Low molecular weight compounds from mushrooms as potential Bcl-2 inhibitors: Docking and Virtual Screening studies

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∆G: binding energy

3D: Three Dimensional

Å: Angström

ABT-199: Venetoclax

ABT-263: Navitoclax

AD4: AutoDock4

ADT: AutoDockTools

AT-101: Gossypol

BAD: Bcl-2-associated death promoter

Bak: Bcl-2 antagonist/Killer

Bax: Bcl-2 associated X protein

Bcl-2: B-cell lymphoma-2

Bcl-A1: Bcl-2 related protein A1

Bcl-w: Bcl-2 like protein2

Bcl-xL: B-cell-lymphoma extra Large

BH: Bcl-2 homology

BID: BH3 interacting-domain

BIM: Bcl-2-like protein 11

CA: Clavilactone A

CB: Clavilactone B

CD: Clavilactone D

HepG2: Human hepatocellular liver carcinoma

HMW: High Molecular Weight

HT-29: Human colon adenocarcinoma

KDa: KiloDalton

Ki: Constant Inhibition

LBVS: Ligand Based Virtual Screening

LMW: Low Molecular Weight

MCF-7: Human breast adenocarcinoma

Mcl-1: myeloid cell leukemia sequence 1

MOM: Mitochondrial Outer Membrane

nM: nanoMolar

NMR: Nuclear Magnetic Resonance

NOXA: Phorbol-12-myristate-13-acetate-induced protein 1

PDB : Protein Data Bank

Pdbqt format: protein data bank, partial charge (q), and atom type (t) format

pKi: $-\log_{10}Ki$, difference between estimated and experimental Ki values

PPI: Protein-protein interaction

PubChem: database of chemical compound

PUMA: p53 upregulated modulator of apoptosis

SBDD: Structure Based Drug Design

SBVS: Structure Based Virtual Screening

Sdf format: Structure data file format

TM: TransMembranane

VS: Virtual screening

Abstract

Mushrooms have the ability to promote apoptosis in tumor cell lines, but the mechanism of action is not quite well understood. Inhibition of the interaction between Bcl-2 and pro-apoptotic proteins could be an important step that leads to apoptosis. Therefore, the discovery of compounds with the ability to inhibit Bcl-2 is an ongoing research topic in drug discovery. In this study, we started by analyzing Bcl-2 experimental structures that are currently available in Protein Data Bank database. After analysis of the more relevant Bcl-2 structures, 4 were finally selected. An analysis of the best docking methodology was then performed using a cross-docking and re-docking approach while testing 2 docking softwares: AutoDock 4 and AutoDock Vina. Autodock4 provided the best docking results and was selected to perform a virtual screening study applied to a dataset of 40 Low Molecular Weight (LMW) compounds present in mushrooms, using the selected Bcl-2 structures as target. Results suggest that steroid are the more promising family, among the analyzed compounds, and may have the ability to interact with Bcl-2 and this way promoting tumor apoptosis. The steroids that presented lowest estimated binding energy (ΔG) were: Ganodermanondiol, Cerevisterol, Ganoderic Acid X and Lucidenic Lactone; with estimated ΔG values between -8,45 and -8,23 Kcal/mol. A detailed analysis of the docked conformation of these 4 top ranked LMW compounds was also performed and illustrates a plausible interaction between the 4 top raked steroids and Bcl-2, thus substantiating the accuracy of the predicted docked poses. Therefore, tumoral apoptosis promoted by mushroom might be related to Bcl-2 inhibition mediated by steroid family of compounds.

Sumário

Os cogumelos apresentam a capacidade de promover a apoptose em linhas células tumorais, No entanto o seu mecanismo de ação não é completamente conhecido. A inibição da interação entre Bcl-2 e proteínas pro-apoptóticas pode ser um passo importante na iniciação do processo de apoptose tumoral. Por essa razão, a descoberta de compostos que inibam a proteína Bcl-2 é uma área importante na descoberta de novos fármacos antitumorais. Neste estudo, começou-se por analisar as estruturas experimentais de Bcl-2 atualmente presentes na base de estruturas Protein Data Bank. Após análise das estruturas de Bcl-2 mais relevantes, 4 foram escolhidas. Um estudo de "cross-docking" e "re-docking" foi então realizado para escolher a metodologia de "docking" mais adequada. Testaram-se 2 softwares, o AutoDock 4 e o AutoDock Vina, e verificou-se que o AutoDock 4 apresentava melhores resultados, tendo sido o selecionado para realizar os ensaios de "screening" virtual dos 40 compostos de baixo peso molecular presentes em cogumelos, utilizando as 4 estruturas selecionadas. Os resultados obtidos sugerem que os esteroides são a família de compostos mais prometedores de entre as famílias de compostos estudadas. Os esteroides que apresentaram valores de energia de ligação (ΔG) mais baixos foram: Ganodermanondiol, Cerevisterol, Ácido Ganoderico X and Lactona Lucidénica, com valores de ΔG estimado entre -8,45 e -8,23 Kcal/mol. Uma análise detalhada da conformação de ligação foi também realizada dos 4 melhores compostos de baixo peso molecular melhor classificados. Esta análise demonstra um modo de interação plausível entre os compostos e a estrutura da Bcl-2, consubstanciando a eficácia dos resultados obtidos por "docking". Conclui-se que o processo inibição de apoptose tumoral observada em cogumelos pode estar relacionado com a inibição da Bcl-2 por esteroides presentes nos cogumelos.

1. INTRODUCTION

1.1. Mushrooms as potential source of bioactive compounds

For centuries mushrooms have been used as human food and have been appreciated for texture and flavours as well as for medicinal purposes. However, the use of mushrooms as an important source of biological active substances with medicinal value has only recently been worthy of note. Several bioactivities of mushrooms have been studied including antibacterial, antitumor, antioxidant, antifungal, antiviral and anti-inflammatory, to name a few (Chang & Miles, 2004; Daba & Ezeronye, 2003). In the present work we will focus on antitumor activity of mushrooms, especially as a potential source of compounds with antitumor activity.

Mushrooms components vary in their chemical nature and include High Molecular Weight (HMW) and Low Molecular Weight (LMW) compounds. Both types of compounds may be involved in the antitumor activity of mushrooms. In this work, because we are studying the potential of mushrooms compounds as inhibitors of the Bcl-2 protein target, we will focus on LMW compounds present in mushrooms, although HMW compounds will also be referred (Ferreira *et al.*, 2010).

1.1.1. High Molecular Weight (HMW) compounds present in mushrooms

HMW mushrooms compounds with antitumor potential are structurally characterized as having long-chains, and include homo and hetero polysaccharides, glycoproteins, glycopeptides, proteins and RNA-protein complexes (Ferreira *et al*., 2010; Patel & Goyal., 2012).

Several phytochemicals have been isolated from medicinal mushrooms and three of those, which are carcinostatic polysaccharide drugs, have been developed from mushrooms in Japan. These are “Krestin” (PSK), from the cultured mycelium of Kawaratake (*Trametes versicolor*), “Lentinan” from the fruiting bodies of Shiitake (*Lentinus edodes*) and “Schizophyllan” (Sonifilan) from the culture fluid of Suehirotake (*Schizophyllum commune*). Lentinan and schizophyllan are pure-glucans, whereas PSK is a protein bound polysaccharide. The biological activity of these three products is related to their immunomodulating properties, which enhance the host’s defense against various forms of infectious disease (Zaidman *et al*., 2005).

More recent studies have shown other mushrooms HMW compounds with antitumor activity. For example proteoglycan, a heavily glycosylated protein purified from *Phellinux linteus*, has shown anti-proliferative effect on human hepatocellular liver carcinoma (HepG2), human colon adenocarcinoma (HT-29), human lung cancer (NCIH 460) and human breast adenocarcinoma (MCF-7) cell lines. *In vitro* anti-proliferative activities of water-soluble polysaccharides extracted from the fruiting body and mycelium of *Pleurotus tuber* regium have also been discovered. Also, the isolation of lectin, a homodimeric 32.4 kDa glycoprotein with specific binding sites for sugars, isolated from fresh fruiting bodies of *Pleurotus citrinopileatus* have been shown to cause 80% inhibition of tumor growth (Ferreira *et al*., 2010; Patel & Goyal, 2012).

1.1.2. Low Molecular Weight (LMW) compounds present in mushrooms

LMW compounds present in mushrooms with known antitumor potential are usually secondary metabolites and include: quinones and hydroquinones, isoflavones, catechols, amines and amides, sesquiterpenes and steroids. The current knowledge of mushrooms LMW compounds with some type of antitumor activity have been reviewed by Ferreira *et al.*, 2010, and are presented in Table 1, with the respective chemical representation of the compounds presented in Figure 1. Although there are most likely a large number of mushroom LMW compounds with antitumor activity yet to be discovered, the list of 40 LMW compounds is a very good starting point, and will be our mushroom LMW compound dataset to be analyzed in this study, as potential Bcl-2 inhibitors (Froufe *et al.*, 2012).

