THAIS APARECIDA SALES

PHOSPHOLIPASES A_2 (PLA_2) AS TARGET ENZYMES FOR NEW ANTI-INFLAMMATORY DRUGS: a theoretical and experimental study

LAVRAS- MG

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Dissertation presented to the Federal University of Lavras, as part of the requirements of the Postgraduate Program in Agrochemistry, area of concentration in Computational Chemistry, to obtain the title of Master

Advisor: Teodorico de Castro Ramalho
Co-advisor: Silvana Marcussi

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"About all, don't fear the difficult moments. The best comes from them"

Rita Levi-Montalcini

Resumo

Tendo em mente o complexo processo de design de drogas, técnicas que possam auxiliar na pesquisa e fornecer dados que facilitam esse processo são de grande valia. Neste contexto, muitos recursos computacionais se destacam. Por meio deles, é possível obter dados sobre o mecanismo de ação desses medicamentos, novas sugestões de estruturas melhoradas para síntese, seletividade, esclarecimento sobre os efeitos colaterais, etc. Além disso, com o uso da química computacional torna-se mais fácil encontrar melhores moléculas ou inibidores, que são capazes de exercer sua atividade de maneira mais eficiente, com menor dose. Isso ajuda a desenvolver compostos cada vez mais específicos, com o objetivo de melhorar muitos problemas relacionados aos efeitos colaterais das drogas. Uma classe de fármaco que é amplamente utilizada em toda a população mundial, mas que tem possui diversos efeitos colaterais associados, como gastrotoxicidade, hepatotoxicidade e doenças cardiovasculares são anti-inflamatórios não esteroides (AINEs), que inclui a classe de inibidores seletivos de COXIBs. Sendo assim, o desenvolvimento de fármacos com atividade anti-inflamatória, mas que atue de forma diferente dos inibidores existentes, com o objetivo de minimizar o dano ao organismo, é de grande importância. Neste contexto, o uso de inibidores de fosfolipase A_2 secretadas como potenciais anti-inflamatórios se destacam. As fosfolipases A_2 são enzimas que iniciam a cascata de ácido araquidônico, dando origem a mediadores inflamatórios. O objetivo deste trabalho foi apresentar, de forma resumida, as etapas do processo inflamatório e o papel das enzimas da fosfolipase A_2, bem como trazer alguns inibidores existentes e seu potencial. Além disso, foram realizados cálculos teóricos e resultados experimentais para avaliar o potencial das fosfolipases A_2 de peçonhas para substituir enzimas humanas em testes experimentais. Os dados teóricos encontrados juntamente com os dados experimentais corroboram que a enzima de peçonha BthTX-II pode ser usada como modelo experimental para a fosfolipase A_2 humana HGIIA, o que pode ajudar muito no desenvolvimento de fármacos anti-inflamatórios, uma vez que as enzimas são mais facilmente obtidas.

Palavras-chave: Química computacional, design de fármacos, anti-inflamatórios, fosfolipase A2

Abstract

Having in mind the complex process of drug design, techniques that can help in research and provide data that facilitate this process be of great value. In this context, many computational resources have been stands out. By mean of them, it is possible to obtain data about mechanism of action of these drugs, new suggestions of improved structures for synthesis, selectivity, clarify about the side effects, etc. In addition, using the computational chemistry becomes easier to find the best molecules, or inhibitors, which are capable of exert its activity of a more efficient way, with less dose. This helps to develop increasingly specific compounds, aiming to improve many problems related to the side effects of the drugs. A class of drugs that is widely used throughout the world population, but which has several side effects, such as gastrotoxicity, hepatotoxicity and cardiovascular diseases are Nonsteroidal Anti-inflammatory (NSAIDs), which includes the class of selective COXIBs inhibitors. Thus, the development of drugs with anti-inflammatory activity, but that act of different form of the existing inhibitors, aiming to minimize the damage to the organism, are of great importance. In this context, the use of secreted phospholipase A_2 inhibitors as potential anti-inflammatory drugs has been highlighted. Phospholipases A_2 are the enzymes that initiate the cascade of arachidonic acid, which gives rise to inflammatory mediators. The objective of this work was to present, in a summarized way, the steps of the inflammatory process and the role of the phospholipase A_2 enzymes, as well as to bring some existing inhibitors and their potential. In addition, theoretical calculations and experimental results were also carried out to evaluate the potential of snake venom phospholipases A2 to replace human enzymes in experimental trials. The theoretical data found together with the experimental data corroborate that the BthTX-II snake venom enzyme can be used as an experimental model for human HGIIA phospholipase A2, which may greatly aid in the development of anti-inflammatory drugs, since enzymes are more easily obtained.

Keywords: Computational chemistry, drug design, anti-inflammatory, phospholipase A2

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Sumário

First Part - General Introduction

1 Introduction

Researches considering the drugs design are not much older than a century. During the beginning of the 20th century, drug research was enriched by several new technologies, which have gained prominence in drug discovery and therapy (Drews, 2000). For developing a new drug, it is estimated a cost of US$2.6 billion (Tresadern, Rombouts, Oehlrich, Macdonald, & Trabanco, 2017). This process, besides very costly, is a time-consuming process (Liao, Sitzmann, Pugliese, & Nicklaus, 2013). Taking account from the initial project to the drug product, a time of 12-24 years are estimated, for designing a single new drug (Lombardino and Lowe III, 2004).

Aiming to reach better and faster results, computational medicinal chemistry have been employed in many stages of the drug discovery. The computational drug design can provide data about action mechanisms, new chemical structures for the synthesis, pharmacokinetic, selectivity, side effects, among others (Liao, Sitzmann, Pugliese, & Nicklaus, 2013). Actually, in the field of medicinal chemistry, proteins become targets for inhibitors, with a pharmacologic potential. In this context, the search for key proteins with biological functions associated to diseases have increased in the last decades (Broomhead & Soliman, 2017).

One class of key inflammatory enzymes that have been target is the secretory phospholipases A_2 ($sPLA_2$). The $sPLA_2$ acts in the broken of membrane phospholipids, giving origin to arachidonic acid, which, in turn, gives origin to the inflammatory mediators, such as prostaglandins and leukotrienes (Joshi, 2016). Taking account its importance in the inflammatory cascade, $sPLA_2$enzymes have been considered promising targets in the development of new anti-inflammatory drugs (QUACH et al. 2014).

The design of new anti-inflammatory drugs, with alternative mechanisms of action, it is of great importance, once anti-inflammatory drugs are the most used drugs in the world (Badri et al., 2016). It is important to mention that the current anti-inflammatory drugs are known for its high gastrotoxicity, hepatotoxicity, cardiovascular complications, among other side effects (Blanca-Lopez, et al., 2017; Modley 2008). In this way, the goal of this study is to provide a better understanding about the $sPLA_2$ enzymes and their inhibitors as possible new anti-inflammatory drugs. In addition, to evaluate by means of theoretical and experimental methods, the possibility of using snake venoms $sPLA_2$ as a model for human $sPLA_2$, developing rational modified human $sPLA_2$ inhibitors from the vanillic acid.

2 Theoretical Referential

2.1 Medicinal Chemistry in the drugs development

The production of new drugs can be categorized into three major phases, which are drug development/discovery, clinical trials and marketing. For drug design, the final stage is necessary a work in scale and an interdisciplinary scientific involvement. Even with all such care, the final product may still be susceptible to biological variability among users (Caldwell, 2015). To reach the preclinical stage, the candidate molecule must have pharmacokinetic parameters well defined, its pharmacodynamics must be optimized, a low toxicity profile, among other characteristics. In this context, the computational chemistry can be useful in key aspects in the development of new drugs, such as the identification and optimization of the initial preferential structure and the best ligand, mechanisms of action, etc. (ALONSO et al., 2006; RABAL et al., 2011; SHENG and ZHANG, 2013).

The availability of databases and computational chemistry programs are fundamental tools in the designing of drugs, since they allow fast analysis of their physicochemical properties in relation to their biological properties, for a series of molecules of medical-scientific interest (CARVALHO et al., 2003). Several methods of computational chemistry are employed in drug design. Thus, it is desirable that the researcher uses all the software and resources that are necessaries. Many of the computational techniques used are cheaper and faster than the experimental techniques, which makes possible the theoretical study of the compounds before the *in vitro* and mainly *in vivo* tests, for example (LIAO et al., 2011).

The theoretical analysis from molecular modeling techniques, such as structure-activity in three-dimensional and molecular dynamics simulations can be used in the development of new therapeutic agents (CARVALHO et al., 2003). In fact, some of the most used techniques in the study of biological macromolecules are Molecular Dynamics Simulation (MD) and Molecular Docking (Morgon and Coutinho, 2007). These techniques have contributed exhaustively to rational design of drugs, in many stages of the process (NAMBA, SILVA and SILVA, 2008). In relation to the development of new compounds with anti-inflammatory properties as well as the study of physical-chemistry properties of current drugs (BANERJEE et al., 2015; GRZYBOWSKA et al., 2010; HADAD et al., 2011; LOCKHART et al., 2012; TAKEDA et al., 2010; YOUSEFPOUR et al., 2013).

2.1.1 Molecular Modeling

According to IUPAC, molecular modeling is defined as the investigation of molecular structures through computational chemistry and other graphic visualization techniques, in order to provide a three-dimensional structure under a given set of circumstances (CARVALHO et al., 2003). The Molecular Modeling has been used to predict the behavior of molecules under unusual conditions, such as high pressures and temperatures, and to describe the processes that cannot be observed by experimental techniques. These techniques are useful for understanding the complex invisible world of atoms and molecules, optimizing time and money (ZHAO et al., 2016).

Theories and methods of molecular modeling can be classified, in a simplified form, in methods based on quantum mechanics (QM) and methods based on classical mechanics (MM). The first group consists of *ab initio* methods, semi-empirical approaches and density functional theory (DFT). The second group uses molecular mechanics (MM) calculations, employing functions of simple potential energies, such as Coulombic potentials and harmonic oscillator. Molecular mechanics approaches are used in molecular dynamics (MD) simulations, in the refinement of molecular structures, in ligand-receptor docking simulations, Monte Carlo simulations (MC), among others (ADCOCK and MCCAMMON, 2006).

2.1.2 Molecular Dynamics

The molecular dynamics (MD) simulation method was developed more than 30 years ago (WARSHEL and LEVITT, 1976; MCCAMMON et al., 1977). This is a technique based on statistical mechanics and classical physics, which becomes possible to examine molecular systems, such as proteins and nucleic acids. Based on molecular mechanics (MM), the molecules are treated as a set of atoms, described by Newtonian forces (NAMBA, SILVA E SILVA, 2008). As mentioned, the MD simulations are applied to systems where classical interactions are considered in a given range of thermodynamic conditions. This technique can ignore the quantum effects, or incorporate them into an effective classical approximation, (MORGON and COUTINHO, 2007).

In a MD simulation, it is necessary to prepare an initial model of the system, by means of Nuclear Magnetic Resonance (NMR), crystallography, or other method, determining the coordinates of the constituent atoms of the system. The atoms are described only by the coordinates of the

nucleus, following the Born-Oppenheimer approximation (ADCOCK and MCCAMMON, 2006). In addition, different solvation models are employed, including explicit or implicit solvent (CHANG et al., 2016). The simulation is carried out by means of the numerical solution of the Newtonian equations of motion, for each atom, using the evolution in the time intervals from *n* to *n + 1*, where a long series of these steps generates a trajectory through space. For an atom *i*, with mass m_i, the Newtonian equation of motion is given by Equation 1:

$$\frac{d\vec{p}_i}{dt} = \vec{F}_i \quad \textbf{(1)}$$

The relation between velocity and momentum is given by Equation 2, shown below:

$$\frac{d\vec{r}_i}{dt} = \frac{\vec{p_i}}{m_i} \quad \textbf{(2)}$$

Where the three-dimensional vector r_i indicates the position of the atom. The net force that the system exerts on the atom *i* can also be obtained by the negative gradient of the potential energy function in relation to the position of the atom *i*, as shown in Equation 3:

$$\vec{F}_i = \frac{d\vec{V}}{d\vec{r}_i} \quad \textbf{(3)}$$

The Newton's second law provides the acceleration of the system (Equation 4).

$$\frac{d^2x(t)}{dt^2} = \frac{\vec{F}_x}{m} \quad \textbf{(4)}$$

By obtaining the values of *x (t), v (t)* and acceleration, it is possible to solve numerically the classical equations of motion (ADCOCK and MCCAMMON, 2006). The numerical integrations of the equations of motion can be solved in several ways and there are several algorithms with different approaches able to solve them, such as the Verlet's algorithm (Verlet 1967). These equations for all simplified systems are so complex that their integration must be done numerically over several small discrete time intervals rather than continuous analysis. The approximations assume that for any discrete time interval, the atomic coordinates are fixed. The fixed coordinates are then used to calculate the intra and intermolecular interaction potentials, which determine the forces acting on each atom, such as atomic partial charge, bond length, bond angle, etc. (BECK and DAGGETT, 2004).

It is generally assumed that the interaction potential between two molecules can be described as the sum of interactions between each pair of atoms, and that interaction depends only on the distance between these atoms. In addition, in the cases of internal motions of the molecule, terms related to the deformation of the molecular geometry are included. The set of parameters needed to describe all these interactions is called the force field. (MORGON and COUTINHO, 2007). To

describe the parameters, most force fields divide the potential energy function in terms of the contribution of bound atoms (Equation 5) and unbound atoms (Equation 6), as shown below.

$$V_{Bond} = \sum_{i=1}^{N_b} \frac{1}{2} K_{bi}(b_n - b_{0i})^2 + \sum_{i=1}^{N_\theta} \frac{1}{2} K_{\theta i}(\theta_n - \theta_{0i})^2 + \sum_{i=1}^{N_\varepsilon} \frac{1}{2} K_{\varepsilon i}(\varepsilon_n - \varepsilon_{0i})^2 + \sum_{i=1}^{N_\varphi} \frac{1}{2} K_{\varphi i}[1 + \cos(n_i \varphi_i - \delta_i)] \quad \textbf{(5)}$$

$$V_{unbond} = \sum_{i<j}^{N} 4\varepsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] + \sum_{i<j}^{N} \frac{q_i q_j}{D r_{ij}} \quad \textbf{(6)}$$

Where

Kb = harmonic constant of energy associated with the chemical bond between two atoms and b_o is the equilibrium distance of said bond;

Kθ = harmonic constant of energy associated with the angle between two chemical bonds and θ_0 is the equilibrium angle;

Kε = harmonic energy constant associated with the angle formed between two planes (defined by four atoms) and ε_0 is the equilibrium angle between these two planes;

Kφ = energy constant associated with the torsional term between two planes defined by four atoms and φ is the torsion angle between these two planes;

n = multiplicity;

δ = phase angle;

r_{ij} = distance between the atoms i and j;

ε_ij and σ_{ij} = Lennard-Jones parameters;

q_i and q_j = partial charges located on the respective atoms

D = effective dielectric constant of the medium

There are several force fields available for MD simulations, which have appropriate parameters for different types of molecules, and the right choice of this set of parameters is crucial for an accurate determination of the potential energy function, which will give reliability to MD simulation. The most well parameterized force fields used for MD simulations of biomolecules in general, such as proteins, nucleic acids and lipids, are AMBER, GROMOS and CHARM (CORNELL et al., 1996; MACKERELL et al., 1998; CHRISTEN et al., 2005).

2.1.3 Molecular Docking

One of the most commonly used computational tools in rational drug design is the ligand-receptor Docking method. By docking calculations, it is possible to predict the interaction mode of the ligand molecule in the binding region of a molecular target, for example the active site of the specific enzyme, which can be inhibited or activated. Molecular Docking allows the definition of orientation and conformation of the ligand (pose) when interacting with its receptor. Besides the prediction of how this molecule will bind to its target (adopted conformation), the affinity of these

bonds is also quantified. The recognition of the preferred arrangement adopted by the receptor-ligand complex, which corresponds to the best interaction energy, depends on several properties of the substrate or inhibitor (MORGON e COUTINHO, 2007).

During the prediction of the protein-ligand structures, the programs that perform the docking calculations obtain a better model of evaluation of the free energy called the scoring function. This will serve to predict the affinity of the interaction between the ligand and the receptor, to select the best poses. This function is usually based on the combination of empirical functions together with geometric and energetic functions, such as bonding lengths, bonding angles, Van der Waals forces, Lenard-Jones, electrostatic interactions, among others. Most of docking programs use simple models of potential energy functions, usually based on molecular mechanics force fields, such as AMBER or CHARM. The function should be fast enough to analyze a large number of energies, and should be efficient to rank each one of them (TAYLOR, JEWSBURY & ESSEX, 2002; STARK e POWERS, 2012).

Equation 7 defines the scoring function values. E_{inter} (Equation 8) is related to the interaction that occurs between the ligand and the protein and E_{intra}, represented in Equation 9, is the internal energy of the ligand.

$$E_{score} = E_{inter} + E_{intra} \quad \textbf{(7)}$$

$$E_{inter} = \sum_{i \in ligand} \sum_{j \in protein} \left[E_{PLP}(r_{ij}) + 332.0 \frac{q_i q_j}{4 {r_{ij}}^2} \right] \quad \textbf{(8)}$$

In the above equation, the first term E_{PLP} corresponds to the potential energy of the inhibitor, which includes both hydrogen bonds and Van der Waals interactions. The second term is a Coulombian potential that has a distance dependent dielectric constant, which describes the electrostatic interactions between atoms. In this Coulombian term, q corresponds to the charges of particles.

$$E_{intra} = \sum_{i \in Ligant} \sum_{j \in protein} \left[E_{PLP}(r_{ij}) + \sum_{flexible\ bonds} A[1 - \cos(m\theta - \theta_0)] + E_{clash} \right] \quad \textbf{(9)}$$

The first two terms of the E_{intra} are related to all pairs of atoms of the ligand that are not connected by double bond. How θ is the torsional angle of the bond, the term with this variable is

related to torsional energy. The last term E_{clash} is a correction term for the nonexistent conformations of the ligand (GIACOPPO et al., 2014).

In addition to the specificity in the scoring function, the docking methods also differ in relation to the degree of flexibility of the ligand, besides the optimization method used to analyze the potential energy hypersurface. In terms of flexibility, the ideal would be as flexible as possible to ensure a good description of the system. However, this requirement becomes impracticable, since the system haves a very large number of degrees of freedom of the receptor molecule, and the largest system indicates a higher computational cost. To overcome this problem, a semi-rigid docking calculation is generally adopted, where only a few regions are considered flexible. Flexibility can be treated implicitly or explicitly (BONVIN, 2006; MORGON e COUTINHO, 2007).

As can be observed, the molecular docking technique is of crucial importance for the rational design of drugs. However, when the phenomenon involves breaking of chemical bonds, it should not be employed. In this case, hybrid QM/MM techniques are used (GIACOPPO et al., 2014).

2.2 Phospholipases A_2

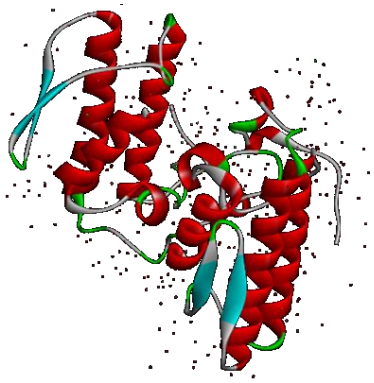
The phospholipases A_2 (PLA_2) are hydrolases enzymes that catalyze the broken of the membrane phospholipids, at the *sn*-2 position. This class of enzymes is important for a series of cellular activities, such as membrane modeling and cell signaling (BALIETTI et al., 2016; DAN, ROSENBLAT and YEDGAR, 2012; REID, 2005). The mechanism of action of these enzymes, proposed by Scott and collaborators (2010) suggests that the His48 / Asp99 pair abstracts a proton from a water molecule, which acts as a nucleophile on the attack at the *sn*-2 position of the membrane phospholipid. The Ca^{2+} cofactor stabilize the oxyanion, the reaction intermediary.

Currently, more than 30 PLA_2 isoforms are known (QUACH, ARNOLD and CUMMINGS, 2014). These are subdivided into six groups, the calcium-independent ($iPLA_2$), cytosolic ($cPLA_2$), secreted ($sPLA_2$), platelet activating factor acetylhydrolase (PAF-AH), the lysosomal ($lyPLA_2$), and a recently discovered, named "Adipose-specific PLA_2" (Ad-PLA_2. For the division of the groups, their molecular weights, disulfide bonds, calcium requirement, among other criteria are taken into account (QUACH, ARNOLD and CUMMINGS, 2014).

The secreted phospholipases A_2 ($sPLA_2$) group were the first types of PLA_2 discovered. They are low molecular weight enzymes (approximately 14 KDa), and are characterized by containing six disulfide bonds, histidine at their catalytic site, and dependent on mM concentrations of calcium.

These enzymes are found in various animal poisons, synovial fluid, and various mammalian tissues. Until now, 16 types of $sPLA_2$ are described in the literature, named by IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA and XIIB PLA_2 (QUACH, ARNOLD and CUMMINGS, 2014). The tree dimensional structure of a human PLA_2 of the IIA group can be seen in the Figure 1.

Figure 1 - Three dimensional structure of human $sPLA_2$ HGIIA. PDBID 3U8D.



Of all class of PLA_2, the $sPLA_2$ group have been considered as "inflammatory enzymes" since they are involved in pathophysiological processes of various inflammation-related diseases (DAN, ROSENBLAT and YEDGAR, 2012). The expression of $sPLA_2$ is related to several inflammatory processes, such as allergic inflammatory processes, pancreatitis, atherosclerosis and cancer (DE LUCA et al., 2013; MURAKAMI and TAKETOMI, 2015; YAMAMOTO et al., 2011; YEDGAR, COHEN and SHOSEYOV, 2006). In particular, the expression of the IIA group is attributed to several pathologies, such as obesity, arthritis, atherosclerosis and cancer, and one of its activities is the recruitment of other $sPLA_2$, such as those of group X and V (MURAKAMI et al., 2015). All of these factor in addition to the discovery of the oncogenic role and involvement in neurological diseases of $sPLA_2$ motivate the study of new inhibitors for this class of enzymes (BURKE and DENNIS, 2009; JIANG et al., 2002; PUCER et al., 2013; YAGAMI, YAMAMOTO and KOMA, 2014; YAMASHITA, YAMASHITA and OGAWA, 1994).

2.2.1 Experimental methods for phospholipase activity analysis

One experimental method that is widely used is the indirect hemolytic assay, proposed by Gutierrez and collaborators in 1988. This method is an alternative technique used firstly for determination of potency of antivenoms, which is based on the measurement of the hemolytic haloes induced by venom on agarose gels containing erythrocytes and egg yolk. However, the preparation of the medium without the addition of the erythrocytes allows to evaluate the phospholipase activity

directly. The egg yolk is a source of phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine, thus constituting a propitious and low cost substrate for the analysis of phospholipase activity (PRICE et al., 1982).

3 References

ADCOCK, S. A.; MCCAMMON, J. A. Molecular dynamics: survey of methods for simulating the activity of proteins. **Chem Rev**, v. 106, n. 5, p. 1589-615, 2006. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/16683746 >.

ALONSO, H.; BLIZNYUK, A. A.; GREADY, J. E. Combining docking and molecular dynamic simulations in drug design**. Med Res Rev**, v. 26, n. 5, p. 531-68, Sep 2006. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/16758486 >.

BADRI, W., MILADI, K., NAZARI, Q. A., GREIGE-GERGES, H., FESSI, H., & ELAISSARI, A. Encapsulation of NSAIDs for inflammation management: Overview, progress, challenges and prospects. **International Journal of Pharmaceutics**, v. 515, p.757–773, 2016. Available at: http://doi.org/10.1016/J.IJPHARM.2016.11.002

BALIETTI M. et al. Cognitive Stimulation Modulates Platelet Total Phospholipases A2 Activity in Subjects with Mild Cognitive Impairment. **J. Alzheimers.** Dis. v. 50, n. 4, p. 957–962, 2016.

