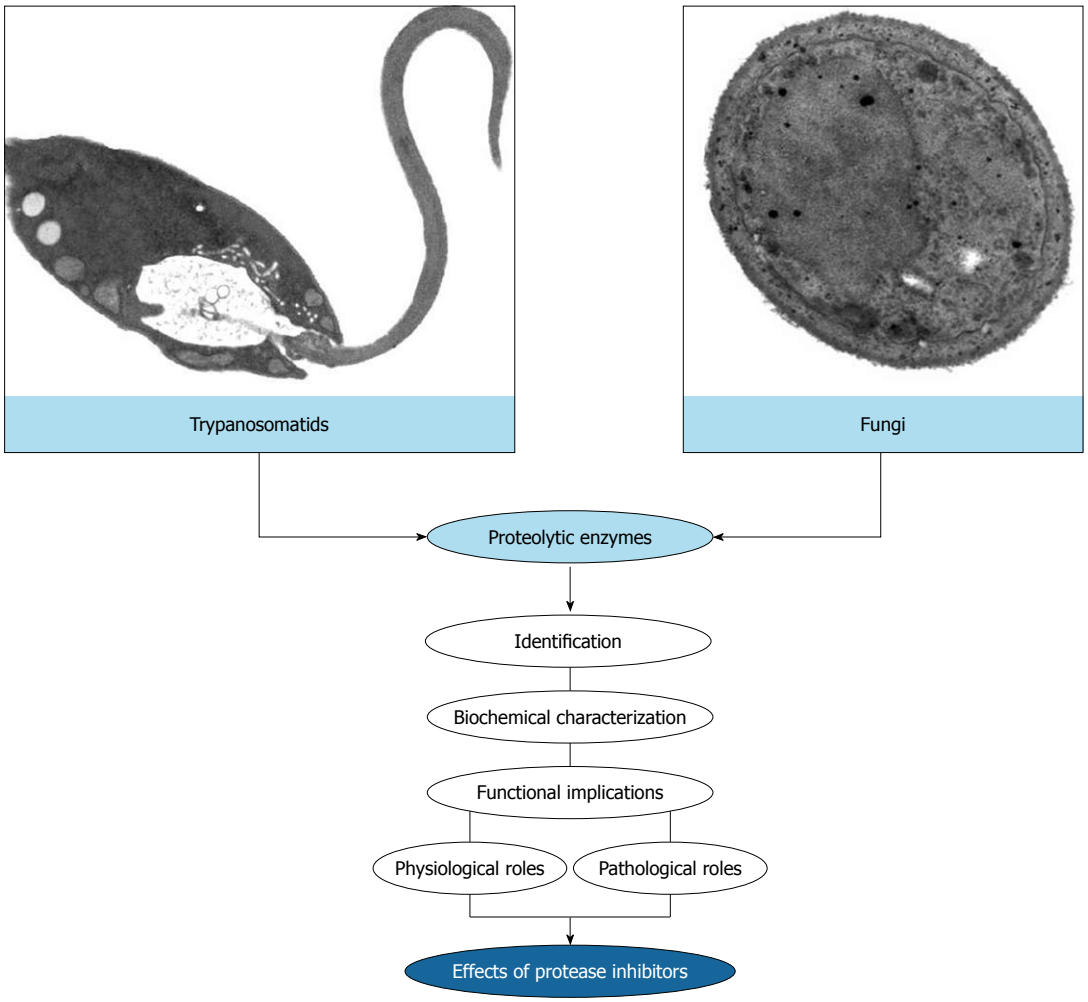

Figure 3 Rationale of the research works developed in the André Santos' laboratory. The main purpose of our study focuses on the identification and biochemical characterization of cellular and/or extracellular proteases produced by eukaryotic microorganisms, especially trypanosomatids and fungi. Subsequently, we have focused on the discovery of possible biological functions for these hydrolytic enzymes in both the social context of the microbial cell and the participation in interaction events with biotic and abiotic substrates. Finally, we have used the protease inhibitors in an attempt to block vital processes in microbial cells, thus preventing a successful infection.

