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Review Article

The ways for ginsenoside Rh2 to fight against cancer: the molecular evidences *in vitro* and *in vivo*.

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ABSTRACT

Cancer is a global public health issue that becomes the second primary cause of death globally. Considering the side effects of radio- or chemo-therapy, natural phytochemicals are promising alternatives for therapeutic interventions to alleviate the side effects and complications. Ginsenoside Rh2 (GRh2) is the main phytochemical extracted from Panax ginseng C.A. Meyer with anticancer activity. GRh2 could induce apoptosis and autophagy of cancer cells and inhibit proliferation, metastasis, invasion, and angiogenesis *in vitro* and *in vivo*. In addition, GRh2 could be used as an adjuvant to chemotherapeutics to enhance the anticancer effect and reverse the adverse effects. Here we summarized the understanding of the molecular mechanisms underlying the anticancer effects of GRh2 and proposed future directions to promote the development and application of GRh2.

1. Introduction

Cancer has become a global public health issue with its increasing morbidity and mortality [1]. The operation, radio- and chemo-therapy are major current cancer therapeutic strategies. However, the operation is only therapeutic in early-stage cancer, and radio- or chemo-therapy still exists short-term toxicity and long-term consequences, such as alopecia [2], cognitive decline [3], skin erythema, mucositis, nausea, and diarrhea [4].

It has been generally recognized in recent years that natural phytochemicals are promising alternatives for therapeutic interventions intended to alleviate the side effects and complications in conventional cancer therapy. Ginseng, the root of Panax ginseng C.A. Meyer, is widely used as a natural health supplement in Asian countries. Ginsenosides are the main bioactive compounds extracted from ginseng. To date, there are more than one hundred naturally ginsenosides identified in ginseng [5]. And these ginsenosides have been proved to have multiple pharmacological activities (eg. anti-diabetes [6], reversing myocardial Ischemia-reperfusion injury [7], promoting cerebral angiogenesis [8], and improving chronic inflammatory arthritis [9]).

In preclinical studies, several types of ginsenosides have also been found to exert anticancer function *in vivo* and *in vitro* [10], and some have demonstrated the potential clinical application on therapy for non-small cell lung cancer [11] and rectal cancer [12]. Ginsenosides exert the anticancer effects mainly via inducing apoptosis, autophagy, and inhibiting cell proliferation, metastasis, and angiogenesis [13]. A large number of studies have revealed that the typical signaling pathways or molecules (eg. PI3K/Akt/mTOR, ERK/MAPK, Wnt/β-Catenin, and STAT3) have participated in the anticancer effects induced by ginsenosides (eg. Rg3, 20(S)-25-OCH3-PPD and CK) [13–15]. However, the mechanisms for triggering these pathways are still unclear, and the relationships between each independent molecules have not been well elucidated, which becomes an obstacle for further clinic trials.

The same dilemma occurred on studies on ginsenoside Rh2, a protopanaxadiol-type ginsenoside. Ginsenoside Rh2 has been shown to have superior cytotoxic potency to cancer cells among various types of ginsenosides [16]. Certainly, ginsenoside Rh2 can regulate various signaling pathways associated with anticancer [17]. However, the clinical applications of ginsenoside Rh2 are still under restrictions due to the inexplicit mechanisms underlying its anticancer effects. Therefore, the regulation patterns of ginsenoside Rh2 on anticancer-associated molecules should be summarized

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systematically and logically. This review (1) focuses on recent advances in the understanding of the molecular mechanisms of Rh2-induced apoptosis, anti-proliferation, autophagy, anti-angiogenesis, and anti-metastasis effects on cancer cells, and summarizes associated key mediators into cascades, (2) introduces the combinations of ginsenoside Rh2 and other anticancer agents, (3) discusses the potential modification of ginsenoside Rh2 to improve its performance on anticancer, (4) proposes the potential applications of ginsenoside Rh2.

