Published in final edited form as:

Phytochemistry. 2015 June ; 114: 114–124. doi:10.1016/j.phytochem.2014.10.010.

Accessing biological actions of *Ganoderma* secondary metabolites by *in silico* profiling

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Abstract

The species complex around the medicinal fungus *Ganoderma lucidum* Karst. (Ganodermataceae) is widely known in traditional medicines as well as in modern applications such as functional food or nutraceuticals. A considerable number of publications reflects its abundance and variety in biological actions either provoked by primary metabolites such as polysaccharides or secondary metabolites such as lanostane-type triterpenes. However, due to this remarkable amount of information, a rationalization of the individual *Ganoderma* constituents to biological actions on a molecular level is quite challenging. To overcome this issue, a database was generated containing meta-information, *i.e.* chemical structures and biological actions of hitherto identified *Ganoderma* constituents (279). This was followed by a computational approach subjecting this 3D multi-conformational molecular dataset to *in silico* parallel screening against an in-house collection of validated structure- and ligand-based 3D pharmacophore models. The predictive power of the evaluated *in silico* tools and hints from traditional application fields served as criteria for the model selection. Thus, we focused on representative druggable targets in the field of viral infections (5) and diseases related to the metabolic syndrome (22). The results obtained from this *in silico* approach were compared to bioactivity data available from the literature to distinguish between true and false positives or negatives. 89 and 197 *Ganoderma* compounds were predicted as ligands of at least one of the selected pharmacological targets in the antiviral and the metabolic syndrome screening, respectively. Among them only a minority of individual compounds (around 10%) has ever been investigated on these targets or for the associated biological activity. Accordingly, this study discloses putative ligand target interactions for a plethora of *Ganoderma* constituents in the empirically manifested field of viral diseases and metabolic syndrome which serve as a basis for future applications to access yet undiscovered biological actions of *Ganoderma* secondary metabolites on a molecular level.

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Keywords

Ganoderma lucidum; Ganodermataceae; pharmacophore profiling; virtual screening; triterpenes; antiviral targets; metabolic syndrome

1 Introduction

The species complex of *Ganoderma* is one of the most important sources for medicinal mushrooms (Lindequist et al., 2010). Especially Asian countries, like the People's Republic of China, Japan, and Korea have developed a long-standing and strong tradition in using *Ganoderma* preparations for the prevention and treatment of various diseases (Paterson, 2006). Major medicinal properties related to this macro-fungal source include *e.g.* anticancer, antibiotic, and antiviral activities as well as immune response-stimulating, anti-hypertensive, and blood lipid lowering effects (Barros et al., 2008). In many studies bioactivities have been determined on a phenotypic level (primary bioassays) performed with complex multi-component mixtures, without chemical characterisation or without specifying the molecular target (secondary bioassays). This might be due to time-consuming isolation and identification processes, as well as cost-intensive target-specific in-depth pharmacological tests. However, in regard to analytical quality control measures of commercially relevant *Ganoderma* preparations or in the search for novel drug leads, there is an urgent need to especially assess the biological effects of secondary metabolites in a fast and straightforward way.

In the case of *Ganoderma* constituents the amount of available structural information is substantial; in the category of secondary metabolites, more than 200 different lanostane-type triterpenes have been described (Paterson, 2006). Moreover, in past years many additional bioactive secondary metabolites were described for this fungal material due to considerable progress in techniques for dereplication, isolation, analysis, and pharmacological evaluation. Major *Ganoderma* constituents are triterpenes of the fungal cell membrane, *e.g.* ergosterol and derivatives such as ergosterol peroxide, γ -ergosterol, α -dihydroergosterol, ergosta-4,6,8(14),22-tetraen-3-one, or (3 β ,5 α ,8 α ,22E)-5,8-epidioxy-ergosta-6,9(11),22-trien-3-ol. Furthermore, according to quantification studies, ganoderic acids A, AM1, B, C1, C2, D, DM, F, G, H, K, Me, Mk, S, T, TR, Y, and ganoderenic acids A, B, D, as well as ganoderols A, B, ganoderiol F, ganoderatriol, ganoderol A, Me ganoderate D, ganoderate G, and lucideric acid A, can be considered as major lanostane-type triterpenes (Liu et al., 2012; Yan et al., 2013; Zhao et al., 2006). *Ganoderma* constituents which are not often reported in literature and thus are considered as minor components include *e.g.* lucialdehyde E, ganoderiol C, lucidenic acid J, or ganodermaside A.

In general, these secondary metabolites are isolated only in low amounts and therefore are not commercially available, which represents a bottleneck for in-depth biological characterisation. To overcome these issues and moreover as a way to close the knowledge gap of this large pool of pharmacologically almost untapped pure constituents, the application of *in silico* based methods might be a promising strategy for prioritising bioassays.

The generation of pharmacophore models, which can be used for virtual screening to predict and rationalize a compounds' biological activity, requires structural data of either the protein or of known ligands; at best both is available. In an attempt to increase the propensity of identifying bioactive molecules it is further crucial to properly validate the models, to understand and to critically review the obtained *in silico* results (Scior et al., 2012). This study is focused on a selection of pharmacologically relevant targets for which already sufficient data is available, *i.e.* druggable targets. Here, two distinctly different disease areas, *i.e.* viral infections and pathological conditions related to the metabolic syndrome, have been chosen on the basis of the traditional application of *Ganoderma* products in these fields (Lindequist et al., 2010; Sanodiya et al., 2009).

Metabolic syndrome is an umbrella term that comprises multiple individual pathological conditions, *e.g.* obesity, elevated blood pressure, glucose intolerance, and altered lipid metabolism, with the primary outcome of increasing the risk for cardiovascular disease (Grundy et al., 2004). Accordingly, the pathogenesis of the metabolic syndrome is very complex and involves many pharmacological targets of which we selected 22 well-characterised ones for this study.