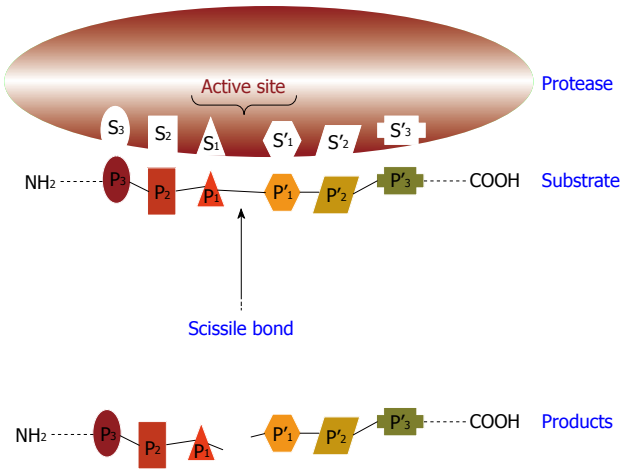

Figure 4 Schematic representation of binding region and catalytic site of a protease. This hypothetical protease possesses six subsites (S_1-S_3 and S_1'-S_3') in its catalytic site and, consequently, is able to recognize and bind to a sequence of six amino acids (P_1-P_3 and P_1'-P_3') in the proteinaceous substrate. After proteolysis, at least two smaller peptides are generated as the reaction products.

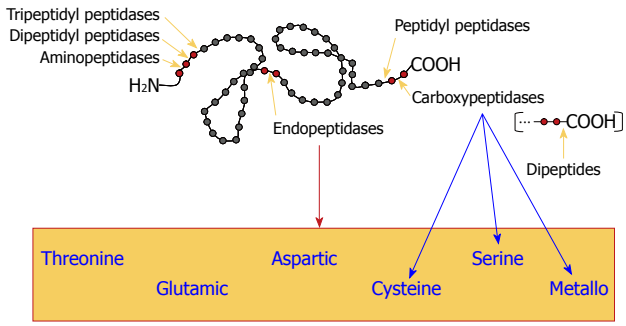

Figure 5 Classification of proteases. Gray circles represent amino acids and red circles indicate the amino acid sequence that is bound to the proteolytic enzyme. Yellow arrows point to the site of cleavage. The blue arrows indicate the classification of carboxypeptidases and the red arrow shows the box containing all the classes of endopeptidases, according to the chemical group present in their catalytic sites.

anti-trypanosomatidal drugs is an imperative requirement. A number of new strategies to obstruct fungal/trypanosomatid biological processes have emerged; one of them

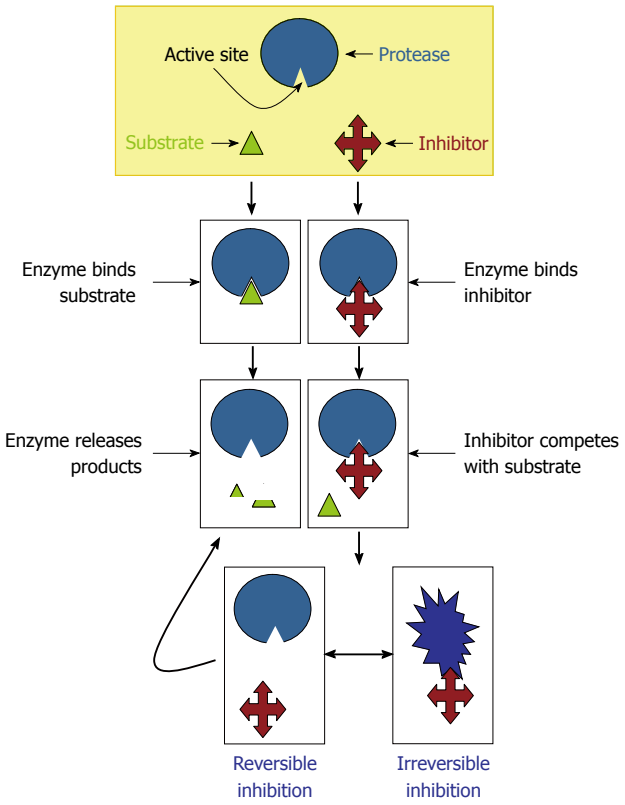

Figure 6 Mechanisms of protease inhibition. The protease inhibitor competes with the substrate to bind to the active site of a protease and two distinct possibilities arise: (1) substrate binds to the catalytic site and then is cleaved by the protease, which releases the products or (2) inhibitor binds to the active site and by steric hindrance blocks the substrate attachment. In this last case, the inhibitor can promote an irreversible (the conformational structure of the protease is completely lost) or reversible inhibition (when the inhibitor disconnects from the enzyme, the substrate can bind to it).