2. The stereoisomerism of ginsenoside Rh2

Ginsenoside Rh2 is one of the triterpene saponins and exists as two stereoisomers: 20(S)-ginsenoside Rh2 and 20(R)-ginsenoside Rh2. The two stereoisomeric pairs were differentiated by R- or S-configuration at carbon-20 (Table 1). This isomerism seems to have no effects on the physicochemical properties of ginsenoside Rh2 (Table 1). However, the stereoisomerism might determine the disparity in pharmacological effects between these two stereoisomers. The 20(S)-ginsenoside Rh2 showed stronger anticancer effects than its 20(R)-stereoisomer in various cancer cell lines [18–20]. The differences on pharmacokinetics might contribute to the differences on the pharmacology. It was reported that the uptake rate of 20(S)-ginsenoside Rh2 was 3-fold higher than 20(R)-ginsenoside Rh2 in Caco2 cell model, and the efflux ratio of 20(S)-ginsenoside Rh2 was significantly lower than the 20(R)-isomer [21]. And in rat model, the plasma concentration of 20(S)-ginsenoside Rh2 was 10-fold higher after intragastric administration with the same dose as the 20(R)-isomer [22]. The protopanoxadiol (PPD), as the important metabolite of Rh2 *in vivo*, has been proved to possess better anticancer activity than Rh2 [20]. And literature have demonstrated that 20(S)-PPD, but not 20(R)-PPD, was observed in plasma of rats which were administered with 20(S)-ginsenoside Rh2, and the plasma concentration ratio of 20(S)-PPD: 20(S)-ginsenoside Rh2 was about 1:4 [22]. On the contrary, PPD could be hardly detected in the rat plasma after administration with the 20(R)-ginsenoside Rh2. Taken together, higher absoption rates and higher concentration of metabolite PPD might contribute to the better anticancer activity of 20(S)-ginsenoside Rh2 (hereinafter referred to as GRh2). However, more evidences should be provided by further *in vivo* studies to certify this inference.

3. Pharmacology of GRh2 in cancer model

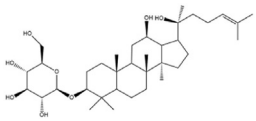
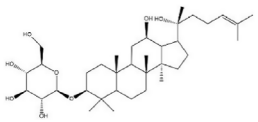
3.1. GRh2-induced apoptosis

The apoptosis induced by GRh2 could be observed in a majority of common cancer models, and like other conventional anticancer drugs, caspases regulation plays a critical role in this apoptotic process (Fig. 1) [23–33]. Caspases are a conserved family of enzymes that can cleave an aspartate residue in their substrates [34]. Once effector caspases (which include caspase-2, -4, -8, -9) are activated [25,27,35], the caspase cascade will be initiated and effector caspases like caspase-3 will cleave various cellular targets like poly ADP-ribose polymerase (PARP) [30], which results in cell death eventually. However, the activation of the caspase-dependent pathway seems not the only way for GRh2 to trigger apoptosis, some caspase-independent pathways associated with NF-κB are also involved in GRh2-induced apoptosis [36,37].

3.1.1. Death-receptor (extrinsic) pathway

There have been a large number of studies reporting that GRh2-induced apoptosis was associated with activation of death receptors (and/or associated ligands) (eg. Fas/FasL, D4/TRAIL, and D5/TRAIL) and the following caspase cascades (Fig. 1 & Table 2). Recent evidences showed that GRh2 could activate death receptor-related proteins Fas/FasL and D5/TRAIL through up-regulating Nur77 expression in acute myeloid leukemia (AML) cell HL-60, which was followed by the activation of caspase-8 and caspase-3 (Fig. 1) [23]. Rather, it seems that the types of activated death receptors in GRh2-induced apoptosis can be cell-type-dependent. For example, GRh2 could only activate D4/TRAIL death receptor-related proteins and did not influence Fas/FasL pathway in lung adenocarcinoma A549 cells [27]. On the contrary, the GRh2 induced the overexpression of death receptor Fas not D4/D5 in HeLa cells but this up-regulating could be attenuated in p53-silence HeLa cells or p53-mutated SW480 cells (Fig. 1), which demonstrated GRh2-induced Fas overexpression was mediated by p53 [38]. However, the activation of p53 might not be solely responsible for Fas activation, as GRh2 can cause lipid rafts disruption which then initiates ligand-independent Fas activation [39]. The cell-type-dependent regulation of death receptors might be related to that the membrane protein composition differs from various cell lines, and death receptors are predominately located in the plasma membrane. Therefore, considering the different death receptors composition in

Table 1
The Structure and Physicochemical Properties of Ginsenoside Rh2

	20(S)-ginsenoside Rh2	20(R)-ginsenoside Rh2
Structure		
CAS Registry Number	67400-17-3	112246-15-8
Molecular Formula	$C_{36}H_{62}O_8$	$C_{36}H_{62}O_8$
Molecular Weight	622.873	622.873
Density	1.21 g/cm^3	1.20 g/cm^3
Melting point	225 °C	228 °C
Boiling Point	726.4 ± 60.0 °C at 760 mmHg	726.4 ± 60.0 °C at 760 mmHg
Flashing Point	365 ± 28 °C	365 ± 28 °C
Water solubility	65.5 μmol/L	65.3 μmol/L
LogP	5.62	5.62
Surface Tension	54.5 dyn/cm	54.5 dyn/cm
Polarizability	67.7 Å^3	67.7 Å^3
Index of Refraction	1.572	1.572

The data is from PubChem and United States Environmental Protection Agency.

