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ORIGINAL PAPER

Partial contribution of Rho-kinase inhibition to the bioactivity of *Ganoderma lingzhi* and its isolated compounds: insights on discovery of natural Rho-kinase inhibitors

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Abstract Recent studies identified Rho-kinase enzymes (ROCK-I and ROCK-II) as important targets that are involved in a variety of diseases. Synthetic Rho-kinase inhibitors have emerged as potential therapeutic agents to treat disorders such as hypertension, stroke, cancer, diabetes, glaucoma, etc. Our study is the first to screen the total ethanol extract of the medicinal mushroom *Ganoderma lingzhi* with thirty-five compounds for Rho-kinase inhibitory activity. Moreover, a molecular binding experiment was designed to investigate the binding affinity of the compounds at the active sites of Rho-kinase enzymes. The structure–activity relationship analysis was investigated. Our results suggest that the traditional uses of *G. lingzhi* might be in part due to the ROCK-I and ROCK-II inhibitory potential of this mushroom. Structure–activity relationship studies revealed some interesting features of the lanostane triterpenes that potentiate their Rho-kinase inhibition. These findings would be helpful for further studies on the design of Rho-kinase inhibitors from natural sources and open the door for contributions from other researchers for optimizing the development of natural Rho-kinase inhibitors.

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Introduction

Approximately one-third of today's best-selling drugs are either natural products or were developed based on lead structures obtained from natural sources. The use of natural products for their therapeutic purposes has a long-standing history and compounds derived from natural products have made a strong impact on the pharmaceutical industry [1]. Among these compounds, enzyme inhibitors play a significant role in the drug discovery process. For example, acetylcholinesterase inhibitors have potent application in treating Alzheimer's disease. Glutathione S-transferase inhibitors are emerging as promising therapeutic agents to overcome resistance amongst anticancer and antiparasitic agents. Moreover, compounds inhibiting the activity of α-glucosidase are used to treat type 2 diabetes mellitus and obesity problems. Of the most essential enzymes in our body, Rho-associated protein kinases (ROCK-I and ROCK-II) regulate a wide range of fundamental cell functions. Evidence has demonstrated that over-expression of ROCK is involved in the pathogenesis of a variety of diseases and that the inhibition of the ROCK pathway might provide a means of therapy for diseases such as hypertension, glaucoma, asthma, erectile dysfunction, diabetes, central nervous system disorders and tumor metastasis [2–6]. Rho-associated protein kinases (ROCK-I and ROCK-II), belonging to the serine/threonine kinases family, regulate a wide range of fundamental cell functions [7]. ROCK activity is mediated by phosphorylating a series of downstream targets, including myosin light chain (MLC), p-Lin-11/Isl-1/Mec-3 kinases, ezrin/radixin/moesin, adducin, calponin, myristoylated alanine-rich C-kinase substrate

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and collapsin response mediator protein-2. ROCK also plays a part in several signaling pathways that are involved in autoimmunity and inflammation. It has been shown to act in the activation of nuclear factor (NF)-κB, a crucial factor that leads to the production of tumor necrosis factor (TNF) and other inflammatory cytokines. ROCK inhibitors effectively prevent TNF-α secretion from macrophages in addition to inhibiting the secretion of interleukin (IL)-1β, IL-6 and IL-17, and limit the inflammatory actions of mediators such as IL-1β on intestinal epithelium by preventing the activation of transcription factors such as NF-κB and activator protein-1 [4]. ROCK was originally discovered in 1996 as the first downstream effector of Rho and found to be responsible for the formation of stress fibers and focal adhesions by phosphorylating MLC [8]. Until now, two ROCK isoforms have been confirmed, namely ROCK-I (also known as Rho-kinase β/p160ROCK/ROKβ) and ROCK-II (also known as Rho-kinase α/ROKα) [9]. The two isoforms share approximately 60% overall amino acid identity and approximately 90% identity within the N-terminal kinase domain. In 1996, Narumiya et al. reported the discovery of ROCK-I and ROCK-II, and confirmed through Northern blot analysis the expression of ROCK-I in many human tissues, e.g., the heart, pancreas, lung, liver, skeletal muscles, and kidney but not the brain. However, in their investigation on mouse tissues, they showed ubiquitous mRNA expression of both ROCK-I and ROCK-II in all tissues, including the brain, with ROCK-I preferentially expressed in the liver, spleen, and kidney and ROCK-II preferentially expressed in the brain and skeletal muscle [10–13].

With regard to the development of ROCK inhibitors, due to the increasing number of inhibitors emerging, the screening of natural sources is an effective approach. In our research, we focus on natural resources for finding ROCK inhibitors. Of these sources, we examined *Ganoderma lingzhi* with thirty-five compounds for Rho-kinase inhibitory activity. Members of *Ganoderma* genus have attracted considerable attention because they produce many biologically active triterpenoids. Among them, *G. lingzhi* has long been used in Traditional Chinese Medicine (TCM) for the promotion of longevity and maintenance of vitality. The lanostane-type triterpenoids are reported to have numerous therapeutic activities including anticancer, antihypertensive, antiviral, antidiabetic, anti-androgenic and immunomodulatory activity [14–17]. Nowadays, *G. lingzhi* is still widely prescribed by TCM doctors for the treatment of debility and weakness, insomnia, hepatitis, cardiovascular diseases, diabetes, cancer, etc. [18]. Accumulative evidence has suggested that the Rho-kinase pathway is widely involved in the pathogenesis of a variety of diseases. At the same time, many different biological activities have been reported for *G. lingzhi* and many researchers have found *G. lingzhi* to be an effective medicinal mushroom for treating many diseases. On that basis, we conducted our study to evaluate and check whether or not the multiple biological activities of *G. lingzhi* are mediated through ROCK inhibition and find the bioactive compounds which are responsible for such activity.