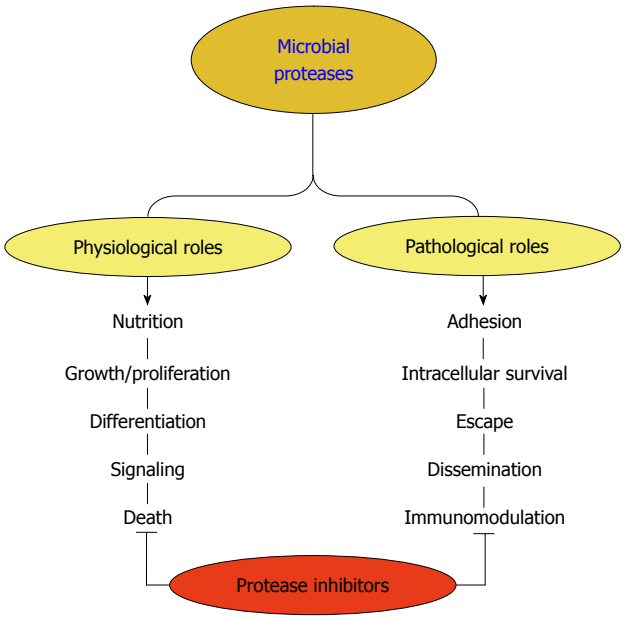

Figure 7 Possible functions played by microbial proteases. Surface and/or secreted proteases are able to cleave different host components such as serum proteins, antimicrobial peptides, surface molecules and structural proteinaceous compounds. The degradation of host proteins can help the microorganisms in several steps of their life cycle and pathogenesis including dissemination, adhesion, escape, nutrition and immunomodulation of the host immune response. These proteases can also contribute to maintaining basic metabolic processes in a microbial cell, which govern crucial events like proliferation, differentiation, signaling and death pathways. Proteolytic inhibitors are able to block one or several of these fundamental events.

is focused on protease inhibition. Currently, the main approach has been to obtain good inhibitors of the target protease, in the belief that inhibition of the activity will be therapeutic. In this context, our research group has published some works that corroborate this premise[1-6,10,29-39].

Aspartic protease inhibitors used in anti-human immunodeficiency virus therapy present anti-microbial properties

Lessons from the yeast *Candida albicans* (*C. albicans*), the filamentous fungus *Fonsecaea pedrosoi* (*F. pedrosoi*) and the protozoan *Leishmania amazonensis* (*L. amazonensis*) are illustrated as follows.

***C. albicans*:** *C. albicans* is both a successful commensal and pathogen of humans that can infect a broad range of body sites[40]. The transition from commensalism to parasitism requires a susceptible host, which includes individuals with humoral and/or cellular deficiencies as well as persons submitted to different immunosuppressive procedures. Candidiasis is the most common fungal infection diagnosed in humans[41-43]. Due to the emergence of pathogens resistant to conventional antifungals and the toxicity of some antimycotics, intense efforts have been made to develop more effective antifungal agents for clinical use[44-48]. The pathogenesis of *C. albicans* is multifactorial and different virulence attributes are important during the various stages of infection[20,21,49-55]. Secreted aspartic proteases (Saps) play a role in several infection stages of *C. albicans*, being the most important virulence factors expressed by this opportunistic fungus. Actually, *C. albicans* possesses ten different *SAP* genes (*SAP1* to *SAP10*), which are expressed according to distinct environments and host conditions[56-60]. Therefore, Saps are potential targets for the development of novel anti-*C. albicans* drugs[1,2,34,35]. In this context, several groups have demonstrated that aspartic protease inhibitors, including pepstatin A and the first generation of protease inhibitors used in anti-human immunodeficiency virus (HIV) therapy (nelfinavir, saquinavir, ritonavir and indinavir), are able to restrain Sap activity (especially Sap1, Sap2 and Sap3) as well as arrest crucial events of *C. albicans* yeast cells such as proliferation and adhesion to both abiotic (e.g. plastic and acrylic substrates) and biotic structures (e.g. surface of different epithelial cell lineages)[61-72]. Our results showed that amprenavir[72] (unpublished data) and lopinavir (unpublished data), two HIV aspartic protease inhibitors of the second generation, significantly inhibited the hydrolytic activity of Sap2 and also blocked the yeasts into mycelia transformation, an essential step during the candidiasis pathogenesis. In addition, scanning electron microscopy revealed prominent ultrastructural alterations of yeast