For the investigation of *Ganoderma* antiviral activities we here focus on druggable targets of the human rhinovirus (HRV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and the influenza virus.

Especially in natural product research, with an abundance of possible structural scaffolds, which can interact with a huge number of pharmacological targets, a rationalised strategy for the identification of bioactivity and the discovery of leads is essential. *In silico* prediction tools such as ChemGPS and PASS or the concept of protein fold topology have proven to be especially effective in this respect (Goel et al., 2011; Larsson et al., 2005; McArdle and Quinn, 2007). Moreover as reported before, the technique of pharmacophore-based parallel screening has been successfully applied to natural compound databases in our research group (Rollinger, 2009; Rollinger et al., 2009). Here, each pharmacological target of interest is represented by one or more validated pharmacophore models (structure- or ligand-based). Each model is composed of steric and electronic features which are necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger or block its biological response (Wermuth et al., 1998). Hence, when screening a compound structural database against a well-defined set of pharmacophore models, pharmacological profiles of these molecules can be predicted.

In sum, the concept of *in silico* parallel screening allows for combining two spaces, both the biological space (*i.e.* the pharmacological target) and the chemical space (*i.e.* the compound structure), and thus might give valuable hints for the straightforward access of biological activities of *Ganoderma* compounds. Moreover, with this strategy we aim to systematically access the longstanding tradition of applying this fungal remedy for viral infections and disorders related to metabolic syndrome on a molecular level.

2 Results and discussion

2.1 Ganoderma database

As a starting point for this study, an extensive literature search was performed to collect structural as well as available bioactivity information of *Ganoderma* secondary metabolites.

The genus *Ganoderma* contains several species which are not easily distinguishable; thus, it is often referred to as species complex (Szedlay, 2002). In most cases, reliable species delimitation is only possible by interpretation of morphological-ecological characteristics in combination with molecular phylogenetic data. In literature, a clear identification of species is often neglected. However, traditionally used *Ganoderma* species (*e.g.* *G. lucidum*, *G. applanatum*, *G. tsugae*) contain a significantly similar pattern of constituents (*e.g.* lanostane-type triterpenes) and therefore the whole genus *Ganoderma* was considered for evaluation.

This survey led to the generation of a 3D multi-conformational *Ganoderma* molecular structure database (*i.e.* *Ganoderma* DB) containing a total number of 279 constituents (see Table S1 in Supporting Information; the sd-file of the database is available as supplementary material). For about 45% of *Ganoderma* compounds only structural information was collected since no data on biological activity was reported exclusively for individual constituents. Concerning structure classes, the *Ganoderma* DB is mainly composed of lanostane-type triterpenes (97%). This chemical class can be subdivided in 188 triterpene acids and esters, 66 triterpene alcohols and ketones, 3 lanostane peroxides, 2 glycosidic lanostanes, and 7 miscellaneous terpenoids (see Figure 1 for examples). Among other scaffolds, 9 long-chain saturated and unsaturated fatty acids and derivatives thereof, one benzofuran and two hydroquinone derivatives are reported as constituents from *Ganoderma* sp. (Table S1).

2.2 Pharmacophore model collection and in silico profiling

In silico profiling of the *Ganoderma* DB was performed using a set of previously generated 3D chemical feature-based pharmacophore models. In general, a pharmacophore model represents the binding mode of a certain compound to a specific drug target. A model represents chemical features such as H-bond donors or acceptors, hydrophobic groups, and positive or negative ionizable moieties which encode chemical functionalities of a ligand. However, ligands for the same target may adopt different binding modes; thus, multiple models might be required to cover all of these binding modes (Schuster, 2010). A major selection criterion for choosing a pharmacological target for the generation of a robust pharmacophore model is the availability of sufficient data.

Accordingly, we focused on targets in the field of viral infections and the area of diseases related to the metabolic syndrome. This has led to the selection of an array of well-defined in-house structure- and ligand-based pharmacophore models from the Inte:Ligand PharmacophoreDB (<http://www.inteligand.com/pharmdb/>) (Markt et al., 2007a; Steindl et al., 2006a; Steindl et al., 2006b). For most targets, more than one model was included in this study to putatively embrace different binding modes. Tables 1 and 2 give an overview on selected targets, the corresponding number of used pharmacophore models, information on

their experimental validation, and references to the original articles where the models have first been described.

In total, 529 models were selected for parallel screening of the *Ganoderma* DB. All models were theoretically validated and proved to find active compounds from the literature. Moreover, 44% of all models were experimentally validated, which means that they successfully identified novel active compounds in prospective screening campaigns. A brief description of the pharmacological targets selected for the virtual profiling is given in chapters 2.3 and 2.4 for targets related to viral infections and metabolic syndrome, respectively.

2.3 Selected targets related to viral infections

The common cold is one of the most abundant viral infections worldwide. Although it usually exhibits a mild disease progress, it has a large socioeconomic impact. The main viral agents causing the common cold are HRVs, which are positively stranded RNA-viruses. These viruses lack an envelope, but are protected by a protein coat that is also involved in functions like host cell attachment and uncoating of the RNA. This coat contains a hydrophobic pocket, to which small drug-like molecules, *e.g.* pleconaril, can bind. This interaction disrupts proper functioning of the protein coat, and pleconaril was already tested in up to phase III clinical trials for the treatment of rhinovirus-caused common cold (Ledford et al., 2004). HCV infection affects approximately 170-200 million people worldwide, potentially leading to liver cirrhosis and cancer in this population. A combination of pegylated interferon alpha and ribavirin was the standard of care during the last decade, and may now be expanded with newly approved NS3/4A inhibitors, however, many adverse effects occur. The HCV NS5B RNA polymerase is crucial for viral replication and therefore accepted as validated target for anti-HCV therapy. Several different allosteric sites have been identified in NS5B (Alexopoulou and Papatheodoridis, 2012) of which three are targeted within this study. The human influenza virus is responsible for the annual epidemic seasonal influenza outbreaks. Besides vaccination, two antiviral compound classes are available for the treatment of influenza at the moment. However, the effects of adamantines, matrix-protein inhibitors, are limited due to high resistance rates. The second drug class inhibits the enzyme neuraminidase, which mediates the release of virions through cleavage of sialic acid (Kamali and Holodniy, 2013). Several therapeutic strategies are pursued for the treatment of AIDS/HIV infection, with the HIV protease and the reverse transcriptase (RT) among the most important targets. The RT promotes the conversion of viral RNA to DNA, which can then be integrated into the host genome. Expression of the target genes produces polyproteins, which have to be further processed by the HIV protease in order to get fully functional viral proteins (Arts and Hazuda, 2012).