BANERJEE, A. G. et al. Synthesis, characterization, evaluation and molecular dynamics studies of 5, 6-diphenyl-1,2,4 triazin-3(2H)-one derivatives bearing 5-substituted 1,3,4-oxadiazole as potential anti-inflammatory and analgesic agents. **European Journal of Medicinal Chemistry**, v. 101, p. 81-95, 2015. Available at: <<Go to ISI>://WOS:000360771900009 >.

BECK, D. A. C.; DAGGETT, V. Methods for molecular dynamics simulations of protein folding/unfolding in solution. **Methods**, v. 34, n. 1, p. 112-120, 9// 2004. Available at:< http://www.sciencedirect.com/science/article/pii/S1046202304000568 >.

BLANCA-LOPEZ, N., PEREZ-ALZATE, D., CANTO, G., & BLANCA, M. . Practical approach to the treatment of NSAID hypersensitivity. **Expert Review of Clinical Immunology**, v. 13, n. 11, p. 1017–1027, 2017. Available at: http://doi.org/10.1080/1744666X.2017.1377072

BROOMHEAD, N. K., & SOLIMAN, M. E. Can We Rely on Computational Predictions To Correctly Identify Ligand Binding Sites on Novel Protein Drug Targets? Assessment of Binding Site Prediction Methods and a Protocol for Validation of Predicted Binding Sites**. Cell Biochemistry and Biophysics**, v. 75, n. 1, p. 15–23, 2017. Available at: http://doi.org/10.1007/s12013-016-0769-y

BONVIN, A. M.J.J. Flexible protein–protein docking. **Current Opinion in Structural Biology**. v. 16, p. 194–200, 2006.

BURKE J.E. AND DENNIS E.A. Phospholipase A2 Biochemistry. **Cardiovasc. Drugs Ther.** v. 23, n. 1, p. 49–59, 2009.

CALDWELL, G. W. In silico tools used for compound selection during target-based drug discovery and development. **Expert Opinion on Drug Discovery**, v. 10, n. 8, p. 901-923, 2015. Available at: < https://www.scopus.com/inward/record.uri?eid=2-s2.0-84937695369&partnerID=40&md5=3c98a31c802678e3c5378556dd79fb22 >.

CARVALHO, I. et al. Introduction to molecular modeling of drugs in the experimental course of pharmaceutical chemistry. **Quim. Nova**, v. 26, n. 3, p. 428-438, 2003. Available at: < http://www.scielo.br/pdf/qn/v26n3/15672.pdf >.

CHANG, C. E. A. et al. Investigation of Structural Dynamics of Enzymes and Protonation States of Substrates Using Computational Tools. **Catalysts**, v. 6, n. 6, 2016. Available at: <<Go to ISI>://WOS:000378839100007 >.

CHRISTEN, M. et al. The GROMOS software for biomolecular simulation: GROMOS05. **J Comput Chem**, v. 26, n. 16, p. 1719-51, 2005. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/16211540 >.

CORNELL, W. D. et al. A second generation force field for the simulation of proteins, nucleic acids, and organic molecules . **Journal of the American Chemical Society**, v. 118, n. 9, p. 2309-2309, Mar 1996.. Available at: <<Go to ISI>://WOS:A1996TY69800032 >.

COSTAL-OLIVEIRA, F., et al. General biochemical and immunological characteristics of the venom from Peruvian scorpion *Hadruroides lunatus*. **Toxicon,** v. 60, n. 5, p. 934–942, 2012 Available at: http://doi.org/10.1016/J.TOXICON.2012.06.013

DAN, P.; ROSENBLAT, G.; YEDGAR S. Phospholipase A2 activities in skin physiology and pathology. **European J. Pharm**. v. 691, p. 1-8, 2012.

DE LUCA, D. et al. Clinical and biological role of secretory phospholipase A2 in acute respiratory distress syndrome infants. **Critical Care.** v. 14, p. 163, 2013.

DE MENEZES, R. R. P. P. B Involvement of Nitric Oxide on *Bothropoides insularis* Venom Biological Effects on Murine Macrophages In Vitro. Involvement of Nitric Oxide on *Bothropoides Insularis* Venom Biological Effects on Murine Macrophages **In Vitro. PLoS ONE**, v. 11, n.3, 2016. Available at: http://doi.org/10.1371/

DREWS, J. Drug discovery: a historical perspective. **Science** (New York, N.Y.), v. 287, n. 5460, p. 1960–4, 2000. Available at: http://doi.org/10.1126/SCIENCE.287.5460.1960

GIACOPPO, J. O. S.; LIMA, W. E. A.; KAMIL, K.; FRANÇA, T. C. C.; da CUNHA, E. F. F.; RAMALHO, T. C. Guerra Química: Perspectivas no Estudo de Reativadores da Enzima Acetilcolinesterase Inibida por Organofosforados. **Rev. Virtual Quim.,** v. 6 n. 3, p. 653-670, 2014. Available at : < http://s3.amazonaws.com/academia.edu.documents/45065297/Chemical_Warfare_Perspectives_on_Reactiv20160425-1023-1brt4yn.pdf?AWSAccessKeyId=AKIAIWOWYYGZ2Y53UL3A&Expires=1488387174&Signature=GBJQlIza3V5X8OmoZqs5gnXT7Jk%3D&response-content-disposition=inline%3B%20filename%3DChemical_Warfare_Perspectives_on_Reactiv.pdf >

GRZYBOWSKA, K. et al. Molecular Dynamics and Physical Stability of Amorphous Anti-Inflammatory Drug: Celecoxib. **Journal of Physical Chemistry B,** v. 114, n. 40, p. 12792-12801, 2010. ISSN 1520-6106. Available at: <<Go to ISI>://WOS:000282546200004 >.

HADAD, A. et al. Two-Level Adsorption of Ibuprofen on C-60 Fullerene for Transdermal Delivery: Classical Molecular Dynamics and Density Functional Theory Computations. **Journal of Physical Chemistry C**, v. 115, n. 50, p. 24501-24511, 2011. ISSN 1932-7447. Available at: <<Go to ISI>://WOS:000297947700008 >.

JIANG, J. et al. Expression of group IIA secretory phospholipase A2 is elevated in prostatic intraepithelial neoplasia and adenocarcinoma. **Am. J. Pathol**. v. 160, n. 2, p. 667–671, 2002.

JOSHI, V. Dimethyl ester of bilirubin exhibits anti-inflammatory activity through inhibition of secretory phospholipase A_2, lipooxygenase and cyclooxygenase. **Archives of Biochemistry and Biophysics**, n. 598, p. 28-39, 2016.

LIAO, C., SITZMANN, M., PUGLIESE, A., & NICKLAUS, M. C. (n.d.). Software and resources for computational medicinal chemistry. http://doi.org/10.4155/fmc.11.63DUNCAN, R. E. et al. Identification and functional characterization of adipose-specific phospholipase A_2 (AdPLA**). J Biol Chem.** v. 12;283(37):25428-36, 2008.

LIAO, C. Z. et al. Software and resources for computational medicinal chemistry. **Future Medicinal Chemistry**, v. 3, n. 8, p. 1057-1085, 2011. ISSN 1756-8919. Available at: <<Go to ISI>://WOS:000294459700017 >.

LOCKHART, C.; KIM, S.; KLIMOV, D. K. Explicit Solvent Molecular Dynamics Simulations of A beta Peptide Interacting with Ibuprofen Ligands. Journal **of Physical Chemistry B**, v. 116, n. 43, p. 12922-12932, 2012. ISSN 1520-6106. Available at: <<Go to ISI>://WOS:000310482800004 >.

LOMBARDINO, J. G. AND LOWE III, J. A. The role of the medicinal chemist in drug discovery — then and now. **Nature reviews| drug discovery**, v. 3, 2004. Available at: http://doi.org/10.1038/nrd1523

MACKERELL, A. D. et al. All-atom empirical potential for molecular modeling and dynamics studies of proteins. **J Phys Chem B**, v. 102, n. 18, p. 3586-616, 1998. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/24889800 >.

MCCAMMON, J. A.; GELIN, B. R.; KARPLUS, M. Dynamics of folded proteins. **Nature**, v. 267, n. 5612, p. 585-590, 1977. Available at: < http://europepmc.org/abstract/MED/301613 >.Available at: < http://dx.doi.org/10.1038/267585a0 >.

MOODLEY, I. Review of the cardiovascular safety of COXIBs compared to NSAIDS. **Cardiovascular Journal of Africa,** v. 19, n. 2, p. 102–7, 2008. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18516356.

MORGON, N.H.; COUTINHO, K. Methods of theoretical chemistry and molecular modeling. São Paulo: **Publisher Physics Bookstore**, 2007, 539 p.

MURAKAMI, M. et al. Recent progress in phospholipase A2 research: From cells to animals to humans. **Prog. Lipid Res**. v. 50, p. 152–192, 2011.

MURAKAMI M. AND TAKETOMI Y. Secreted phospholipase A2 and mast cells. **Allergol. Int**. v. 64, n. 1, p. 4–10, 2015.

NAMBA, A. M.; SILVA, V. B. da; SILVA, C. H. T. P. Molecular dynamics: theory and applications in drug planning. **Eclética Química, São Paulo**, v. 33, n. 4, p. 13-24, 2008. Available at: < http://www.scielo.br/pdf/eq/v33n4/v33n4a02.pdf>.

OLIVEIRA, C. H. DE M., ASSAID SIMÃO, A., & MARCUSSI, S. Inhibitory effects of ascorbic acid, vitamin E, and vitamin B-complex on the biological activities induced by *Bothrops* venom. **Pharmaceutical Biology**, v. 54, n. 5, p. 845–852, 2016. Available at: http://doi.org/10.3109/13880209.2015.1087038

PRICE, M. F.; WILKINSON, I. D.; GENTRY, L. O. Plate method for detection of phospholipase activity in *Candida albicans*. **Sabouraudia**, v. 20, n. 1, p. 7-14, 1982. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/7038928 >.

PUCER, A et al. Group X secreted phospholipase A2 induces lipid droplet formation and prolongs breast cancer cell survival. **Mol. Cancer**. v. 12, n. 1, 2013.

QUACH, N. D.; ARNOLD, R. D.; CUMMINGS, B. S. Secretory phospholipase A_2 enzymes as pharmacological targets for treatment of disease. **Biochemical Pharmacology**, v. 90, n. 4, p. 338-348, 2014. Available at: < https://www.scopus.com/inward/record.uri?eid=2-s2.0-84904638907&partnerID=40&md5=6702a004656956db600cd9217c54b2fc >.

RABAL, O.; URBANO-CUADRADO, M.; OYARZABAL, J. Computational medicinal chemistry in fragment-based drug discovery: what, how and when. **Future Medicinal Chemistry**, v. 3, n. 1, p. 95-134, 2011. Available at: <<Go to ISI>://WOS:000287887900015 >.

REID, R.C. Inhibitors of secretory phospholipase A2 group IIA. Curr. Med. Chem. v. 12, n. 25, p. 3011–26, 2005.

SCOTT, K.F. et al. Emerging roles for phospholipase A2 enzymes in cancer. **Biochimie**. v. 92, n. 6, p. 601–610, 2010.

SHENG, C.; ZHANG, W. Fragment informatics and computational fragment-based drug design: an overview and update. **Med Res Rev**, v. 33, n. 3, p. 554-98, 2013. Available at: < https://www.ncbi.nlm.nih.gov/pubmed/22430881 >.

STARK, J. L.; POWERS R. Application of NMR and Molecular Docking in Structure-Based Drug Discovery. **Top Curr Chem**. v. 326, p. 1–34, 2012.

TAKEDA, T. et al. Nonsteroidal Anti-inflammatory Drug Naproxen Destabilizes A beta Amyloid Fibrils: A Molecular Dynamics Investigation. **Journal of Physical Chemistry B,** v. 114, n. 46, p. 15394-15402, 2010. Available at: <<Go to ISI>://WOS:000284287700071 >.

TAYLOR, R.D.; JEWSBURY, P. J.; ESSEX, J.W. A review of protein-small molecule docking methods. **Journal of Computer-Aided Molecular Design**, v. 16, p. 151–166, 2002.

TRESADERN, G., ROMBOUTS, F. J. R., OEHLRICH, D., MACDONALD, G., & TRABANCO, A. A. Industrial medicinal chemistry insights: neuroscience hit generation at Janssen. **Drug Discovery Today**, v. 22, n. 10, p. 1478–1488, 2017. Available at: http://doi.org/10.1016/J.DRUDIS.2017.05.013

VERLET, L. Computer "Experiments" on Classical Fluids. I. Thermodynamical Properties of Lennard-Jones Molecules. **Physical Review**, v. 159, n. 1, p. 98-103, 1967. Available at: < http://link.aps.org/doi/10.1103/PhysRev.159.98 >.

WARSHEL, A.; LEVITT, M. Theoretical studies of enzymic reactions: Dielectric, electrostatic and steric stabilization of the carbonium ion in the reaction of lysozyme. **Journal of Molecular Biology**, v. 103, n. 2, p. 227-249, 1976. Available at: < http://www.sciencedirect.com/science/article/pii/0022283676903119 >.

YAGAMI, T.; YAMAMOTO; Y.; KOMA H. The role of secretory phospholipase A_2 in the central nervous system and neurological diseases. **Mol. Neurobiol**. v. 49, n. 2, p. 863–76, 2014.

YAMAMOTO, K. et al. Secreted phospholipase A2, lipoprotein hydrolysis, and atherosclerosis: Integration with lipidomics. **Analytical and Bioanalytical Chemistry**. v. 44, n. 7 p. 1829-1842, 2011.

YAMASHITA, S.; YAMASHITA, J.; AND OGAWA, M. Overexpression of group II phospholipase A2 in human breast cancer tissues is closely associated with their malignant potency. **Br. J. Cancer**. v. 69, n. 1993, p. 1166–1170, 1994.

YEDGAR, S.; COHEN, Y.; SHOSEYOV D. Control of phospholipase A2 activities for the treatment of inflammatory conditions. **Biochim Biophys Acta.** v. 1761, n. 11, p. 1373–1382, 2006.

YOUSEFPOUR, A. et al. Molecular dynamics simulation of nonsteroidal antiinflammatory drugs, naproxen and relafen, in a lipid bilayer membrane. **International Journal of Quantum Chemistry**, v. 113, n. 15, p. 1919-1930, 2013. Available at: < https://www.scopus.com/inward/record.uri?eid=2-s2.0-84879248940&partnerID=40&md5=367c7e360b68482cb1828795535b4c12 >.

ZHAO, L. et al. Molecular Modeling for Petroleum-Related Applications. In: XU, C. e SHI, Q. (Ed.). Structure and Modeling of Complex Petroleum Mixtures. **Cham: Springer Int Publishing Ag**, v.168, p. 121-177, 2016.

Second part - Articles

Phospholipase A2 inhibitors in the design of new anti-inflammatory drugs: a review of 2012 - 2018

Abstract: The inflammatory process is a series of complex processes that occurs in response to damage agents, for the defense of the organism. However, this process can become a chronic process, causing much harm to the organism. To controll this condition, over the years many anti-inflammatory drugs have been developed aiming to decrease the concentrations of inflammatory mediators in the organism. The principal target of conventional anti-inflammatory drugs is the cyclooxygenase (COX) enzyme, but its use implies a series of side effects. Thus, based on these problems, many studies have been done, aiming to create new drugs, with new action mechanisms. In this context, the phospholipase A2 (PLA2) enzymes stand out. Off all existent isoforms, secretory PLA2 is the major target for inhibitor development, since many studies have proven that this enzyme participates in many inflammatory conditions, such as cancer, Alzheimer and arthritis, for instance. Aiming at the production of anti-inflammatory drugs that are sPLA2 inhibitors, many molecules have been developed. This review presents an overview of inflammatory processes and mediators, the available anti-inflammatory drugs, and also shows a little about the PLA2 enzymes, as well as the diverse structural array of the newest sPLA2 inhibitors as a possible target for the production of new anti-inflammatory drugs.

1. INTRODUCTION

The contact of mammalian organisms with noxious agents brings an immediate and non-specific response called acute inflammatory response [1]. Those injurious agents are broadly divided into endogenous and exogenous agents, and can be physical, chemical or biological compounds. Among all the complex processes that occur, one of the steps is the inflammatory cascade. This step uses the arachidonic acid to originate important inflammatory mediators which play different and very important roles in inflammatory response [1].

The reactions begin with the oxidation of the membrane phospholipids, by the phospholipases A2 (PLA2), giving rise to the fatty acids, such as arachidonic acid (AA), and lysophospholipids. The AA degradation is performed by cyclooxygenases (COX) and lypooxygenases (LOX) enzymes, and originates prostaglandins, thromboxanes and leukotrienes. Furthermore, the other product of membrane phospholipids degradation,, the lysophospholipids, originate other important mediators, which are the platelet-activating factors (PAF) [2].

Currently, the treatment of inflammatory conditions is performed considering one of the final products of this arachidonic cascade, the prostaglandins [3]. The Non-steroidal Anti-inflammatory Drugs (NSAIDs) are one of the class of drugs that are most utilized by the global population [4]. These drugs act inhibiting COX enzymes, blocking the production of prostaglandins, as mentioned. However, due to the non selectivity of these compounds in inhibiting only the COX-II isoforms, that are most related to the inflammatory response, the NSAIDs bring a series of side effects, such as gastrotoxicity and hepatotoxicity, among others [5, 7].

Having in mind that pain and inflammation are mediated by COX-II, and gastro protection by the COX-I isoform, new anti-inflammatory drugs, selective for COX-II, were developed. The specific COX-II inhibitors (COXIBs), are a class of anti-inflammatory drugs that selectively inhibit the COX-II isoform, decreasing the gastrotoxicity [7, 8]. However, many studies have pointed out that, besides decreasing of some undesirable effects, these drugs result in many side effects related to the cardiovascular system [9].

These cardiovascular problems occur due to blocking of the COX-II enzyme, reducing the inflammation process. But it also reduces the vasodilation and anti-platelet effects in the vascular wall, since it decreases the production of prostacyclin (PGI2) that prevents platelet aggregation and vasoconstriction. At the same time, the COX-I isoform maintains the production of Thromboxane A2 (TxA2), which can lead to vasoconstriction and platelet aggregation, and these effects cause a mediator production disequilibrium. This disequilibrium between vasoconstriction and vasodilatation in the vascular system could promote hypertension and thrombosis, and increase cardiovascular risk [10]. In this way, a decrease of both COX pathway products can be interesting.

Apart from all those factors, it is necessary to consider that the COX inhibitors do not have any effect in the leukotrienes and platelet activating factor production. These other products of membrane phospholipids degradation are also involved in the inflammation process and the blockage of the COX pathway may accentuate the production of these products [10]. If the first stage of membrane phospholipids degradation is interrupted, the production of all pro-inflammatory mediators could theoretically be controlled. In this context, PLA2 inhibitors are very promising for the development of new anti-inflammatory drugs, which could be more effective in the treatment of inflammation [11].

1.1. Inflammatory process

Inflammation is the term used to describe a series of events that occur in response to infection or tissue damage. This process has been known to humanity for at least a few thousand years, and was first introduced as "redness and swelling with heat and pain", or loss of function", describing the improper operation of cells when exposed to a stressful stimulus [12]. After some studies on inflammation, it was concluded that inflammatory responses are not only vital for host defense, but also imperative for natural tissue homeostasis, being the innate immune system inducers of a broad range of remodeling process. Currently, inflammation has been understood as "a perpetual and essential immune response, that maintains tissue homeostasis under a variety of noxious conditions" [13]

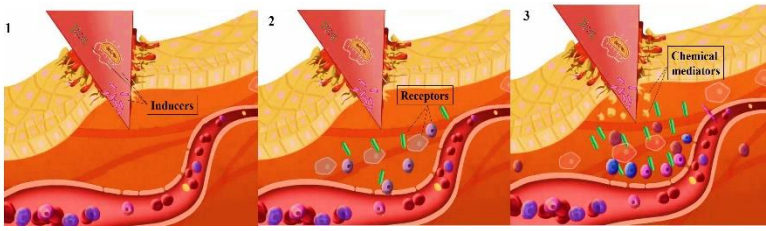
Inflammation begins with the action of injury agents in the living tissue, leading to a series of molecular events and cellular signalizations. This process is fundamental to the survival of the organism, since it provides its protection against these noxious agents and mechanical injuries [1, 14]. However, the inflammatory process can lead to negative consequences for the host, and brings the progression of many chronic conditions, such as diabetes, atherosclerosis, autoimmune diseases, and cancer [13].

Four functional categories of molecules or cells are identified in the acute inflammatory response, which are inducers, sensors, mediators and effectors, as can be seen in Figure 1 [12]. Firstly, inducers initiate the inflammatory response. These inducer compounds can be exogenous or endogenous agents that can act chemically, physically or biologically. Afterwards, the inflammation inducers act on cell-resident receptors, such as the Toll-like receptor (TLRs), which detects them, producing the chemical mediators, such such as eicosanoids (prostaglandins, platelet activating factor, and leukotrienes), and promoting vascular and cellular events in the organism. Each component takes part in distinct inflammation pathways, depending on the nature of the trigger agent [1].

The acute inflammatory response is successful when it results in elimination of the infectious agents, and in tissue repair. This repair phase is mediated by tissue-resident and recruited macrophages. If all this inflammatory process fails to eliminate the pathogen, the inflammatory process persists and acquires new characteristics that can differ depending on the effector classes that are present. The persistence of inflammation may result in a chronic process, sepsis, multiple organ failure and death [15].

Therefore, the inflammation can be viewed as a two-sided track. On one side, acute inflammation is essential for mediating protective functions, eliminating pathogenic agents that cause much damage to the host organism, repairing the tissues. On the other side, when this process fails, chronic inflammation occurs. When this process is prolonged, it causes tissue destruction; deregulates the organism, causing much harm. Thus, the development of new drugs is so important for the control of this chronic process, aiming to improve the life quality and life expectancy of the population [16].

Figure 1- Components of inflammatory response: The inducer agents initiate the molecular events and so they are detected by the receptors of the innate immune systems, as an example the Toll-Like receptors (TLRs). These receptors are expressed in specialized cells, inducing the production of the mediators, which act in the specific tissue, which are damaged to elicit changes in their functional states.



1.1.1. Pro-inflammatory mediators and their role in the inflammatory process

The production of the inflammatory mediators is triggered by the inflammation inducers [1]. These compounds alter the functionality of many tissues and organs. Most of them act in the vasculature and leukocyte recruitment [15]. Some mediators are inactive precursors in the plasma, and others are stored in granulates of mast cells, platelets and basophils [1]. At the same time, other mediators can be produced directly in response to appropriate stimulation that causes inflammation [15].

According their biochemical properties, the inflammatory mediators are classified into seven groups, which are lipid mediators, cytokines, chemokynes and proteolytic enzymes, vasoactive peptides, vasoactive amines and fragment of complement components [15]. Of all these mediators, the lipid mediators stand out; that are eicosanoids (4, 5, 6) and platelet-activating factors (7). The eicosanoids, mainly those coming from the COX pathway, are the major targets for anti-inflammatory drug development, as for example NSAIDs [17].