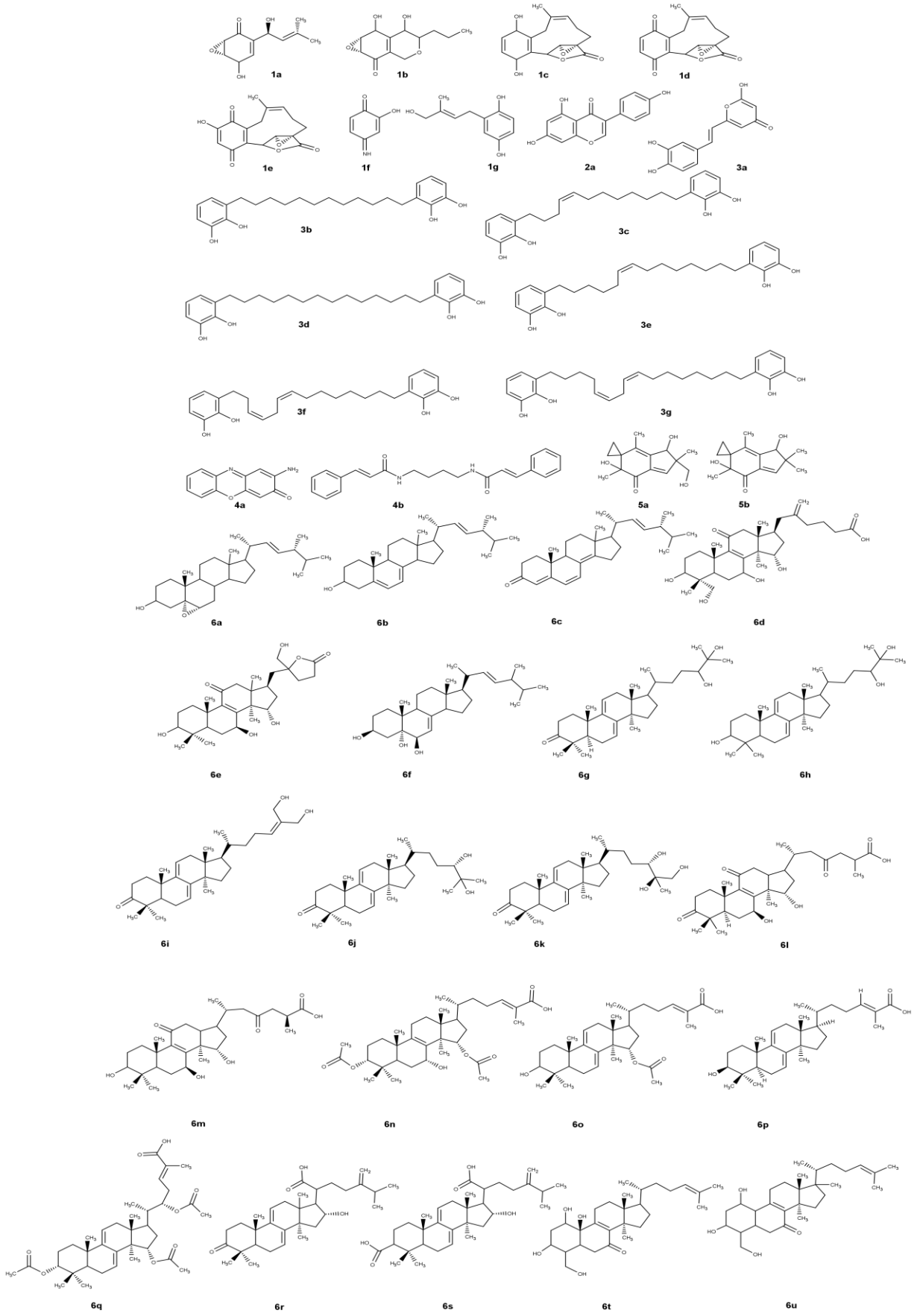

Figure 1. Chemical structure of the LMW compounds with anti-cancer potential isolated from mushrooms.

The known antitumor activity of the 40 LMW compound dataset will now be briefly overviewed. The LMW dataset includes 3 macrolidic fungal metabolites produced by *Clitocybe clavipes*: clavilactones A (CA), clavilactones B (CB), and clavilactones D (CD). These compounds were identified as inhibitors of protein tyrosine kinases. Also our LMW database include 2 epoxy compounds; panepoxydone (isolated from *Panus conchatus*, *Panus rudis*, and from *Lentinus crinitus*) and cycloepoxydon (isolated from a Xylaria strain); that are known to inhibit NF-κB, a transcription factor that is activated in cancer disease.

The isoflavonoid genistein have been shown to inhibit proliferation of breast cancer and prostate cancer cells. Genistein have been shown to act by inhibition of protein tyrosine kinase activity, regulating the proliferation of cancer cells and consequently inhibiting Cdc2 kinase.

The gerronemins A-F extracts of a Gerronema species showed Cyclo-Oxygenase 2 enzyme inhibition and 2-Aminophenoxazin-3-one (Questiomycin A). Belonging to amines and amides family of compounds, they have shown antitumor potential as a non-steroid aromatase inhibitor (Zaidman *et al.*, 2005).

The sesquiterpenes illudin S and illudin M, obtained from the mushrooms *Omphalotus illudens* and Lampteromyces japonicas, behave as alkylating agents of protein and DNA (Zaidman *et al.*, 2005).

Ganoderma lucidum contains a high amount of steroid compounds. Currently around 20 different steroids have been isolated with therapeutic effects. Lucidenic acid O and Lucidenic lactone have shown DNA polymerase α, ß inhibition activity, while Cerevisterol presented DNA polymerase α inhibition.

Also Lucidumol A, B and F, Ganodermondiol and Ganodermontriol presented anti-tumor activity against several tumor cell lines. Ganoderic acids A presented NF-KB and Activator Protein1 (AP-1) inhibition activity while Ganoderic acid F has been shown to prevent invasion of metastatic cells.

Also Ganoderic acid W, X, Y, T presented DNA topoisomerase inhibition and anticancer activities (Patel & Goyal, 2012; Sliva, 2004).

Finally steroid compounds extracted from *Grifola frondosa*: ergosterol, ergostra-4, 6, 8(14), 22-tetraen-3-one; have shown Cyclo-Oxygenase enzyme inhibition (Zaidman *et al.*, 2005).

Table 1. Mushrooms LMW compounds with anticancer bioactivity (adapted from Ferreira *et al.*, 2010).

Compound familly	Compound	Anticancer bioactivity
Quinones and hydroquinones	Panedoxine (1a)	NF-kB inhibitor
	Cycloepoxydol (1b)	
	Clavilactones A (1c)	Protein tyrosine kinases inhibitor
	Clavilactones B (1d)	
	Clavilactones D (1e)	
	490 Quinone (1f)	DNA polymerase α inhibitor
	Hydroquinone (1g)	Matrix Metallo-Proteinase (MMPs) inhibitors
Isoflavones Catechols	Genistein (2a)	Cdc2 kinase modulator
	Hispidin (3a)	PKCß inhibitor
	Gerronemins A (3b)	Cyclo-OXygenase-2 (COX-2) inhibitor
	Gerronemins B (3c)	
	Gerronemins C (3d)	
	Gerronemins D (3e)	
	Gerronemins E (3f)	
	Gerronemins F (3g)	
Amines and amides	2-Aminophenoxazin-3-one (Questiomycin A) (4a)	Aromatase inhibitor
	Putrescine-1,4-dicinnamide (4b)	Apoptosis inducer
Sesquiterpenes	Illudin S (5a)	DNA alkylating agent
	Illudin M (5b)	
Steroids	5,6-Epoxy-24(R)-methylcholesta-7,22-dien-3β-ol (6a)	Sulfatase inhibitor
	Ergosterol (6b)	Cyclooxygenase inhibitor
	Ergosta-4,6,8(14),22-tetraen-3-one (6c)	
	Lucidenic acid O (6d)	DNA polymerase α, ß inhibitions
	Lucidenic lactone (6e)	
	Cerevisterol (6f)	DNA polymerase α inhibition
	Lucidumol A (6g)	Anticancer activity against some cell lines
	Lucidumol B (6h)	
	Ganoderiol F (6i)	
	Ganodermanondiol (6j)	
	Ganodermanontriol (6k)	
	Ganoderic acid A (6l)	NF-KB and AP-1 inhibitor
	Ganoderic acid F (6m)	prevention of invasion of metastatic cells
	Ganoderic acid W (6n)	DNA topoisomerase inhibitor
	Ganoderic acid X (6o)	
	Ganoderic acid Y (6p)	
	Ganoderic acid T (6q)	
	Polyporenic acid C (6r)	MMPs inhibitor
	Dehydroebriconic acid (6s)	DNA topoisomerase II inhibitor
	Fomitellic acid A (6t)	DNA polymerase α and ß inhibitors
	Fomitellic acid B (6u)	

1.2. Bcl-2 as a potential protein target for drug discovery

Protein-protein interactions (PPI) play an important role in signaling pathways that regulate biological processes in the cell (Dong *et al.*, 2014). One of the biological processes that are tightly regulated is apoptosis and a large number of proteins, some known and some still unknown, are involved in this signaling pathway. Bcl-2 (B cell lymphoma-2) protein is the best studied of the proteins involved in the regulation of apoptosis and that is the reason why Bcl-2 protein also names the family of proteins that it belongs to. Several studies have shown that overexpression of Bcl-2 leads to cancer and numerous Bcl-2 inhibitors have been developed with antitumor activity (Kang & Reynolds., 2009).

Understanding the PPI mechanisms of Bcl-2 with other proteins, also involved in the regulation of apoptosis, is thus of great interest for the discovery of potential new anticancer drugs. Still, the discovery of drugs that target Bcl-2 using Cheminformatic tools remains a major challenge.

1.2.1. The Bcl-2 protein family and its role in apoptosis

The Bcl-2 protein family is composed of important protein mediators of the apoptotic response. The proteins belonging to this family are structurally related and include pro-apoptotic and anti-apoptotic proteins that interact with each other. The common feature that all Bcl-2 proteins share is the presence of a conserved short sequence of amino acids, known as Bcl-2 homology (BH) domain. The BH domain plays an important role in each Bcl-2 family protein function. In addition, the C-terminal region of Bcl-2 proteins are dominated by the presence of hydrophobic residues, not well conserved, that are known as the transmembrane (TM) region, important for membrane attachment (Czabotar *et al.*, 2014; Kvansakul & Hinds., 2015).

The Bcl-2 family members are classified into 3 main functional groups (Figure 2):

(1) anti-apoptotic or pro-survival proteins

(2) pro-apoptotic effector proteins (pro-apoptotic effectors)

(3) pro-apoptotic activator proteins (pro-apoptotic activators)

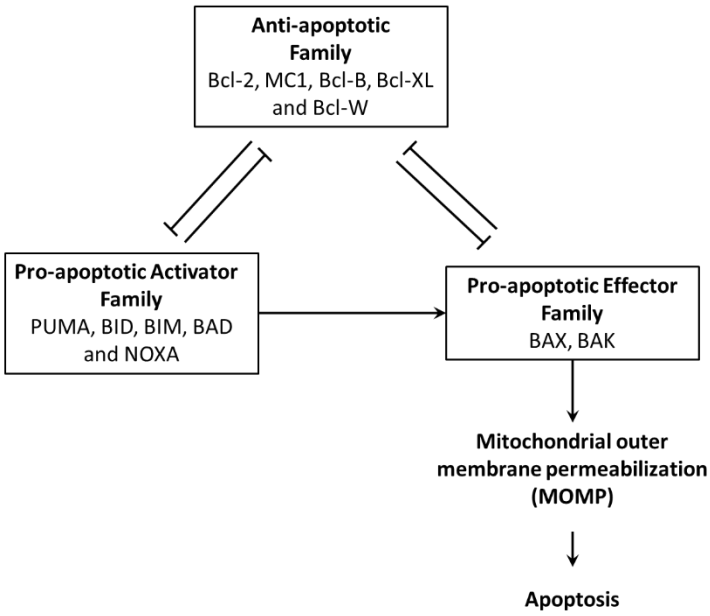

Figure 2: Apoptosis process and the interaction between proteins belonging to the 3 Bcl-2 families. Anti-Apoptotic Family: MCl-1 (Myeloid Cell Leukemia) ; Bcl (B Cell Lymphoma) B, Bcl-XL and Bcl-W. Pro-Apoptotic Activator: PUMA (p53 upregulated modulator of apoptosis); BID (BH3 interacting-domain); BIM (Bcl-2-like protein); BAD (Bcl-2-associated death promoter); NOXA (Phorbol-12-myristate-13-acetate-induced protein. Pro-Apoptotic Effector: BAX (Bcl-2-Associated X Protein); BAK (Bcl-2 homologous antagonist/killer).