2.4 Selected targets related to the metabolic syndrome

One of the most common targets for the treatment of dyslipidemia is the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, which is the rate-limiting step in endogenous cholesterol synthesis. The inhibition of HMG-CoA reductase with statins improves the cholesterol-lipoprotein profile, *e.g.* decreases low density lipoprotein (LDL) cholesterol and serum

triglyceride, and increases high density lipoprotein (HDL) cholesterol levels. The composition of the various lipoproteins is also modified by the cholesteryl ester transfer protein (CETP), which mediates the exchange of cholesteryl esters against triglycerides between HDL and very low density lipoprotein (VLDL) and LDL. Inhibition of CETP is considered as promising strategy to increase HDL levels, and several efficacious compounds are currently investigated (Chapman et al., 2010). Microsomal triglyceride transfer protein (MTP) initiates the loading of apolipoprotein B (apoB) with lipids, and is therefore responsible for the formation of apoB containing lipoproteins. Patients with MTP deficiency have decreased levels of VLDL and chylomicrons, and the treatment with the recently approved MTP inhibitor lomitapide was shown to decrease LDL-cholesterol and total cholesterol levels in patients suffering from familial hypercholesterolemia (Goldberg, 2013). Hormone- and neurotransmitter triggered lipolysis, in contrast, is mainly mediated via the hormone sensitive lipase (HSL). Activation of β -adrenergic receptors increase cAMP levels and stimulate protein kinase A (PKA), resulting in the activation of HSL and hydrolysis of triglycerides. Further, HSL can also be activated by the extracellular signal regulated kinase (ERK) pathway. HSL is a major factor responsible for the mobilization of fatty acids and modulating its function may exert beneficial effects in metabolic disorders (Scott et al., 2014).

Adipose tissue produces the hormone leptine, encoded by the obese gene, which regulates the body weight. It activates the janus kinase (JAK)2 receptor, leading to activation and nuclear shuttling of the transcription factor signal transducer and activator of transcription 3 (STAT3). Gene transcription is further increased by the activity of p38 (also known as MAPK14). Leptine signaling is disrupted by protein tyrosine phosphatase 1 (PTP1B), which dephosphorylates and thereby inactivates its targets, in this case JAK2 (La Cava and Matarese, 2004). However, also the insulin receptor and several growth factor receptors are negatively regulated via this pathway (Johnson et al., 2002), thereby linking PTP1B also to insulin resistance and DM.

DM is associated with multiple complications like atherothrombotic cardiovascular disease, nephropathy, or neuropathy, often caused by increased levels of reactive oxygen species (ROS). One source of ROS is the aldose reductase (AR) dependent polyol-pathway, which mediates the conversion of glucose to fructose. Inhibition of AR is considered as valuable strategy to reduce the intracellular oxidative stress burden and thereby also the occurrence of diabetic complications (Tang et al., 2012). In addition, the inhibition of AR activity was shown to also prevent nuclear factor kappa B (NF- κ B) activation in rat lens epithelial cells (Nambu et al., 2008), which is a major signal transducer in inflammation signaling. Upon activation of the inflammatory pathway the inhibitor of kappa B (I κ B) gets phosphorylated by the inhibitor of kappa B kinase (IKK), which leads to the release of NF- κ B and induction of NF- κ B regulated target genes. Recent evidence suggests also a major impact of NF- κ B target genes in energy metabolism, insulin resistance, and other pathological disorders of the metabolic syndrome (Tornatore et al., 2012).

The endocannabinoid system regulates energy balance via multiple pathways dependent on the organ and tissue. Activation of the cannabinoid receptor 1 (CB1) for instance stimulates de novo lipogenesis in the liver, induces hypothalamus-mediated food intake, and leads to

cell growth and differentiation in adipocytes. However, the first CB1 antagonist, rimonabant, approved for weight reduction, was withdrawn from the market because of its severe psychiatric side effects.

The phenotype of Cushing's syndrome has provided important insights in glucocorticoid function. This disease is characterized by excessive levels of cortisol, caused by adenomas in the pituitary gland, and linked elevated cortisol levels to pathological conditions like obesity, altered lipid metabolism, type 2 DM, insulin resistance, and atherosclerosis. Glucocorticoids exert their effects via binding and subsequent activation of the glucocorticoid receptor (GR), which regulates the expression of multiple target genes involved in the pathology of the metabolic syndrome. Cortisol, one of the most important glucocorticoids, is converted to inactive cortisone by the enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD) 2, however, it can be recycled by 11 β -HSD1. This enzyme was identified as important target in the treatment of the metabolic syndrome (Odermatt and Kratschmar, 2012), and by now several selective 11 β -HSD1 inhibitors are investigated in clinical trials. 11 β -HSD2 plays a crucial role in preventing cortisol binding to the mineralocorticoid receptor (MR). The MR has a similar binding affinity for aldosterone and cortisol, and cortisol inactivation by 11 β -HSD2 allows for almost exclusive binding of aldosterone (Gathercole et al., 2013). In tissue with low 11 β -HSD2 abundance, however, glucocorticoids represent the major ligands. There is increasing evidence for the involvement of the MR in, for example, insulin dysregulation, adipocyte differentiation, and endothelial remodeling. It may therefore also contribute to the pathogenesis of the metabolic syndrome. Similar to the GR, the MR is a nuclear receptor responsible for the regulation of target gene expression (Marzolla et al., 2012). Beside these two, several other nuclear receptors are considered druggable targets for the treatment of the metabolic syndrome, among them are the peroxisome proliferator-activated receptors (PPARs) α , β/δ , and γ , the liver x receptors (LXR) α and β , the farnesoid X receptor (FXR), retinoid X receptor (RXR) (D'Amore et al., 2013), and the thyroid hormone receptor (TR) β (Bryzgalova et al., 2008).