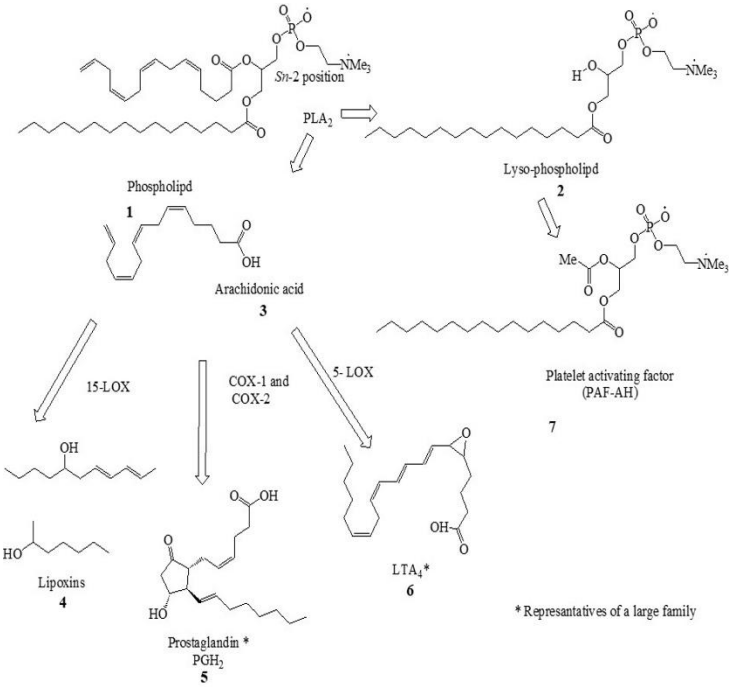
The eicosanoids are a family of 20-carbon bioactive oxygenated metabolites formed via enzymatic and non-enzymatic pathways. This family includes Prostaglandins (PGs), thromboxanes (TX) leukotrienes (LT), lipoxins (LX) epoxides, hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs) [18]. These compounds are capable to initiate many biological responses, such as edema, platelet aggregation and smooth muscle contraction, and seem to be involved in the majority of physiological events. Position (5-, 8-,12-, or 15-), number (mono or di) and stereospecificity (R or S) of oxygen insertion into the substrate and the type of receptors expressed in corresponding cell types are responsible for their structural and functional diversity.

In the mammalian system, every tissue and cell system can synthesize these mediators, since their production is initiated by the phospholipid membrane (1) degradation [16],[19]. For the synthesis of the eicosanoids, the production of arachidonic acid (AA) (3) is necessary. AA is one of the products of phospholipid oxidation, which occurs by the action of the

PLA2 enzyme. The succinct mechanism of this process can be observed in Figure 2.

This series of reactions is called arachidonic acid cascade. The hydrolysis of the phospholipids generates lysophospholipids (2) and fatty acids, such as AA (3), which is the substrate of the cyclooxygenases (COX-I and COX-II) enzymes and lipooxygenases (LOX). The metabolism of AA by these enzymes produces potent inflammatory mediators, such as prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs) [10]. Moreover, the other cleavage product, lysophospholipids, can also be converted to another inflammatory mediator, the platelet activating factor (PAF) (7) [10, 20, 21].

Figure 2- Arachidonic acid cascade. The rupture of the phospholipid membrane occurs by the action of PLA2 enzymes, originating lysophospholipids and fatty acids (e.g. arachidonic acid). After this, by the action of cyclooxygenases (COX) and lypooxygenases (LOX) enzymes on the arachidonic acid structure, the inflammatory mediators (Prostaglandins, PGs and leukotrienes, LTs) are obtained.



Adapted from Reid, 2005 [10].

1.2. Current Anti-inflammatory drugs

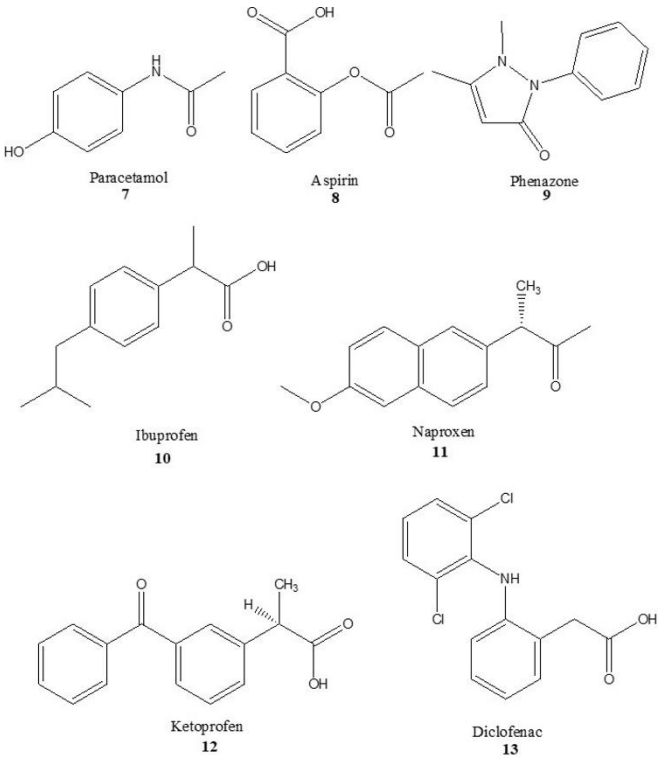
The products of the arachidonic acid cascade are the major targets for development of anti-inflammatory drugs [3]. The action mechanism of Non Steroidal Anti-inflammatory Drugs (NSAIDs), which include the Selective COX-II inhibitors (COXIBs), are based on the COX-II inhibition, decreasing the prostaglandin production.

Anther drug class that is one of the most used in the world is the corticosteroids [22]. Besides their anti-inflammatory effects, these compounds exerts diverse cellular functions such as homeostasis, metabolism and cognition[22]. The corticosteroids acts indirectly on phospholipases A2 enzymes inhibition, and this action mechanism involves a series of processes [23].

1.2.1. NSAIDs

The Non Steroidal Anti-inflammatory drugs (NSAIDs) include both the oldest NSAIDs, which are unspecific inhibitors of COX enzymes, and the newest specific COX-II inhibitors (COXIBs) [24]. NSAIDs have different chemical structures, but these pharmacologically active compounds have similar therapeutic and side effects. The compounds Paracetamol (7), aspirin (8) and phenazone (9), whose chemical structures can be observed in Figure 3, were the first NSAIDs developed, more than 100 years ago [25]. Currently, more than 50 different NSAIDs are known. The most popular drugs of this class include ibuprofen (10), naproxen (11), aspirin (8), ketoprofen (12) and diclofenac (13), whose structures are presented in Figure 3 [25].

Figure 3- Some of the most popular Non Steroidal Anti-inflammatory Drugs (NSAIDs)



In general, NSAIDs are the drugs most commonly used for their antipyretic, analgesic and anti-inflammatory activity, as well as their use in the treatment of other diseases, such as Alzheimer's and cancer [26]. The therapeutic effects, coupled with the spread of rheumatic diseases and increased life expectancy, make NSAIDs one of the most prescribed medications [26]. Each day, approximately 30 million people use NSAIDs around the world [27]. In addition, 7.7% of all pharmaceutical prescriptions in Europe are NSAIDs, 70 million NSAIDs prescriptions in the United States, 20 million in Great Britain and 10 million in Canada [25]. Of all users, 90% are people over 65 years of age [27].

Besides the differences in chemical structures, all molecules of this group possess a steroidal frame. NSAIDs can be classified by the plasma half-life and by the chemical structure. Its structural classification includes 8 groups, which are oxicams, acid derivatives, phenylacetic derivatives, phenylpropionics, sulfonanilides, indoleacetic acid derivatives, pyrazolone derivatives, salicilates and paraminophenol derivatives. In relation to the action mechanism, all NSAIDs are COX enzyme inhibitors. These drugs are not selective and inhibit both isoforms of the COX enzymes (COX-I and COX-II). Since the COX enzymes act in the production of PGs, as mentioned, these compounds play their role by means of the reduction of PGs and, consequently, minimizing the inflammation effects. The target for inhibition is the COX-II isoform, which plays a critical role in inflammatory events. The PGs coming from the COX-I pathway act in normal physiological functions as gastroprotection, in the vascular system, among others [27].

There are many problems due to inhibition of the COX-I isoform pathway [28]. Despite its prolonged use, NSAIDs cause a series of side effects, including gastrointestinal toxicity, ulcers and hepatotoxicity, among others. In addition, together with antirheumatic drugs, NSAIDs are the class of drugs that most cause hepatotoxicity [3, 28]. Considering a range of 1683 confirmed cases with drug hypersensitivity, 42% are caused by NSAIDs [29]. Beyond the problems caused by nonselectivity, other complications arise with the inhibition of the COX pathway. The decrease in the production of prostaglandins by the inhibition of COXs may increase the activity of lipooxygenases and consequently increase the production of leukotrienes, which are also involved in inflammatory processes [30, 31].

Taking into account the latest problems, the inhibition of PLA2 can be interesting. If the anti-inflammatory action occurs through the inhibition of PLA2, a decrease in the production of the pro-inflammatory mediators should occur, as desired, but at the same time the side effects due to the inhibition only of the COX pathway are solved [10]. In this context, given the important participation of PLA2 in the inflammatory process, the inhibition of these enzymes is an alternative and promising way to block inflammatory processes, aiming the design of new drugs.

1.2.1.1. COXIBs

The selective COX-II inhibitors or COXIBs are a class of NSAIDs developed in the 1990s. The COXIBs were created in order to reproduce pharmacologic properties of common NSAIDs, while decreasing the side effects related to COX-I inhibition, such as gastrotoxicity. Until now, five molecules have been approved for marketing. These molecules are presented in Figure 4 and are named celecoxib (14), rofecoxib (15), etoricoxib (16), valdecoxib (17) and lumiracoxib (18) [32].

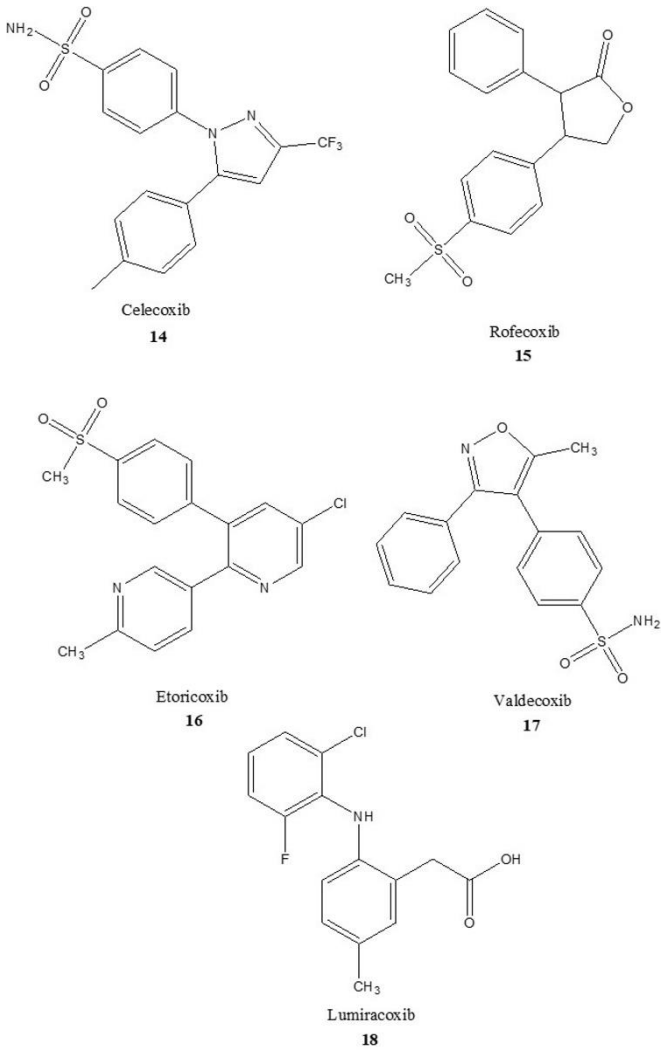
Despite the gastrointestinal side effects having been resolved, there is a concern that this class of drugs increases the risk of cardiovascular diseases. There are reports in the literature that COXIBs, such as rofecoxib, increased deaths due to the cardiovascular complications [33]. This feature occurs because both COX-I and COX-II production pathways are equilibrated processes that keep the vasoconstriction and vasodilatation in order. Blocking the COX-II enzyme, the inflammation process decreases. However, the COX-II inhibition also reduces the vasodilation and anti-platelet effects in the vascular wall, since it decreases the production of prostacyclin (PGI2) that prevents platelet aggregation and vasoconstriction. At the same time, the COX-I isoform maintains the production of Thromboxane A2 (TXA2) that can lead to vasoconstriction and platelet aggregation, and this causes a mediator production disequilibrium. This disequilibrium between vasoconstriction and vasodilatation in the vascular system could promote hypertension and thrombosis, and increase the cardiovascular risk [9].

1.2.2. Corticosteroids

The term "corticosteroid" is generally used to refer to glucocorticoids (GCs). However, the corticosteroids class also includes the mineralocorticoids [22]. The corticosteroids are molecules derived from cholesterol, and are small lipophilic molecules [23]. The GCs are a class of steroid hormones, widely used due to their strong immunosuppressive and anti-inflammatory activity [34].

The anti-inflammatory effect, as well as the other pharmacological and phisiological properties of GC result from the binding on the glucocorticoid receptor (GR) on multiple signaling pathways [35]. To produce GCs, the inflammatory citokynes, such as TNF-α or IL-β, secreted by macrophages or activated lymphocytes, induce the expression and release of corticotrophin-releasing hormone (CRH), besides activating the components of the inflammatory system. Then, the CRH increases the production of adrenocorticotropic hormone (ACTH), which stimulates GC secretion [36].

Figure 4 - Comercially available COXIBs



The produced GC displays a predominantly genomic mechanisms, but these compounds also exert non genomic effects [34]. When the formed GC-GR complex enters the cell nucleus, the modulation of various DNA transcription factors occurs, promoting a decrease in the production of pro-inflammatory proteins while leading to an increase in the production of anti-inflammatory proteins, such as annexin [34]. These compounds act in many cell types, such as macrophages, neutrophils, mast cells and epithelial cells, among others [36].

The genomic effects of synthetic GC compounds are slow, while the fast anti-inflammatory effects occur by the non-genomic mechanisms [37]. The known mechanisms are the physicochemical interactions with membrane-bound GR or cytosolic GR. These interactions occur in seconds or minutes after the GR activation, and do not require protein synthesis [22]. The GR activation releases proteins, such as annexin, which act in signaling cascades [22, 36]. The annexin inhibits PLA2 enzymes, decreasing the arachidonic acid (AA) levels [35, 37].

Based on this idea, the synthetic GCs are used to treat immune and anti-inflammatory diseases such as rheumatoid arthritis and asthma, among others [36]. However, long therapies with GC promotes tissue-specific resistance [22]. Furthermore, the binding of synthetic GC in steroidal receptors results in many side effects, such as diabetes, hypertension, cataracts, osteoporosis, skin atrophy, glaucoma, abdominal obesity, avascular necrosis and infection and growth retardation [22, 36]. Thus, it is possible to infer that, considering that the GCs are primary stress hormones which regulate many physiologic process, there is an advantage in using specific inhibitors of PLA2. By the action of sPLA2 inhibitors, the AA levels will decrease as desired, but the effects related to the interaction between GCs and receptors will not be observed.

1.3. Phospholipases A2

1.3.1. Overview

As mentioned, phospholipases A2 (PLA2) are hydrolase enzymes that catalyze the hydrolysis of the membrane phospholipids, at the sn-2 position. This class of enzymes is important for a series of cellular activities, such as membrane modeling and cell signaling [10, 19, 38]. The action mechanism of these enzymes, proposed by Scott and collaborators (2010) [39], can be seen in Figure 5. It suggests that the His48 / Asp99 pair (19) removes a proton from a water molecule (20), which acts as a nucleophile on the attack at the sn-2 position of the membrane phospholipid (1). The Ca^{2+} cofactor stabilizes the oxyanion, the reaction intermediary.

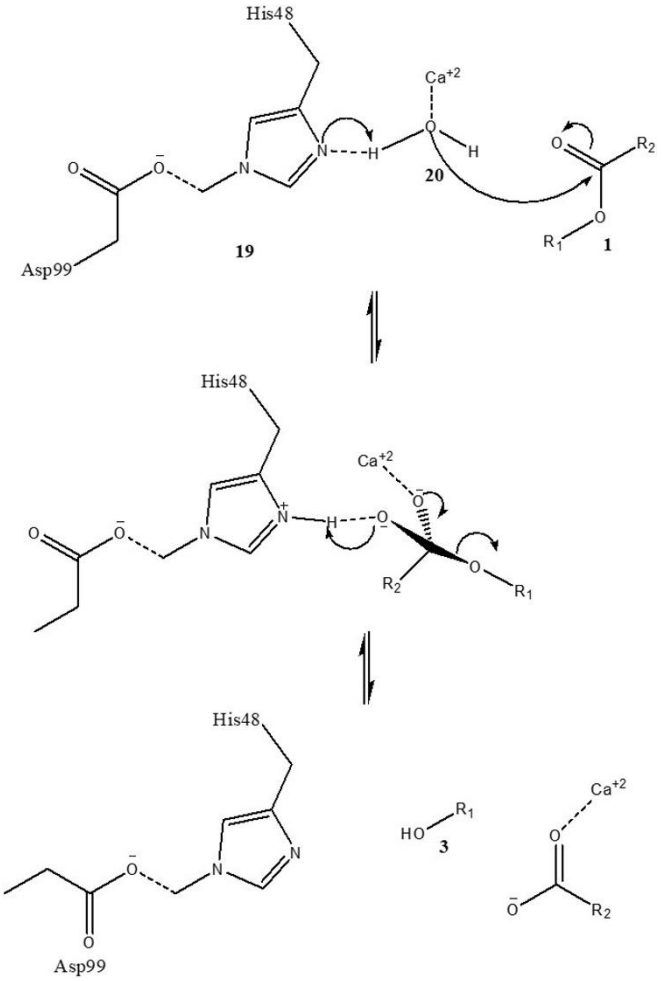
Currently, more than 30 PLA2 isoforms are known [11],and are subdivided into six groups, the calcium-independent (iPLA2), cytosolic (cPLA2), secreted (sPLA2), platelet activating factor acetylhydrolase (PAF-AH), the lysosomal (lyPLA2), and one recently discovered, named "Adipose-specific PLA2" (Ad-PLA2). These groups are presented in Table 1. For the division of the groups, their molecular weights, disulfide bonds, calcium requirement, among other criteria are taken into account [11].

The group of cPLA2 contains six isoforms (IVA cPLA2α groups), IVB (cPLA2β), IVC (cPLA2γ), IVD (cPLA2δ), IVE (cPLA2ε) and IVF (cPLA2ζ), which have between 24 and 51% of sequential identity, different catalytic activities and expression in tissues [40]. The active site of this class contains the pair Ser/Asp, which çis specific for phospholipids at the sn-2 position, in addition to requiring micromolar concentrations of calcium ions for their functioning [41]. The first group, IV, of cPLA2 was found in 1986 [41]. Since then, many isoforms have been sequenced and studied [20, 38].

IPLA2 belongs to group VI of PLA2. There are six isoforms of iPLA2, which, similarly to cPLA2, utilize the serine residue present at its catalytic site as a nucleophile to exert its catalytic function. The crystalline structures of IPLA2 are still not well elucidated and the molecular weight of this group ranges from 85 to 88 KDa. Unlike cPLA2, iPLA2 has no preference for sn-2 positions of fatty acids. These proteins are involved in signaling. In addition, they are

related to some diseases, for example diabetes and skin inflammations, among others [20, 38, 42].

Figure 5 - Suggested action mechanism of PLA2 in the breaking of phospholipids.



PLA2 PAF-AH are enzymes responsible for the hydrolysis of the acetyl group at the sn-2 position of the platelet-activating factor, a potent phospholipid mediator. In addition, hydrolyzes oxidized phospholipids are produced during the LDL oxidation, inflammation and oxidative stress [43]. This group of PLA2 is also known as Lp-PLA2, because it circulates in plasma associated with lipoproteins. Like i-PLA2, these enzymes do not need available calcium ions to perform their function. However, unlike the other PLA2, PAF-AH is specific for short acidic esterified groups, at the sn-2 position of their substrate [43].

The fourth group, LyPLA2 has this name because they are located in the lysosome. Currently, two enzymes have been identified. The first Ly-PLA2 identified has a preference for substrates phosphatidylcholine and phosphatidylethanolamine, high homology with the enzyme lecithin cholesterol acyltransferase, independence of calcium ions and optimum pH of 4.5 for its activity [44]. This enzyme is highly expressed in alveolar macrophages, but can also be found in different cell types. This makes it play a key role in surfactant metabolism, acting on the catabolic homeostasis of pulmonary surfactants [11].

The Adipose-specific PLA2 (Ad-PLA2) is a class of recently discovered phospholipase [45]. These enzymes belong to the lecithin retinol acyltransferase family., Studies suggest that to carry out their activity the residues His 23 and Cys 113 are indispensable [45, 46]. In addition to hydrolyzing phospholipids at the sn-2 positions, these enzymes are also able to hydrolyze phospholipids at the sn-1 position, and depending on the condition, the latter may be more efficient than the former [43].

The last group, but no less important, it is composed of secreted phospholipases A2 (sPLA2), which were the first types of PLA2 discovered. They are low molecular weight enzymes (approximately 14 KDa), and are characterized by containing six disulfide bonds, Histidine at their catalytic site, and dependent on mM concentrations of calcium. These enzymes are found in various animal poisons, synovial fluid, and various mammalian tissues. Until now, 16 types of sPLA2 are described in the literature, named as IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA and XIIB PLA2. In general, these enzymes have catalytic preference for negatively charged phospholipids, in particular phosphatidylserine, phosphatidylglycerol and phosphatidylethanolamine, but this preference varies according to each isoform. This preference is an important feature, for example, to act in the defense of the organism, since these phospholipids are present in the membrane of bacteria [11].

In addition to membrane phospholipids, sPLA2 may also act on "non-cellular" phospholipids, such as those found in pulmonary surfactants, microvesicles, and lipoproteins. This variation may explain the great molecular evolution and the amount of isoforms existing in this group, in relation to the other PLA2 [20, 38, 47].

Table 1- General classification of Phospholipases A2 and their associated diseases [11].

PLA_2 Family	Group	Organism	Weight (KDa)	Catalytic residues		Associated diseases
$cPLA_2$	IVA to F	Human/ murine	60-85	Ser/Asp		-
$iPLA_2$	VIA to F	Human/ murine	28-146	Ser/Asp		-
PAF-AH	VIIA and VIIB VIIIA IIIB	Human/ murine /swine/bovine	40-45 ~26	Ser/His/Asp		- -
$LyPLA_2$	XV	Human	~45	Ser/His/Asp		-
$AdPLA_2$	XVI	Human adipocytes	~18	His/Cys		-
$sPLA_2$	IA	Swine	~14	His/Asp		
		IB	Human/ swine pancreas	~14	His/Asp	Pancreatic acinar carcinoma and dry eye disease
		IIA	Human synovial fluid /snakes	~14	His/Asp	-
		IIB	Gabon viper	~14	His/Asp	-
		IIC	Mouse / murine testes	~14	His/Asp	Obstructive chronic lung disease and asthma
		IID	Human / murine pancreas / spleen	~14	His/Asp	Ulcerative colitis and chronic rhinosinusitis
		IIE	Uterus / brain / human / murine heart	~14	His/Asp	Atopic dermatitis and colorectal cancer
		IIF	Human / murine embryos and testes	~14	His/Asp	Colorectal cancer and atherosclerosis
		III	Human / murine lizards / bees	~55	His/Asp	Arthritis, atherosclerosis, sepsis, cancer and chronic hepatitis
		V	Human / murine	~14	His/Asp	-
		IX	Heart / lung / macrophages	~14	His/Asp	-
		X	Leukocytes / human spleen	~17	His/Asp	-
		XIA e XIB	Sprouts of green rice	~13	His/Asp	-
		XIIA	Human/murine	~14	His/Asp	-
		XIIB	Human / murine	~14	His/Asp	Acute pancreatitis
		XIII	Parvovirus	<10	His/Asp	-
		XIV	Symbiotic fungi / bacteria	13-19	His/Asp	-

1.3.2. Secretory PLA2 as targets for inhibitors

Several; studies have demonstrated the alteration of PLA2 levels, especially sPLA2, during inflammatory disorders, such as rheumatoid arthritis, atherosclerosis, asthma, pancreatitis, Alzheimer's disease, and respiratory tract diseases and cancer [48]. In this way, sPLA2 inhibitory molecules are a source of possible anti-inflammatory drugs, different from NSAIDs and other known anti-inflammatory drugs.