cells, which corroborated the inhibition of cellular division by these protease inhibitors. Several surface and/or secreted molecules have had their expression/production significantly diminished including (1) mannose- and sialic acid-rich surface glycoconjugates, which are directly involved in adhesive properties and biofilm formation; (2) sterol content, which controls the membrane fluidity; (3) secretion of lipases (e.g. esterases and phospholipases), which are related to the host membrane disruption; and (4) catalase activity, which reduces the ability of yeasts to escape from oxidative stress generated by hydrogen peroxide, for example, released by host phagocytes[72] (unpublished data). However, it is also important to note that the inhibitory effects of HIV protease inhibitors both in *in vitro* and *in vivo* experimental models were observed at concentrations (μmol/L range) much higher than those needed for HIV protease inhibition (nmol/L range). This probably reflects a much lower affinity of these drugs for Sap than that for HIV protease[31,34]. Another explanation is that, in contrast to the very small and structurally simplified HIV protease, Saps are larger and more complex[60,73]. They possess a relatively large active site which might be responsible for the broader substrate specificity and also their susceptibilities to distinct aspartic protease inhibitors[60]. Nevertheless, the above concentrations may be achieved under current highly active antiretroviral therapy (HAART) regimens both in the blood[31], in human saliva (at least for indinavir)[74] and in lungs (at least for lopinavir)[75]. In this sense, our group has showed that lopinavir at 10 mg/kg promoted a therapeutic effect in an experimental murine model of disseminated candidiasis, with an efficacy comparable to that of fluconazole, a recognized anti-candidal drug (unpublished results).

F. pedrosoi: *Fonsecaea* is a genus containing pigmented filamentous fungus isolated from soil, rotten wood and decomposing plant material. *F. pedrosoi* is one of the major causative agents of chromoblastomycosis, a post-traumatic and chronic infection of subcutaneous tissues in humid tropical areas specially South America and Japan[8,76-79]. *F. pedrosoi* is a valuable model in cell biology, since its life cycle comprises different morphological states that include reproduction structures (conidia) and fungal forms usually found in the saprophytic (mycelia) and parasitic stage (sclerotic bodies)[8]. The first report on protease production by *F. pedrosoi* was described by our group[80], which demonstrated that the pattern of protease production and secretion by *F. pedrosoi* conidial cells was closely dependent on the culture medium composition: metalloproteases were induced after cultivation in complex culture medium, while aspartic proteases were detected under chemically defined growth conditions. Mycelia[81] and sclerotic cells (unpublished results) of *F. pedrosoi* were also able to secrete aspartic-type proteases. The aspartic proteases produced by conidia and mycelia were capable of degrading relevant host serum proteins (e.g. IgG and albumin) as well as extracellular matrix components (e.g. laminin, fibronectin and collagen)[80,81]. For that reason, the extracellular hydrolytic enzymes produced by *F. pedrosoi* cells, such as proteases and lipases[82], could support the initial development of this fungus inside the host, and the existence of two biochemically distinct secreted proteases makes it possible to cover a wide range of host conditions. The effect of saquinavir, ritonavir, indinavir and nelfinavir on the secreted proteases of *F. pedrosoi* was evaluated[81,83]. These compounds inhibited the extracellular aspartic proteolytic activity produced by both conidial and mycelial forms in a dose-dependent manner. Nelfinavir was the best inhibitor of the aspartic protease activity secreted by conidia and mycelia, restraining the hydrolytic activities around 80% at 50 μmol/L. Interestingly, recent isolated strains of *F. pedrosoi* produced higher levels of extracellular protease activity when compared with a laboratory-adapted strain[81,83], suggesting that the production of secreted aspartic-type proteases may be stimulated by interaction with the host. HIV aspartic protease inhibitors and pepstatin A also arrested the growth of conidial forms as well as transformation into mycelia[83], an essential step during the *F. pedrosoi* life cycle and virulence[8]. Pepstatin A showed a significant inhibition of conidial viability even at low concentration (0.1 μmol/L); however, the HIV protease inhibitors were toxic only at high concentrations (ranging from 50 to 200 μmol/L). The synergistic action on proliferation behavior between nelfinavir (25 μmol/L) and amphotericin B (3 μg/mL), when both were used at sub-inhibitory concentrations, was also observed[83]. Interestingly, HIV protease inhibitors-treated conidial cells presented irreversible ultrastructural alterations, as shown by transmission electron microscopy images such as invaginations in the cytoplasmic membrane and withdrawal of the cytoplasmic membrane from within the cell wall, disorder and detachment of the cell wall, rupture of internal organelles, detection of large and irregular cytoplasmic vacuoles, some of them containing small vesicles, abnormal cellular division and breakage of cell wall. Furthermore, the aspartic protease inhibitors drastically reduced the adhesion and endocytic indexes during the interaction between *F. pedrosoi* conidia and epithelial cells of the Chinese hamster ovary lineage, fibroblasts or macrophages. Aspartic protease inhibitors also promoted a significant increase in the susceptibility killing by macrophage cells, promoting a significant reduction in the number of viable intracellular conidia after the treatment of infected macrophage monolayers with indinavir, nelfinavir and ritonavir at 6.25 μmol/L for 24 h[83].