Materials and methods

Fungal material

The dried ethanol extract of the fruiting bodies of *G. lingzhi* was kindly provided by Toyotanshien Co Ltd (3-1 Kitanijyonishi, Chuo-ku, Sapporo 060-0002, Japan).

Tested compounds

Some of the tested compounds were isolated from the fruiting bodies of *G. lingzhi* as previously described [19, 20, unpublished data]. Other compounds were purchased from Chemfaces (Wuhan, Hubei, China) with HPLC purity >98%. The structures of the purchased compounds were reconfirmed through 1D, 2D-NMR and mass analysis.

Chemicals and reagents

Homogeneous time-resolved fluorescence (HTRF) (HTRF® KinEASE™ STK S2 Kit; Cisbio Bioassays, Japan) [containing STK substrate 2-biotin, streptavidin-XL665 (acceptor), STK antibody cryptate (donor)], Rho-kinase enzymes (Carna Biosciences, Japan), ROCK inhibitor Y-27632 (LC Laboratories, USA), and 96-well black plate (Corning, USA) were used. Adenosine triphosphate (ATP), (±)-dithiothreitol (DTT) and magnesium chloride hexahydrate were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Reagent preparation

The contents of the HTRF kit were reconstituted as per the instructions given. In brief, the 5× kinase buffer was diluted to 1× buffer containing 5 mM $MgCl_2$*$6H_2O$ and 1 mM DTT. STK substrate 2 was reconstituted in distilled water to obtain a 500 µM stock solution. Streptavidin (SA)-XL665 was reconstituted in distilled water to obtain a 16.67 µM stock solution. The detection buffer was used directly for the preparation of STK antibody–cryptate. After reconstitution, the reagents were aliquoted and stored at −20 °C until further use. The ROCK enzymes were reconstituted in 100 µL of 1x kinase buffer, aliquoted and stored at −80 °C until use. A stock solution of Y-27632

was prepared in distilled water, aliquoted and stored at −20 °C until use. 5 mM stock solution of ATP was prepared in water and diluted to working stock with 1× kinase buffer. The extracts/compounds were prepared fresh every time in dimethyl sulfoxide (DMSO) and further diluted to a working solution in 1× kinase buffer.

ROCK inhibition assay by the HTRF method

The inhibition assay was prepared as per kit protocol and reported earlier [21, 22]. The assay was performed using an HTRF assay kit that is based on the time-resolved fluorescence resonance energy transfer principle as mentioned in the HTRF® KinEASE™ STK S2 Kit protocol. The HTRF technique is preferred over the ELISA-based technique as it does not involve several washing steps as required in assays based on the ELISA method. It also eliminates the interference of extracts having antioxidant effects; the horseradish peroxidase-based detection system in ELISA can lead to false positive results [21–23]. Y-27632 was used as the reference/standard inhibitor and it is a non-specific inhibitor of both ROCK-I and ROCK-II [24]. The final concentrations for both the compounds and the positive control (Y-27632) are 100 and 1.6 µM, respectively. Briefly, in total enzymatic reaction mixture 50, 20 µL of 4 µM Y-27632 or 10 mM of the tested compound or 100 mg/mL total extract, 10 µL of 3.5 mM substrate, 10 µL (0.5 ng) enzyme and 10 µL of 350 mM ATP were mixed together. In control wells, the inhibitor was replaced by buffer. Wells without enzyme were considered as negative control/blank. The reaction mixture was incubated for 30 min at 37 °C, followed by the addition of 25 µL of 175 nM SA-XL665 and 25 µL of STK antibody–cryptate, and further incubated at 25 °C for 60 min. The HTRF signal was measured using FlexStation 3 Microplate Reader (Molecular Devices, USA). A vehicle control for 2.5% DMSO was tested to check the interference of DMSO in the assay.

Molecular docking

The docking experiment was used to investigate the binding affinity of the isolated compounds to the binding residues of the ROCK enzymes. The crystal structures of Rho-kinase I and II were downloaded from Protein Data Bank (www.rcsb.org) with access code 2ETR for Rho-kinase I [25] or access code 2H9V for Rho-kinase II [26] and imported into the work place of CLC Drug Discovery Workbench 3.0 software. The docking experiment was adjusted as previously described [27]. The three-dimensional structures of ligands were drawn using Chem3D Pro 12.0 software and saved as mol2 formats. Compounds were docked inside a sphere with a 15 Å radius centered at the largest cavity detected by the program.

Statistical analysis

Rho-kinase inhibition assay against the ROCK-I and ROCK-II enzymes was performed twice and mean values are presented ±SD.