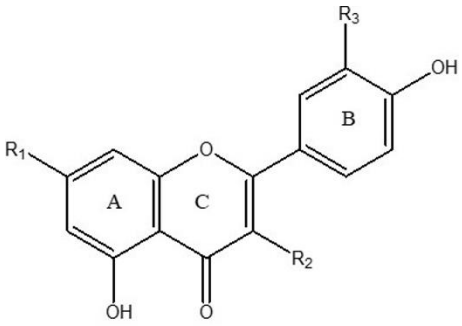
Of all PLA2 groups, sPLA2 has been considered as one of "inflammatory enzymes" since they are involved in pathophysiological processes of various inflammation-related diseases [38]. The expression of sPLA2 is related to several inflammatory processes, such as allergic inflammatory processes, pancreatitis, atherosclerosis and cancer [47, 49–51]. In particular, the expression of the IIA group is attributed to several pathologies, such as obesity, arthritis, atherosclerosis and cancer, and one of its activities is the recruitment of other sPLA2, such as those of group X and V [11, 43]. Furthermore, the PLA2 inhibition in general, as well as its mechanisms, are widely studied using sPLA2. Since they are more abundant and are present in venoms of snakes and the pancreas, they can facilitate its acquisition and availability [52].

Given the importance of these enzymes in inflammatory processes, sPLA2 is an interesting target for research in several areas such as medicinal chemistry, biochemistry and pharmacology [20, 38, 43]. These studies are aimed at elucidating inhibition mechanisms at the molecular level for the design of novel sPLA2-specific inhibitor compounds, such as in the treatment of diseases related to inflammatory processes [53]. In addition, the discovery of the sPLA2 oncogenic role and involvement in neurological diseases has also motivated the study of new inhibitors for this class of enzymes [21, 54–57].

1.3.3. The newest sPLA2 inhibitors as potential anti-inflammatory drugs

Many molecules are already known to inhibit sPLA2 activity. In the 2012-2018 period, much research has been focused on the design of sPLA2 inhibitors, for the development of new anti-inflammatory drugs. In relation to natural products, many compounds are found by inhibiting the sPLA2 enzymatic activity. Belchor et al. (2017) [58] studied the inhibitory effect, *in vitro* and *in vivo*, of natural compounds Rhamnetin (Rhm) (21), 3-O-methylquercetin (3MQ) (22) and Rhamnazin (Rhz) (23), which are quercetin (24) derivatives. The basic structure and derivatives are displayed in Figure 6.

Figure 6 - Quercetin derivatives as inhibitors of sPLA2: Rhamnetin (Rhm), 3-O-methylquercetin (3MQ) and Rhamnazin (Rhz).



Substituents

Name	R1	R2	R3
21 3MQ	OH	O-CH3	OH
22 Rhm	O-CH3	OH	OH
23 Rhz	O-CH3	OH	O-CH3
24 q	OH	OH	OH

For the inhibitory analysis, the authors employed a snake venom PLA2 from *Bothrops jararacussu* [58, 59]. In addition, the creatine kinase (CK) levels and cytotoxicity were evaluated. Both compounds evaluated show inhibitory activity. However, compounds 3QM and Rhz increased the CK levels. Rhm shows good inhibition, no toxicity and decreased CK levels, indicating its potential to decrease muscle injury. The authors attribute its anti-inflammatory activity to the presence of 3OH on C-ring, while the increase of cell viability and low CK levels induced by sPLA2 were attributed to methylation of ring A.

The potential of a major constituent of Korea edible mushroom *Polyozellus multiplex*, polyozellin, to inhibit sPLA2 was tested by Ku et al. (2016) [60]. The polyozellin was able to inhibit the phosphorylation of the cytosolic PLA2, which consequently modulates the sPLA2 expression and activity. Other natural compounds, baicalin, baicalein and wogonin, isolated from Chinese herb Huang Qui [61], and vicenin-2 and scolymoside, isolated from Cyclopia subernata [62], were able to inhibit the sPLA2-IIA activity by the same mechanism. Similarly, the GLP-1 receptor against exendin-4 (EX4), a 39-residue peptide, isolated from the salivary secretions of the lizard *Heloderma suspectum*, was also capable of suppressing LPS-induced activation of cPLA2 and extracellular signal-regulated kinase (ERK) 1/2 [63].

Another possibility for the development of specific and strong sPLA2 inhibitors is the modification of natural compounds. Based on the active site residues, in the past year Sales and collaborators [59] developed modified compounds using vannilic acid, a natural compound found in the species *Sambucus williamsii Hance*. By means of theoretical tools, the

authors developed two possible analogues, which show stronger interactions in relation to vanillic acid. In addition, the author show that is possible to employ some snake venom sPLA2 as an experimental model, in order to facilitate the experimental process. The inhibitory effect over sPLA2 enzymes from synthetic compounds has also been evaluated. Bukhari et al. (2016) [64] evaluated, by theoretical and experimental studies, the inhibitory effects of 35 benzimidazole derivatives on group 5 sPLA2 (25) (Figure 7) and found that all synthesized compounds are effective to inhibit the enzymatic activity of PLA2. The compounds 27-30, 41 and 44 presented a strong inhibition, with IC_{50} less than 10 μM. Of these, compound (28) was the most potent sPLA2 inhibitor, with an IC_{50} of 3.22 μM.

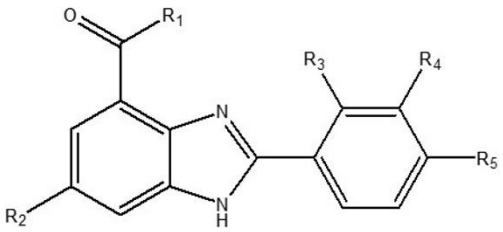
One drug used in the treatment of metastatic melanoma therapy, Dabrafenib, also demonstrates potential to regulate sPLA2 activity. Jung, Kim and Bae (2016) [65] performed in vivo and in vitro tests for evaluating this hypothesis. As a result, Dabrafenib remarkably suppressed the activity of a II-A group PLA2 by the inhibition of phosphorylation of cPLA2, thus causing a regulation of sPLA2 activity.

A novel group of sPLA2 and Sphingomyelin Synthase (SMS) inhibitors (60-67) based on α-amino cyanide fragments and indole molecules (Figure 8) were developed by Gao et al. (2013) [66]. In vitro assays were performed and as a result, all compounds provided inhibitory effects at a concentration between 14-32 μmol.L^{-1}.

In 2013, Dileep and collaborators [67] studied the inhibitory activity of 160 indole derivatives. Of these, four presented high interaction energies with the sPLA2 of porcine pancreas. These four inhibitors, CID2324681 (68), CID8617 (69) (indolebutyric acid or IBA), CID22097771 (70) and CID802 (71) (indoleacetic acid or IAA) can be seen in Figure 9. With all analyses performed, it was possible to conclude compounds 71 and 69 are promising candidates as anti-inflammatory drugs.

Another interesting class of inhibitors recently developed are the synthetic specific peptide inhibitors [68]. Based on important peptide fragments of sPLA2, specific sequences of aminoacids are employed. As described by the authors, for the human sPLA2 IIA, the specific sequence ^{17}AALSYGFYG^{25} was identified as a potentially forming region of amyloid-like aggregates. These regions cold mediate PLA2-PLA2 interactions, which results in formation of oligomers with high activity. The idea is that these specific synthetic sequences can interfere in oligomerization of these proteins, which decrease the catalytic activity of sPLA2. The result shows that there was approximately 36% inhibition at an AALSYGFYG concentration of 80nM. The shift in oligomerization can occur via non-specific and specific interactions [68]. The use of class specific peptides is a promising alternative, since these compounds are derived from biomolecules, which decrease its toxicity. Identification of these sequence structures could be used in the design of many specific and potent peptide inhibitors, for many different enzymes [68].

Figure 7- General structure and derivatives of benzimidazole inhibitors.



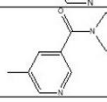
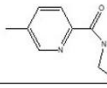
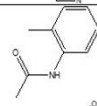
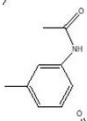
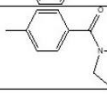
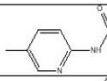
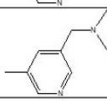
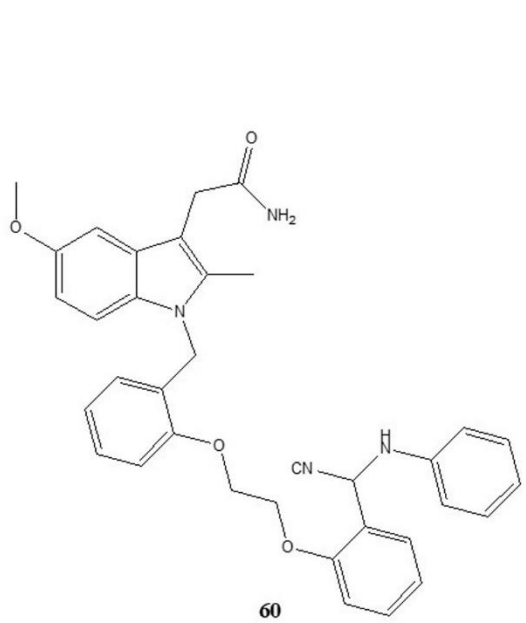
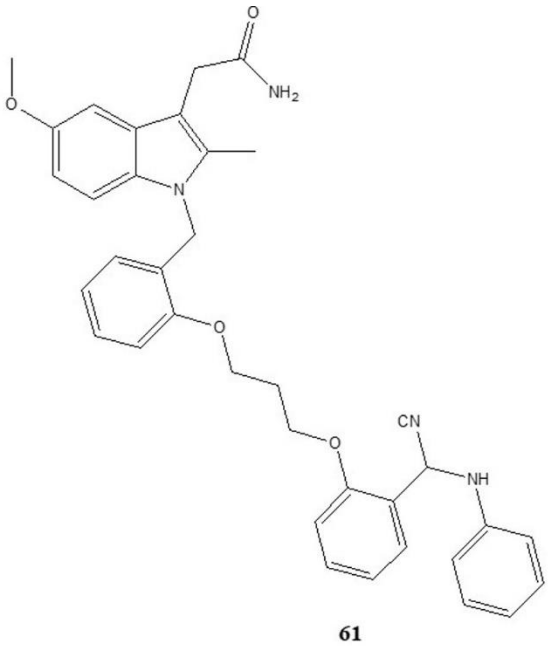
Entry	R_2	R_1	R_3	R_4	R_5
25	H	NH_2	H	H	
26	CH_3	NH_2	H	H	
27	H	NH_2	H	H	$-OCH_3$
28	CH_3	NH_2	H	H	
29	H	NH_2	H	H	$-CF_3$
30	CH_3	NH_2	H	H	
31	CH_3	NH_2	H	H	-CN
32	H	NH_2	H	H	
33	H	NH_2	H	H	
34	H	NH_2	H	H	
35	CH_3	NH_2	H	H	
36	H	NH_2	H	H	
37	CH_3	NH_2	H	H	
38	H	NH_2	H	H	
39	H	NH_2	H	H	
40	H	NH_2	H	H	
41	H	NH_2	H	H	
42	H	NH_2	H	H	
43	CH_3	NH_2	H	H	
44	H	NH_2	H	H	
45	H	OH	H	H	

Figure 8- α-amino cyanide fragments and indole-based sPLA2 inhibitors



60



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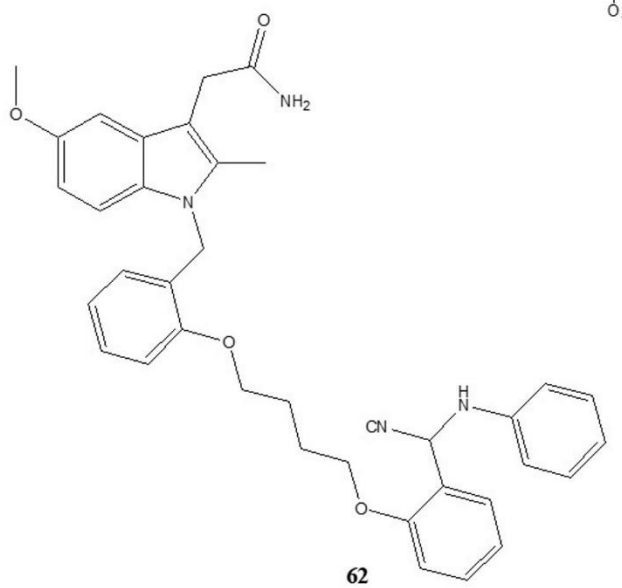

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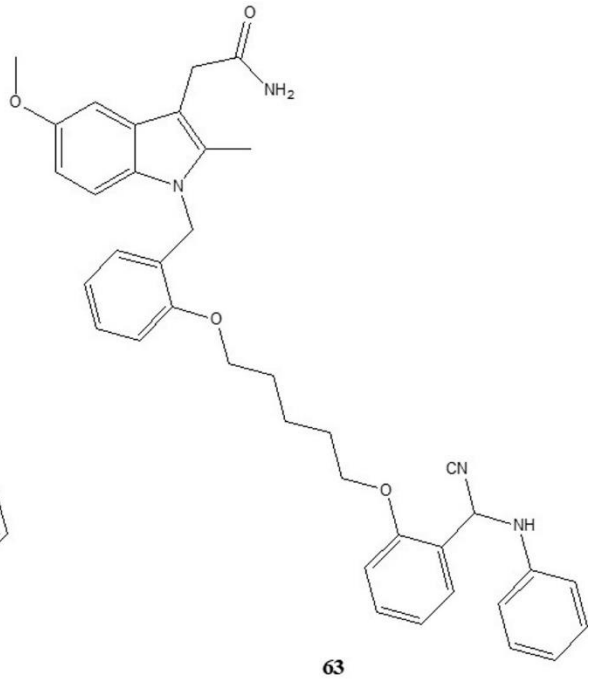

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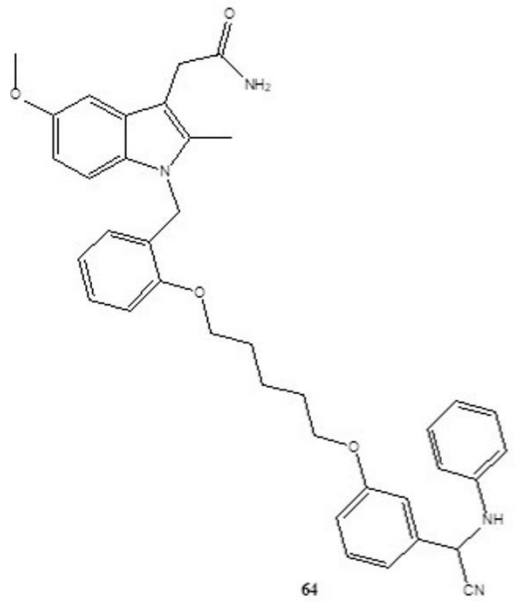

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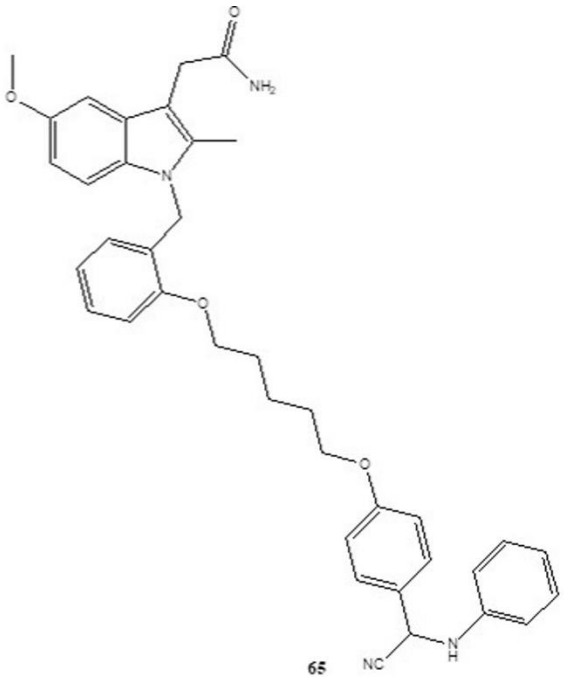

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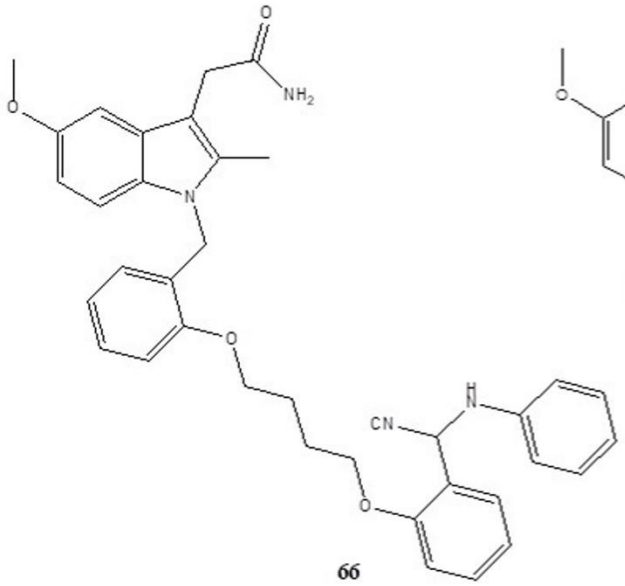

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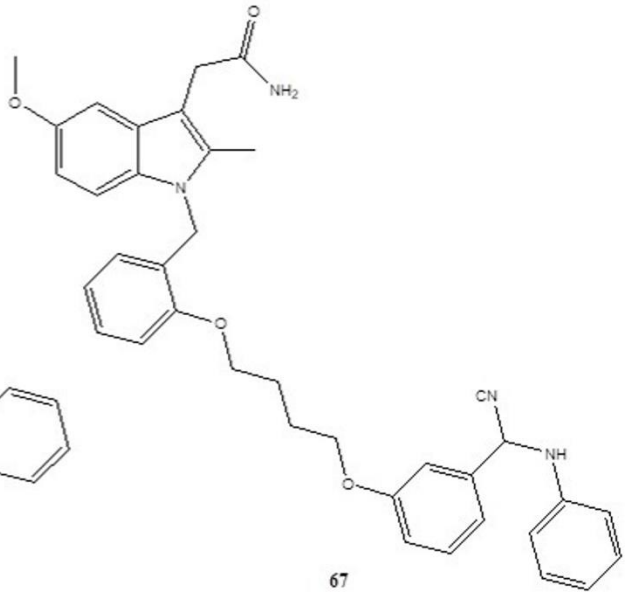
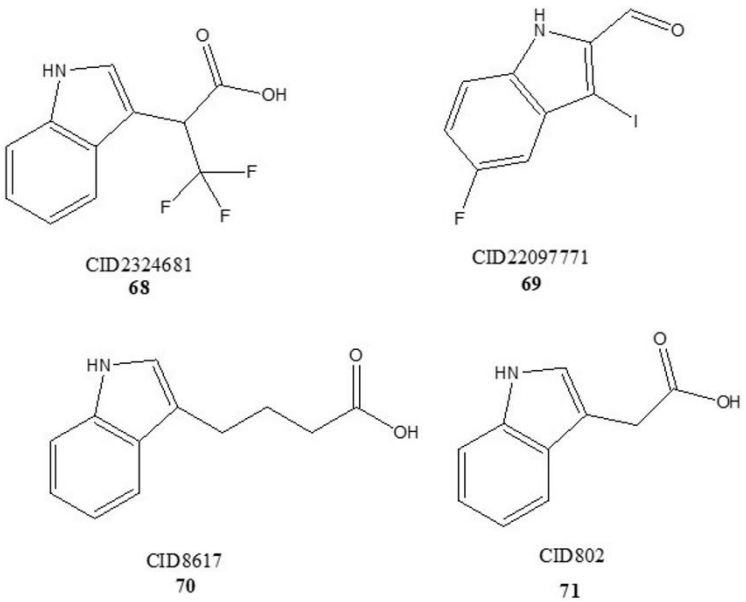


67

Figure 9- Indole derivatives inhibitors



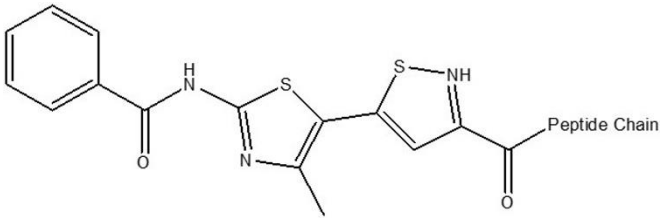
Focusing on the interaction between sPLA2IIA and integrinαvβ3, other peptide based inhibitors were identified using heterocycle-capped compounds. The authors used the One-Bead-One-Compound (OBOC) for identification of biologically active compounds [69]. The interaction of sPLA2 with this integrin induces the cell proliferation and pro-inflamatory activation of extracellular responsive kinase 1 and 2 (ERK1/2). In this way, the authors used this interaction as a target for inflammatory intervention, testing pyrazolylthiazole-tethered peptide inhibitors (Figure 10). These compounds showed more than 70% inhibition effect against the interaction between sPLA2-IIA and integrin αvβ3.

1.3.4. The 2012-2018 patents

Due to the large number of existing sPLA¬2 inhibitors, this paper focuses only in inhibitors developed in the last six years. The patent described by Tamarit and Theze (2017) [80] relates indole-based compounds (85-104), including its salts, esters, hydrates, racemates, enantiomers, prodrugs or metabolites. The base structure and radical substituents of the patent can be seen in Figure 11. The suggested compounds are able to inhibit sPLA2 of the IB group. According to the authors, the indole-based compounds exhibit potent PLA2 inhibitory effect. In addition, the inhibitors have effects on the treatment of AIDS by many mechanisms, and can help the immune system.

The patent developed by Luo Ruixue in 2016 [81] reports the application of pleurolactone for the preparation of anti-inflammatory drugs. In vivo studies have been employed and the results obtained by the authors indicate that pleurolactone has an IC_{50} close to that of indole-based sPLA2 inhibitors.

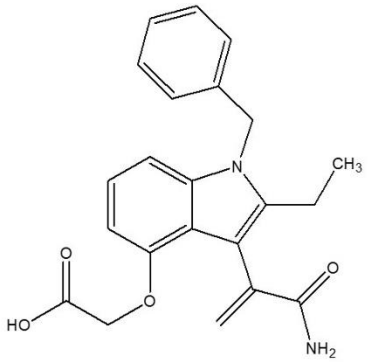
Figure 10 - pyrazolylthiazole-tethered peptide hits and their analogs



Compound	Peptide Chain
72	R-OH
73	R-Ala
74	R-Ala-Trp
75	R-Ala-Trp-Asp*
76	Ala-Trp-Asp*-Ile
77	R-Ala-Trp-Asp*-Ile
78	R-Gly
79	R-Gly-Arg*
80	R-Gly-Arg*-Gly
81	R-Gly-Arg*-Gly-Asp*
82	R-Gly-Arg*-Gly-Asp*-Asp*
83	Gly-Arg*-Gly-Asp*-Asp*-Asp*
84	R-Gly-Arg*-Gly-Asp*-Asp*-Asp*

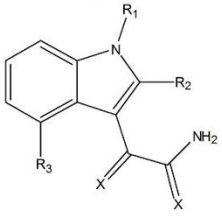
* D-amino acids.

Figure 11- Indole-based sPLA2 inhibitors. The top structure is the principal molecule of the patent and the general structure below shows its derivatives.



3-(2-amino-1,2-dioxoethyl)-2-ethyl-1-(phenylmethyl)-1H-indol-4-yl)oxy)acetic acid

85

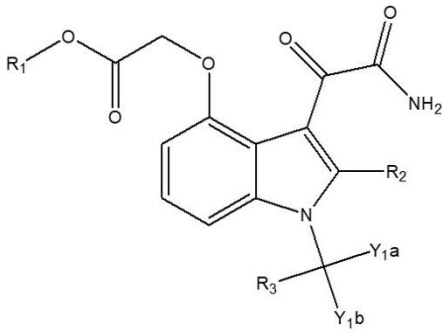


X= O	**86**
X=S	**87**
R1= -(CH2)n	**88**
R1= -Ar1; n=0 1 or 2;	**89,90,91**
R1= Ar1 = 5-7C aromatic group	**92,93,94**
R2= 1-4C alkyl;	**95,96,97,98**
R3= -(O)m(CH2)pCOOR4; m= 0 or 1	**99,100**
; p= 0-2;	**101,102**
R4= H	**103**
R4= 1-3 alkyl	**104**

In 2013, Mehendale [82] developed an sPLA2 inhibitor that is also efficient in protecting or ameliorating liver damage caused by a lethal dose of hepatotoxicant. The compound, named 5-(4-benzyloxyphenyl)-4S-(7-phenylhepatonoylamino) pentanoic acid, was tested in male Swiss Webster mice, at a dose of 20 mg/kg. The results showed that the use of sPLA2 inhibitor resulted in more deaths caused by the administration of lethal dose of hepatotoxicant (acetaminophen, carbon tetrachloride or thioacetamide). Only 10% of mortality was observed. In addition to the specific compound, sodium (3-aminooxyalyl-1-benzyl-2-ethyl-6-methyl-1 H-indol-4-yloxy)-acetic acid), ochnaflavone, para-bromophenacyl bromide or other benzophenone oximes derivatives are described in this patent. In 2014, Dennis and collaborators [83] developed PLA2 inhibitors using amides (105-131) (Figure 12).