L. amazonensis: *Leishmania* are digenetic protozoan parasites that live as promastigotes in the digestive tract of sand flies and as amastigotes in the phagolysosomes of mammalian macrophages. They cause a wide spectrum of clinical manifestations (generically known as leishmaniasis), and its clinical manifestations are dependent on both parasite species and immune response of the host[84-89]. The increase in the incidence of the disease, associated with higher morbidity rates, the spread of some forms of leishmaniasis to new geographical areas and *Leishmania*-HIV co-infection, has become an important public health

problem in the world[90-93]. However, the incidence of HIV-*Leishmania* co-infections has been decreasing since the introduction of HAART, in which aspartic-type protease inhibitors were included[94,95]. These findings instigated the research to confirm the possible connection between aspartic protease expression and basic molecular processes in *Leishmania*[96-103]. Our group showed that HIV protease inhibitors were able to impair *in vitro* proliferation of *L. amazonensis* promastigotes in a dose-dependent manner and in different extensions, in which nelfinavir (IC_{50} = 15.1 ± 1.1 μmol/L), lopinavir (IC_{50} = 16.5 ± 0.8 μmol/L) and amprenavir (IC_{50} = 62.0 ± 2.1 μmol/L) were the most potent compounds[103]. These three protease inhibitors (at the IC_{50} value) caused profound changes in the leishmania ultrastructure, including cytoplasm shrinking, increase in the number of lipid inclusions and some cells with the nucleus closely wrapped by endoplasmic reticulum, resembling an autophagic process, as well as chromatin condensation that is suggestive of apoptotic death. The treatment with HIV protease inhibitors of either the promastigote forms preceding the interaction with macrophage cells or the amastigote forms inside macrophages drastically reduced the association indexes (when inhibitors were used at 50 μmol/L) and the number of intracellular amastigotes (when inhibitors were used at 3.12 μmol/L)[103]. The hydrolysis of HIV protease substrate by *L. amazonensis* extract was fully inhibited by pepstatin A and HIV protease inhibitors at 10 μmol/L, suggesting that an aspartic protease may be the parasite target of the inhibitors. Despite all these beneficial effects, the HIV protease inhibitors induced an increase in the expression of cysteine protease b (cpb)[19] and the metalloprotease gp63[24], two well-known virulence factors expressed by *Leishmania* spp., probably in an attempt to compensate the parasite aspartic protease inhibition[103].

Proposals of the molecular mechanisms of the aspartic protease inhibitors on the aspartic protease produced by microorganisms