2.5 Evaluation of hit lists

As a result of the parallel *in silico* screening approach, hit lists of 89 and 197 *Ganoderma* constituents were obtained in the antiviral and the metabolic syndrome screening experiments, respectively (see Tables S2 and S3 in Supporting Information). Most of the virtual hits (VHs) belong to the structure class of lanostane-type triterpenes. Interestingly, 20% of compounds have been predicted to be active on more than one target in the antiviral screening (total number of predictions: 131). This multi-target phenomenon has also been observed for 23% of compounds in the metabolic screening (total number of predictions: 546). In this context, it has not been taken into account whether there was more than one hit model for the same target.

From all *Ganoderma* VHs resulting from the antiviral screening, the major part was predicted as ligands for targets of HIV (69) and hepatitis C virus (28). Concerning the metabolic syndrome screening, the highest number of compounds was predicted to interact with the nuclear receptors FXR (62) and LXR- β (54) as well as receptors involved in the endocannabinoid system (122). Interestingly, no hits were predicted for the hormone-

sensitive lipase (HSL), the inhibitor of κ B kinase2 (IKK2), the microsomal triglyceride transfer protein (MTP), and the peroxisome proliferator-activated receptor alpha (PPAR α). The combination of this structural information with meta-information obtained from the public domain allowed for a first evaluation of hits (Figure 2 and Figure 3).

***Ganoderma* constituents predicted as ligands of antiviral targets**

Among the antiviral targets, influenza neuraminidase (NA) has been identified as hit target for several compounds from *Ganoderma* sp. (see Table S2). Out of 279 molecules in the database, 13 lanostane-type triterpenes were predicted to interact with the active site of the influenza NA. The predicted structures share an unsaturated lanostane scaffold with a carboxyl group in position 24 (lucidenic acids) or 26 (ganoderic acids). Furthermore, the VHS are characterised by a double bond at position 8 accompanied by a hydroxyl or oxo group in position 7 and a hydrogen or oxo group at position 11.

In literature, very few studies deal with *Ganoderma* extracts or pure compounds with respect to an interaction on influenza NA. So far, only results obtained by phenotypic assays have been published. For instance, three lanostane-type triterpenes isolated from *G. pfeifferi*, i.e. ganoderadiol, lucidadiol, and applanoxidic acid G, showed antiviral activity against influenza virus type A in a dye uptake assay (Mothana et al., 2003). In MDCK cells the ED₅₀ against influenza virus is 0.22 mmol/L or higher for ganoderadiol and lucidadiol. The corresponding ED₅₀ for applanoxidic acid G is 0.19 mmol/L (Mothana et al., 2003). A *G. lucidum* water extract and polar fractions thereof were inactive in a cell-based cytopathic effect (CPE) inhibition assay against an influenza A virus strain (A/Equine/Miami/1/63) (Eo et al., 1999). In MDCK cells, ganoderone C, lucialdehyde B, ergosta-7,22-dien-3 β -ol revealed IC₅₀s of 2.6, 3.0, and 0.78 μ g/mL, respectively, against influenza A (Niedermeyer et al., 2005).

Whether these constituents of *Ganoderma* identified to exhibit a general anti-influenza activity target the NA or another influenza target remains to be elucidated by target-specific assays. Based on our pharmacophore-based virtual screening, the above mentioned constituents, by our hypothesis, have not been predicted to interact. We used a highly restrictive *in silico* workflow in this study in order to limit the resulting hits to only such compounds that have a pronounced likelihood to interact with the investigated targets (cherry-picking approach). Therefore, we only applied restrictive models that did not retrieve a high number of false positive hits in the theoretical validation. This approach, however, as a matter of fact also represents a limitation to the hit list, which might explain why none of the already known active compounds mapped to the influenza NA models. In a next step, we also subjected more general models, which are less restrictive, to the profiling, and indeed the known NA inhibitor applanoxidic acid G was correctly predicted.

In our virtual parallel screening experiments, HCV polymerase has clearly been identified as hit target for several compounds from *Ganoderma* sp. (see Table S2). Besides one benzofuran, one hydroquinone, and two fatty acids, the 28 VHS are lanostane-type triterpenes. The degree of unsaturation varies between one double bond in position 8 or two double bonds in positions 7 and 9. Moreover, there is also no common pattern observed for the side chain; some VHS comprise a carboxyl group in position 26 which can also be

esterified whereas some VHS bear an alcohol or aldehyde function instead of the carboxyl group.

Interestingly, there is no experimental work reported in literature where these compounds have been evaluated on HCV polymerase. However, commercial extracts of *G. tsugae* inhibited HCV replication at 1 mg/mL in Huh-7 human hepatoma cells containing a HCV subgenomic replicon (Huang et al., 2009).

Our predictions propose a number of different lanostane-type triterpenes (Table S2) as putative ligands for HCV polymerase. As an example, Fig. 3 shows mapped interactions of ganoderic acid DM and lucidadiol with one of the HCV polymerase models.