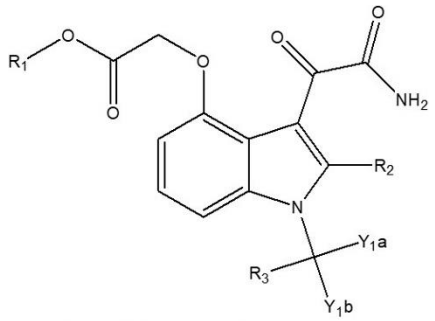
Liu, in 2012 [84], created new sPLA2 inhibitors based on (3-aminooxalyl-1H-indol-4-yloxy) acetic acid and its salts (132-141). The basic structure is represented in Figure 13. These compounds demonstrate therapeutic effect on several disorders, such as acute bronchitis, bronchiolitis, acne, acute coronary syndrome, allergic conjunctivitis, allergic rhinitis, angina, cancer, gastritis, contact dermatitis, esophagitis, dermatoses and cystitis [84]. All these activities are attributed to inhibition of PLA2 enzymes.

Figure 12- Amide derivatives PLA2 inhibitors



R1= -CD3	**132**
R1= -CD2	**133**
R1= -CH3	**134**
R1= H	**135**
R2= ehtyl	**136**
R2= deuterium	**137**
Y1a= H	**138**
Y1a= D	**139**
Y1b= H	**140**
Y1b= D	**141**

Figure 13- PLA2 inhibitors based on (3-aminooxalyl-1H-indol-4-yloxy) acetic acid.

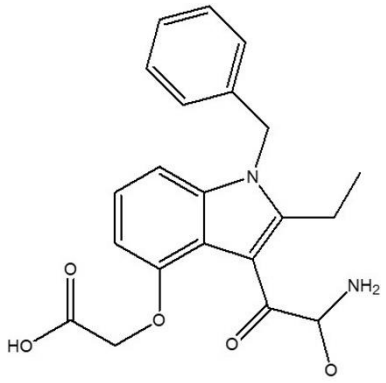


R1= -CD3	**132**
R1= -CD2	**133**
R1= -CH3	**134**
R1= H	**135**
R2= ehtyl	**136**
R2= deuterium	**137**
Y1a= H	**138**
Y1a= D	**139**
Y1b= H	**140**
Y1b= D	**141**

As can be seen, in recent years many sPLA2 inhibitors have been developed. All these studies prove that the use of sPLA2 as target enzymes is quite promising. However, the drug design is a very costly and a time-consuming process [70]. To reach the preclinical stage, the candidate molecule must have well defined pharmacokinetic parameters, its pharmacodynamics must be optimized and have a low toxicity profile, among other pharmacological characteristics [71].

Sometimes, the compounds are potent inhibitors but cannot be commercialized due to side effects presented in pre-clinical texts. An example, the potent sPLA2 inhibitor Varespladib (Figure 14) (142) (LY315920), developed in 1996 [72].

Figure 14- Chemical structure of Varespladib



142

The IC_{50} found for this compound was 0.009μM [72, 73]. Since then, many studies have been made for testing the pharmaceutical potential of Varespladib, investigating its use for the treatment and prevention of Sickle Cell Disease, Vaso-occlusive Crisis, Acute Coronary Syndrome, among others [72, 74, 75]. Varespladib was a promising sPLA2 inhibitor that reached clinical phase trials (clinicaltrials.gov Identifier: NCT01130246). However, it was not commercialized because some undesirable side effects were observed. The side effects arise due to the potentially inadequate penetration of Varespladib into vascular cells to inhibit pro-inflammatory intracellular mediators, and also its the non-specificity, since it inhibits many sPLA2 isoforms [76].

To assist in all phases of this complex process of drug design, the crystallography and the computational chemistry is of great value [77]. The use of computational chemistry techniques in the study of biomolecules can be of great value not only in the process of drug design but also in the development of other technologies [78]. In relation to the sPLA2 study, several computational medicinal chemistry techniques, such as structure–activity relationship, molecular modeling studies and theoretical drug development based on the crystal structures of sPLA2, have been used [59, 79]. In an attempt to find new promising pharmacologically active molecules, computational drug design methodologies can be employed to provide data about action mechanisms, molecular modifications for chemical structures, pharmacokinetics, selectivity and side effects, among others [70].

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CONCLUSION

Having in mind the large incidence of inflammatory conditions, and the many side effects caused by recent available anti-inflammatory drugs, the secretory phospholipases A2 (sPLA2) enzyme constitutes a promising target for designing new anti-inflammatory drugs. Several potent, bioavailable and selective sPLA2 inhibitors have been developed over the years, which attest to the continued interest in designing new anti-inflammatory drugs. In addition to molecules developed specifically for inhibiting the sPLA2, drug repurposingt is also a promissing technique, since the drug is already characterized pharmacologically, and this provides a time gain in many stages of the drug design process. In this scenario, we could cite, as an example, Dabrafenib, used in the treatment of metastatic melanoma therapy. We strongly feel, then, that Secretory Phospholipases A2 can be considered a molecular target for the development of new anti-inflammatory drugs and that many of the compounds presented herein may become, in the future, efficient and non-toxic anti-inflammatory drugs, but many studies still need to be done.

LIST OF ABBREVIATIONSPLA2 – Phospholipase A2

AA – Arachidonic acid
COX - Cyclooxygenase
LOX - Lipoxygenase
PAF – Platelet activating factor
NSAID – Non steroidal anti-inflammatory drugs
COXIBS – Selective cox-ii inhibitors
PGI2 – Prostaglandin I2
TxA2 – Thromboxane A2
TLR – Troll-like receptor
TX - Thromboxane
LT - Leukotriene
LX - Lipoxin
HETE - Hydroxyeicosatetraenoic acids
EET - Epoxyeicosatrienoic acids
PG - Prostaglandins
GC - Glucocorticoids
GR – Glucocorticoid receptor
CRH - Gorticotrophin-releasing hormone
ACTH - Adrenocorticotropic hormone
PAF-AH – Platelet activating factor
CK – Creatine kinase
RHM - Rhamnetin
RHZ - Rhamnazin
MQ - Methylquercetin

REFERENCES

[1] Karmarkar, D. *Modulators of the Acute Inflammatory Response: A Dissertation.* University of Massachusetts Medical School : GSBS Dissertations and Theses. Paper 656, February 5, **2013**.

[2] Joshi, V.; Umashankara, M.; Ramakrishnan, C.; Nanjaraj Urs, A. N.; Suvilesh, K. N.; Velmurugan, D.; Rangappa, K. S.; Vishwanath, B. S. Dimethyl Ester of Bilirubin Exhibits Anti-Inflammatory Activity through Inhibition of Secretory Phospholipase A2, Lipoxygenase and Cyclooxygenase. *Arch. Biochem. Biophys*. **2016**, 598, 28–39.

[3] Rafaniello, C.; Ferrajolo, C.; Sullo, M. G.; Sessa, M.; Sportiello, L.; Balzano, A.; Manguso, F.; Aiezza, M. L.; Rossi, F.; Scarpignato, C.; et al. Risk of Gastrointestinal Complications Associated to NSAIDs, Low-Dose Aspirin and Their Combinations: Results of a Pharmacovigilance *Reporting System. Pharmacol. Res*. **2016**, 104, 108–114.

[4] Ahmadi, A.; Khalili, M.; Olama, Z.; Karami, S.; Nahri-Niknafs, B. Synthesis and Study of Analgesic and Anti-Inflammatory Activities of Amide Derivatives of Ibuprofen. *Mini-Reviews Med. Chem*. **2017**, 17 (9), 799–804.

[5] Cronstein, B.; Weissmann, G. Targets for Antiinflammatory Drugs. *Annu. Rev. Pharmacol. Toxicol*. **1995**, 35, 449–462.

[6] Yousefpour, A.; Amjad Iranagh, S.; Nademi, Y.; Modarress, H. Molecular Dynamics Simulation of Nonsteroidal Antiinflammatory Drugs, Naproxen and Relafen, in a Lipid Bilayer Membrane. Int. *J. Quantum Chem.* **2013**, 113 (15), 1919–1930.

[7] Marnett, L. J. The COXIB Experience: A Look in the Rearview Mirror. Annu. Rev. *Pharmacol. Toxicol*. **2009**, 49 (1), 265–290.

[8] Ramalho, T. C.; Rocha, M. V. J.; da Cunha, E. F. F.; Freitas, M. P. The Search for New COX-2 Inhibitors: A Review of 2002 - 2008 Patents. Expert Opin. Ther. Pat. **2009**, 19 (9), 1193–1228.

[9] Christopher P. Cannon; Paul J. Cannon. COX-2 Inhibitors and Cardiovascular Risk. Science **2012**, 336 (6087), 1386–1387.

[10] Reid, R. C. Inhibitors of Secretory Phospholipase A2 Group IIA. *Curr. Med. Chem*. **2005**, 12 (25), 3011–3026.

[11] Quach, N. D.; Arnold, R. D.; Cummings, B. S. Secretory Phospholipase A2 Enzymes as Pharmacological Targets for Treatment of Disease. *Biochemical Pharmacology.* **2014**, 90 (4), 338–348.

[12] Medzhitov, R. Inflammation 2010: New Adventures of an Old Flame. *Cell* **2010**, 140 (6), 771–776.

[13] Allen, J.; Sun, Y.; Woods, J. A. Exercise and the Regulation of Inflammatory Responses. *Prog. Mol. Biol. Transl. Sci*. **2015**, 135, 337–354.

[14] Zweifach, B. W.; Grant, L.; McCluskey, R. T. *The Inflammatory Process*.; Elsevier Science, **2014.**

[15] Medzhitov, R. Origin and Physiological Roles of Inflammation. *Nature* **2008**, 454 (7203), 428–435.

[16] Agarwal, S.; Reddy, G. V.; Reddanna, P. Eicosanoids in Inflammation and Cancer: The Role of COX-2. *Expert Rev. Clin. Immunol*. **2009**, 5 (2), 145–165.

[17] Khanapure, S.; Garvey, D.; Janero, D.; Gordon Letts, L. Eicosanoids in Inflammation: Biosynthesis, Pharmacology, and Therapeutic Frontiers. *Curr. Top. Med. Chem.* **2007**, 7 (3), 311–340.

[18] Elizabeth Berry, Yanzhou Liua, Li Chenc, A. M. G. Eicosanoids: Emerging Contributors in Stem Cell-Mediated Wound Healing. *Prostaglandins Other Lipid Mediat.* **2017**, 132, 17–24.

[19] Balietti, M.; Giuli, C.; Fattoretti, P.; Fabbietti, P.; Postacchini, D.; Conti, F. Cognitive Stimulation Modulates Platelet Total Phospholipases A2 Activity in Subjects with Mild Cognitive Impairment. *J. Alzheimers. Dis.* **2016**, 50 (4), 957–962.

[20] Burke, J. E.; Dennis, E. A. Phospholipase A2 Biochemistry. *Cardiovasc. Drugs Ther***. 2009**, 23 (1), 49–59.

[21] Ong, W.-Y.; Farooqui, T.; Kokotos, G.; Farooqui, A. A. Synthetic and Natural Inhibitors of Phospholipases A2: Their Importance for Understanding and Treatment of Neurological Disorders. *ACS Chem. Neurosci***. 2015**, 6 (6), 814–831.

[22] Ramamoorthy, S.; Cidlowski, J. A. Corticosteroids Mechanisms of Action in Health and Disease. *Rheum. Dis. Clin. NA* **2016**, 42, 15–31.

[23] Riedemann, T.; Patchev, A. V; Cho, K.; Almeida, O. F. Corticosteroids: Way Upstream The Protagonists and Their Roles. *Mol. Brain* **2010**, 3 (2).

[24] Cata, J. P.; Guerra, C. E.; Chang, G. J.; Gottumukkala, V.; Joshi, G. P. Non-Steroidal Anti-Inflammatory Drugs in the Oncological Surgical Population: Beneficial or Harmful? A Systematic Review of the Literature. *Br. J. Anaesth*. **2017**, 119 (4), 750–764.

[25] He, B.; Wang, J.; Liu, J.; Hu, X. Eco-Pharmacovigilance of Non-Steroidal Anti-Inflammatory Drugs: Necessity and Opportunities. *Chemosphere* **2017**, 181, 178–189.

[26] Boggara, M. B.; Mihailescu, M.; Krishnamoorti, R. Structural Association of Nonsteroidal Anti-Inflammatory Drugs with Lipid Membranes. *J. Am. Chem. Soc*. **2012**, 134 (48), 19669–19676.

[27] Badri, W.; Miladi, K.; Nazari, Q. A.; Greige-Gerges, H.; Fessi, H.; Elaissari, A. Encapsulation of NSAIDs for Inflammation Management: Overview, Progress, Challenges and Prospects. *Int. J. Pharm.* **2016**, 515 (1–2), 757–773.

[28] Anelli, M. G.; Scioscia, C.; Grattagliano, I.; Lapadula, G. Old and New Antirheumatic Drugs and the Risk of Hepatotoxicity. *Therapeutic Drug Monitoring*. **2012**, 34 (6), 622–62.

[29] Blanca-Lopez, N.; Perez-Alzate, D.; Canto, G.; Blanca, M. Practical Approach to the Treatment of NSAID

Hypersensitivity. *Expert Rev. Clin. Immunol*. **2017**, 13 (11), 1017–1027.

[30] Gaddipati, R. S.; Raikundalia, G. K.; Mathai, M. L. Dual and Selective Lipid Inhibitors of Cyclooxygenases and Lipoxygenase: A Molecular Docking Study. *Med. Chem. Res*. **2014,** 23 (7), 3389–3402.

[31] Pyasi, K.; Tufvesson, E.; Moitra, S. Evaluating the Role of Leukotriene-Modifying Drugs in Asthma Management: Are Their Benefits "losing in Translation"? *Pulmonary Pharmacology and Therapeutics*. **2016**, 41, 52–59.

[32] Patrono, C. Cardiovascular Effects of Nonsteroidal Anti-Inflammatory Drugs. *Curr. Cardiol. Rep.* **2016**, 18 (3), 25.

[33] Moodley, I. Review of the Cardiovascular Safety of COXIBs Compared to NSAIDS. *Cardiovasc. J. Afr*. **2008**, 19 (2), 102–107.

[34] Ozbakir, B.; Crielaard, B. J.; Metselaar, J. M.; Storm, G.; Lammers, T. Liposomal Corticosteroids for the Treatment of Inflammatory Disorders and Cancer. *J. Control. Release* **2014**, 190, 624–636.

[35] Rhen, T.; Cidlowski, J. A. Antiinflammatory Action of Glucocorticoids — New Mechanisms for Old Drugs. N. *Engl. J. Med.* **2005**, 353 (16), 1711–1723.

[36] Smoak, K. A.; Cidlowski, J. A. Mechanisms of Glucocorticoid Receptor Signaling during Inflammation. *Mechanisms of Ageing and Development*, **2004**, 125, 697-706.

[37] Buttgereit, F.; Straub, R. H.; Wehling, M.; Burmester, G.-R. Glucocorticoids in the Treatment of Rheumatic Diseases: An Update on the Mechanisms of Action. *Arthritis Rheum*. **2004**, 50 (11), 3408–3417.

[38] Dan, P.; Rosenblat, G.; Yedgar, S. Phospholipase A2 Activities in Skin Physiology and Pathology. *European Journal of Pharmacology.* **2012**, 691 (1-3), 1–8.

[39] Scott, K. F.; Sajinovic, M.; Hein, J.; Nixdorf, S.; Galettis, P.; Liauw, W.; de Souza, P.; Dong, Q.; Graham, G. G.; Russell, P. J. Emerging Roles for Phospholipase A2 Enzymes in Cancer. *Biochimie* **2010**, 92 (6), 601–610.

[40] Wang, H.; Klein, M. G.; Snell, G.; Lane, W.; Zou, H.; Levin, I.; Li, K.; Sang, B. C. Structure of Human GIVD Cytosolic Phospholipase A2 Reveals Insights into Substrate Recognition. *J. Mol. Biol*. **2016**, 428 (13), 2769–2779.

[41] Kramer, R. M.; Checani, G. C.; Deykin, A.; Pritzker, C. R.; Deykin, D. Solubilization and Properties of Ca2+-Dependent Human Platelet Phospholipase A2. *Biochim. Biophys. Acta (BBA)/Lipids Lipid Metab.* **1986**, 878 (3), 394–403.

[42] Mouchlis, V. D.; Limnios, D.; Kokotou, M. G.; Barbayianni, E.; Kokotos, G.; McCammon, J. A.; Dennis, E. A. Development of Potent and Selective Inhibitors for Group VIA Calcium-Independent Phospholipase A2 Guided by Molecular Dynamics and Structure-Activity Relationships. *J. Med. Chem*. **2016,** 59 (9), 4403–4414.

[43] Murakami, M.; Taketomi, Y.; Miki, Y.; Sato, H.; Hirabayashi, T.; Yamamoto, K. Recent Progress in Phospholipase A2 Research: From Cells to Animals to Humans. *Prog. Lipid Res.* **2011**, 50, 152–192.

[44] Hiraoka, M.; Abe, A.; Lu, Y.; Yang, K.; Han, X.; Gross, R. W.; Shayman, J. A. Lysosomal Phospholipase A2 and Phospholipidosis. *Mol. Cell. Biol.* **2006**, 26 (16), 6139–6148.

[45] Duncan, R. E.; Sarkadi-Nagy, E.; Jaworski, K.; Ahmadian, M.; Hei, S. S. Identification and Functional Characterization of Adipose-Specific Phospholipase A2 (AdPLA*). J. Biol. Chem.* **2008**, 283 (37), 25428–25436.

[46] Murakami, M.; Taketomi, Y.; Sato, H.; Yamamoto, K. Secreted Phospholipase A2 Revisited. *J. Biochem.* **2011**, 150 (3), 233–255.

[47] Murakami, M.; Taketomi, Y. Secreted Phospholipase A2 and Mast Cells. *Allergol*. Int**. 2015**, 64 (1), 4–10.

[48] Leistad, L.; Feuerherm, A. J.; Faxvaag, A.; Johansen, B. Multiple Phospholipase A2 Enzymes Participate in the Inflammatory Process in Osteoarthritic Cartilage. *Scand. J. Rheumatol*. **2011**, 40 (4), 308–316.

[49] De Luca, D.; Lopez-Rodriguez, E.; Minucci, A.; Vendittelli, F.; Gentile, L.; Stival, E.; Conti, G.; Piastra, M.; Antonelli, M.; Echaide, M.; et al. Clinical and Biological Role of Secretory Phospholipase A2 in Acute Respiratory Distress Syndrome Infants. *Critical Care*; **2013**, 17 (4), 163.

[50] Yamamoto, K.; Isogai, Y.; Sato, H.; Taketomi, Y.; Murakami, M. Secreted Phospholipase A2, Lipoprotein Hydrolysis, and Atherosclerosis: Integration with Lipidomics. *Analytical and Bioanalytical Chemistry.* **2011**, 400 (7), 1829–1842.

[51] Yedgar, S.; Cohen, Y.; Shoseyov, D. Control of Phospholipase A2 Activities for the Treatment of Inflammatory Conditions. *Biochim Biophys Acta* **2006**, 1761 (11), 1373–1382.

[52] Dennis, E. A. Introduction to Thematic Review Series: Phospholipases: Central Role in Lipid Signaling and Disease. *J. Lipid Res.* **2015**, 56 (7), 1245–1247.

[53] Margarucci, L.; Monti, M. C.; Chini, M. G.; Tosco, A.; Riccio, R.; Bifulco, G.; Casapullo, A. The Inactivation Mechanism of Human Group IIA Phospholipase A2 by Scalaradial. *ChemBioChem* **2012**, 13 (15).

[54] Jiang, J.; Neubauer, B. L.; Graff, J. R.; Chedid, M.; Thomas, J. E.; Roehm, N. W.; Zhang, S.; Eckert, G. J.; Koch, M. O.; Eble, J. N.; et al. Expression of Group IIA Secretory Phospholipase A2 Is Elevated in Prostatic Intraepithelial Neoplasia and Adenocarcinoma. *Am. J. Pathol*. **2002**, 160 (2), 667–671.

[55] Pucer, A.; Brglez, V.; Payré, C.; Pungerčar, J.; Lambeau, G.; Petan, T. Group X Secreted Phospholipase A2 Induces Lipid Droplet Formation and Prolongs Breast Cancer Cell Survival. *Mol. Cancer* **2013**, 12 (1).

[56] Yagami, T.; Yamamoto, Y.; Koma, H. The Role of Secretory Phospholipase A_2 in the Central Nervous System and Neurological Diseases. *Mol. Neurobiol*. **2014**, 49 (2), 863–876.

[57] Yamashita, S.; Yamashita, J.; Ogawa, M. Overexpression of Group II Phospholipase A2 in Human Breast Cancer Tissues Is Closely Associated with Their Malignant Potency. *Br. J. Cancer* **1994**, 69, 1166–1170.

[58] Novo Belchor, M.; Hessel Gaeta, H.; Fabri Bittencourt Rodrigues, C.; Ramos da Cruz Costa, C.; de Oliveira Toyama, D.; Domingues Passero, L.; Dalastra Laurenti, M.; Hikari Toyama, M. Evaluation of Rhamnetin as an Inhibitor of the Pharmacological Effect of Secretory Phospholipase A2. *Molecules* **2017**, 22 (9), 1441.

[59] Sales, T.; Marcussi, S.; da Cunha, E.; Kuca, K.; Ramalho, T. Can Inhibitors of Snake Venom Phospholipases A2 Lead to New Insights into Anti-Inflammatory Therapy in Humans? A Theoretical Study. *Toxins (Basel).* **2017**, 9 (11), 341.

[60] Ku, S.-K.; Yang, E.-J.; Kang, H.; Jung, B.; Bae, J.-S. Inhibitory Effect of Polyozellin on Secretory Group IIA Phospholipase A2. *Arch. Pharm. Res*. **2016**, 39 (2), 271–278.

[61] Ku, S.-K.; Lee, H. G.; Bae, J.-S. Inhibitory Effect of Baicalin, Baicalein and Wogonin on Secretory Group IIA Phospholipase A2. *Arch. Pharm. Res.* **2015**, 38 (10), 1865–1872.

[62] Lee, I.-C.; Bae, J.-S. Inhibitory Effect of Vicenin-2 and Scolymoside on Secretory Group IIA Phospholipase A 2. Animal Cells Syst. *(Seoul).* **2015**, 19 (5), 305–311.

[63] Lee, W.; Kwak, S.; Lee, H.-S.; Na, D. H.; Lee, Y.-M.; Bae, J.-S. Inhibitory Effect of Exendin-4 on Secretory Group IIA Phospholipase A2. *Biochem. Biophys. Res. Commun.* **2015**, 459 (4), 650–654.

[64] Bukhari, S. N. A.; Lauro, G.; Jantan, I.; Fei Chee, C.; Amjad, M. W.; Bifulco, G.; Sher, H.; Abdullah, I.; Rahman, N. A. Anti-Inflammatory Trends of New Benzimidazole Derivatives. *Future Med. Chem*. **2016,** 8 (16), 1953–1967.