Direct actions - inhibition of aspartic proteases: The binding of the aspartic protease inhibitor to the active site of an aspartic protease blocks the binding of substrate to the enzyme. Therefore, the substrate remains intact and no peptides and/or amino acids are generated. Obviously aspartic protease inhibition will be more or less drastic depending on several parameters like the inhibitor affinity constant for the active site, its ability to reversibly or irreversibly bind to the enzyme, and the ratio of inhibitor in relation to the available substrate and enzyme. (1) The inhibition of secreted and/or surface aspartic proteases can result in an inability of the microorganism to obtain peptides and amino acids to its nutrition, leading to a reduction or a complete interruption in the proliferation rate. This phenomenon is clearly observed in *C. albicans* yeast cells when cultured under chemically defined medium containing large proteins (e.g. albumin and hemoglobin) as a unique nitrogenous source, but not when *Candida* cells are cultured in a medium containing an unlimited nitrogenous source[104-108]; (2) Some intracellular aspartic proteases produced by microorganisms also control the cleavage of important own proteins in order to promote protein activation and/or perfect functioning of a biosynthetic route; their inhibition can arrest signaling events and/or metabolic pathways, as a result inhibiting some crucial biological processes for microbial cells such as morphogenesis or expression of surface molecules responsible for adhesion or fungal protection. For example, some of these aspartic protease inhibitors alter the lipid biosynthesis, including ergosterol, resulting in altered membrane permeability[68,72,83]. These inhibitory actions will depend on the ability of the aspartic protease inhibitors to (a) enter in the microbial cells and (b) accumulate inside them; and (3) Some surface aspartic proteases participate in the assembly and organization of the microbial surface. For instance, in contrast to all other members of the Sap family, the proteases Sap9 and Sap10 are bound to the *C. albicans* cell surface by a glycosylphosphatidylinositol anchor motif. Sap9 seems to be predominantly located in the cell membrane, and Sap10 is located in the cell wall and membrane[109]. Recently, Schild *et al*[110] demonstrated that Sap9 and Sap10 cleave covalently linked cell wall proteins, including chitinase Cht2 and the glucan-cross-linking protein Pir1. Deletion of the *SAP9* and *SAP10* genes resulted in a reduction of cell-associated chitinase activity similar to that upon deletion of *CHT2*, suggesting a direct influence of Sap9 and Sap10 on Cht2 function. The treatment with amprenavir[72] and lopinavir (data not shown) promoted the removal of the amorphous layer that covers the entire surface of *C. albicans*, turning the rough surface into a smooth one. Moreover, surface aspartic proteases can promote microorganism adhesion (by functioning as an adhesive molecule or by destroying some receptors at the host surface, exposing and/or facilitating the adhesion event); therefore, their inhibition can diminish the ability of a microorganism to interact with host structures.

Indirect actions - binding to unrelated molecules: The possibility of aspartic protease inhibitors binding to or interfere with other molecules than aspartic proteases can not be excluded[35]. In this context, these compounds can generate irreversible toxic effects by perturbing the homeostasis of the microbial cells, culminating in death of microorganisms.

CONCLUSION

Microbial pathogenesis is a multifactorial process and different virulence factors are important during the various phases of infection. Some virulence attributes, such as the aspartic proteases, play a role in several infection stages and the inhibition of one of the many stages probably will contribute to the containment of the pathogen and thus should help in the treatment of disease. Therefore, aspartic proteases synthesized by pathogenic fungi and trypanosomatids are prospective targets for the development of new chemotherapeutic compounds. Both *in vitro*

and *in vivo* studies demonstrated that the use of HIV protease inhibitors promoted a drastic reduction in the presence of both fungal and trypanosomatid opportunistic diseases as well as clearly revealing that these inhibitors are able to arrest vital events in microbial cells presenting eukaryotic architecture, including proliferation, differentiation and nutrition. These inhibitors also impair the development of infection in culture or animal models due to their capability of blocking adhesion, internalization, evasion and escape of host responses. Together, all these beneficial effects culminate in death of the microorganism and/or its inadequate ability to develop an efficient and successful infection. Future studies must investigate combination drug therapy, which may reduce the incidence of toxicity due to individual drugs and may also delay the emergence of drug resistance. In addition, the purification of aspartic proteases produced by fungi and trypanosomatids, the knowledge of its biochemical properties and the crystallization of the tertiary structure will contribute to better understanding of the functioning of these proteolytic enzymes as well as allowing the design of more specific inhibitors. At least for *C. albicans*, the crystal structure of Sap2 complexed with pepstatin A has been known since 1993[111], whereas the crystal structure of Sap3 and its complex with pepstatin A was first presented in 2007[112]. The secondary structures of Sap2 and Sap3 as well as Sap1 and Sap5 were recently described[113]. These data could help in the development of novel and more effective anti-*C. albicans* compounds.

I really hope that all these findings together arouse the curiosity and the enthusiasm of other researchers in order to look for novel compounds with the ability to inhibit aspartic proteases produced by fungi and trypanosomatids. These novel compounds must be more specific, powerful and with reduced side effects, in an attempt to increase our armamentarium to treat fungal and trypanosomatid diseases.

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It is so difficult to put in words my scientific trajectory; firstly, because it is difficult to write about myself and, secondly, because some of the professional moments that I came through were intimately associated with my personal life, which has been difficult. But all of these difficult times were compensated by satisfactory and unforgettable moments. In this sense, I am grateful to the past and present members of my laboratory for their contributions to our studies. I also wish to express my gratitude to several colleagues and investigators for their collaborations, in especial Marta Helena Branquinha and Claudia Masini d'Avila-Levy, with whom I share all my scientific achievements and victories and to whom I dedicate this publication.