For the HRV coat protein 20 out of 279 *Ganoderma* secondary metabolites have been predicted as VHS (see Table S2). They include a triterpene lactone, four fatty acids, a benzofuran, a hydroquinone derivative, and 14 lanostane-type triterpenes. Interestingly, three of the latter group are esters of long chain fatty acids. Remarkably, according to the literature, *Ganoderma* sp. extracts or constituents thereof have never been evaluated as agents against HRV.

With 40 predicted structures, HIV-1 protease is the predominant target identified for *Ganoderma* constituents in the area of viral infections (see Table S2). For this antiviral target, all of the predicted VHS belong to the class of lanostane-type triterpenes with one double bond at position 7 or 8 or two double bonds at position 7 and 9. In general, a high number of oxo or hydroxyl groups can be observed involved in H-bond interactions of the protease binding site. In literature, anti-HIV-1 protease activity of a number of triterpenes from *G. sinense* or *G. lucidum* was evaluated. Ganoderiol F, 20-hydroxylucidinic acid N, ganoderic acid GS-2, and 20(21)-dehydrolucidinic acid N showed the most promising inhibition of this key enzyme of HIV proliferation with IC₅₀s of 22, 25, 30, and 48 µM, respectively (Sato et al., 2009). Furthermore, by investigation of this panel of triterpenes first structure activity relations could be derived; among 24(25) unsaturated ganoderic acids, derived from *G. sinense*, the 3-oxo derivatives revealed more potent inhibition than the 3-hydroxy derivatives. Concerning lucidinic acids, the 3-hydroxy compounds showed stronger inhibition than the 3-oxo ones. With the *Ganoderma* alcohols, 24(25) unsaturated derivatives showed higher inhibition than the 24-hydroxy compounds. Interestingly, the 23-oxo ganoderic acids derived from *G. lucidum* revealed no activity against HIV-1 protease (Sato et al., 2009).

Furthermore, the farnesyl hydroquinone ganomycin I and another hydroquinone derivative ganomycin B as well as schisanlactone A isolated from *G. colossum* showed significant anti-HIV activity (El Dine et al., 2009). Ganomycin I and B inhibit HIV-1 protease with IC₅₀ values in the range of 7.5 and 1.0 µg/mL, whereas schisanlactone A is a dimerization inhibitor with an IC₅₀ value of 5.0 µg/mL (El Dine et al., 2009). Further *G. colossum* triterpenoids, *i.e.* colossolactone E, colossolactone V, and colossolactone VII, inhibit HIV-1 protease with IC₅₀ values of 8.0, 9.0, and 13.8 µg/mL, respectively (El Dine et al., 2008). Also El Dine and co-workers established first structure activity relationships for their isolated compounds. Concerning triterpenes with seven-membered and six-membered

lactone rings (colossolactones VIII, E, G, and schisanlactone A), the presence of a hydroxyl group at C-23 or C-5 reduced the activity. Colossolactone V with an unsaturation at C-8 showed higher activity than colossolactone VI with an unsaturation at C-7, C-8 and C-9, C-11. A lactone ring attached to ring D does not reduce the activity compared to a compound with a side chain connected to ring D (El Dine et al., 2008).

Further 13 isolated triterpenoids from *G. lucidum* were investigated for their anti-HIV-1 and anti-HIV-1 protease activity (El-Mekkawy et al., 1998). Ganoderiol F and ganodermanontriol were effective against HIV-1 proliferation with inhibitory concentrations of 7.8 µg/mL. Ganoderic acid B, ganoderiol B, ganoderic acid C1, 3β-5α-dihydroxy-6β-methoxy-ergosta-7,22-diene, ganoderic acid α, ganoderic acid H and ganoderiol A were moderately active against HIV-1 protease with IC₅₀ values in the range of 0.17 to 0.23 mM (El-Mekkawy et al., 1998). Furthermore, Min and co-workers investigated the HIV-1 protease inhibitory activities of 10 triterpenes isolated from *G. lucidum* spores (Min et al., 1998). Ganoderic acid β, lucidumol B, ganodermanondiol, ganodermanontriol, and ganolucidic acid A showed moderate inhibition with IC₅₀ values of 20, 50, 90, 70, and 70 µM, respectively. In this study, hydroxyl groups at C-3 or C-24 and C-25 were found to be essential for HIV-1 protease inhibition (Min et al., 1998).

In our *in silico* approach, none of these experimentally investigated compounds were directly predicted to interact with this target, however, HIV-1 protease has clearly been identified as possible hit target for a set of analogues of these constituents not yet assayed. The pharmacophore models for HIV-1 protease which were included in the model database share the same binding mode as the active constituents described here. This is valuable information and stresses that further model refinement steps are required to also cover those compounds with the restrictive models applied within this study. Nevertheless, as described already for influenza NA above, we further investigated the known active compounds also with the general models we originally excluded from this study. Similar to NA, several of the known active compounds were predicted as ligands for HIV-1 protease with these less restrictive models. In detail, 20-hydroxylucidic acid N, 20(21)-dehydroxylucidic acid N, ganomycin B, ganoderic acid B, ganoderiol A, ganoderic acid β, and ganolucidic acid A mapped a HIV-1 protease model, thereby covering seven out of 24 active compounds reported in the literature. Concerning the target HIV-1 RT, 29 *Ganoderma* structures have been predicted by the *in silico* screening (see Table S2). Apart from two hydroquinone derivatives and one fatty acid, all VHs are lanostane-type triterpenes. For this HIV target, not much literature data dealing with *Ganoderma* extracts or pure compounds is available. So far, lucidic acid O and lucidic lactone isolated from *G. lucidum* showed an inhibition with IC₅₀ values of 67 and 69 µM (Mizushima et al., 1999). The considerable number (29) of *Ganoderma* constituents predicted to inhibit HIV-1 RT renders experimental evaluation of these VHs highly relevant.