[65] Jung, B.; Kim, J.; Bae, J.-S. Dabrafenib, as a Novel Insight into Drug Repositioning Against Secretory Group IIa Phospholipase A2. *Int. J. Pharmacol*. **2016**, 12 (4), 415–421.

[66] Gao, X.; Gong, H.; Men, P.; Zhou, L.; Ye, D. Design, Synthesis, and Biological Evaluation of Novel Dual Inhibitors of Secretory Phospholipase A2 and Sphingomyelin Synthase. *Chinese J. Chem*. **2013**, 31 (9), n/a-n/a.

[67] Dileep, K. V.; Remya, C.; Tintu, I.; Haridas, M.; Sadasivan, C. Interactions of Selected Indole Derivatives with Phospholipase A2: In Silico and in Vitro Analysis. J. *Mol. Model*. **2013**, 19 (4), 1811–1817.

[68] Ajay K. Mahalka, P. K. J. K. Class Specific Peptide Inhibitors for Secretory Phospholipases A2. *Biochem. Biophys. Res. Commun*. **2013**, 436, 349–353.

[69] Ye, L.; Dickerson, T.; Kaur, H.; Takada, Y. K.; Fujita, M.; Liu, R.; Knapp, J. M.; Lam, K. S.; Schore, N. E.; Kurth, M. J.; et al. Identification of Inhibitors against Interaction between pro-Inflammatory sPLA2-IIA Protein and Integrin αvβ3. *Bioorg. Med. Chem. Lett***. 2013**, 23 (1), 340–345.

[70] Liao, C.; Sitzmann, M.; Pugliese, A.; Nicklaus, M. C. Software and Resources for Computational Medicinal Chemistry. *Futur. Med. Chem*. **2011**, 3 (8), 1057–1085.

[71] Rabal, O.; Urbano-Cuadrado, M.; Oyarzabal, J. Computational Medicinal Chemistry in Fragment-Based Drug Discovery: What, How and When. Future Med. Chem. **2011**, 3 (1), 95–134.

[72] Draheim, S. E.; Bach, N. J.; Dillard, R. D.; Berry, D. R.; Carlson, D. G.; Chirgadze, N. Y.; Clawson, D. K.; Hartley, L. W.; Johnson, L. M.; Jones, N. D.; et al. Indole Inhibitors of Human Nonpancreatic Secretory Phospholipase A 2 . 3. Indole-3-Glyoxamides. *J. Med. Chem*. **1996**, 39 (26), 5159–5175.

[73] Kokotou, M. G.; Limnios, D.; Nikolaou, A.; Psarra, A.; Kokotos, G. Inhibitors of Phospholipase A 2 and Their Therapeutic Potential: An Update on Patents (2012-2016). *Expert Opin. Ther. Pat.* **2017**, 27 (2), 217–225.

[74] Holmes, M. V.; Simon, T.; Exeter, H. J.; Folkersen, L.; Asselbergs, F. W.; Guardiola, M.; Cooper, J. A.; Palmen, J.; Hubacek, J. A.; Carruthers, K. F.; et al. Secretory Phospholipase A2-IIA and Cardiovascular Disease: A Mendelian Randomization Study. *J. Am. Coll. Cardiol.* **2013**, 62 (21), 1966–1976.

[75] Paul M. Ridker1, 2* and Thomas F. Lu¨ scher3. Anti-Inflammatory Therapies for Cardiovascular Disease. *Eur. Heart J.* **2014**, 35, 1782–1791.

[76] Nicholls, S. J.; Kastelein, J. J. P.; Schwartz, G. G.; Bash, D.; Rosenson, R. S.; Cavender, M. A.; Brennan, D. M.; Koenig, W.; Jukema, J. W.; Nambi, V.; et al. Varespladib and Cardiovascular Events in Patients With an Acute Coronary Syndrome. *JAMA* **2014,** 311 (3), 252.

[77] Lombardino, J. G. and Lowe III, J. A. The role of the medicinal chemist in drug discovery — then and now. *Nat. Rev. | DRUG Discov*. **2004**, 3.

[78] Ramalho, T. C.; de Castro, A. A.; Silva, D. R.; Silva, M. C.; Franca, T. C. C.; Bennion, B. J.; Kuca, K. Computational Enzymology and Organophosphorus Degrading Enzymes: Promising Approaches Toward Remediation Technologies of Warfare Agents and Pesticides. *Curr. Med. Chem*. **2016**, 23 (10), 1041–1061.

[79] Wang, P.; Li, Y.; Shao, Q.; Zhou, W.; Wang, K. Targeting Human Secretory Phospholipase A2 with Designed Peptide Inhibitors for Inflammatory Therapy. *J. Drug Target*, **2015**, 23 (2), 140–146.

[80] Tamarit B, Theze J. Use of indole-based compounds to induce or stimulate immune response to treat AIDS in HIV-infected subject, and suppress or reverse HIV-mediated immunodeficiency and restore cluster of differentiation 4 T cell function. WO2017037041-A1, **2017**.

[81] Luo Ruixue. Application of pleurolactone to preparation of drugs for treating inflammations CN105213371 (A), **2016**.

[82] Mehendale, H. M. Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences (NIEHS), US 20130253060 A1, **2013**.

[83] Dennis, E. A.; Kokotos, G.; Constantinou-kokotou, V. ; David, S. Amides as inhibitors of human secreted phospholipase A2, US8759392B2**, 2014,** United States.

[84] Liu J F. New (3-aminooxalyl-1H-indol-4-yloxy)-acetic acids, are secretory phospholipase A2 inhibitors, useful for treating e.g. acne, acute bronchitis, bronchiolitis, acute congestive heart failure, acute lung injury, and acute coronary syndrome. WO2012027579-A1, **2012.**

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Article 2- Can Inhibitors of Snake Venom Phospholipases A_2 Lead to New Insights into Anti-Inflammatory Therapy in Humans? A Theoretical Study

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Abstract: Human phospholipase A_2 (*h*PLA_2) of the IIA group (HGIIA) catalyzes the hydrolysis of membrane phospholipids, producing arachidonic acid and originating potent inflammatory mediators. Therefore, molecules that can inhibit this enzyme are a source of potential anti-inflammatory drugs, with different action mechanisms of known anti-inflammatory agents. For the study and development of new anti-inflammatory drugs with this action mechanism, snake venom PLA_2 (*sv*PLA_2) can be employed, since the *sv*PLA_2has high similarity with the human PLA_2 HGIIA. Despite the high similarity between these secretoryPLA_2s, it is still not clear if these toxins can really be employed as an experimental model to predict the interactions that occur with the human PLA_2 HGIIA and its inhibitors. Thus, the present study aims to compare and evaluate, by means of theoretical calculations, docking and molecular dynamics simulations, as well as experimental studies, the interactions of human PLA_2 HGIIA and two *sv*PLA_2s, *Bothrops* toxin II and Crotoxin B (BthTX-II and CB, respectively). Our theoretical findings corroborate experimental data and point out that the human PLA_2 HGIIA and *sv*PLA_2 BthTX-II lead to similar interactions with the studied compounds. From our results, the *sv*PLA_2 BthTX-II can be used as an experimental model for the development of anti-inflammatory drugs for therapy in humans.
Keywords: experimental model; *sv*PLA_2; vanillic acid

1. Introduction

The inflammatory process involves a complex cascade of biochemical and cellular events, and it is an innate reaction of the organism that occurs in tissue in response to any cell injury from any dangerous agent: physical, chemical or biological. One of the stages of the inflammatory process is the breakdown of membrane phospholipids by phospholipases A_2 (PLA_2), which generates fatty acids, such as arachidonic acid (AA) and lysophospholipids. Oxidation of AA generates inflammatory mediators, such as prostaglandins, thromboxanes and leukotrienes, through the action of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. In addition to AA, the breakdown of membrane phospholipids generates lysophospholipids, a precursor of platelet-activating factor (PAF), another potent inflammatory mediator [1,2].

For the treatment of these inflammatory conditions, the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are the most commonly employed drugs [3]. Their wide use throughout the world is due to the large number of diseases involving inflammatory disorders, the spread of rheumatic diseases, and an increase in the life expectancy of the population. Despite their widespread utilization, the prolonged use of this class of anti-inflammatory drugs causes several side effects, such as gastrointestinal toxicity and hepatotoxicity, among other diseases [4–6]. For this reason, there is great interest in the development of new compounds that can act as anti-inflammatory drugs, but with fewer side effects.

Despite their structural differences, all NSAIDs have a similar action mechanism, and are inhibitors of COX enzymes [7]. Recent studies have reported that the anti-inflammatory action of NSAIDs occurs by inhibition of the COX-2 isoform. However, the other products of the inflammatory cascade are also involved in inflammatory conditions, and the inhibition of the COX pathway may accentuate the LOX activity, and consequently increases leukotrienes production, the other product of arachidonic acid degradation [8–10]. In this way, the inhibition of the PLA_2, which can act at the top of the cascade, is a promising alternative, since at the same time that it decreases the COX pathway, it also regulates the production of leukotrienes and the PAF-AH. Despite having great importance, only a few theoretical studies have been

devoted to this topic and currently secreted PLA_2 enzymes have not been explored as a molecular target by medicinal chemistry [11].

Among the various classes of existing PLA_2, the human secreted PLA_2 of the IIA group (HGIIA) belongs to the group of PLA_2 that is the most associated with diseases and consequently are the target enzyme for inhibition [11]. Since human enzymes are difficult to obtain, some experimental models are generally employed for their study [12–16]. In this line, some enzymes that would possibly serve as an experimental model are the snake venoms PLA_2 (*sv*PLA_2). The secreted PLA_2 from snake venoms are distributed in Subgroups I and II of the secreted PLA_2 group. Of these, the crotalid and bothropic PLA_2 are part of the II group, which is the same group as the HGIIA, which is a human PLA_2. Crotoxin B (CB) (PDB ID 2QOG, UniProtKB AC: P24027) is the basic part of the Crotoxin (Cro), and its toxic part. Crotoxin was the first animal toxin to be purified and crystallized and is the main protein present in venom of the *Crotalus durissus terrificus* (South American rattlesnake) [17]. The *Bothrops* toxin II (BthTX-II) is another basic PLA_2 isolated from *Bothrops jararacussu* venom. This myotoxic toxin is also known for its edematogenic and hemolytic effects and for its ability to induce platelet aggregation and secretion [18].

The use of *sv*PLA_2 for understanding the activity and action mechanisms of the human PLA_2 HGIIA has been proposed, as there is a high similarity between *sv*PLA_2 and HGIIA PLA_2 [19]. Moreover, the use of snake venom toxins could also be justified, because they are rich in Group I and II secreted PLA_2, especially Elapidae and Viperidae families [20]. It should be kept in mind that despite this high similarity, it is not clear if these enzymes can perform similar interactions and if the *sv*PLA_2 can really be employed as an experimental model to describe the HGIIA interactions, since some works reveal the contrary [21]. Thus, the objective of this work was to evaluate, experimentally, the phospholipasic activity of vanillic acid (VA) on *sv*PLA_2 enzymes, such as BthTX-II and CB. In addition, we compare, theoretically, the interactions of these enzymes with the interaction of the same compound with HGIIA. Finally, two molecules rationally modified from the VA molecule were proposed to improve their interaction of the VA with HGIIA and to develop new potential anti-inflammatory drugs.

2. Results

2.1. Experimental Assays

Figure 1 contains the percent inhibition of both *sv*PLA$_2$ by VA in relation to the different concentrations of this molecule. It is possible to observe that *sv*PLA$_2$ BthTX-II presented a higher activity and value of inhibition percentage by the VA. In relation to the halo of activity (Figure S1 of the support material), the sample with the highest proportion of VA presents the lowest activity halo for both enzymes, which indicates that VA decreases the activity of both *sv*PLA$_2$. In addition, the inhibition percentage values also indicate that vanillic acid is able to inhibit *sv*PLA$_2$. Figure 1 shows that the highest proportion of VA tested was responsible for the highest percentage of inhibition for both enzymes, equivalent to 23.7% for the BthTX-II and 20% for CB.

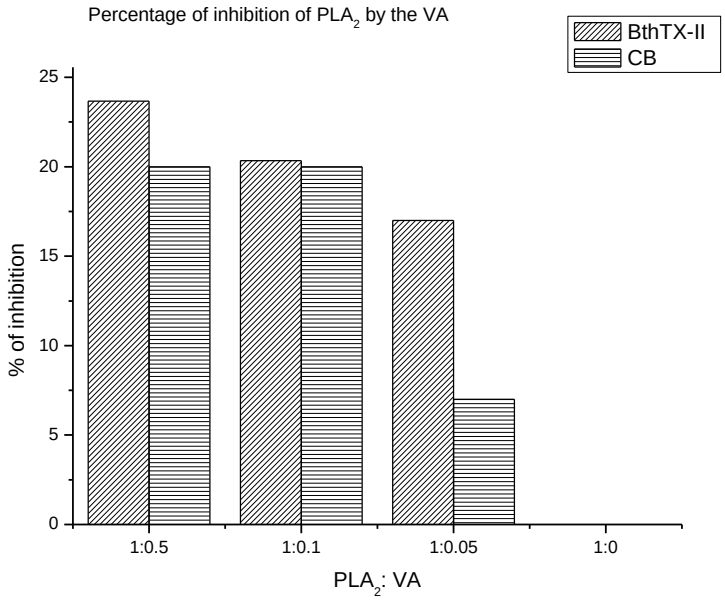


Figure 1. Percent inhibition of phospholipase A$_2$ activity caused by vanillic acid (VA), for the phospholipases A$_2$ isolated from snake venom *Bothrops* toxin II (BthTX-II) and Crotoxin B (CB).

2.2. Alignments of Amino Acid Sequences of svPLA2 and Human PLA2

Two alignments of primary amino acid sequences of BthTX-II (PDBID 3JR8), CB (PDBID 2QOG), and HGIIA (PDBID 3U8D) were performed and can be seen in Figures S2 and S3 of the support material. One is for calculating the percentages of identity and similarity between the enzymes, while the other is focusing on charge

distributions and hydrophobicity of the three PLA_2. The results of the first alignment showed 64.0% identity (84.4% similar) for alignment BthTX-II vs. CB (3-143:4-144); 53.1% identity (72.0% similar) for HGIIA vs. BthTX-II (3-145:3-143); and 55.2% identity (81.1% similar) for the alignment of the HGIIA vs. CB (3-145:4-144). For the second alignment, it is possible to observe that, although there are a few differences in some residues, the enzymes present groups (hydrophobic, negatively or positively charged residues) that behave similarly in the same positions, in most cases.

2.3. Theoretical Calculations

2.3.1. Molecular Docking Calculations

To validate the methodology, re-docking was performed on the human enzyme, under the same conditions, to compare the structure obtained in the theoretical calculation with the ligand structure of the U8D present in the crystal. The root-mean-square deviation (RMSD) obtained in re-docking was zero for all structures, which means that the structures obtained presented little alteration in relation to the average structure, which is satisfactory. The overlap of the obtained poses with the U8D active ligand are shown in Figure 2. As can be seen, there was no significant variation in the structures theoretically obtained with the active ligand structure of the 3U8D complex. Therefore, this result can validate our docking study.

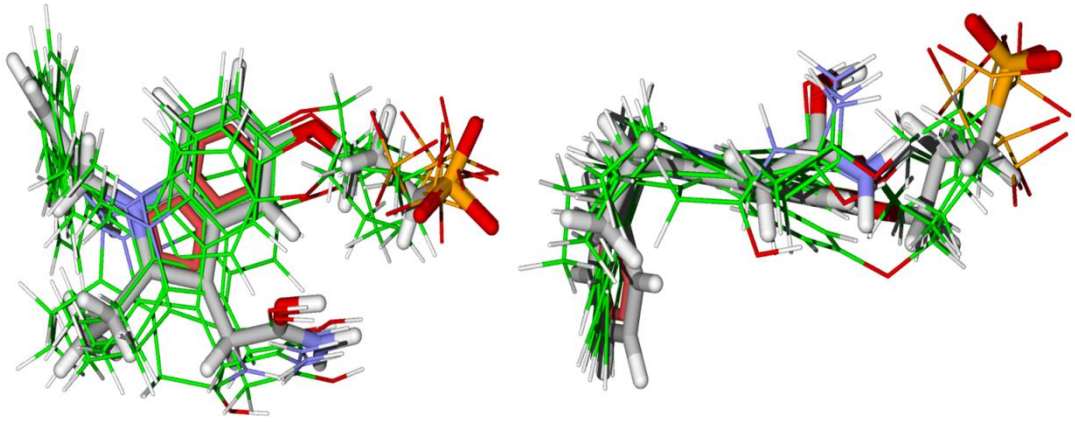


Figure 2. Superposition of the obtained poses with the active ligand U8D, obtained by re-docking calculation.

Afterwards, the docking analysis of the vanillic acid with all enzymes was performed, and the results are reported in Table 1. As can be seen in Table 1, the interaction energies and the score function obtained for the enzyme HGIIA and BthTX-

II were very close. This result does not apply to the third phospholipase CB. Both *sv*PLA_2 have high similarity to the human enzyme HGIIA, however, only CB has four subunits composed of two equal dimmers, as seen in Figure 3. Each of these two dimmers is similar to the other PLA_2 studied. BthTX-II and HGIIA enzymes have only two subunits in their active conformation, and this small structural difference can make the BthTX-II enzyme a little more appropriate to help describe the interactions that occur in the human enzyme.

Table 1. Values obtained for the Score Binding Functions, Interaction Energy, and Hydrogen bonds for docking calculation of human phospholipase A_2 (*h*PLA_2)of the IIA group PLA_2 (HGIIA), BthTX-II, and CB.

Enzyme	MolDock Score	Rerank Score	Interaction	HBond
		hPLA_2		
HGIIA	−69.38	−58.93	−75.35	−2.21
		svPLA_2		
BthTX-II	−71.22	−57.02	−79.45	0.00
CB	−37.87	−35.17	−44.91	−0.02

In relation to the hydrogen bond energies, the *sv*PLA_2 had the lowest values, different from the HGIIA that have approximately −2.21 KJ mol^{-1}. Despite of this difference in energies, just one hydrogen bond between the Histidine 47 residue of HGIIA and VA occurs, which can be seen in Figure 4. The bond length is 2.601 Å, and bonds longer than 2.5 Å are not very stable [22].

Through the superposition of the VA and the active ligand U8D, in Figure S4 of the support material, it is possible to deduce that VA leads to similar hydrophobic interactions. This means interactions between U8D and the hydrophobic residues of HGIIA, since the aromatic rings of VA are localized very close to the aromatic ring of the U8D. In addition, the oxygen atoms of the VA carboxyl group are also near the oxygen groups of the U8D molecule. Figure S5 shows the vanillic acid molecule inside the cavity of the HGIIA enzyme. As can be seen, VA occupies only a part of the cavity.

If new radical groups are rationally added in a vanillic acid molecule to take up all the cavity space, it is possible that the interaction of these compounds increases. Based on this idea, and considering the composition of the residues that are in the active

site, two VA modified molecules were supposed, and docking analysis of their energies was performed. The proposed modifications are shown in Figure 5.

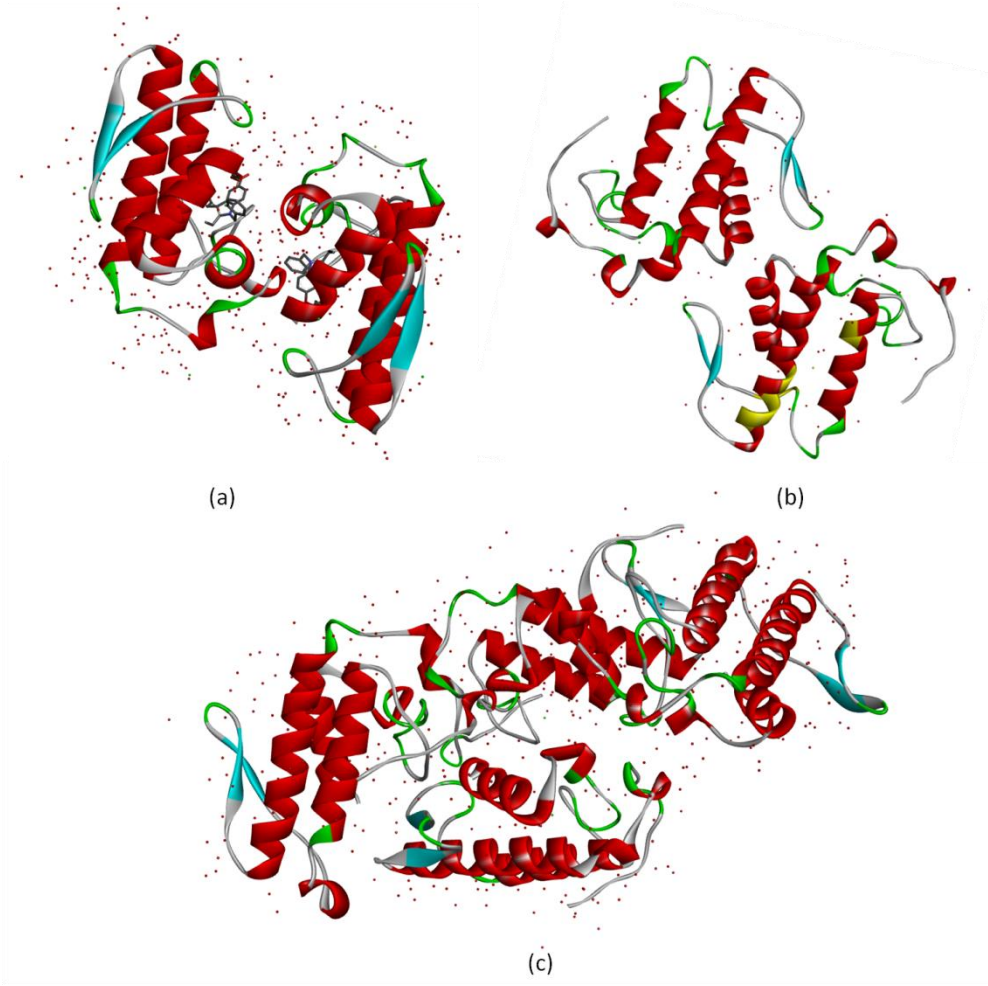


Figure 3. Three dimensional structures of secretory phospholipases A_2:(**a**) represents the structures of HGIIA, with 3U8D PDB code; (**b**) represents the BthTX-II structure, with 3JR8 PBD code; and (**c**) is the 3D structure of CB, PDB code 2QOG.

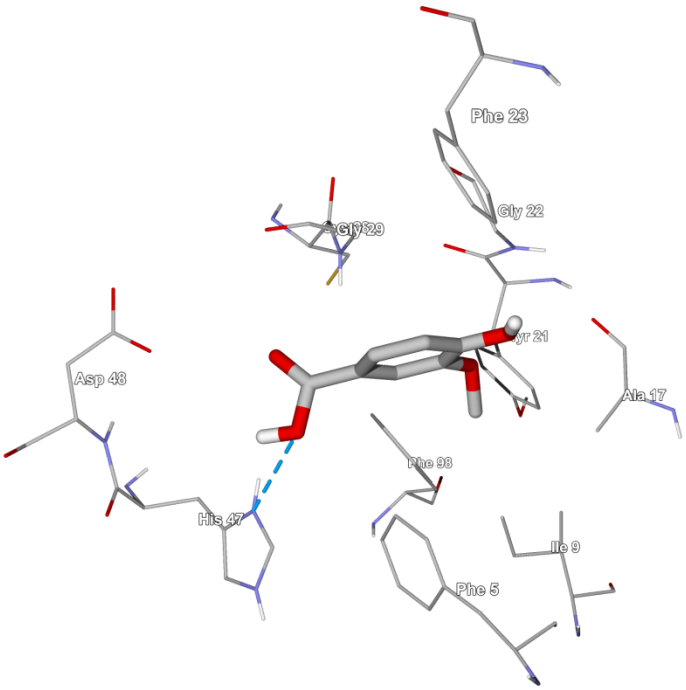


Figure 4. Hydrogen bond made between a vanillic acid molecule and the His 47 residue of PLA_2 HGIIA, whose length is 2,601 Å.