Members of the 3 Bcl-2 families interact with each other and together help regulate the apoptotic process. The pro-apoptotic effectors are closely associated with the mitochondrial membrane and promote the formation of pores in the mitochondrial membrane, initiating the apoptotic program. The pro-apoptotic activators are important mediators in the cellular response to stresses such as DNA damage and act by stimulating pro-apoptotic effectors. The anti-apoptotic protein act by directly interacting and inhibiting the apoptotic promoting effects from both pro-apoptotic effectors and pro-apoptotic activators. The dynamic balance that occurs between Bcl-2 anti-apoptotic and Bcl-2 pro-apoptotic proteins helps determine whether the cell initiates apoptosis (Figure 2).

The human anti-apoptotic proteins present multiple BH-domains that support the most conserved BH1 and BH2 domains, a BH4 domain and may also support BH3 domain and include: Bcl-2, Bcl-xL, Bcl-w, Mcl-1 (myeloid cell leukemia sequence 1), A1 and Bcl-B.

Like the pro-survival proteins, the pro-apoptotic effectors also include multi BH-domains and with the mains members being: Bax (Bcl-2 associated X protein), Bak (Bcl-2 antagonist/Killer) also support BH1 and BH3 domain and obligatory contain BH3 domain that define the apoptotic behavior. The pro-apoptotic activators contain only one BH3 domain and

include PUMA (p53 upregulated modulator of apoptosis); BID (BH3 interacting-domain); BIM (Bcl-2-like protein 11); BAD (Bcl-2-associated death promoter); NOXA (Phorbol-12-myristate-13-acetate-induced protein 1) (Kvansakul & Hinds., 2015). (Figure 2)

1.2.2. Bcl-2 as a potential protein target for drug discovery

The overexpression of Bcl-2 is common in several human cancers including prostate, lung, gastric, renal, epithelial, non-Hodgkin's lymphoma, acute and chronic leukemia cancer with chemotherapeutic resistance (Kirkin *et al.*, 2004). Although the number of protein members of the 3 Bcl-2 families implies that the apoptosis regulation is a complex and tightly process, the overexpression of Bcl-2 family probably promotes the inhibition of pro-apoptotic proteins thus stopping apoptosis in tumoral cells. Overexpression of Bcl-2 protein is thus a brake for what would otherwise be a healthy process of tumoral apoptosis. Inhibition of the PPI between Bcl-2 and pro-apoptotic proteins is thus an important step that leads to apoptosis of tumoral cells. By inhibiting Bcl-2, the free pro-apoptotic effector proteins will then promote MOM permeabilization and consequent tumoral apoptosis (Kang & Reynolds., 2009). Therefore, the discovery of compound with the ability to inhibit Bcl-2 is an ongoing research topic.

1.2.3. Current knowledge on known Bcl-2 inhibitors

The disruption of PPIs between anti-apoptotic and pro-apoptotic Bcl-2 family protein has been successfully established as an anticancer therapy, with the development of small molecule inhibitors that have entered in clinic trial and act by directly targeting the hydrophobic groove of the Bcl-2 protein and potentially restoring apoptosis (Souers *et al.*, 2013; Walensky, 2006).

The first compound identified that has shown inhibition against Bcl-2 was gossypol (AT-101, Ascenta), currently in Phase I/II clinical trials. Gossypol analog, apogossypol (Burnham Institute), is in preclinical development (Kang & Reynolds, 2009). Navitoclax (ABT-263) is an orally bioavailable compound with a high affinity for both Bcl-2 and Bcl-xL and it's currently in phase 2 of clinical trial and activating the intrinsic apoptotic pathway (Waldman *et al.*, 2016).

Venetoclax (ABT-199), an inhibitor which specifically targets Bcl-2, shows similar target-driven activity, is significantly more potent than navitoclax (Souers *et al.*, 2013) (Figure 3).

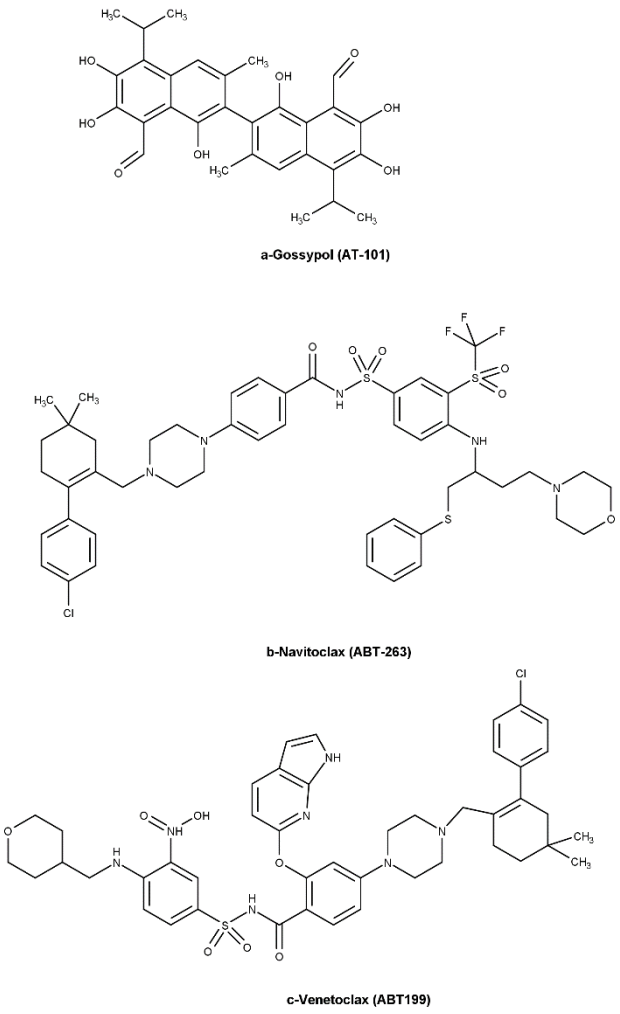

Figure 3. Synthetic inhibitors of Bcl-2 in clinical trials. a- Gossypol (AT-101); b- Navitoclax (ABT263); c- Venetoclax (ABT199).

1.3. Molecular Docking in Drug Discovery

The discovery of new drugs has been an increasingly more difficult proposition for the pharmaceutical industry. The costs of producing a new drug have been increasing each year and this is the driving source for developing new more cost efficient tools. The use of bioinformatic and chemoinformatic tools to aid the drug discovery process is now common in the pharmaceutical industry as well as in public institutions (Grinter & Zou., 2014). In this study we use the bioinformatics methodology called molecular docking (or just docking) (Froufe *et al.*, 2011).

Molecular docking belongs to the Structure Based Drug Design (SBDD) methodologies, where experimental structure information of the protein target of interest is mandatory. Docking simulation can only be performed when 3D (Three Dimensional) proteins structures, obtained either by X-ray crystallography or Nuclear Magnetic Resonance (NMR) techniques, are available (Ferreira, L *et al.,* 2015).

1.3.1. Methods and challenges of molecular docking

Trying to predict if a given compound interacts with a protein target of interest, using only bioinformatic tools, is not an easy task. The docking tools must find the optimum binding orientation for the compound in the active site of the protein. This means that it must predict the correct ligand conformation and orientation, usually term the POSE. In addition the *in silico* method must also try to calculate the relative affinity of the compound. This quantitative value is usually referred as the SCORE. Many docking methods and programs have been developed and tested as docking applications. Docking POSE accuracy is usually evaluated by the ability to reproduce the experimentally determined binding mode of a ligand. The best docking programs correctly dock around 70–80% of the docked ligands, when tested on large sets of protein–ligand complexes, although these percentages are highly dependent of protein structures available and the accuracy of a given software. It is widely accepted that different docking softwares, because they use different POSE search algorithms, performed better for different protein structures, so it is always a sound methodology to use and test more than one docking software in a drug discovery project (Verdonk et al., 2008).

The docking SCORE accuracy is usually evaluated by predicting the binding energy (ΔG) or the constant inhibition (Ki) values for a number of known inhibitors of the protein target studied, and comparing them to known experimental values. A good correlation between predicted and experimental values will demonstrate a good performance of a given docking software in predicting POSE and SCORE of other tested compounds (Grinter & Zou., 2014; Ferreira, L *et al.,* 2015).

Finding out the best POSE for each tested compound into the binding site of the protein structure, and evaluating and comparing the SCORES of each docked compound are thus the main object to determine the potential of the studied compounds as inhibitors of a given protein target of interest (Ferreira. L *et al.,* 2015; Kroemer., 2007).

1.3.2. Molecular docking softwares: AutoDock 4 and AutoDock Vina

There are a number of docking softwares, either commercial or free for academic use. Among the latter, two of the most used softwares are AutoDock 4 (AD4) and AutoDock Vina (Vina). As all docking softwares, and SBDD methodologies in general, the knowledge of the 3D experimental structures of the protein target of interest is essential. Both AD4 and Vina require the knolwledge of the 3D "search space" that must include the binding site of the target protein.

AD4 is maintained by the Molecular Graphics Laboratory, Scripps Research Institute, La Jolla. AutoDock 4 uses a Lamarckian Genetic algorithm to get fast predictions of the POSE and the SCORE as free energy of binding. This type of algorithm simulates the genetic selection that occurs in nature. A number of conformations of the ligand are generated (population) and evaluated, and the ligand structure with the best binding energy are selected and used to generate the next population. This process is performed millions of times till eventually the docked pose of the ligand with the best SCORE and POSE is obtained. In order to search efficiently the selected 3D conformational space and to speed up the interaction energy calculation, AD4 prepare grid map for each possible atom in the ligand or protein structure (Morris et al., 2009). AD4 is one of the first softwares to be developed and is one of the more widely used as there is a large number of studies that use AD4 (Meng *et al.*, 2011; Morris *et al.*, 2009).

Vina is another docking software that is free for academic use that is also maintained by the Molecular Graphics Laboratory on The Scripps Research Institute, La Jolla. Vina uses a different algorithm that calculates automatically, quickly and without generating three dimensional grid map, a binding energy (ΔG). It´s a more recent docking software and is currently very popular because a docking simulation is easier to prepare and each docking run is much faster than AD4. (Abreu et al., 2012; Trott & Olson., 2010).