2.7 *Ganoderma* constituents predicted as ligands of targets involved in metabolic syndrome

From a general point of view, several studies dealing with *Ganoderma* extracts report hypoglycemic, cholesterol-lowering, anti-diabetic, anti-obesity, and hypotensive effects of

G. lucidum water and organic extracts *in vitro* and *in vivo* without specifying the molecular target (Bastami et al., 2007; Berger et al., 2004; Lee and Rhee, 1990; Mohammed et al., 2009; Seto et al., 2009; Thyagarajan-Sahu et al., 2011).

Concerning selected targets involved in diseases related to the metabolic syndrome, only a small number has been experimentally evaluated in relation to *Ganoderma* multi-component mixtures or pure compounds, *i.e.* AR, FXR, HMG-CoA R, PPAR, and PTP1B.

In our *in silico* screening campaign, 7 *Ganoderma* lanostane-type triterpenes have been predicted to interact with aldose reductase (AR) (see Table S3). First literature data confirm this hypothesis; on the level of multi-component mixtures, a methanol extract of *G. applanatum* revealed potent rat lens AR inhibition *in vitro* (Jung et al., 2005). Also an ethanol extract of *G. lucidum* has shown significant *in vitro* inhibition of AR with an IC₅₀ of 66.4 µg/mL (Fatmawati et al., 2009).

Furthermore, Fatmawati and co-authors investigated 17 triterpene acids and esters from *G. lucidum* for their AR inhibiting potential (Fatmawati et al., 2010a, b; Fatmawati et al., 2011). As a result, ganoderic acid Df and ganoderic acid C2 showed IC₅₀ values of 22.8 and 43.8 µM, respectively. The findings of this research group suggest that a hydroxyl group at C-11 and the carboxylic group in the side chain are essential for an AR inhibitory activity. An improvement of activity can be observed for compounds with a double bond moiety at C-20 and C-22 in the side chain (Fatmawati et al., 2011). Furthermore, Lee and co-workers investigated eight constituents isolated from *G. applanatum* for their AR activity on rat lens (Lee et al., 2006) with ergosterol peroxide being the most active compound (IC₅₀ of 15.4 µg/mL) (Lee et al., 2006). However, none of these known active compounds mapped the models applied here. Our results suggest triterpenes with 2 hydrophobic and either three or five hydrogen bond acceptor features to act as potential ligands of AR. This is in accordance with experimentally verified constituents mentioned above.

Among the selected targets related to the metabolic syndrome, the nuclear receptor FXR has been identified as hit target for 62 compounds from *Ganoderma* sp. (see Table S3).

The predicted structures comprise a fatty acid, a benzofuran, a hydroquinone derivative, and 59 triterpenes sharing a lanostane scaffold.

In a previous pharmacophore-based study, triterpenes predicted to interact with FXR led to the selection of *G. lucidum* for mycochemical and pharmacological investigation. This prompted us to investigate a *Ganoderma* extract and its isolated compounds for their effects on FXR. As a result, ergosterol peroxide, lucidumol A, ganoderic acid TR, ganodermanontriol, and ganoderiol F, were successfully identified as modulators of this nuclear receptor (Grienke et al., 2011). Finally, an investigation of the putative binding mode by molecular docking revealed crucial interactions between the lanostane-type triterpenoids and a water-mediated H-bond to Arg331 of the receptor's active site. Figure 5 shows ganoderiol F mapped with one of the FXR pharmacophore models.

Furthermore, HMG-CoA-R has been identified as hit target for 14 lanostane-type triterpene acids including lucidenic and ganoderic acids (see Table S3).

According to the literature, 7-oxo-ganoderic acid Z and ganoderic acid TR, isolated from *G. lucidum*, were experimentally evaluated and showed HMG-CoA-R inhibition with IC₅₀ values of 22.3 μ M and 21.7 μ M, respectively (Li et al., 2006). Whether the predicted VHs (see Fig. 5 for an example) exert an inhibition on this target still needs to be evaluated experimentally.

Concerning subtypes of PPARs, only PPAR- δ and PPAR- γ have been identified as potential hits for *Ganoderma* constituents, whereas no VHs were predicted for PPAR- α . In sum, 12 VHs have been predicted for PPAR- δ and 45 for PPAR- γ (see Table S3) comprising four fatty acids, a benzofuran, a hydroquinone derivative, and several lanostanes.

Concerning the experimental evaluation on PPARs Shimojo and colleagues report that an extract of *G. lucidum* has indeed PPAR- α and PPAR- γ activating properties (Shimojo et al., 2011). Furthermore, lipid fraction of *G. lucidum* spores was investigated for the induction of these receptors. However, this fraction mainly containing unsaturated fatty acids induced the activity of PPAR- α but not PPAR- γ or PPAR- δ (Huang et al., 2011). According to our *in silico* results, there is still an enormous potential to discover in particular PPAR- γ modulators from *Ganoderma* sp. primarily from the rich pool of lanostane triterpenes which can form extensive interactions with the hydrophobic branches of the Y-shaped binding pocket of PPAR- γ and in addition, can be anchored with at least one hydrogen bond acceptor in the third branch. Moreover, PTP1B has been identified as hitting target for *Ganoderma* constituents. In total, 40 lanostane-type triterpenes (mainly triterpene acids) have been predicted to interact with this target (Table S3).

Concerning literature data, the macromolecular acidic proteoglycan FYGL-a was reported as an inhibitor of PTP1B from *G. lucidum* (Pan et al., 2014; Teng et al., 2011; Teng et al., 2012; Wang et al., 2012). However, no secondary metabolites from *Ganoderma* sp. have been reported as PTP1B inhibitors so far.