Results and discussion

This study evaluated the effects of the ethanol extract of *G. lingzhi* and its isolated compounds on ROCK-I and ROCK-II inhibition assays using the HTRF method. The HTRF® KinEASE™ STK assay format involves two steps—the first is the enzymatic step, in which the kinase phosphorylates the substrate. The STK substrate-biotin is incubated with the kinase. ATP is added to start the enzymatic reaction. The second step is the detection step in which the detection reagents catch the phosphorylated substrate. The resulting signal is proportional to the phosphorylation level. The STK-antibody labeled with Eu^{3+}-cryptate and streptavidin-XL665 are mixed in a single addition with EDTA (used to stop the kinase activity).

The activity of the total ethanol extract of *G. lingzhi* at a concentration of 1 mg/mL was tested against the two enzymes (ROCK-1 and ROCK-II). The inhibition percentages against ROCK-I and ROCK-II were 62.7 ± 3.6 (IC_{50} 933.4 ± 9.8 µg/mL) and 60.2 ± 3.3 (IC_{50} 966.1 ± 8.4 µg/mL), respectively. Thirty-five compounds were tested against the ROCK enzymes to find the compounds which are responsible for the activity of the extract. In that way, we hope to provide the scientific community with a new natural ROCK inhibitor as a lead compound. The results revealed some structurally important characteristics of the lanostane triterpenes with regard to their Rho-kinase inhibition. Among the compounds, ten lanostane triterpenes showed approximately 40–50% inhibition of Rho-kinases enzymes (ROCK-I and ROCK-II) at 100 µM. These compounds deserve to get more attention in further studies as natural Rho-kinase inhibitors. Information on the structure–activity relationship can also provide some valuable hints for designing a Rho-kinase inhibitor with multiple biological activities. A molecular binding experiment was designed to investigate the binding affinity of the compounds at the active sites of Rho-kinase enzymes. The in silico docking experiment of small molecules into the macromolecule targets is a commonly used technique in hit identification and lead optimization. The structural details of the compounds used in the study are presented in Table 1 and Figs. 1 and 2, while their

Table 1 Structural details of the compounds used in the study

Compounds		R_1	R_2	R_3	$R_{3'}$	R_4	R_5	Type[a]
Skeleton T								
1	Ganoderic acid A	=O	β–OH	=O	–H	α–OH	A1	**I**
2	Ganoderic acid B	β–OH	β–OH	=O	–H	=O	A1	**I**
3	Ganoderic acid C1	=O	β–OH	=O	–H	=O	A1	**I**
4	Ganoderic acid C2	β–OH	β–OH	=O	–H	α–OH	A1	**I**
5	Ganoderic acid C6	β–OH	=O	=O	β–OH	=O	A1	**I**
6	Ganoderic acid F	=O	=O	=O	β–$OCOCH_3$	=O	A1	**I**
7	Ganoderic acid H	β–OH	=O	=O	β–$OCOCH_3$	=O	A1	**I**
8	Ganoderic acid K	β–OH	β–OH	=O	β–$OCOCH_3$	=O	A1	**I**
9	Ganoderenic acid C	β–OH	β–OH	=O	–H	α–OH	A2	**I**
10	Ganoderenic acid D	=O	β–OH	=O	–H	=O	A2	**I**
11	12β-acetoxy-7β-hydroxy-3,11,15,23-tetraoxo-5α-lanosta-8,20-dien-26-oic acid	=O	β–OH	=O	β–$OCOCH_3$	=O	A2	**I**
12	Ethyl ganoderenate D	=O	β–OH	=O	–H	=O	A3	**I**
13	12β-acetoxy-7β-hydroxy-3,11,15,23-tetraoxo-5α-lanosta-8,20-dien-26-oic acid ethyl ester	=O	β–OH	=O	β–$OCOCH_3$	=O	A3	**I**
14	Ganoderic acid DM	=O	=O	–H	–H	–H	A4	**I**
15	Ganoderic acid GS-1	=O	β–OH	=O	–H	=O	A4	**I**
16	Ganoderic acid S	=O	–H	–H	–H	–H	A4	**II**
17	Ganoderic acid TN	β–OH	–H	–H	–H	α–$OCOCH_3$	A4	**II**
18	Ganoderic acid T-Q	=O	–H	–H	–H	α–$OCOCH_3$	A4	**II**
19	Ganoderic acid TR	=O	–H	–H	–H	α–OH	A4	**II**
20	Ganoderic acid LM2	=O	β–OH	=O	–H	=O	A5	**I**
21	12β-acetoxy-3,7,11,15,23-pentaoxo-5α-lanosta-8-en-26-oic acid ethyl ester	=O	=O	=O	β–$OCOCH_3$	=O	A6	**I**
22	Ganoderol A	=O	–H	–H	–H	–H	B1	**II**
23	Ganoderol B	β–OH	–H	–H	–H	–H	B1	**II**
24	Ganoderiol F	=O	–H	–H	–H	–H	B2	**II**
25	Ganodermanondiol	=O	–H	–H	–H	–H	C1	**II**
26	Lucidumol A	=O	=O	–H	–H	–H	C1	**I**
27	Lucidumol C	=O	=O	=O	–H	–H	C1	**I**
28	Ganodermanontriol	=O	–H	–H	–H	–H	C2	**II**
29	Methyl lucidenate A	=O	β–OH	=O	–H	=O	D1	**I**
30	Ethyl lucidenate A	=O	β–OH	=O	–H	=O	D2	**I**
Skeleton S								
31	Ergosterol	–	–	–	–	–	–	**A1**
32	Ergosta-7,22-dien-3β-ol	–	–	–	–	–	–	**A2**
33	Ergosterol peroxide	–	–	–	–	–	–	**A3**
34	Ergosterol palmitate	–	–	–	–	–	–	**A4**
35	Ergosta-7,22-dien-3β-yl palmitate	–	–	–	–	–	–	**A5**

[a] Structure type **I** bears double bond at $\Delta^{8,9}$; type **II** bears double bonds at $\Delta^{7,8}$ and $\Delta^{9,11}$

ROCK activity and the docking scores of the tested compounds are presented in Table 2.