AD4 and Vina use a specific PDBQT file format, which is an extension of the pdb file format. The pdbqt format can easily be opened by most molecular modeling softwares, including AutoDockTools (ADT) and Pymol, used in this work (Lill & Danielson., 2011; Trott & Olson., 2010).

1.3.3. Virtual screening using AutoDock 4 and AutoDock Vina

The concept of virtual screening is to find and prioritize the potential active compounds from a virtual library of compounds in the context of a computer aided drug discovery project (Kumar *et al.,* 2007). The virtual screening methodologies are usually classified in two categories: ligand-based virtual screening (LBVS) and structure-based virtual screening (SBVS). The availability of docking softwares that can screen millions of compounds in a short time, and that can successfully predict the suitable conformation of a compound into the binding site of a protein target, makes docking one of the most used SBVS methodologies for virtual screening projects (Kumar *et al.,* 2007).

Specifically in anti-cancer drug discovery, because there are always new 3D protein structures being determined, VS methodologies have been widely used. Many of the recently discovered potential inhibitors of relevant protein targets have, at least in part of the drug discovery project, used some type of VS tool, and in most cases molecular docking methodologies were used. The main advantage of using this VS tools are: the increase in speed to the process, the possibility of automation of the process and specially the decrease of the costs associated with the drug discovery process.

In this work, a number of tools have been used to perform VS of the selected LMW mushroom dataset of compounds using AD4 and Vina as docking tools. These tools include AutoDockTools (ADT) and MOLA.

ADT is a graphical user interface that is part of the MGLTools suite and was implemented by the Molecular Graphics Laboratory at the Scripps Research Institute. ADT performs the preparation of the grid with the appropriate parameters to be used for AD4 docking studies. It prepares the protein structures in PDBQT format, needed for using AD4 and Vina. (Morris *et al.*, 2008).

MOLA is a free software for VS using AD4 and Vina on computer clusters. It's integrated in a customized Live-CD LINUX operating system and was developed in our group (Abreu *et al.*, 2010). MOLA is able to prepare large datasets of compounds for screening and also automates the complete VS project in computer clusters, using either AD4 or Vina as docking tools. It is especially useful when large datasets of compounds are going to be screened as it would be impossible to perform each docking run manually.

1.4. Objectives

In order to better understand the possible mechanism of action of mushrooms anti-cancer bioactivity at a molecular level, a dataset of 40 low molecular weight (LMW) compounds, present in mushrooms, will be virtually screened against Bcl-2 (B cell /lymphoma-2), a pro-apoptotic proteins known to be involved in several cancer situations.

In this study, the LMW dataset of compounds will be virtually screened using two docking software tools: AutoDock 4 and AutoDock Vina. The validation of the selected Bcl-2 experimental structures as targets, will be performed using a re-docking and cross-docking approach. The docking studies of the LMW dataset of compounds will then be performed using the selected Bcl-2 structures as targets. Compounds with the lowest predicted binding energy (predΔG) are expected to be the more potent Bcl-2 inhibitors.

2. MATERIALS AND METHODS

2.1. Ligand structures preparation (LMW compound dataset)

The majority of the 40 LMW compound structures (Figure 1) were sketched using MarvinSketch version.1.25 (www.chemaxon.com), and saved in sdf file format. When available, some structures were downloaded, in sdf file format, from the PubChem compound database, belonging to the National Center for Biotechnology Information. Compound structures were then verified from the original articles (Wang Y *et al.*, 2010).

The chemical structures in sdf file format were then converted into pdb file format, using PyMol, an open-source structure visualization software. Next, AutoDockTools 1.5.2 (ADT) was used to perform the following procedures on each ligand structure: merge nonpolar hydrogen, add Gasteiger charges, set up rotatable bonds and convert the ligands into pdbqt file format, adequate for AutoDock4 (AD4) and AutoDock Vina (Vina) use (Gasteiger *et al.*, 2005).

2.2. Protein structure preparation

The Bcl-2 experimental structures were all downloaded from the PDB database (Protein DataBank) (http://www.rcsb.org), with their respective PDB id's code being: 4IEH, 4AQ3, 4LVT and 4LXD. These Bcl-2 protein structures were experimentally determined using X-Ray diffraction methods, and made available in PDB for all researches to use. For docking studies, the inhibitors of each protein structure were separated by removing the coordinates of the respective atoms from the pdb file. The water molecules atom coordinates, include in the co-crystallized protein structure, were also removed. When there was more than one protein chain represented in the pdb file, chain A was selected for docking. ADT software was then used to prepare the input files necessary for AD4 and Vina, by performing the following procedures: assignment of polar hydrogens, calculation of Gasteiger charges to the protein structures and conversion of the protein structures from pdb file format into pdbqt file format (Morris *et al.*, 2009).

2.3. Virtual screening using molecular docking

The docking studies were performed using two molecular docking softwares: AutoDock 4.2 version (AD4) and AutoDock Vina (Vina) (Morris *et al.,* 2009). Both AD4 and Vina require the specification of the 3D "search space", centered on the interaction site of the protein structure (Trott O. *et al.,* 2010).

The size of the grid box for Vina was 22*22*22 Å, for the X, Y, Z dimensional coordinates, applied for each protein structure interaction site. The default setting for spacing was 1 Å, and exhaustiveness value used was 16. For each PDB structures, the center coordinate was obtained from the analysis of the co-crystallized inhibitors for each protein structure (Abreu *et al.*, 2012).

For AD4, ADT was used to create atom grid affinity maps, for each atom type present in each of the 4 protein structures, using AutoGrid4 algorithm. ADT prepared the grid maps with the following parameters: the number of grid points for the X, Y, Z dimensional coordinates were 88*88*88, with 0.250 Å regular spaces within the grid space selected. AD4 resolution of the grid space resulted in 88*0.250 = 22 Å grid space, the same grid space used for Vina. All affinity grid maps were centered on the active site and coordinates were selected in order to encompass the complete active site for each protein structure (Goodsell *et al.,* 2008). AD4 used the Lamarckian genetic algorithm (Morris *et al.,* 1998), to perform the Bcl-2/compounds molecular docking studies. Docking parameters selected for AD4 runs were as follows: 50 docking runs, population size of 200, random starting position and conformation, translation step ranges of 2.0 Å, mutation rate of 0.02, crossover rate of 0.8, local search rate of 0.06, and 2.5 million energy evaluations. The entire virtual screening experiment was performed on Core AMD 2.0 GHz computers using MOLA (Abreu et al., 2010), a custom designed software to perform virtual screening studies using AD4 and Vina (Abreu *et al.,* 2010).

Estimated constant inhibition (Ki) for all compounds were calculated as follows: Ki = exp ((ΔG*1000)/(Rcal*TK)), where ΔG is the estimated binding energy, calculated by AD4 and Vina, Rcal is 1.98719 and TK is 298.15. (Trott O. *et al.,* 2009). All structural analysis and figures with structure representations were produced and analyzed using PyMOL software (Seelige *et al.,* 2010).

3. RESULTS AND DISCUSSION

3.1. Bcl-2 molecular docking protocol validation

Before performing virtual screening of our mushroom LMW dataset of compounds against Bcl-2 protein structures, a docking protocol or methodology must be selected and validated. Essentially this means that we must first select experimental 3D (three dimensional) structures of the protein target being studied; in this case Bcl-2, and then we must select suitable docking software to be used in the virtual screening study.

Docking experiments are only possible if experimental structures of the protein target are available; preferably complexed with a known ligand or inhibitor. The selection of the experimental protein structures is an essential step of molecular docking studies as reliable docking results are highly dependent on the quality of the protein structures used. Once these protein structures are selected, the feasibility of using them can be evaluated by performing docking studies of the complexed ligands into the protein binding site and then analysing the docked conformation obtained. These docking studies are usually called re-docking and can be considered a control on the quality of the protein structure to be used for docking studies. If more than one experimental structure of the target with co-crystalized ligands is available, docking experiments can also be performed by using all ligands against all the selected protein structures. These docking studies are usually termed cross-docking.

On this study we started by selecting adequate Bcl-2 crystal structures and then validating them by performing re-docking and cross-docking studies. Two docking softwares were used in this study: AutoDock 4 (AD4) and AutoDock Vina (Vina). Although both softwares were developed in the same laboratory, they use different approaches and algorithms. Each docking software may work better depending on the type of protein target and the objectives of the docking project. For this reason a decision was made to test both software's on this re-docking and cross-docking stage.

3.1.1. Bcl-2 experimental structure selection

A total of 13 human Bcl-2 experimentally obtained structures are currently available at the PDB protein structure database (Table 2). In order to maintain this work into a more manageable timeframe, due to computational constrains, it was decided to use a total of 4 Bcl-

2 structures. First a decision was made to use only structures obtained by X-ray crystallography methodology. Structures obtained by Nuclear Magnetic Resonance (NMR) can also be used for docking studies but our group has more experience using X-ray structures. Also NMR structures were determined a long time ago and as a rule newer structures are preferred as in principle they have better resolution. From the 13 initial structures 7 presented X-ray determined structures.

Table 2. Experimental structures of Bcl-2 available on Protein DataBank (PDB).

PDB Code	Method	Resolution (Å)	Co-crystallized LMW Inhibitor	Experimental Ki value	Reference
5AGW	X-ray	2,69	No	No	Smith *et al*. 2015
5AGX	X-ray	2,24	No	No	Smith *et al*. 2015
4MAN	X-ray	2,07	Yes	No	Park *et al.* 2013
4LVT	**X-ray**	**2,05**	**Yes**	**0,044 nM**	**Park *et al.* 2013**
4LXD	**X-ray**	**1,90**	**Yes**	**59 nM**	**Park *et al.* 2013**
4IEH	**X-ray**	**2,10**	**Yes**	**14 nM**	**Xie *et al.* 2013**
4AQ3	**X-ray**	**2,40**	**Yes**	**37 nM**	**Bertrand *et al.* 2012**
2O21	NMR	N. A.	Yes	No	Bruncko *et al.* 2007
2O22	NMR	N. A.	Yes	67 *nM*	Bruncko *et al.* 2007
2O2F	NMR	N. A.	Yes	No	Bruncko *et al*. 2007
1YWN	NMR	N. A.	Yes	30 *nM*	Oltersdorf *et al*. 2005
1GJH	NMR	N. A.	No	No	Petros *et al.* 2001
1G5M	NMR	N. A.	No	No	Petros *et al.* 2001

As this study evaluates the potential Bcl-2 inhibition activity of LMW compounds, only structures with co-crystallized LMW inhibitors were considered. From the 7 available Bcl-2 structures, 5 presented LMW inhibitors.