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Meetings

Events Calendar 2011

January 19-20,
BioBusiness
London, United Kingdom

January 27-28
Predictive Human Toxicity and ADME/Tox Studies 2011
Brussels, Belgium

January 29-February 2
LabAutomation 2011
Palm Springs, United States

February 1-2
2011 Pharma Market Research Conference
Parsippany, United States

February 6-8
5th Drug Discovery for Neurodegeneration
San Diego, United States

February 7-10
3rd International Conference and Exhibition on Drug Discovery and Therapy
Dubai, United Arab Emirates

February 13-16
Natural Products Conference 2011
Sharm el Sheikh, Egypt

February 14-17
Therapeutic Approaches to Neurodegeneration - Age Modifiers, Proteostasis, and Stem Cells
Nassau, Bahamas

February 16-19
Electrochemistry Conference 2011
Sharm el Sheikh, Egypt

February 21-23
World Antibody Drug Conjugate Summit
Frankfurt, Germany

February 22-24
2011 International Conference on Bioinformatics and Computational Biology III ROUND
Haikou, China

February 22-25
Medicinal Chemistry Conference 2011
Sharm el Sheikh, Egypt

February 23-25
International Conference on Bioscience, Biotechnology, and Biochemistry
Penang, Malaysia

February 26-28
2011 International Conference on Bioscience, Biochemistry and Bioinformatics
Sentaosa, Singapore

March 4
Discussion Workshop: Perfecting the ELISPOT - a time for answers
London, United Kingdom

March 4-11
Inorganic Reaction Mechanisms Gordon Research Conferences
Galveston, United States

March 7-8
Fragments 2011 - Third RSC-BMCS Fragment-based Drug Discovery meeting
Stevenage, United Kingdom

March 9-13
10th International Conference on Alzheimers and Parkinsons Diseases
Barcelona, Spain

March 13-18
Pittcon 2011
Atlanta, United States

March 17-20
EMBO | EMBL Symposia: Seeing is Believing - Imaging the Processes of Life
Heidelberg, Germany

March 20-22
The molecular biology of inflammatory bowel diseases
Durham, United Kingdom

March 21-23
World Congress on Biotechnology
Hyderabad, India

March 23-25
BIT's 4th Annual Protein and Peptide Conference
Beijing, China

March 25-27
2011 3rd International Conference on Bioinformatics and Biomedical Technology 3rd round call for paper
Sanya, China

March 27-April 2
EMBO Practical Course - Methods in Chemical Biology
Heidelberg, Germany

April 6-8
Faraday Discussion 150: Frontiers in Spectroscopy
Basel, United States

April 6-8
Membrane Proteins: Structure and Function
Oxford, United Kingdom

April 11-12
7th SCI-RSC symposium on Proteinase Inhibitor Design
Basel, United States

April 11-14
First EuCheMS Inorganic Chemistry Conference (EICC-1)
Manchester, United Kingdom

April 18-19
Analysis of free radicals, radical modifications and redox signalling
Birmingham, United Kingdom

April 20-21
BioFine Europe Exhibition 2011
Cambridge, United Kingdom

May 1-6
46th EUCHEM Conference on Stereochemistry
Brunnen, United States

June 1-5
EMBO Conference Series - Chromatin and Epigenetics
Heidelberg, Germany

June 15-17
Spectroscopy - Detective in Science
Rostock, Germany

June 15-18
3rd International Symposium on Metallomics
Münster, Germany

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Ubiquitin Conference
Philadelphia, United States

July 17-22
Charge Transfer in Biosystems - ESF-LFUI Conference
Obergurgl, United States

July 18-20
2nd International Congress on Analytical Proteomics
Ourense, United States

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From beads on a string to the pearls of regulation: the structure and dynamics of chromatin
Cambridge, United Kingdom

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Instructions to authors

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The columns in the issues of *WJBC* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Articles: To report innovative and original findings in biochemistry and molecular biology; (9) Brief Articles: To briefly report the novel and innovative findings in biochemistry and molecular biology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJBC*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of biochemistry and molecular biology; and (13) Guidelines: To introduce Consensuses and Guidelines reached by international and national academic authorities worldwide on the research in biochemistry and molecular biology.

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stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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