For skeleton T (triterpenes) The most active compounds with Rho-kinase inhibitory activity are those characterized by the presence of double bond substitution at $\Delta^{24,25}$ while $R_6 = H$ and $R_7 = H$ (Fig. 1; Table 1). These compounds showed approximately 40−45% inhibition of enzyme activity at a concentration of 100 μM. At the same time, the binding energy of these compounds with the targeted enzymes was better than the binding of the standard inhibitor Y-27632. These compounds include ganoderic acid TN **(17)**, ganoderic acid T-Q **(18)** and ganoderic acid TR

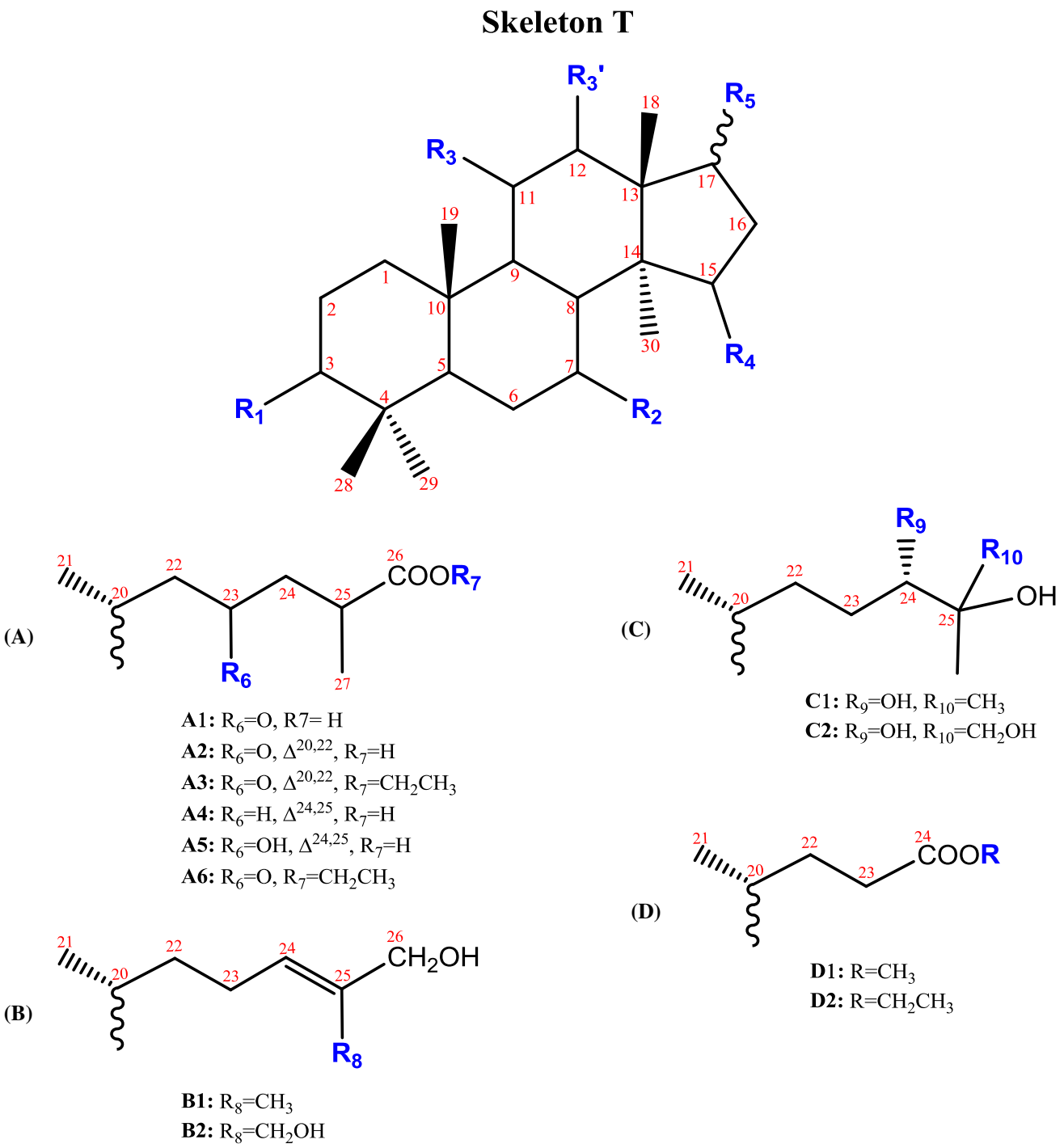

Fig. 1 Basic structure of Skeleton T (triterpenes) mentioned in Table 1

(19). However, some compounds with the same structural features, e.g., ganoderic acid DM **(14)**, ganoderic acid GS-1 **(15)** and ganoderic acid S **(16)**, did not show the same activity observed with **17**, **18** and **19**. On careful investigation of these phenomena, we noticed that the presence of the α-hydroxy group or α-acetyl group at C-15 (R_4) is essential for Rho-kinase inhibitory activity, since compounds **14, 15** and **16**, while sharing the same structural features with the active compounds **17**, **18** and **19** but lacking the α-hydroxy group or α-acetyl group at C-15, showed weak inhibition percentages against the targeted enzymes. Reviewing the literature, a study by Zhu et al.