To reduce to 4 the number of Bcl-2 structures, only X-ray structures with experimental Ki (Inhibitory Constant) values were considered, with the following PDB structures: 4IEH, 4AQ3, 4LVT and 4LXD (PDB entries) that are presented in bold on Table 2. The 4 PDB structures used present co-crystallized inhibitors from known families of Bcl-2 inhibitors: a heteroaryl-sulfonamide derivative in 4IEH, a phenylacyl-sulfonamide derivative in 4AQ3, and two benzamide derivative: Navitoclax drug in 4LVT and a Navitoclax analog in 4LXD (Figure 4).

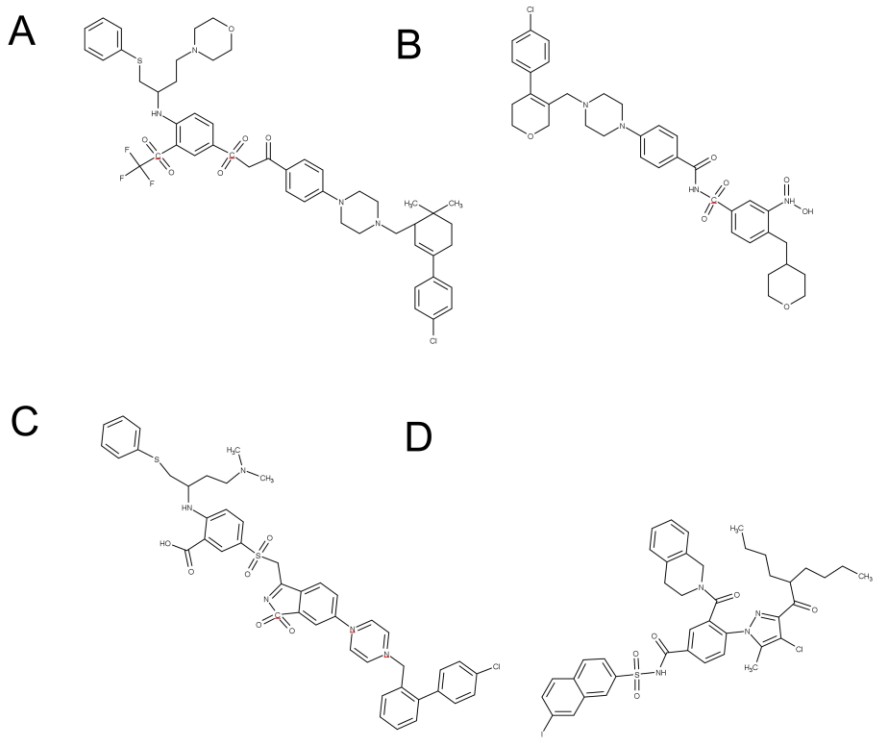

Figure 4: Chemical representation of the co-crystalized ligands used for the docking control analysis. A: 4LVT, B: 4LXD, C: 4IEH, D: 4AQ3.

3.1.2. Bcl-2 re-docking and cross-docking using AD4 and Vina

In order to validate the docking approach for the Bcl-2 selected structures, the inhibitors were re-docked to the respective structure and then cross-docked to the other 4 selected structures. The docking scores obtained using both AD4 (Table 3) and Vina (Table 4) are present as estimated average binding energy (ΔG) and estimated inhibition constant (Ki) values. Tables 3 and 4 also present the experimental Ki values obtained from the literature, and the difference between estimated and experimental Ki values are calculated as pKi difference (Estimated pKi – Experimental pKi), with pKi values calculated using the formula: $pKi = -\log_{10}Ki$. The ideal scenario will be that the pKi difference values were null. This would mean that the estimated inhibition scores obtained as estimated Ki values exactly matched the experimental Ki values.

Comparing the results from AD4 and Vina, we can observe that the pKi differences are in general much smaller for AD4 compared to Vina. For AD4 the pKi difference values were: -0,104; -0,058; -1,890 and -0,193; and for Vina; -0,341;-0,322; -3,741 and 0,138 for 4IEH, 4AQ3, 4LVT and 4LXD inhibitors, respectively. For both AD4 and Vina, the higher pKi

difference was for Navitoclax (4LVT inhibitor). These results shows that both AD4 and Vina had difficulty in predicting the much higher potency of Navitoclax as a Bcl-2 inhibitor (experimental Ki value of 0,044 nM), when compared to the inhibitors of the other 3 structures. Still the difference was much smaller for AD4.

Table 3. AutoDock 4 re-docking and cross-docking results using the selected Bcl-2 crystal structures.

Compound	Estimated ΔG (Kcal/mol)					Estimated Ki (nM)	Estimated pKi**	Experi-mental Ki (nM)	Experi-mental pKi**	pKi difference ***
	4IEH	4AQ3	4LVT	4LXD	Average					
Inhibitor (4IEH)	-9,91*	-10,96	-10,32	-11,10	-10,57	17,8	7,75	14,0	7,85	-0,104
Inhibitor (4AQ3)	-9,19	-10,73*	-11,23	-9,72	-10,22	32,4	7,49	37,0	7,43	-0,058
Inhibitor (4LVT)	-9,13	-12,39	-13,13*	-13,13	-11,55	3,4	8,47	0,044	10,34	-1,890
Inhibitor (4LXD)	-10,33	-8,46	-10,63	-11,08*	-10,13	37,9	7,42	59,0	7,23	-0,193

*re-docking studies; **pKi= $-\log_{10}$Ki; ***pKi difference = (Estimated pKi – Experimental pKi)

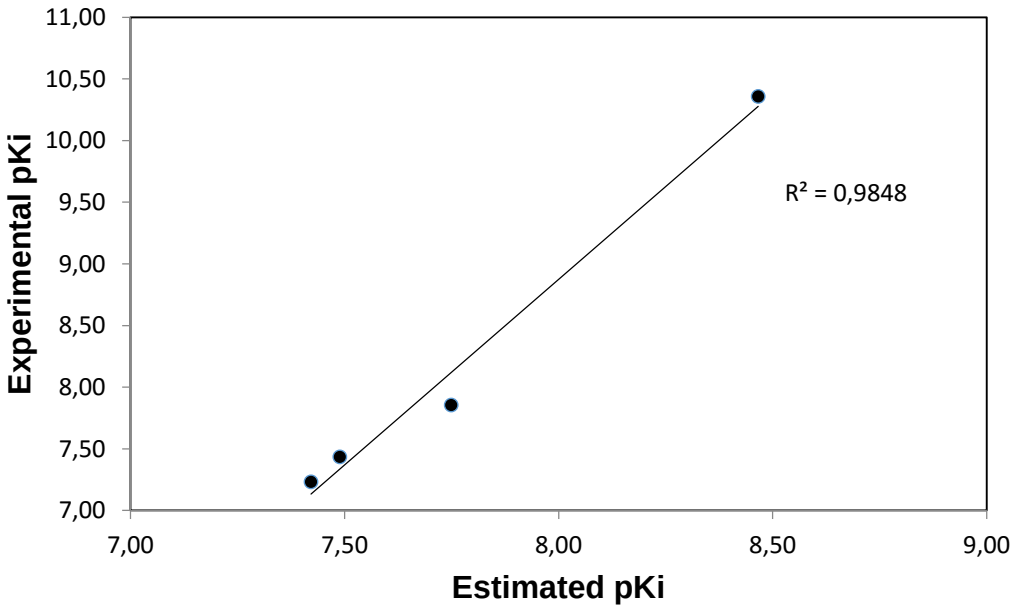

Figure 5. Correlation between experimental pKi and estimated pKi values, obtained using AD4, for the co-crystallized inhibitors present in the 4 selected PDB structures (4IEH, 4AQ3, 4LVT and 4LXD).

We then plotted the average experimental and estimated pKi values for both AD4 and Vina and presented the graph in figure 5 and 6. We can observe that AD4 presented a much better correlation between estimated and experimental values with a correlation coefficient of

0,9848. This means that the more potent inhibitor (with the lowest experimental Ki, hence the higher pKi) was predicted by AD4 to be the more potent Bcl-2 inhibitor. In fact, AD4 ranked correctly all inhibitors from the more potent Navitoclax (4LVT) to less potent Navitoclax analogue (4LXD). For Vina, the correlation coefficient was 0,7239 but the correlation was inverted. This means that the more potent inhibitor (with the lowest experimental Ki, hence the higher pKi) was predicted by Vina to be the least potent Bcl-2 inhibitor. The ligand that is mainly responsible for the difference of results between AD4 and Vina is again Navitoclax and the inability, speciality of Vina, to predict Navitoclax astounding inhibition potency (3 orders of magnitude more potent than the other inhibitors used).

Another aspect that should be taken into account, when comparing AD4 and Vina performance, is the time it takes to perform the docking simulations. Considering the parameters used, an AD4 docking run for each inhibitor against each protein structure takes on average 90 minutes, while for Vina the same docking run takes on average 15 minutes. This means that the cross-docking and re-docking runs for just the 4 inhibitors used took about 24 hours of processing time for AD4 and just 4 hours for Vina. Considering the 40 compounds from our mushrooms LMW library, to be used in the next virtual screening step, using AD4 and Vina means a processing time of about 10 and 1,5 days, respectively. This essentially means that, if docking accuracy for both softwares is similar, Vina is usually the selected docking software due to the much lower computer processing time needed.

Still, taking into account all results for both AD4 and Vina, the accuracy of the estimated values results point to AD4 performing much better than Vina, and for this reason AD4 was selected for the next virtual screening step.

Table 4. Vina re-docking and cross-docking results using the selected Bcl-2 crystal structures.