Remarkably, for all other selected and hit targets in the field of metabolic syndrome, primarily LXR- β , receptors of the endocannabinoid system, MAPK14, 11 β HSD-1, GR, and MR, experimental data confirming or disproving our predictions is still missing which highlights the high potential of pharmacological studies in these areas.

2.8 Experimental access and challenges involved

A classical *in silico* driven workflow for natural lead discovery usually aims at the identification of bioactive compounds of all types of natural sources including various chemical scaffolds and analogues based on the prediction of VHs for a certain well-characterised pharmacological target (Rollinger and Wolber, 2011). In this study, the focus is narrowed down to metabolites of basically one structural scaffold (lanostane-type triterpenes) within one natural source (*Ganoderma* sp.). Accordingly, by using the described parallel *in silico* screening approach, limitations in the experimental evaluation have to be faced.

In this study, remarkably, 97% of all hit predictions in the antiviral as well as the metabolic syndrome screening have not yet been confirmed by literature data. This might be due to

various reasons, *e.g.* availability of pure compounds, lack of interdisciplinary collaborations (natural product research groups often have limited knowledge and facilities to perform pharmacological tests), and limited financial resources. Moreover, when considering the large number of compounds predicted to be bioactive on selected targets, the time needed for experimental evaluation is also an issue that might not be neglected.

However, bioactivity results from already performed *Ganoderma* extract screenings might give an additional hint in order to prioritise target-based assays. Various antiviral activities (Kim et al., 1997; Lai et al., 2010) and *in vitro* or even *in vivo* anti-hypertension, anti-diabetes, and lipid-lowering effects have already been reported for *Ganoderma* multi-component mixtures (Fatmawati et al., 2009; Kabir et al., 1988; Kagaya et al., 2001; Kanmatsuse et al., 1985; Seto et al., 2009). The herein presented approach of parallel *in silico* screening provides insight into ligand-target interactions. This allows to access and to identify the molecular targets and the putatively involved natural compounds acting as ligands.

However, *in silico* based projects always have to face some challenges as can also be observed in this study. For some targets, many of the already known active compounds were not predicted by the applied models. This can be explained by several reasons: First, for some active molecules, the exact target was not confirmed, *e.g.* the influenza NA, thereby suggesting that the active constituents possess a different mode of action. Second, as already mentioned above, we applied a rather restrictive workflow to only prioritize compounds for biological testing that have a very high probability to be active. This of course may lead to the exclusion of additional active compounds, but helps to avoid high rates of false positive hits. Third, most of the models applied within this study were generated and validated using synthetic compounds. Since plant constituents often represent a different chemical space than those compounds (Feher and Schmidt, 2003), it can sometimes be observed that fewer molecules map the models in comparison to compounds originating from a commercial synthetic library.

Several strategies can be employed to circumvent this limitation, like the usage of less restrictive models or a defined number of omitted features, which means that not all features of the model have to map to count as hit. However, this inevitably increases the number of false positive compounds in the hit list. Considering the rate of false positive and false negative predictions in general, not all hits can be confirmed in the biological evaluation. Nevertheless, the hit lists obtained with *in silico* tools show a much higher enrichment of active compounds compared to random experimental screening (Schuster et al., 2010) and, as shown in many examples, it is possible to correctly predict a hit scaffold or structure class.

3 Concluding remarks

The medicinal fungus *G. lucidum* (incl. related species), commonly called “mushroom of immortality”, produces a tremendous amount of diverse lanostane-type triterpenes. Its application as multi-component mixture or in complex formulas has without doubt a longstanding tradition in many Asian countries. Since the market for *Ganoderma* products

has now expanded also to the Western world, where natural extracts are usually narrowed down to the active principle or to single components, a large scientific interest is focused on the evaluation of bioactive *Ganoderma* constituents. Due to their complexity, both in number and type, classical chromatographic separation is extremely challenging.

By the generation of our 3D multi-conformational structural database we created a virtual *Ganoderma* “avatar” giving clues which molecular mechanisms might be involved in the plethora of biosynthesized lanostane-type triterpenes from this fungus. Even if there are only small differences between the structures, a certain substitution pattern might give valuable hints about ligand target interactions in the sense of SAR. However, such hypotheses can only be made in combination with experimental results.

The presented approach highlights that parallel *in silico* pharmacophore-based profiling provides a helpful tool to unravel which constituents of *Ganoderma* sp. might be responsible for a certain biological activity and which *in vitro* test systems should be prioritised to corroborate these results.

4 Experimental

Compilation of *Ganoderma* database - Preparation of the screening dataset. In the course of an extensive literature survey, 279 different structures of *Ganoderma* constituents were collected. Absolute stereochemistry data was available for most, but not all of the compounds. For all compounds with incomplete stereochemistry data, all possible stereoisomers were created. The 2D structure of every compound was drawn in ChemBioDraw Ultra 11.0 (PerkinElmer, 2008), and saved as cdx-file. The cdx-files were converted into an sd-file with PipelinePilot 8.5 (2011) using the components ChemDraw Reader and SD Writer, and one low-energy 3D input conformation was calculated with OMEGA2.3.2 (3.0.0, 2014; Hawkins and Nicholls, 2012; Hawkins et al., 2010). Each molecule was manually checked to ensure proper structural representation. For each compound, a maximum number of 500 conformations was calculated, respectively, with the BEST algorithm of Discovery Studio 3.5 (2012).

Hardware setup. All processes and predictions were performed on a multi-core workstation with 2.4+ GHz, 8 GB of RAM, and a high-end NVIDIA graphical processing unit. All programs run on the Windows 7 platform.

Pharmacophore models for parallel screening. In order to generate pharmacophore models capable of discriminating active and inactive molecules, sufficient data concerning the protein structure or already known ligands is required (see Tables S4 and S5 for PDB entries used for the generation of pharmacophore models). Especially published bioactivity data for compounds, including inactive ones, is highly valuable for the proper theoretical validation of the created models. In this study, only carefully optimized and restrictive pharmacophore models were included to avoid high numbers of false positive hits. In addition to the theoretical assessment of the model quality, several of the used pharmacophore models have also been validated experimentally.