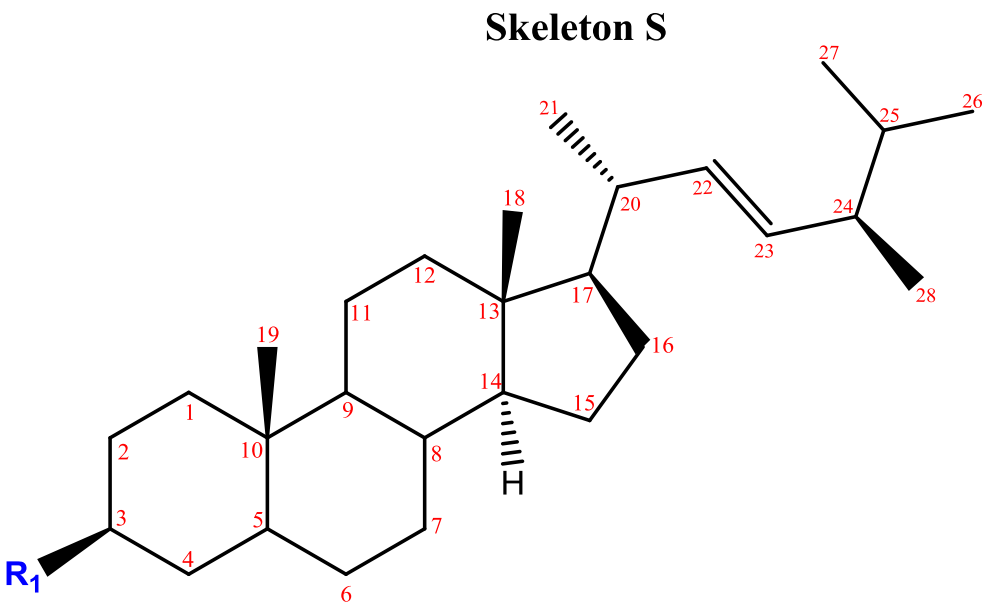

Fig. 2 Basic structure of Skeleton S (steroids) mentioned in Table 1

A1: R_1=OH, $\Delta^{5,6}$, $\Delta^{7,8}$
A2: R_1=OH, $\Delta^{7,8}$
A3: R_1=OH, 5α,8α-epidioxy, $\Delta^{6,7}$
A4: R_1=*O-palmitate*, $\Delta^{5,6}$, $\Delta^{7,8}$
A5: R_1=*O-palmitate*, $\Delta^{7,8}$

[17] revealed the potential antiviral activity of **18** against influenza neuraminidases, particularly H5N1 and H1N1 NAs. Based on this report and a study by Haidari et al. who revealed the involvement of the Rho/Rho-kinase pathway in influenza virus proliferation especially H3N2 and H1N1 strains [28], we suggest that **18** exerts its antiviral effect partially through Rho-kinase inhibition. We also suggest that the multiple biological activities related to the compound, e.g., antitumor and anti-inflammatory, might be in part due to its Rho-kinase inhibitory activity [29].

Interestingly, the presence of peripheral hydroxyl groups in the side-chain terminus at R_5, specifically at C-24, C-25 or C-26, increases Rho-kinase inhibitory activity and the activity observed with these compounds reached approximately 50% inhibition. That was very clear from the results obtained with ganoderol A **(22)**, ganoderol B **(23)**, ganoderiol F **(24)**, ganodermanondiol **(25)**, lucidumol A **(26)**, lucidumol C **(27)** and ganodermanontriol **(28)**. The docking study of these compounds revealed excellent binding of these compounds with the targeted enzymes. It is noted that the presence of a hydroxyl group at C-23 decreased the Rho-kinase inhibitory activity as indicated by the activity of ganoderic acid LM2 **(20)**. We presented the binding modes of the top two active compounds, **18** and **22**, with the active sites of ROCK-I and ROCK-II (Figs. 3, 4).

Based on these findings, three main structural features in the lanostane skeleton were found to affect the Rho-kinase inhibitory activity—the double bond substitution at $\Delta^{24,\ 25}$ but without any substitution at C-23, the presence of the α-hydroxy group or α-acetyl group at C-15, and the presence of hydroxyl groups at C-24, C-25 or C-26. These findings indicate that these features are favorable for the Rho-kinase inhibitory activity of lanostane triterpenes. These results suggest that this class of compounds has the potential to be used as lead compounds as Rho-kinase inhibitors. These results indicate that sharing a similar skeleton, a small difference in functional groups or double bond positions might lead to completely different bioactivity.

For Skelton S (steroids) In general, all the compounds showed very weak inhibition of enzyme activity or even no activity at all in case of ergosterol peroxide **(33)** or palmiate esters of ergosterol and ergosta-7,22-dien-3β-ol **(34, 35)** (Fig. 2; Table 1). However, the docking scores of all the compounds with skeleton S are almost more than the standard ligand.