Compound	Estimated ΔG (Kcal/mol)					Estimated Ki (nM)	Estimated pKi**	Experi-mental Ki (nM)	Experi-mental pKi**	pKi difference ***
	4IEH	4AQ3	4LVT	4LXD	Average					
Inhibitor (4IEH)	-10,00*	-11,00	-9,70	-10,30	-10,25	30,7	7,51	14,0	7,85	-0,341
Inhibitor (4AQ3)	-9,20	-10,10*	-10,10	-9,40	-9,70	77	7,11	37,0	7,43	-0,322
Inhibitor (4LVT)	-10,50	-7,60	-7,60*	-10,40	-9,03	242	6,62	0,044	10,36	-3,741
Inhibitor (4LXD)	-10,60	-8,50	-10,60	-10,50*	-10,05	43	7,37	59,0	7,23	0,138

*re-docking studies; **pKi= $-\log_{10}$Ki; ***pKi difference = (Estimated pKi – Experimental pKi)

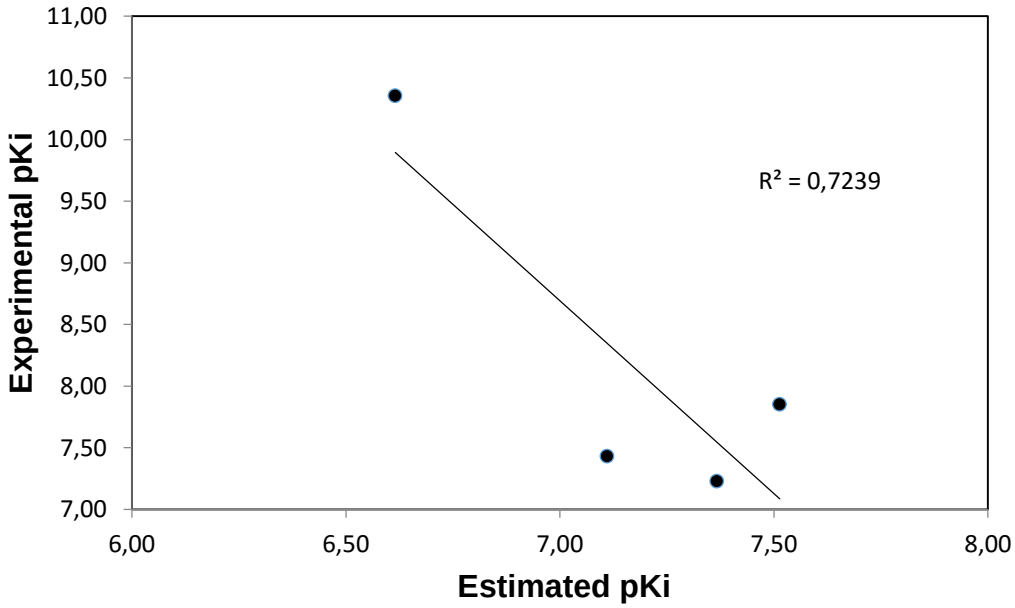

Figure 6. Correlation between experimental pKi values and estimated pKi values, obtained using Vina, for the co-crystallized inhibitors present in the 4 selected PDB structures (4IEH, 4AQ3, 4LVT and 4LXD).

3.1.3. Conformation analysis of Bcl-2 re-docking studies

The 3D docked conformation, predicted by AD4 for each Bcl-2 inhibitor, was also structurally analysed. This docked conformation analysis is usually performed by aligning the experimental conformation of the inhibitor, present in the experimental structure used, with the docked conformation of the same inhibitor predicted by the docking software, in this case AD4. Ideally the better the alignment, the more confident we are that the docking approach used will provide predictions with good accuracy.

This analysis was performed for each of the 4 Bcl-2 inhibitors used. The docked conformation of each ligand was aligned with the experimental conformation bounded to the respective structure (Figure 7). This means that Figure 7 represents the re-docking results obtained using AD4.

We can observe that in general the predicted docked conformation occupies the same binding space as the experimental conformation. This binding space of Bcl-2 structure is composed of several binding pockets that collectively are usually termed Bcl-2 binding cleft, and is the 3D space were Bcl-2 usually binds with the interaction partner of the same Bcl-2 protein family.

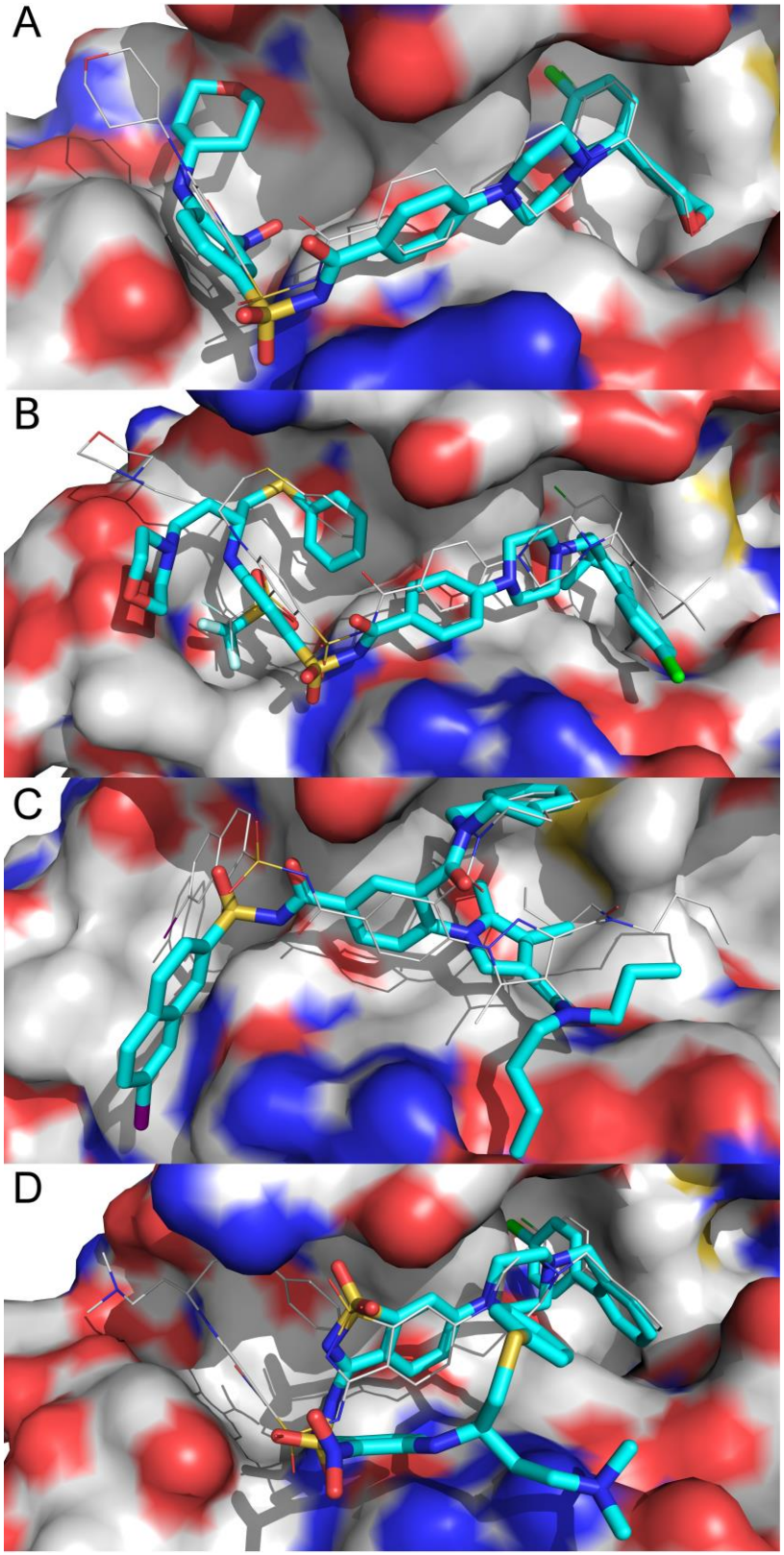

Figure 7. Alignment of experimental (wire representation, white color) and docked conformations (sticks and balls representation, cyan color), obtained using AD4 for: (A) Navitoclax analog present in 4LXD, (B) Navitoclax present in 4LVT, (C) phenylacyl-sulfonamide derivative present in 4AQ3 and (D) heteroalryl-sulfonamide derivative present in 4IEH. Superimpositions obtained by aligning the three Bcl-2 structures using Pymol.

From the alignments we can observe that the main skeleton of the compounds occupies the same Bcl-2 interaction space and with the correct inhibitor orientation. Still some inhibitor docked conformations are better aligned with the experimental structures than others and, in Figure 7, the alignments are presented from better to worst. In figure 7A, we can observe the Navitoclax analogue present in 4LXD structure. The superimposition is near perfect, with all the heteroaryl ring structures and linker groups occupying the same pockets, except for the terminal heteroaryl group that was predicted to be positioned more to the inside of the Bcl-2 structure, when compared to the experimental structure. The Navitoclax alignment is also very good and can be observed in Figure 7B. Again the terminal aryl group occupies a different pocket, in this case more to the exterior of the Bcl-2 structure and the other terminal heteroaryl group with a Fluorine atom being displaced outwards of the pocket instead of inwards. When looking at the other compounds we can observe that the experimental and predicted binding conformation are less aligned with more heteroaryl groups displaced from the experimental binding pockets, still the same 3D space is occupied for both predicted docked and experimental conformation.

These results are quite encouraging as a perfect alignment is not expected for such large compounds and with such a large binding area on Bcl-2 structure surface. In general the bigger the compounds and the interaction area are, the more difficult it is for the docking software to correctly predict docked conformations, as more variables have to be taken into consideration including, more possible rotatable bonds in the compound structure, and more possible binding pockets on the protein structure. Also the interaction site of Bcl-2 is quite exposed to the solvent and, although AD4 takes (de)solvation displacement (water molecules displacement) into consideration when doing the docking algorithm calculations, there are too many structural variables that the software has to account for and that are simplified by AD4 docking algorithm.

At the end of this re-docking and cross-docking methodology selection step, AD4 software was finally selected for the next virtual screening step. Docking studies will be performed using the 4 Bcl-2 structures selected and the average scores obtained will be used.

3.2. Virtual screening of the LMW mushroom compound dataset

Once the best docking approach was established, a virtual screening was performed using the 40 LMW selected dataset of mushroom compounds as ligands, and the 4 selected Bcl-2 structures as targets. AD4 docking runs were performed using exactly the same AD4 docking parameters as with the 4 inhibitors on the re-docking and cross-docking studies. The LMW compounds were ranked according to estimated ΔG and Ki values and the results are present in table 5.

The compounds with best docking scores and affinity were: Ganodermondiol (6j), Cerevisterol (6f), Ganoderic acid X (6o) and Lucidenic lactone (6e) with estimated Ki values of 645, 667, 787 and 935 nM, respectively. All the top ranked compounds belong to the steroid family of mushroom compounds. In fact, all the steroids screened scored better than the other LMW compound families studied (Table 5). Taking a closer look at the steroid structures (Figure 1), we can observe that steroids present a large hydrophobic skeleton, with differences between steroids occurring in terms of the number and position of small hydrophilic groups, usually: hydroxyl (OH) carbonyl groups (C=O) or carboxyl (COOH) groups. Steroids are thus amphipathic compounds with both hydrophobic and hydrophilic regions. We can thus hypothesize that the hydrophobic steroids skeleton probably interacts with the hydrophobic inside pockets of the Bcl-2 interaction site, and the steroid hydrophilic groups probably interacts with more external hydrophilic residues present on the Bcl-2 structure.