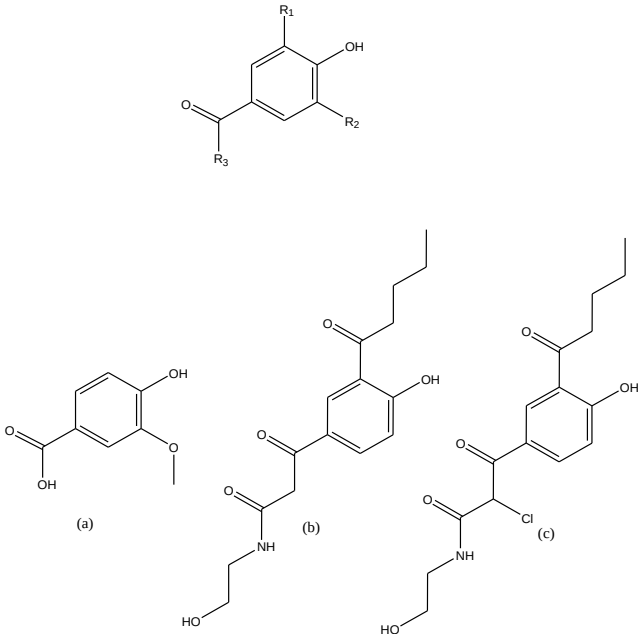


Figure 5. Modifications rationally proposed to improve the inhibitory activity of vanillic acid. On the top of the figure is the general structure; the molecule represented in (**a**) is the vanillic acid (VA), (**b**) is the first modification, named analogue I, and (**c**) is the second modification, named analogue II.

The docking calculation of the modified VA structures (analogues I and II) also were performed, and the results are displayed in Table 2. As can be seen, all results are better than the unmodified VA molecule (Table 1), which means that the modifications are satisfactory. The hydrogen bond energies have improved and are more similar between BthTX-II and HGIIA than the unmodified VA molecule. Moreover, the analogues followed the same interaction pattern, having more affinity for BthTX-II, followed by HGIIA and CB, the energies of the first two being very similar. The interaction energy between analogue I and the enzymes BthTX-II, HGIIA, and CB are −113.82, −107.12, and −71.93 KJ mol^{-1}, respectively. For the interaction of these enzymes and analogue II, the interaction energies were −126.35, −115.38, and −55.64 KJ mol^{-1}, respectively. These data also suggest that the BthTX-II serves as an experimental model to evaluate inhibitions in human secretory phospholipases of the IIA group.

Table 2. Values obtained for the Score, Interaction Energy, and Hydrogen Bond energies of the two analogues tested by docking calculation with the PLA_2 HGIIA, BthTX-II, and CB.

Compound	Enzyme	MolDock Score	Rerank Score	Interaction	HBond
Analogue I	HGIIA	−101.52	−73.99	−107.12	−4.99
Analogue I	BthTX-II	−111.32	−69.64	−113.82	−4.61
Analogue I	CB	−72.26	24.57	−71.94	−5.14
Analogue II	HGIIA	−117.02	−26.35	−115.38	−4.63
Analogue II	BthTX-II	−123.33	−101.81	−126.35	−7.91
Analogue II	CB	−59.03	31.78	−55.64	−9.65

2.3.2. Molecular Dynamics Simulation

After the molecular docking study of VA in both PLA_2, the structures obtained from the enzymes and the poses were analyzed by molecular dynamics. The root-mean square deviation (RMSD) and the number of hydrogen bonds were obtained for both

systems. The first plot (Figure S6) shows the RMSD for each enzyme/inhibitor complex (HGIIA/VA; BthTX-II/VA, and CB/VA). In all systems, both VA and enzymes were stabilized, which indicates that all systems reached equilibrium. The *sv*PLA_2 structures have more fluctuations over time, especially the CB/VA system.

For the HGIIA/VA system, which was the most stable, the equilibrium occurred as early as in the first picoseconds of simulation, and its maximum value was approximately 0.5 nm for the VA and 0.4 nm for the protein, both low values. This indicates that the VA ligand stabilized within the active site of the enzyme and that its interactions with HGIIA are favorable, proving its inhibitory potential. For the BthTX-II/VA system, the equilibrium was reached later for the ligand after 1000 ps, but it also occurred and was relatively maintained over time. Its maximum value was less than 1.2 nm while the RMSD of the BthTX-II enzyme did not reach 0.7 nm, which means that the permanence of the VA in the active site of the PLA_2 BthTX-II is also favorable. The CB/VA complex provided the largest variation in position over time, but despite this, it also stabilized. The ligand varied widely in the active site of the CB enzyme, reaching a maximum RMSD of 2.5 nm for the ligand and 1 nm for the protein. Similar to the behavior adopted in the docking calculations, the BthTX-II enzyme was that which behaved more like the human enzyme HGIIA. This also suggests that BthTX-II is capable of aiding in the description of the experimental behavior of the human enzyme and that the CB PLA_2 does not provide information of interactions between the VA ligand and the human PLA_2.

Comparing the RMSD between the enzymes, the HGIIA human PLA_2 (*h*PLA_2) was the most stable during the simulation, and BthTX-II was relatively stable. At the same time, the structure of CB *sv*PLA_2 had many more fluctuations, as already mentioned. Turning now to the inhibitor in the three enzymes (Figure 6), it can be seen that the VA conformations in the enzymes HGIIA and BthTX-II have similar behaviors, unlike the ligand in the CB active site.

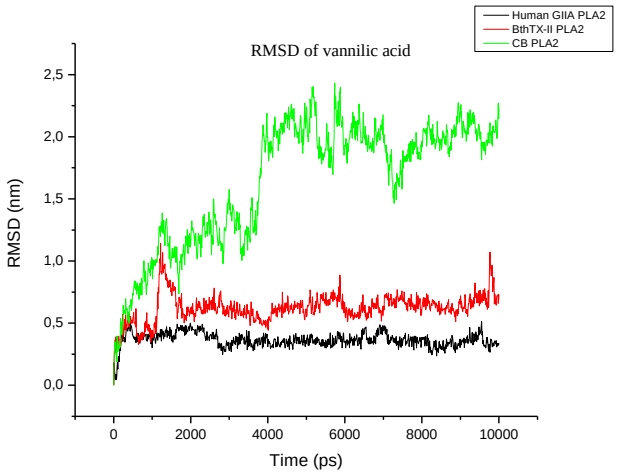


Figure 6. Comparison of root-mean square deviation (RMSD) of VA in each active site.

In relation to the hydrogen bonds carried out over time for the three studied PLA_2 (Figure S7 of the support material), it is possible to observe that the *h*PLA_2 HGIIA performed seven bonds during the molecular dynamics (MD) simulation, with approximately four being maintained most of the time. The BthTX-II *sv*PLA_2, similarly to HGIIA, performed six hydrogen bonds in the MD simulation, four of which are more frequent over time. Regarding CB *sv*PLA_2, unlike the other two phospholipases, CB PLA_2 showed up to eight hydrogen bonds, but these were less stable, since they appear only at a few moments throughout the time.

3. Discussion

3.1. Can svPLA$_2$/Inhibitors Describe the hPLA$_2$ /Inhibitors Interactions?

From the experimental data, it is possible to observe that the BthTX-II enzyme has more affinity with the VA molecule. This pattern was maintained in the docking and MD simulations, which indicate that the theoretical studies carried out are coherent with the experiment and suggest that HGIIA performs similar interactions between BthTX-II and VA. According to the literature, a degree identity over 35% is satisfactory [23]. Despite their similarity, it is important to comment that Kim and collaborators (2017) [21] found that the *sv*PLA$_2$ purified from the venom of *Daboia russelli pulchella* (VRV-PL-VIII) is not appropriate as a model for describing the interactions between the human PLA$_2$ and its inhibitors. As we can see in this work, the *sv*PLA$_2$ CB, despite the high similarity with HGIIA, does not provide information about the interactions that occur between the HGIIA and VA, while the BthTX-II has a behavior similar to the human enzyme. This feature suggests that the structural similarity is a very important factor to consider, but is not the only factor. The other factor which plays an important role is the behavior of the enzyme in solution [24,21]. According to Kim and collaborators (2017) [21], the *sv*PLA$_2$ does not provide any useful foundation for a prediction of the binding mode to specific ligands in a HGIIA complex. The authors conclude this based on the fact that the *sv*PLA$_2$ enzymes have different behavior in solution, and because of this feature can interact with different chains (A or B) in a different mode. They found that the ligand FLSIK, in the HGIIA:FLSIK complex, does not interact with both chains, and as such, the chain B is not necessary for the inhibition activity, since the ligand interacts only with chain A. In other words, the authors found that the HGIIA acts as a monomer in solution. For the *sv*PLA$_2$ that the authors chose (PLA$_2$ purified from the venom of *Daboia russelli pulchella*(VRV-PL-VIIIA, svPLA$_2$, UniProt accession code P59071, with 49% identity to HGIIA), the behavior in solution is different, and because of this, despite the high similarity, this *sv*PLA$_2$ doesnot provide information of HGIIA interactions, as it acts as a monomer and *sv*PLA$_2$ act as a dimmer.

Similarly, for the authors, the simulations with HGIIA in the present work show that the ligand interacts with a single chain of the enzyme, which can be seen in Figure 7. The images represent the frames at the beginning, middle, and end of the simulation

for the HGIIA/VA complex. As can be seen, the VA molecule is maintained in a single chain of the molecule at the three times. However, different from the conclusions of Kim and collaborators (2017) [21], in this work, we found that the *sv*PLA_2 BthTX-II can provide a useful foundation for a prediction of the HGIIA binding mode. This fact is justified because the BthTX-II behavior in solution is similar to the HGIIA (Figure 7), different from the *sv*PLA_2 CB tested in this work and the *sv*PLA_2 tested by Kim and collaborators [21]. In Figure 7, the VA molecule also remains in the only chain of the enzyme most of time. As mentioned above, the CB PLA_2, despite its high primary sequence similarity with HGIIA, acts as a tetramer, different from the other two tested PLA_2. In addition, the PLA_2 tested by Kim et al. (2017) [21], besides having less similarity to HGIIA, does not act as a monomer in solution.

Thus, for the similar interactions between HGIIA and BthTX-II, the similar behavior in solution, and for the high structural similarity of these compounds, it is possible that, experimentally, the vanillic acid acts in HGIIA in the same manner, with inhibition percentage values close to those of the BthTX-II results. Despite the differences in hydrogen bond energies in the docking calculations, the time dependent simulations show that the number of hydrogen bonds of BthTX-II and HGIIA are similar, and are maintained most of time, which also contributes to their similarity in interactions, contributing to the fact that the BthTX-II can be used as an experimental model for HGIIA.

Moreover, the aromatic ring of the VA is in the same position as the active ligand of the 3U8D complex, suggesting that the same hydrophobic interaction can occur. With the structures obtained in the MD simulation, it was possible to create a pharmacophoric map of the HGIIA middle structures, which is approximately correspondent to the BthTX-II interactions. The maps are shown in Figure 8. The enzymes have similar hydrophobic interactions, and these interactions can explain the similar interaction energy obtained in molecular docking. In the map, it is possible to observe that the VA molecule performs π-π stacking interactions with phenylalanine residues and a hydrogen bond with glycine residues in both enzymes. The results obtained in this work are in agreement with the results obtained by Dileep *et al*. (2015) [25], who analyzed the effect of some phenolics on secretory PLA_2 of the swine pancreas. The authors reported that

vanillic acid interacts with this phospholipase by performing an H bond and by hydrophobic interactions with residues Phe 5, Leu 2, Phe 22, and Leu 31. Therefore, one secretory PLA_2 that has more availability and is more easily obtained, which is BthTX-II, can be used as an experimental model for the study of mechanisms and the development of new inhibitors for the HGIIA PLA_2 that are so important in regulations of the arachidonic acid pathway.

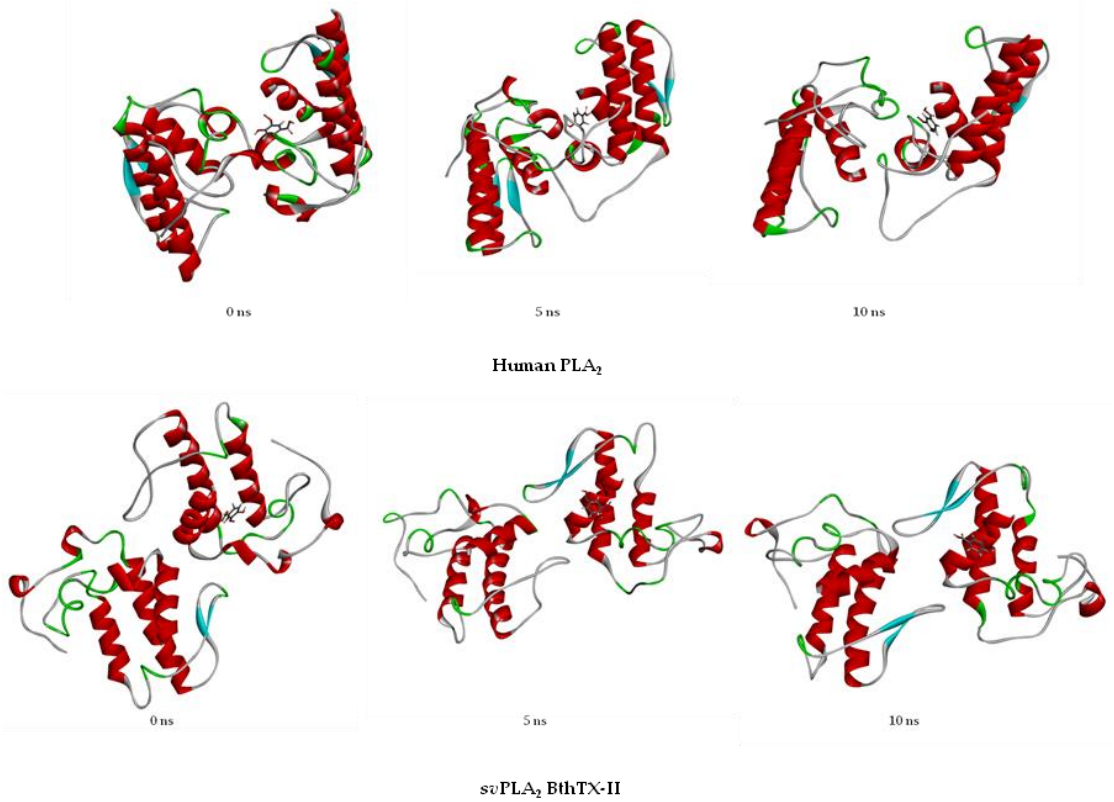


Figure 7. Comparison between the structure of the complex HGIIA/VA at the beginning (0 ns), middle (5 ns), and end (10 ns) of the molecular dynamics simulation, and comparison of the structures of the complex BthTX-II/VA at the beginning (0 ns), middle (5 ns), and end (10 ns) of the molecular dynamics simulation.

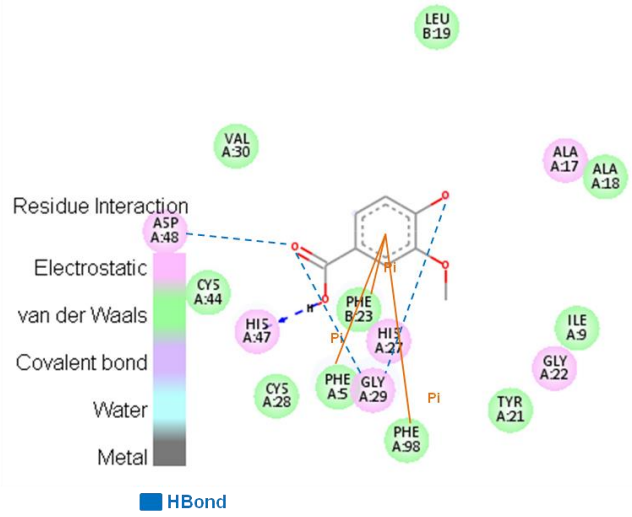
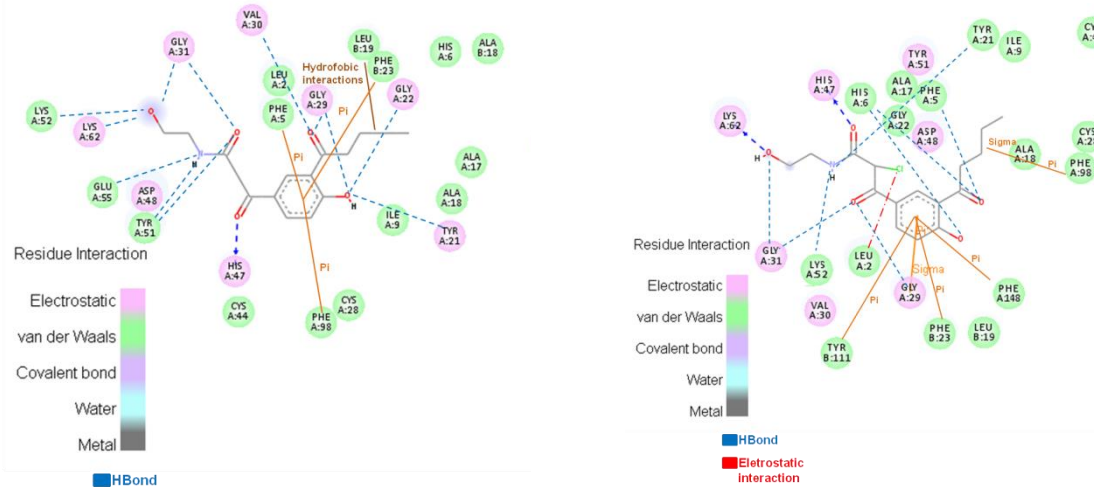


Figure 8. Pharmacophoric map of the interactions between HGIIA and vanillic acid (VA).

3.2. Searching Molecular Interactions of Vanillic Acid Analogs

With the modification of the VA molecule, the interactions increase significantly. As presented in Table 2, the interaction energies increase for both modifications with all PlA_2. Moreover, the hydrogen bond energies for HGIIA and BthTX-II were very similar. Through the modifications of the VA molecule, the majority of the active site was occupied with radicals that interact with specific residues. This modification brings new hydrophobic interactions and hydrogen bonds, as can be seen in Figure 9, the pharmacophoric map. In addition, the chlorine atom in analogue II performs electrostatic interactions with HGIIA. With this, it is possible to conclude that vanillic acid can act as a base molecule for the rational development of new secretedPLA$_2$ inhibitors. With better interaction, these new inhibitors can be more effective and selective for these enzymes, which enables the use of these molecules as possible anti-inflammatory drugs, with a different action mechanism from that of the current commercially available drugs.



(**a**) (**b**)

Figure 9. Interactions between the analogues I and II and HGIIA enzyme: (**a**) presents the interactions of the analogue I with HGIIA; (**b**) shows the interactions of the analogue II with the HGIIA enzyme.

4. Conclusions

In this work, a comparison of the HGIIA and *sv*PLA_2 interactions was performed in order to clarify the discussion about the use of *sv*PLA_2 as a model for analysis of human PLA_2 interactions. In addition, two modified molecules from vanillic acid were theoretically proposed for increasing the inhibition of the VA molecule as well as its inhibitory effect. It is possible to conclude that the enzyme BthTX-II can provide useful information about the interactions of the potential inhibitors with HGIIA *h*PLA_2. The other *sv*PLA_2 tested, the Crotoxin B, or CB, does not present the same results, and so this enzyme cannot be used as an experimental model for HGIIA. It is also concluded that the primary sequence similarity is not the only factor to be considered, and the behavior of the enzyme in solution is an important factor for the comparison of interactions between the structurally similar enzymes.

This work is of great use, because we report a proof-of-principle study that snake venom toxins, more specifically *sv*PLA_2, can be used as tools for studies in human PLA_2, taking care in choosing the correct *sv*PLA_2. Furthermore, it serves as evidence that both structural similarity and enzyme solution behavior are important to describe similarities in interactions of two or more enzymes. Lastly, vanillic acid has potential to inhibit secreted PLA_2, and can be a base molecule for the development of molecules that can interact more strongly and can be more selective. The two rationally modified molecules developed from VA show better interaction energies than VA, which means that the developed molecules are more potent inhibitors than VA, and can be potential-use candidates for new anti-inflammatory drugs.

5. Materials and Methods

5.1. Experimental Assays

For the experimental analysis, the model of secretory *PLA₂* employed was the *sv*PLA_2 isolated from the species *Crotalus durissus terrificus* (CB) and *Bothrops jararacussu* (BthTX-II). The inhibition of phospholipase activity for vanillic acid was assessed using solid medium as described by Gutiérrez et al., 1988 [26], replacing agarose with agar and without the addition of erythrocytes. The substrate used was egg yolk. The egg yolk is a source of phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine, thus forming an affordable and low-cost source for the detection of phospholipase activity [27]. The medium was prepared with 1% bacteriological agar, pH 7.2, and egg yolk diluted in phosphate-buffered saline (PBS) (1:3, vv^{-1}). Also, 0.01 mol L^{-1} of $CaCl_2$ and 0.005% of sodium azide was also added in the medium. After the gel solidified in plates, the treatments were applied in wells of approximately 0.5 cm of diameter. The two PLA_2 isolated from snake venoms (BthTX-II and CB) were used to induce the breakdown of phospholipids. Each PLA_2 and vanillic acid were diluted in $CaCl_2$ solution and previously incubated in a water bath at 37 °C for 30 min, at the following ratios: 1:1, 1:0.5, 1:0.1, and 1:0.05 (PLA_2/vanillic acid, *w/w*). The potential of vanillic acid in inhibiting PLA_2 was evaluated after 18 h of incubation of the plates in a cell culture chamber at that same temperature. Controls containing only PLA_2 were also evaluated. The formation of a clear halo around the well in the gel characterized the phospholipase activity, which was measured according to the halo diameter. The results were expressed as percentages of activity, and inhibition and the controls containing only venom were considered as having 100% phospholipase activity.

5.2. Alignments of Amino Acid Sequences

In order to verify the similarity of these enzymes with the human secretory PLA_2 HGIIA, alignments were made using the LALIGN [28], a dynamic programming algorithm that determines similar regions of two protein sequences and other biomolecules. Additionally, the alignment of the UniProt [29] was employed to verify the presence of positive, negative, and hydrophobic residues. For the alignments, the primary sequences of these secretory PLA_2 were downloaded from Expasy [30] in the

categories of proteomics on the topic of protein sequences and identification, using the UniProtKB database [31]. In order to compare the interactions that occur between the ligands and all secretory PLA_2, the same theoretical calculations were performed for both secretory PLA_2.

5.3. Simulation Methods

5.3.1. Docking Energies Calculations

To calculate the partial charges of ligands, the three-dimensional structures were previously created through the program PC Spartan® (version Pro, Wavefunction, Inc., Ivine, CA)[32], and the calculation was performed by the semi-empirical method AM1. After this, the ligands were docked inside the HGIIA (PDB code 3U8D, that have a resolution of 1.8 Å and are complexed with Ca^{2+}, Cl^-, and 3-{[3-(2-amino-2-oxoethyl)-1-benzyl-2-ethyl- 1H-indol-5-yl]oxy}propyl)phosphonic acid (PDB code U8D)) using the software Molegro Virtual Docker (MVD®, version 2011.4.3.0) [33]. The binding site was restricted into a sphere with a radius of 11 Å, and the residues within a radius of 8 Å were considered flexible. Fifty runs were performed, with 50 poses obtained for the analysis of the ligand-protein interactions and of the overlaps with the U8D inside of the human PLA_2. The best conformation was selected, based on the best overlap and the interaction energy. For the analysis of the *sv*PLA_2, the binding site, identified by the His47, was restricted into a sphere with 7 and 5 Å for the BthTX-II and CB, respectively, according to the size of the cavity. Since these enzyme structures do not have ligands, the best energy of interaction was taken into account. The selected conformations of all were used for the further MD simulation steps.