The pharmacophore model set used for the antiviral activity profiling comprised 320 models for 5 targets. For the metabolic syndrome screening 207 models for 22 related targets were selected. For a detailed list of the selected targets and further information related to the respective models please refer to Tables 1 and 2. The Ligand Profiler tool implemented in Discovery Studio 3.5 was used for the virtual parallel screening, except for the RXR models, which were screened with LigandScout 3.1 (Wolber and Langer, 2005). In principle, all compounds that map to every chemical feature of the respective pharmacophore model were counted as hits. The screening was conducted with default settings or with an interfeature distance of 0.00001 Å.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

U.G. and C.E.M are grateful for their positions funded by the Austrian Science Fund (FWF: P24587). T.K was supported by the foundation “Verein zur Förderung der wissenschaftlichen Ausbildung und Tätigkeit von Südtirolern an der Landesuniversität Innsbruck“. D.S. thanks the University for her position within the Erika Cremer Habilitation Program. We thank OpenEye and Inte:Ligand for providing their software under special academic grant agreements free of charge.

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Figure 1.

Examples for chemical structures of *Ganoderma* triterpenes including an acid, ester, ketone, alcohol, peroxide, and glycosidic constituents.

Figure 2.
Overview on number of hit compounds of the parallel pharmacophore screening against druggable targets in the area of viral diseases.

Figure 3.
Overview on number of hit compounds of the parallel pharmacophore screening against druggable targets in the field of diseases related to the metabolic syndrome.

Figure 4. Ganoderic acid DM (olive) and lucidadiol (grey) map to the HCV polymerase model 2brl-poo-2.40-d-2-s. Hydrophobic features are displayed in blue, hydrogen bond acceptor features in green, and exclusion volumes are represented by grey spheres. The shape of the co-crystallized ligand was added.

Figure 5. Ganoderiol F interacts with Arg331 of FXR *via* a water network (red balls). Hydrogen bond acceptor features are displayed as red arrows, and hydrophobic interactions and exclusion volumes are represented by yellow and grey spheres, respectively.

Figure 6.

Ganoderic acid C2 maps the HMG-CoA-model 1hw8-114-2.10-p-1-s. Hydrophobic features are displayed in light blue, hydrogen bond donor features in pink, negatively ionizable groups in dark blue, and exclusion volumes are represented by grey spheres. The shape of compactin, a well-known HMG-CoA reductase inhibitor, was added.

Table 1
Pharmacophore model collection selected for the antiviral profiling.

Hepatitis C virus (HCV), human immunodeficiency virus (HIV), reverse transcriptase (RT), human rhinovirus (HRV).

target	no. of models	model type	experimentally validated	reference(s)
HCV polymerase	22	structure-based	no	(Steindl et al., 2006a)
HIV-1 protease	154	structure-based	no	(Steindl et al., 2006a)
HIV-RT	43	structure-based	no	(Steindl et al., 2006a)
HRV coat protein	37	structure-based	yes	(Rollinger et al., 2009; Steindl and Langer, 2005; Steindl et al., 2005)
influenza neuraminidase	64	structure-based	yes	(Steindl and Langer, 2004)

Table 2
Pharmacophore model collection selected for the metabolic syndrome profiling.

11 β -hydroxysteroid dehydrogenase 1 and 2 (11 β -HSD1 and 2), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), cholesteryl ester transfer protein (CETP), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), aldosterone reductase (AR), cannabinoid receptor (CB), farnesoid X receptor (FXR), hormone-sensitive lipase (HSL), hydroxymethylglutaryl coenzyme A reductase (HMG-CoA-R), inhibitor of κ B kinase2 (IKK2), microsomal triglyceride transfer protein (MTP), mitogen-activated protein kinase 14 (MAPK14), protein tyrosine phosphatase 1B (PTP1B), thyroid hormone receptor β (TR- β), retinoid X receptor (RXR).

target	no. of models	model type	experimentally validated	reference(s)
11 β -HSD1	4	ligand- and structure-based	yes	(Schuster et al., 2006; Vuorinen et al., 2014)
11 β -HSD2	1	ligand-based	yes	(Schuster et al., 2006; Vuorinen et al., 2014)
PPAR α	5	ligand- and structure-based	yes	(Fakhrudin et al., 2010; Markt et al., 2008; Markt et al., 2007)
PPAR δ	11	structure-based	yes	(Fakhrudin et al., 2010; Markt et al., 2008; Markt et al., 2007)
PPAR γ	13	ligand- and structure-based	yes	(Fakhrudin et al., 2010; Markt et al., 2008; Markt et al., 2007)
LXR- α	6	ligand- and structure-based	yes	(von Grafenstein et al., 2012)
LXR- β	8	structure-based	yes	(von Grafenstein et al., 2012)
CETP	7	ligand-based	yes	(Duwensee et al., 2011)
GR	17	ligand- and structure-based	no	
MR	2	ligand- and structure-based	no	
AR	12	ligand- and structure-based	no	
CB1	5 *	ligand-based	yes	(Markt et al., 2009)
CB2	3	ligand-based	yes	(Markt et al., 2009)
FXR	14	ligand- and structure-based	yes	(Schuster et al., 2011)
HSL	6	ligand-based	no	
HMG-CoA-R	22	structure-based	no	
IKK2	2	ligand- and structure-based	yes	(Noha et al., 2011)
MTP	4	ligand-based	no	
MAPK14	6	structure-based	no	
PTP1B	51	structure-based	yes	unpublished results
TR- β	5	structure-based	no	
RXR	5	structure-based	yes	unpublished results

*one of them is an unselective CB1 and CB2 model