Table 2 ROCK inhibitory activity and docking scores of the tested compounds

	ROCK assay[a]		Docking experiment[b]	
	ROCK-I inhibition	ROCK-II inhibition	2ETR (ROCK-I)	2H9 V (ROCK-II)
1	17.6 ± 1.5	16.9 ± 0.9	−48.74	−45.28
2	19.8 ± 2.4	16.8 ± 0.7	−59.39	−46.39
3	30.2 ± 3.9	28.5 ± 1.9	−53.51	−43.80
4	12.4 ± 1.5	11.9 ± 2.7	−55.90	−39.44
5	15.6 ± 2.9	14.7 ± 1.8	−53.93	−37.72
6	22.8 ± 0.9	20.8 ± 3.6	−46.51	−38.99
7	21.8 ± 1.8	19.9 ± 3.5	−53.20	−36.35
8	18.1 ± 2.6	16.5 ± 3.8	−51.07	−36.74
9	20.7 ± 2.1	19.3 ± 0.9	−51.74	−34.38
10	19.4 ± 3.4	18.9 ± 2.8	−52.32	−34.07
11	16.5 ± 1.9	15.9 ± 3.4	−40.43	−15.80
12	20.2 ± 0.9	19.6 ± 1.5	−44.65	−38.26
13	19.4 ± 2.3	18.7 ± 1.7	−43.16	−21.51
14	20.7 ± 3.4	18.6 ± 2.5	−60.49	−43.72
15	19.8 ± 2.5	20.9 ± 1.9	−57.78	−39.35
16	27.4 ± 2.5	29.9 ± 3.6	−62.46	−45.94
17	**40.6 ± 1.7**	**39.2 ± 3.2**	**−61.74**	**−47.68**
18	**45.7 ± 2.5**	**42.4 ± 0.9**	**−62.46**	**−46.81**
19	**42.4 ± 4.0**	**40.7 ± 2.6**	**−62.43**	**−40.42**
20	22.6 ± 2.7	20.8 ± 0.8	−49.63	−42.19
21	20.7 ± 1.9	19.6 ± 4.2	−54.64	−32.74
22	**49.7 ± 2.8 (IC_{50} 103.4 ± 3.6 μM)**	**47.9 ± 1.3 (IC_{50} 108.1 ± 2.6 μM)**	**−63.21**	**−47.08**
23	**48.2 ± 0.7**	**46.4 ± 1.9**	**−61.69**	**−47.14**
24	**40.9 ± 1.7**	**39.1 ± 2.9**	**−67.06**	**−46.67**
25	**40.9 ± 2.9**	**39.6 ± 0.7**	**−52.94**	**−44.19**
26	**39.9 ± 3.4**	**38.4 ± 2.8**	**−54.43**	**−44.66**
27	**41.6 ± 2.6**	**40.5 ± 4.9**	**−52.57**	**−39.92**
28	**43.8 ± 1.2**	**39.8 ± 2.4**	**−54.47**	**−48.18**
29	22.5 ± 5.4	21.9 ± 2.6	−51.23	−35.11
30	23.4 ± 3.7	22.6 ± 3.9	−58.76	−41.29
31	10.8 ± 1.5	9.4 ± 2.9	−59.84	−40.33
32	13.5 ± 1.9	12.5 ± 0.9	−67.61	−63.27
33	Nil[c]	Nil[c]	−56.93	−41.11
34	Nil[c]	Nil[c]	−69.38	−44.27
35	Nil[c]	Nil[c]	−77.96	−59.82
PC	42.6 ± 3.8	41.8 ± 4.6	−49.36	−49.82

[a] The results are expressed as mean values ± SD ($n = 4$). The final concentration of triterpenoids used in this assay was 100 μM, while the final concentration of positive control (PC) Y-27632 was 1.6 μM

[b] Scores expressed as free energy of binding ΔG in kcal/mol (S) calculated by CLC Drug Discovery Work Bench 3.0 for the tested compounds

[c] Nil—no enzyme inhibition

The bold values represent the top ten active compounds

It is also noted that almost all of the tested compounds except for the top ten ROCK inhibitors, showed varying degrees of weak inhibition ranging from 10–30% inhibition at a final concentration of 100 μM. We suggest that all of these compounds act together in a synergistic effect to give a total 60% inhibition of the ethanol extract at a final concentration of 1 mg/mL (Figs. 5, 6).

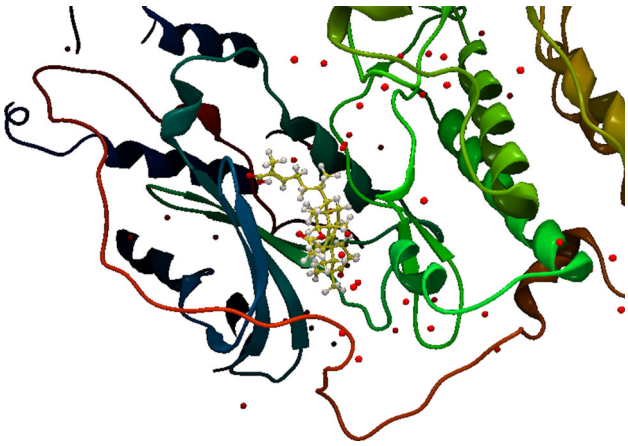

Fig. 3 Ganoderic acid TQ binds to chain A of 2ETR (ROCK-I)