This docking analysis provides strong evidence that the steroid mechanism of inducing apoptosis in tumor cells may be by interacting with Bcl-2 protein, thus preventing Bcl-2 from forming complexes with the respective proapoptotic protein interaction partners, namely Bak, Bax, and Bim. The free pro-apoptotic effector proteins will then be able to promote MOM permeabilization and consequence tumoral apoptosis. It is important to note that, as apoptosis is a complex cellular event, Bcl-2 is most probably just one of the LMW mushroom compounds protein targets for apoptosis promotion. In this study we highlight steroids as a possible new class of Bcl-2 inhibitions, but steroids, and other classes of compounds present in mushrooms, will probably target other potential apoptosis inducting targets. Bcl-2 inhibition is just one of the events that will act synergistically with inhibition or activation of other protein targets to promote tumor apoptosis.

Table 5. Virtual Screening of the LMW mushroom compound database using AutoDock4.

Compound Familly	Compound	Code	Estimated					Ki (nM)	Rank
			4IEH	4AQ3	4LVT	4LXD	Average		
Quinones and Hydroquinones	Panepoxydone	1a	-4,72	-4,65	-4,74	-5,14	-4,81	296760	39
	Cycloepoxydon	1b	-4,78	-4,35	-4,95	-5,27	-4,84	284499	38
	Clavilactones A	1c	-6,86	-7,27	-7,3	-8,17	-7,40	3765	18
	Clavilactones B	1d	-6,22	-7,27	-7,3	-8,17	-7,24	4932	19
	Clavilactones D	1e	-6,32	-7	-7,08	-7,78	-7,05	6854	22
	490 Quinone	1f	-4,27	-4,29	-3,92	-4,51	-4,25	770113	40
	Hydroquinone	1g	-5,35	-5,09	-5,15	-5,86	-5,36	117288	37
Isoflavones	Genistein	2a	-6,44	-5,6	-5,7	-6,2	-5,99	41016	32
Catechols	Hispidin	3a	-5,86	-5,44	-5,97	-5,67	-5,74	62547	34
	Gerronemins A	3b	-6,38	-5,44	-5,76	-5,73	-5,83	53506	33
	Gerronemins B	3c	-5,78	-6,47	-6,92	-5,88	-6,26	25677	30
	Gerronemins C	3d	-6,65	-6,06	-6,65	-5,12	-6,12	32659	31
	Gerronemins D	3e	-7,29	-6,32	-6,86	-6,24	-6,68	12745	25
	Gerronemins E	3f	-6,86	-7,22	-7,03	-6,07	-6,80	10452	23
	Gerronemins F	3g	-5,42	-6,93	-7,3	-5,95	-6,40	20359	29
Amines and amides	(Questiomycin A	4a	-5,57	-4,98	-5,62	-6,68	-5,71	64968	35
	Putrescine-1,4-dicinnamide	4b	-6,56	-6,55	-7,11	-6,68	-6,73	11763	24
Sesquiterpenes	Illudin S	5a	-5,73	-5,66	-5,32	-6,07	-5,70	66916	36
	Illudin M	5b	-6,53	-5,77	-6,96	-6,87	-6,53	16279	28
Steroids	EMCD	6a	-8,56	-7,5	-8,6	-7,15	-7,95	1482	9
	Ergosterol	6b	-7,88	-8,06	-7,93	-7,49	-7,84	1792	11
	Ergosta-4,6,8(14),22-tetraen-3-	6c	-8,96	-8,07	-8,01	-7,55	-8,15	1066	8
	Lucidenic acid O	6d	-8,13	-7,6	-7,73	-7,63	-7,77	2008	13
	Lucidenic lactone	*6e*	*-8,65*	*-8,87*	*-7,76*	*-7,62*	*-8,23*	*935*	*4*
	Cerevisterol	*6f*	*-8,9*	*-8,25*	*-8,73*	*-7,82*	*-8,43*	*667*	*2*
	Lucidumol A	6g	-8,12	-7,85	-8,09	-7,39	-7,86	1725	10
	Lucidumol B	*6h*	*-8,41*	*-7,92*	*-8,6*	*-7,92*	*-8,21*	*955*	*5*
	Ganoderiol F	6i	-8,46	-7,8	-8,64	-7,8	-8,18	1018	6
	Ganodermanondiol	*6j*	*-8,64*	*-8,26*	*-8,69*	*-8,19*	*-8,45*	*645*	*1*
	Ganodermanontriol	6k	-9,15	-6,05	-6,18	-4,86	-6,56	15541	27
	Ganoderic acid A	6l	-7,06	-7,15	-7,25	-6,88	-7,09	6407	21
	Ganoderic acid F	6m	-6,99	-7,44	-8,56	-6,84	-7,46	3417	17
	Ganoderic acid W	6n	-7,26	-8,46	-8,76	-7.51	-8,16	1044	7
	Ganoderic acid X	*6o*	*-8,2*	*-8,5*	*-8,45*	*-8,16*	*-8,33*	*787*	*3*
	Ganoderic acid Y	6p	-7,59	-7,16	-8,5	-7.08	-7,75	2085	16
	Ganoderic acid T	6q	-8,72	-8,38	-8,16	-5,76	-7,76	2068	15
	Polyporenic acid C	6r	-7,74	-7,11	-8,45	-7.38	-7,77	2028	14
	Dehydroebriconic acid	6s	-7,98	-7,63	-8,25	-7,42	-7,82	1853	12
	Fomitellic acid A	6t	-6,65	-6,4	-6,6	-6,99	-6,66	13127	26
	Fomitellic acid B	6u	-7,44	-6,81	-7,88	-6,63	-7,19	5366	20

Although this varies between protein drug targets, in the drug discovery process, the potency of the drug candidates are usually expected to present Ki values in the nanoMolar range, with 100 nM begin a threshold value. Steroids that showed more potent estimated Bcl-2 inhibition, namely Ganodermondiol (6j), Cerevisterol (6f), Ganoderic acid X (6o), Lucidenic lactone (6e), presented estimated Ki in the hundreds of nanoMolar range (between 645 and 935 nM). This fact points to a probable situation where not one but several steroids maybe acting synergistically to inhibit Bcl-2 protein target. It´s important to highlight that the presented study is performed using only computational tools, and that the proposed Bcl-2 inhibition, mediated by steroids, will need future experimental verification. Still several studies points to the fact that steroids, and specifically steroids found in mushrooms, are in fact a family of compound involved in tumoral apoptosis. For example research and clinical studies support the beneficial effect of *Ganoderma lucidum* mushroom species for reducing and preventing cancer risk. *Ganoderma lucidum* yields a series of triterpenoid and steroid compounds with significant cytotoxicity and anti-inflammatory bioactivity including ganodermanondiol and Ganoderic acid X (Than *et al.*, 2013). *Ganoderma lucidum* mushroom with anti-tumor activity has been proved by several experimental studies in cancer therapy (Yue *et al.,* 2009). The steroid apoptosis induction is also supported by several reports stating that steroids induce apoptosis in several tumor cells (Choi *et al.,* 2011; Harhaji *et al.,* 2009).

The top ranked steroids estimated to be the more potent Bcl-2 inhibitors: (Ganodermanondiol, Cerevisterol, Ganoderic acid X, Lucidenic lactone) are all steroids from the triterpenoid group. Triterpenoid is a chemical structure based on lanosterol, an important intermediate with biological and pharmacological effects such as inducing antitumor activity, via induction of apoptosis, and increasing levels of Bax protein. (Luis, *et al*. 2012). These compounds have showed cytotoxic effect on Meth-A (sarcoma) and on other tumor cells. (Sliva, 2004).

Also, previous studies have shown that Ganodermanondiol inhibits the growth of cancer cells, specifically it showed cytotoxicity against HeLa and Meth-A tumoral cell lines (Río *et al.,* 2012).

Cerevisterol is a polyoxygenated ergostanoid that showed cytotoxicity against different types of human cancer cells. A known Cerevisterol protein target is polymerase α (Ferreira *et al.,* 2010). In this study, we hypothesized that Cerevisterol may also exert his anti tumoral activity by targeting Bcl-2.

Ganoderic acids from *Ganoderma Lucidum* are characterized by their hydroxylation in the triterpene lactone structure which could be promising as natural agents for further study of invasive breast cancers (Roupas *et al.,* 2012). These compounds showed cytotoxicity against

hepatoma cells *in vitro* (Sliva, 2004). Ganoderic acid X has the ability to inhibit topoisomerases, to produce apoptosis through degradation of chromosomal DNA, reduction of the antiapoptotic protein Bcl-xL, and disruption of the mitochondrial membrane. (Ríos *et al.*, 2012). Again, our virtual screening showed that Ganoderic acid X may also produce his anti tumoral activity by inhibiting Bcl-2. Although the other Ganoderic acids screened presented lower estimated Bcl-2 inhibition activity, they may also act synergistically with Ganoderic acid X to promote even more potent Bcl-2 inhibition.

Lucidenic lactone prevented not only the activity of calf DNA polymerase α but also those of human immunodeficiency virus type 1 reverse transcriptase (Ferreira *et al.,* 2010). In this study we add Bcl-2 as a probable new target for Lucidenic lactone.

Moreover, previous studies on our research group demonstrated that 48 h treatment of MCF-7 cells (breast carcinoma) with *Suillus collinitus* methanolic extract caused a decrease in Bcl-2, highlighting the antitumor potential of this mushroom species (Vaz *et al.,* 2012).

Despite the promising results in this virtual screening study and considering the mushroom LMW compounds anti-tumor bioactivity evidences presented above, the activity of the top ranked compounds against Bcl-2 must still be experimentally proven. There is still a reasonable degree of uncertainty in docking scoring predictions, and we must have presented that other steroids which were ranked lower in relation to the top four should also be considered as potential Bcl-2 inhibitor.

3.3. Structural analysis of the top ranked steroids as Bcl-2 inhibitors

In order to better understand the possible mechanism of action of the top ranked LMW compounds mushrooms as Bcl-2 inhibitors, a detailed analysis of the estimated docking conformation (pose) was performed. Analysis and visualization of the docking pose of the 4 top ranked compounds into the active site of Bcl-2 was performed using Pymol software. This analysis focused on the predicted interaction bonds and the determination of the key amino acid residues that may interact with the LMW compounds. The docking pose of the compounds with Bcl-2 amino acid residues are presented in the Figure 8, with the residues involved in the Hydrogen bonds (H-bond) and van der Waals interactions presented in Table 6.