5.3.2. Molecular Dynamics Simulations

Initial ligand configurations were produced using the Gaussian 09 Program [34] to construct the structures, and the Automated Topology Builder (ATB) server [35, 36] to generate the topology and structure files. For the simulations, the force field used was GROMOS 96 54a7 [37], GROMACS program [38] (Version 5.1.2, Royal Institute of Technology and Uppsala University, Sweden). The enzyme/inhibitor complexes (HGIIA/VA, BthTX-II/VA and CB/VA) were constructed using the mentioned force field, in a volume simulation box of 645.57, 742.71, and 938.66 nm for each complex,

respectively. For the energy minimization, the steepest descent algorithm was used, minimizing when the maximum force was <10.0 kJ/mol. After the minimization step, the complexes were submitted to molecular dynamics analysis for a time interval of 10 ns, and 1000 conformations were obtained for each complex. The equations of motion were integrated using the leapfrog scheme. The results were analyzed through the VMD® program (version 1.9.2, University of Illinois at Urbana-Champaign) [39] and Discovery Studio® 3.5 (manufacturer, city, abbreviated state (if has), country). The total energy, RMSD, and hydrogen bond graphs were generated to analyze the results through the Origin® program (Version 3.5.0, Accelrys Software Inc.) [40-43].

References

Joshi, V. Dimethyl ester of bilirubin exhibits anti-inflammatory activity through inhibition of secretory phospholipase A2, lipoxygenase and cyclooxygenase. *Arch. Biochem. Biophys.***2016**, *598*, 28–39.

Silva, P. *Farmacologia*, 8th ed.; Guanabara Koogan: Rio de Janeiro, Brazil, 2010; 1024p.

Muri, E.M.; Sposito, M.M.M; Metsavaht, L. Nonsteroidal antiinflammatory drugs and their local pharmacology. *Acta Fisiatr.***2009**, *16*, 186–190.

Anelli, M.G.; Scioscia, C.; Grattagliano, I.; Lapadula, G. Old and new antirheumatic drugs and the risk of hepatotoxicity. *Ther. Drug Monit.***2012**, *34*, 622–628.

Rafaniello, C.; Ferrajolo, C.; Sullo, M.G.; Sessa, M.; Sportiello, L.; Balzano, A.; Manguso, F.; Aiezza, M.L.; Rossi, F.; Scarpignato, C.; et al. Risk of gastrointestinal complications associated to NSAIDs, low-dose aspirin and their combinations: Results of a pharmacovigilance reporting system. *Pharm. Res.***2016**, *104*, 108–114.

Yousefpour, A.; Iranagh, S.A.; Nademi, Y.; Modarress, H. Molecular dynamics simulation of nonsteroidal antiinflammatory drugs, naproxen and relafen, in a lipid bilayer membrane. *Int. J. Quant. Chem.***2013**, *113*, 1919–1930.

Cronstein, B.N.; Weissmann, G. Targets for antiinflammatory drugs. *Ann. Rev. Pharmacol. Toxicol.***1995**, *35*, 449–462.

Gaddipati, R.S.; Raikundalia, G.K.; Mathai, M.L. Dual and selective lipid inhibitors of cyclooxygenases and lipoxygenase: A molecular docking study. *Med. Chem. Res.***2014**, *23*, 3389–3402.

Peters-Golden, M.D.M.; Henderson, M.D.W.R., Jr. Leukotrienes. *N. Engl. J. Med.***2007**, *357*, 1841–1854.

Pyasi, K.; Tufvesson, E.; Moitra, S. Evaluating the role of leukotriene-modifying drugs in asthma management: Are their benefits 'losing in translation'? *Pulm. Pharmacol. Ther.***2016**, *41*, 52–59.

Quach, N.D.; Arnold, R.D.; Cummings, B.S. Secretory phospholipase A2 enzymes as pharmacological targets for treatment of disease. *Biochem. Pharmacol.***2014**, *90*, 338–348.

Tomankova, V.; Anzenbacher, P.; Anzenbacherova, E. Effects of obesity on liver cytochromes P450 in various animal models. *Biomed. Pap.-Olomouc***2017**, *161*, 144–151.

Lerch, M.M.; Adler, G. Experimental animal-models of acute-pancreatitis. *Int. J. Pancreatol.***1994**, *15*, 159–170.

Liu, Y.; Zeng, B.H.; Shang, H.T.; Cen, Y.Y.; Wei, H. Bama Miniature Pigs (Sus scrofa domestica) as a Model for Drug Evaluation for Humans: Comparison of In Vitro Metabolism and In Vivo Pharmacokinetics of Lovastatin. *Comp. Med.***2008**, *58*, 580–587.

Prueksaritanont, T. Use of In Vivo Animal Models to Assess Drug-Drug Interactions. Enzyme- and Transporter-Based Drug-Drug Interactions: Progress and Future Challenges. *Pharm. Res.***2010**, *27*, 283–297.

Siltari, A.; Kivimäki, A.S.; Ehlers, P.I.; Korpela, R.; Vapaatalo, H. Effects of Milk Casein Derived Tripeptides on Endothelial Enzymes In Vitro; a Study with Synthetic Tripeptides. *Arzneimittelforschung* **2012**, *62*, 477–481.

Marchi-Salvador, D.P.; Corrêa, L.C.; Magro, A.J.; Oliveira, C.Z.; Soares, A.M.; Fontes, M.R. Insights into the role of oligomeric state on the biological activities of crotoxin: Crystal structure of a tetrameric phospholipase A2 formed by two isoforms of crotoxin B from *Crotalus durissus terrificus* venom. *Proteins***2008**, *72*, 883–891.

Dos Santos, J.I.; Cintra-Francischinelli, M.; Borges, R.J.; Fernandes, C.A.; Pizzo, P.; Cintra, A.C.; Braz, A.S.; Soares, A.M.; Fontes, M.R. Structural, functional, and bioinformatics studies reveal a new snake venom homologue phospholipase A_2 class. *Proteins***2011**, *79*, 61–78.

Teixeira, C.F.; Landucci, E.C.; Antunes, E.; Chacur, M.; Cury, Y. Inflammatory effects of snake venom myotoxic phospholipases A2. *Toxicon***2003**, *42*, 947–962.

Marcussi, S.; Sant'Ana, C.D.; Oliveira, C.Z.; Rueda, A.Q.; Menaldo, D.L.; Beleboni, R.O.; Stabeli, R.G.; Giglio, J.R.; Fontes, M.R.; Soares, A.M. Snake venom phospholipase A2 inhibitors: Medicinal chemistry and therapeutic potential. *Curr. Top. Med. Chem.***2007**, *7*, 743–756.

Kim, R.R.; Malde, A.K.; Nematollahi, A.; Scott, K.F.; Church, W.B. Molecular dynamics simulations reveal structural insights into inhibitor binding modes and functionality in human Group IIA phospholipase A2. *Proteins***2017**, *85*, 827–842.

Batsanov, S.S. Van der Waals Radii of Elements. *Inorg. Mater.***2001**, *37*, 1031–1046.

Da Cunha, E.E. Barbosa, E.F.; Oliveira, A.A.; Ramalho, T.C. Molecular modeling of Mycobacterium tuberculosis DNA gyrase and its molecular docking study with gatifloxacin inhibitors. *J. Biomol. Struct. Dyn.***2010**, *27*, 619–625.

Golçalves, M.A.; Santos, L.S.; Prata, D.M.; Fernando, P.C.; Cunha, E.F.F.; Ramalho, T.C. Optimal wavelet signal compression as an efficient alternative to investigate molecular dynamics simulations: Aplication to thermal and solvent effects of MRI probes. *Theor. Chem. Acc.***2017**, *136*, 15, doi:10.1007/s00214-016-2037-z.

Dileep, K.V.; Remya, C.; Cerezo, J.; Fassihi, A.; Pérez-Sánchez, H.; Sadasivan, C. Comparative studies on the inhibitory activities of selected benzoic acid derivatives against secretory phospholipase A2, a key enzyme involved in the inflammatory pathway. *Mol. Biosyst.***2015**, *11*, 1973–1979.

Gutiérrez, J.M.; Avila, C.; Rojas, E.; Cerdas, L. An alternative in vitro method for testing the potency of the polyvalent antivenom produced in Costa Rica. *Toxicon***1998**, *26*, 411–413.

Price, M.F.; Wilkinson, I.D.; Gentry, L.O. Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia***1982**, *20*, 7–14.

Huang, X.; Miller, W.; Huang, X.; Miller, W. A time-efficient linear-space local similarity algorithm. *Adv. Appl. Math.***1991**, *12*, 337–357.

Consortium, U. UniProt: A hub for protein information. *Nucleic Acids Res.***2015**, *43*, D204–2D12

Expasy. Available online: http://au.expasy.org/ (accessed on 23/10/2017).

UniProtKB Database. Available online: http://www.uniprot.org/ (accessed on 23/10/2017).

Hehre, W.J.; Deppmeier, B.J.; Klunzinger, P.E. *Pcspartanpro*; Wavefunction, Inc.: Irvine, CA, USA, 1999.

Thomsen, R.; Christensen, M.H. MolDock: A new technique for high-accuracy Molecular docking. *J. Med. Chem.***2006**, *49*, 3315–3321.

Frisch, M.J.; *Gaussian 09*; Gaussian, Inc.: Wallingford, CT, USA, 2009.

Canzar, S.; El-Kebir, M.; Pool, R.; Elbassioni, K.; Mark, A.E.; Geerke, D.P.; Stougie, L.; Klau, G.W. Charge group partitioning in biomolecular simulation. *J. Comput. Biol.***2013**, *20*, 188–198.

Automated Topology Builder (ATB) Server. Available online: http://compbio.biosci.uq.edu.au/atb/(accessed on 23/10/2017).

Van Gunsteren, W.F.; Billeter, S.R.; Eising, A.A.; Hunenberger, P.H.; Krüger, P.; Mark, A.E.; Scott, W.R.P.; Tironi, I.G. *Biomolecular Simulation: The GROMOS96 Manual and User Guide*; VDF Hochschulverlag AG an der ETH Zürich: Zurich, Switzerland, 1996.

Abraham, M.J.; Murtola, T.; Schulz, R.; Pall, S.; Smith, J.C.; Hess, B.; Lindahl, E. GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX*, **2015**, *1–2*, 19−25.

Caddigan, E.J.; *VMD User's Guide*; University of Illinois; Beckman Institute: Urbana, IL, USA, 2004.

Edwards, P.M. Origin 7.0: Scientific graphing and data analysis software. *J. Chem. Inf. Comput. Sci.Wash.***2002**, *42*, 1270–1271.

Mancini, D.T.; Matos, K.S.; da Cunha, E.F.; Assis, T.M.; Guimarães, A.P.; França, T.C.; Ramalho, T.C. Molecular modeling studies on nucleoside hydrolase from the biological warfare agent *Brucella suis*. *J. Biomol. Struct. Dyn*. **2012**, *30*, 125–136.

Martins, T.L.C.; Ramalho, T.C.; Figueroa-Villar, J.D.; Flores, A.F.C.; Pereira, C.M.P. Theoretical and experimental C-13 and N-15 NMR investigation of guanylhydrazones in solution. *Magn. Reson. Chem.***2013**, *41*, 983–988.

De Castro, A. A.; Prandi, I. G.; Kuca; Kamil K; Ramalho, T.C. Organophosphorus degrading enzymes: Molecular basis and perspectives for enzymatic bioremediation of agrochemicals. *Ciencia e Agrotecnologia*, **2017**, *41*, 471-482.

Supplementary Materials: Can Inhibitors of Snake Venom Phospholipases A_2 Lead to New Insights into Anti-Inflammatory Therapy in Humans? A Theoretical Study

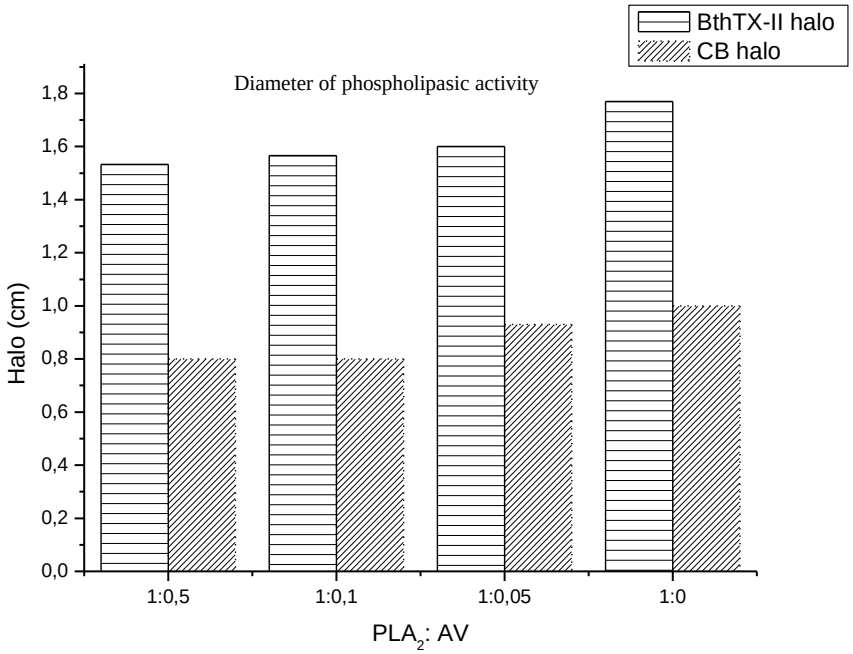
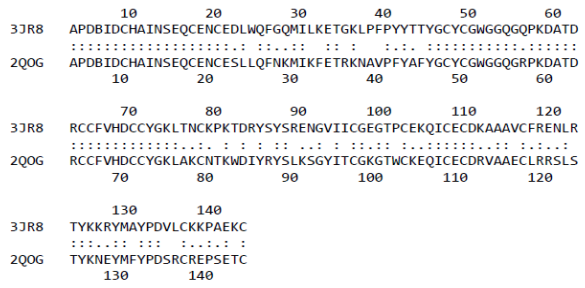
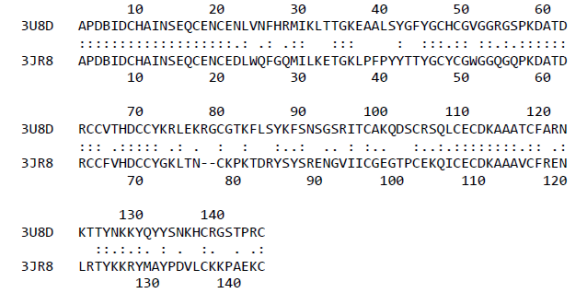


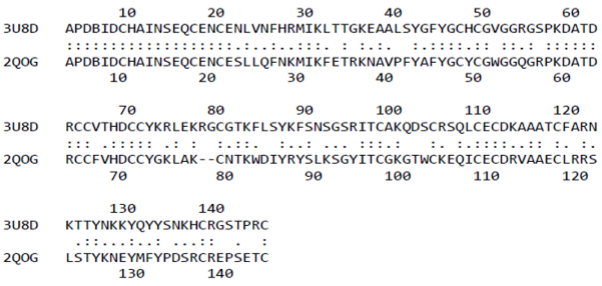
Figure S1. Halo of inhibition, in centimeters, formed by the inhibition of svPLA2svPLA2 isolated from BthTX-II and CB venom, by vanillic acid.



(**a**)



(**b**)



(**c**)

Figure S2. Alignments of Human PLA2 HGIIA (3U8D) aminoacid sequences with the phospholipases A2 BthTX-II (3JR8) and PLA2 CB (2QOG): (**a**) is the *sv*PLA2 aminoacids sequences alignment, (**b**) it's the alignment of the PLA_2 primary sequences HGIIA and BthTX-II and (**c**) represents the alignment performed with HGIIA and CB.

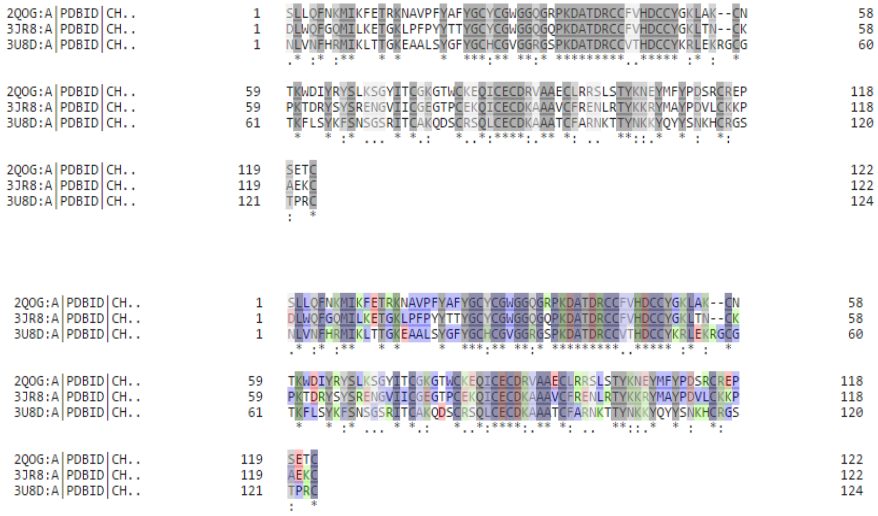


Figure S3. The amino acid sequence comparison between HGIIA, BthTX-II and PLA2 CB focusing on similar distribution of charged amino acid and hydrophobicity. The gray color represents the similarity between the amino acid sequences. The residues in purple represent the hydrophobic parts, in pink represent the negative parts and residues in green correspond to positive charged residues.

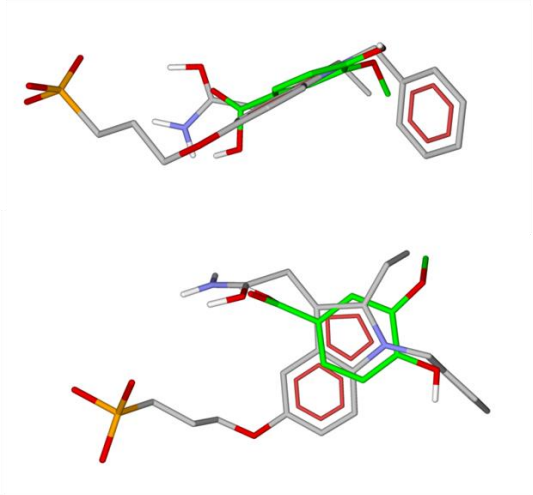


Figure S4. Overlap of the active ligand of the 3U8D complex, of the enzyme HGIIA, with the vanillic acid obtained by the molecular docking.

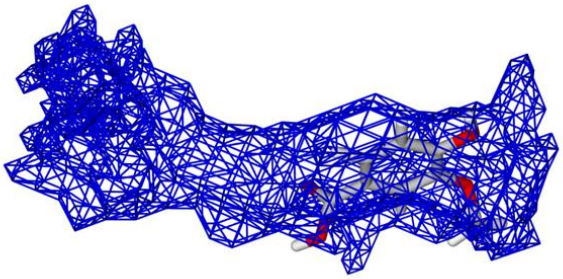
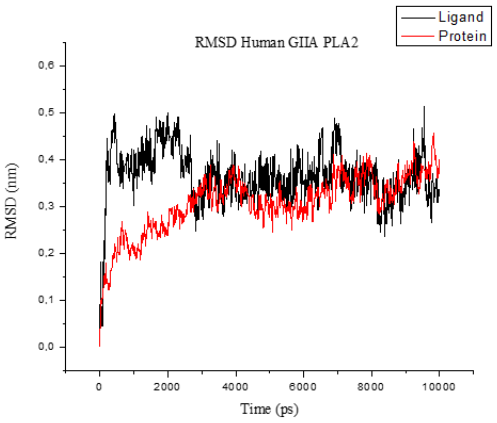
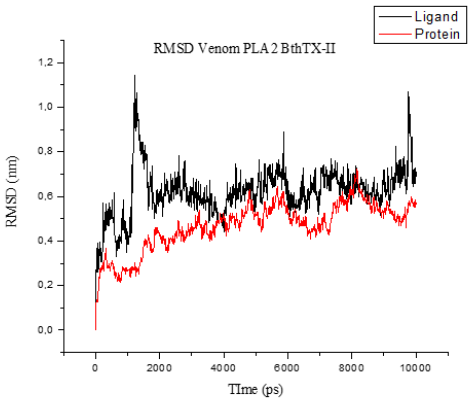


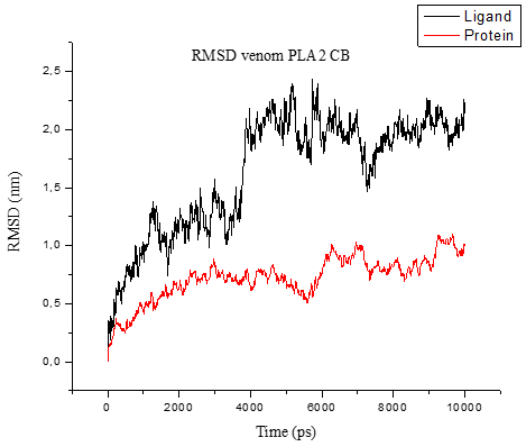
Figure S5. Volume of the cavity of the enzyme HGIIA with the molecule of vanillic acid anchored.



(**a**)

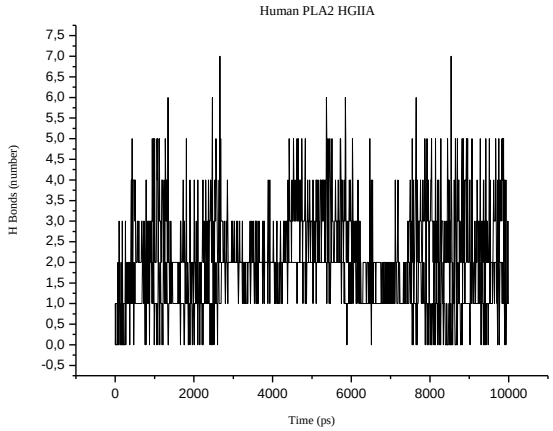


(**b**)

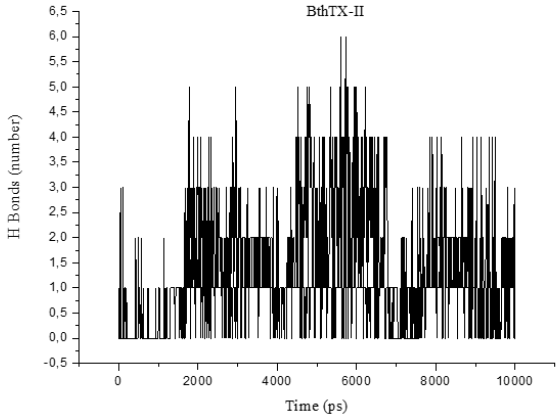


(**c**)

Figure S6. Root Mean square deviation (RMSD) for the HGIIA/VA, BthTX-II/VA and CB/VA complexes: (a) is the plot of RMSD for the HGIIA enzyme from the MD simulation, (b) is the RMSD for the BthTX-II toxin and (c) is the RMSD for the second toxin CB from the MD analysis.



(**a**)



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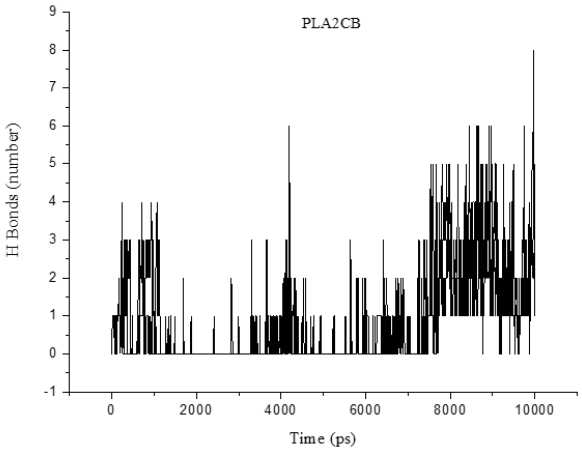

(**c**)

Figure S7. Hydrogen bonds carried out between vanillic acid and PLA2 enzymes. The first plot (**a**) is the Hydrogen bonds made with HGIIA, (**b**) is the Hydrogen bonds made with BthTX-II enzyme and (**c**) is the CB hydrogen bonds.