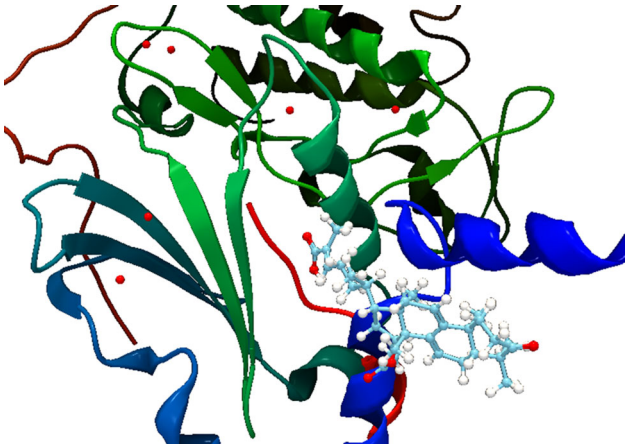

Fig. 4 Ganoderic acid TQ binds to 2H9V (ROCK-II)

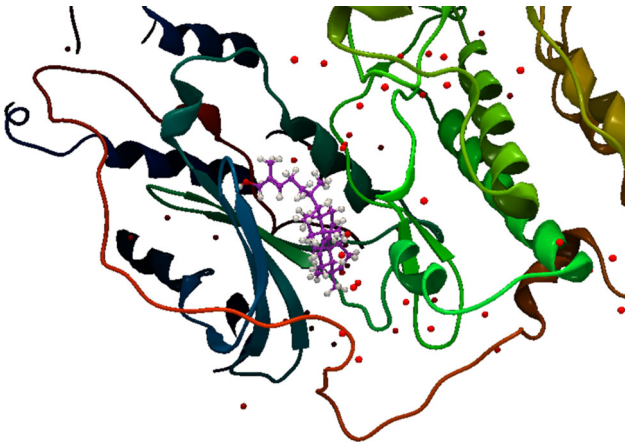

Fig. 5 Ganoderol A binds to chain A of 2ETR (ROCK-I)

Conclusion

The inhibitors of Rho-kinase have attracted a great deal of attention. An increasing amount of effort has been made to study the development of ROCK inhibitors. Many new potential Rho-kinase inhibitors have been proved to be effective in in vivo and in vitro experiments. The present study was undertaken to identify a possible mechanism of action for the oriental fungus *G. lingzhi* through investigation of the Rho-kinase inhibitory activity of the total ethanol extract and thirty-five compounds. The results indicated that the multiple biological activities of *G. lingzhi* might be at least in part due to Rho-kinase inhibitory activity (ROCK-I and ROCK-II). In addition we also report some lanostane triterpenoids as promising natural compounds for further studies on the development of Rho-kinase inhibitors.

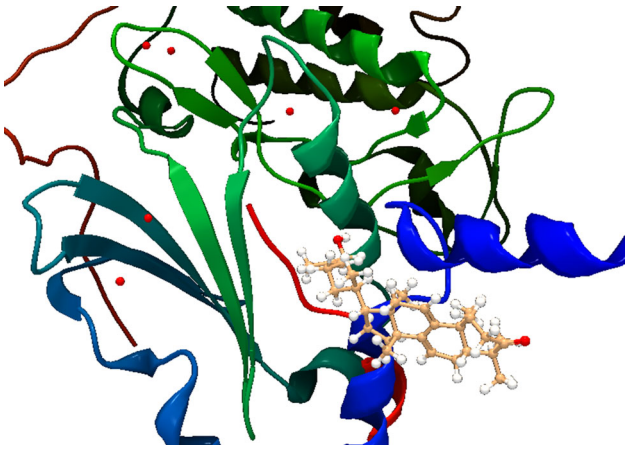

Fig. 6 Ganoderol A binds to 2H9V (ROCK-II)

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Compliance with ethical standards

Conflict of interest The authors have declared that there is no conflict of interest.

References

1. Newman DJ, Cragg GM (2007) Natural products as sources of new drugs over the last 25 years. J Nat Prod 70:461–477. doi:10.1021/np068054v
2. Doggrell SA (2005) Rho-kinase inhibitors show promise in pulmonary hypertension. Expert Opin Investig Drugs 14:1157–9. doi:10.1517/13543784.14.9.1157
3. Honjo M, Inatani M, Kido N et al (2001) Effects of protein kinase inhibitor, HA1077, on intraocular pressure and outflow facility in rabbit eyes. Arch Ophthalmol (Chicago, Ill 1960) 119:1171–1178
4. Pan P, Shen M, Yu H et al (2013) Advances in the development of Rho-associated protein kinase (ROCK) inhibitors. Drug Discov Today 18:1323–1333. doi:10.1016/j.drudis.2013.09.010
5. Rath N, Olson MF (2012) Rho-associated kinases in tumorigenesis: re-considering ROCK inhibition for cancer therapy. EMBO Rep 13:900–8. doi:10.1038/embor.2012.127