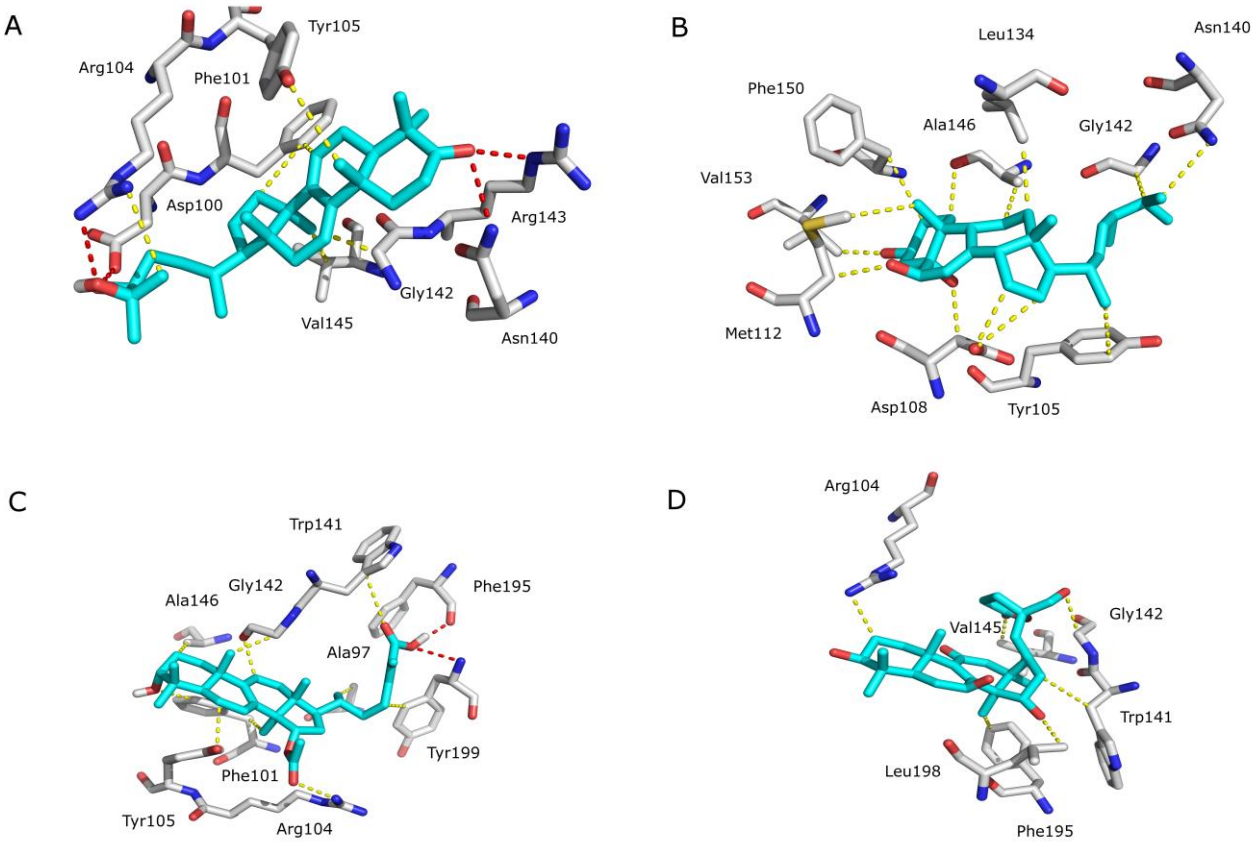

Figure 8. Docked conformations of the top ranked LMW compounds against Bcl-2 structure. Compounds are presented as sticks models (cyan color). A: Ganodermanondiol (6j); B: Cerevisterol (6f); C: Ganoderic acid X (6o) and D: Lucidenic Lactone (6e). The Bcl-2 structure used was PDB: 4LVT, and key amino acid residues are labeled and presented as sticks models (white color). The H-bonds are colored red and the van der Waals interactions are shown in yellow color. Analysis and image preparation was performed using Pymol software.

The first pose to be structurally analyzed was the top ranked Ganodermanondiol (Figure 8A). This steroid presents the characteristic 4 ring steroid hydrophobic skeleton punctuated by 2 hydroxyl groups and 1 carbonyl group. The steroid skeleton was predicted to be located more to the inside of the Bcl-2 interaction site, forming 6 Van der Waals interactions between methyl (CH_3) or methylene (CH_2) groups and key amino acid residues (Figure 8A and Table 6). The docking conformation is also predicted to be stabilized by 4 H-bonds between the hydroxyl groups (OH) or carbonyl group (C=O) and several key amino acid residues. The amino acid residues that form H-bonds are located on more exposed position of Bcl-2 structure, as expected due to the hydrophilic nature of most of them. Overall, this analysis shows a structurally credible scenario, where the hydrophobic regions of Ganodermanondiol are located more to the hydrophobic interior of Bcl-2 interaction site, and the hydrophilic hydroxyl or carbonyl groups are located more in solvent exposed regions. All the individual

predicted interactions contribute to the low estimated binding energy for Ganodermanondiol and, as a general rule, the more the number of predicted interactions, the lower the estimated binding energy.

Table 6. Predicted interactions of the top ranked LMW compounds as Bcl-2 inhibitors.

Compound codes	Estimated ΔG average (Kcal/mol)*	Molecular interactions**					
		Compound Group	Bcl-2 residue	Distance (A°)	Compound Group	Bcl-2 residue	Distance (A°)
		H-Bonds			Van der Waals interactions		
6j	-8,45	OH	Arg104	3,2	CH_3	Arg104	3,5
		C=O	Arg 143	2,8	CH_2	Phe101	3,2
		C=O	Asn140	3,2	CH_2	Phe101	3,4
		OH	Asp100	2,6	CH_3	Val145	3,5
					CH_3	Gly142	3,0
					CH_3	Tyr105	3,6
		Van der Waals interactions					
6f	-8,43	OH	Val153	2,8	CH_2	Ala146	3,1
		OH	Met112	3,2	CH_2	Phe101	3,2
		OH	Asp108	3,0	CH_3	Phe101	3,6
		CH_2	Phe105	3,6	CH_2	Leu134	3,3
		CH_2	Met112	3,5	CH_2	Ala146	3,1
		CH_3	Asn140	3,3	CH_2	Asp108	3,4
		CH_2	Gly142	3,4	CH_2	Asp108	3,0
		H-Bonds			Van der Waals interactions		
6o	-8,33	COOH	Phe195	2,9	COOH	Trp141	3,5
		COOH	Tyr199	2 .8	OH	Arg101	3.0
					CH_2	Tyr199	3.5
					CH_3	Ala97	2.9
					CH_3	Phe101	3.8
					CH_2	Gly142	2.7
					CH_3	Gly142	3.1
					CH_2	Ala146	3.6
					CH_2	Tyr105	2.8
					COOH	Phe101	2.8
		Van der Waals interactions					
6e	-8,30	OH	Leu198	3,2	CH_2	Arg104	3,1
		C=O	Val145	3,3	CH_2	Trp141	3,7
		OH	Gly142	3,0	CH_3	Phe195	3,8

*Results obtained using AD4; **Results obtained using Pymol

Cerevisterol was the second top ranked LMW compound in our virtual screening study, and its docked conformation is presented in Figure 8B. Surprisingly, Cerevisterol was not predicted to perform any H-bond in the docked pose obtained. Still a total of 14 van der

Waals interactions (Table 6) were predicted, and these interactions, although weaker than H-bonds, probably account for Cerevisterol predicted Bcl-2 inhibition ability.

Ganoderic acid X best docked conformation was able to form 2 H-bonds between the carboxyl group and 2 key amino acid residues, Phe195 and Tyr199 (Figure 8C). The steroid skeleton of Ganoderic acid X was then stabilized by 10 van der Waals interactions.

Finally, Lucidenic Lactone was last of the top ranked LMW compounds present in mushrooms, whose docked conformation was structurally analyzed (Figure 8D). Like Cerevisterol, Lucidenic Lactone was also not predicted to perform any H-bond. The docked conformation was only stabilized by a total of 6 van der Waals interactions. This small number of compound-Bcl-2 structure interaction probably accounts for the slightly lower binding energy obtained for Lucidenic Lactone.

4. CONCLUSION AND FUTURE PERSPECTIVES

In this work, a comprehensible study was performed, in order to virtually screen a LMW database of 40 compounds, known to be present in mushrooms, against Bcl-2, a protein target that has been receiving much attention recently in the discovery of new anticancer drugs.

A lot of attention has been given to the initial process of selecting the best virtual screening methodology. Using a cross-docking and re-docking approach, and testing two molecular docking softwares: AutoDock 4 and AutoDock Vina, a computational methodology was stablished, where the average estimated binding energy (ΔG) and binding constant (Ki), obtained by AutoDock 4, were the selected parameters to score and rank the LMW compounds as potential Bcl-2 inhibitors.

The selected virtual screening methodology was then applied to the 40 LMW compounds dataset. The results point to steroids as the compound class with the best docking scores, presenting the lowest ΔG and Ki values. Steroid compounds with best predicted results against Bcl-2 were: Ganodermanodiol, Cerevisterol, Ganoderic Acid X and Lucidenic Lactone; with estimated ΔG values between -8,45 and -8,23 Kcal/mol, and estimated Ki values between 645 and 935 nM. Also, a detailed docking conformation analysis of the 4 top ranked LMW compounds was performed, and shows a plausible three-dimensional interaction, with a large number of estimated molecular interactions between the top ranked steroids and Bcl-2 interaction site.

This analysis provides evidence that steroids present in mushrooms may in fact be responsible, at least partially, for the antitumor and pro-apoptotic activities that has been discovered in a large number of mushroom species; and that the molecular mechanism of these activities may be through promoting Bcl-2 inhibition. Still, it´s important to stress that the reported studies were all performed using bioinformatic tools, and that further studies are needed to experimentally confirm the conclusions of this work.

For future work several directions can be considered:

- The use of molecular dynamic simulations to confirm the plausibility of the docking conformations obtained.
- More Bcl-2 inhibitors, with known experimental Ki values, could be used in the methodology selection step, in order to turn the docking methodology even more robust and reliable.

- Considering the growing number of publications studying mushroom properties and composition, the 40 LMW compound dataset could be enlarged to encompass more compounds. Also it could be considered to go beyond mushrooms and include compounds present in relevant aromatic and medicinal plants.
- More proteins from the Bcl-2 family are being considered as potential targets in the drug discovery process, namely Bcl-XL and Mcl-1, and could also be studied as potential target of the LMW compounds dataset.

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