6. Raad M, El Tal T, Gul R et al (2012) Neuroproteomics approach and neurosystems biology analysis: ROCK inhibitors as promising therapeutic targets in neurodegeneration and neurotrauma. Electrophoresis 33:3659–68. doi:10.1002/elps.201200470
7. Amano M, Nakayama M, Kaibuchi K (2010) Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity. Cytoskeleton (Hoboken) 67:545–54. doi:10.1002/cm.20472
8. Leung T, Chen XQ, Manser E, Lim L (1996) The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol 16:5313–27
9. Reuter S, Kentrup D, Büssemaker E (2010) ROCK Inhibition – A New Therapeutic Avenue in Kidney Protection
10. Feng Y, LoGrasso PV, Defert O, Li R (2016) Rho kinase (ROCK) Inhibitors and their therapeutic potential. J Med Chem 59:2269–300. doi:10.1021/acs.jmedchem.5b00683
11. Satoh K, Fukumoto Y, Shimokawa H (2011) Rho-kinase: important new therapeutic target in cardiovascular diseases. Am J Physiol Heart Circ Physiol 301:H287–96. doi:10.1152/ajpheart.00327.2011
12. Shahin R, Alqtaishat S, Taha MO (2012) Elaborate ligand-based modeling reveal new submicromolar Rho kinase inhibitors. J Comput Aided Mol Des 26:249–66. doi:10.1007/s10822-011-9509-y
13. Tan H-B, Zhong Y-S, Cheng Y, Shen X (2011) Rho/ROCK pathway and neural regeneration: a potential therapeutic target for central nervous system and optic nerve damage. Int J Ophthalmol 4:652–7. doi:10.3980/j.issn.2222-3959.2011.06.16
14. Fatmawati S, Kurashiki K, Takeno S et al (2009) The inhibitory effect on aldose reductase by an extract of *Ganoderma lucidum*. Phytother Res 23:28–32. doi:10.1002/ptr.2425
15. Hai-Bang T, Shimizu K (2015) Structure–activity relationship and inhibition pattern of reishi-derived (*Ganoderma lingzhi*) triterpenoids against angiotensin-converting enzyme. Phytochem Lett. doi:10.1016/j.phytol.2015.04.021
16. Kandabashi T, Shimokawa H, Miyata K et al (2000) Inhibition of myosin phosphatase by upregulated rho-kinase plays a key role for coronary artery spasm in a porcine model with interleukin-1beta. Circulation 101:1319–23
17. Zhu Q, Bang TH, Ohnuki K et al (2015) Inhibition of neuraminidase by Ganoderma triterpenoids and implications for neuraminidase inhibitor design. Sci Rep 5:13194. doi:10.1038/srep13194
18. Xia Q, Zhang H, Sun X et al (2014) A comprehensive review of the structure elucidation and biological activity of triterpenoids from *Ganoderma* spp. Molecules 19:17478–17535. doi:10.3390/molecules191117478
19. Amen YM, Zhu Q, Tran H-B et al (2016) Lucidumol C, a new cytotoxic lanostanoid triterpene from *Ganoderma lingzhi* against human cancer cells. J Nat Med 70:661–6. doi:10.1007/s11418-016-0976-2
20. Amen YM, Zhu Q, Afifi MS et al (2016) New cytotoxic lanostanoid triterpenes from *Ganoderma lingzhi*. Phytochem Lett. doi:10.1016/j.phytol.2016.07.024
21. Goswami SK, Pandre MK, Jamwal R et al (2012) Screening for Rho-kinase 2 inhibitory potential of Indian medicinal plants used in management of erectile dysfunction. J Ethnopharmacol 144:483–9. doi:10.1016/j.jep.2012.07.045
22. Lokesh D, Amitsankar D (2012) Evaluation of mechanism for antihypertensive action of *Clerodendrum colebrookianum* Walp., used by folklore healers in north-east India. J Ethnopharmacol 143:207–12. doi:10.1016/j.jep.2012.06.025
23. Dufau I, Lazzari A, Samson A et al (2008) Optimization of a homogeneous assay for kinase inhibitors in plant extracts. Assay Drug Dev Technol 6:673–682. doi:10.1089/adt.2008.143
24. Dong M, Yan BP, Liao JK et al (2010) Rho-kinase inhibition: a novel therapeutic target for the treatment of cardiovascular diseases. Drug Discov Today 15:622–9. doi:10.1016/j.drudis.2010.06.011
25. Jacobs M, Hayakawa K, Swenson L et al (2006) The structure of dimeric ROCK I reveals the mechanism for ligand selectivity. J Biol Chem 281:260–8. doi:10.1074/jbc.M508847200
26. Yamaguchi H, Miwa Y, Kasa M et al (2006) Structural basis for induced-fit binding of Rho-kinase to the inhibitor Y-27632. J Biochem 140:305–11. doi:10.1093/jb/mvj172
27. Hai-Bang T, Shimizu K (2014) Potent angiotensin-converting enzyme inhibitory tripeptides identified by a computer-based approach. J Mol Graph Model 53:206–11. doi:10.1016/j.jmgm.2014.08.002
28. Mehrbod P, Omar AR, Hair-Bejo M et al (2014) Mechanisms of action and efficacy of statins against influenza. Biomed Res Int 2014:872370. doi:10.1155/2014/872370
29. Akihisa T, Nakamura Y, Tagata M et al (2007) Anti-inflammatory and anti-tumor-promoting effects of triterpene acids and sterols from the fungus *Ganoderma lucidum*. Chem Biodivers 4:224–31. doi:10.1002/cbdv.200790027