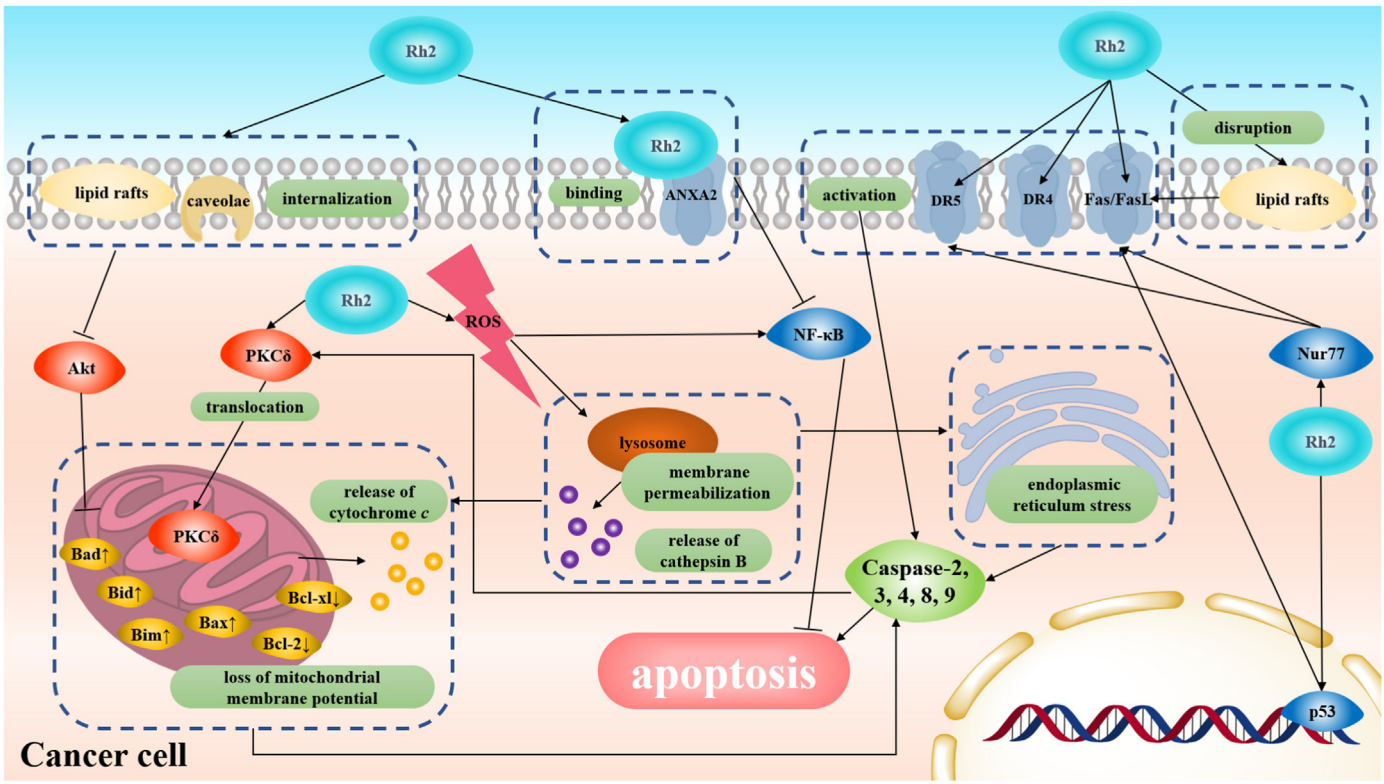


Fig. 1. Molecular mechanisms of GRh2-induced apoptosis.

Table 2
Important Mediators of GRh2-Induced Anticancer Effects

Key molecule(s)	Activity alteration in response to GRh2	Pharmacological effects associated	References
Annexin A2	DOWN	Apoptosis	[37]
Akt	DOWN	Apoptosis, Anti-proliferation	[31,45,49,59,72]
Atg5/7	UP	Autophagy	[44,45,50,75]
Bax	UP	Apoptosis	[24,31–33,35,36,45,46,52]
Bak	UP	Apoptosis	[32]
Bad	UP	Apoptosis	[31,36]
β-catenin	DOWN	Anti-proliferation	[53,73,75]
Bcl-2	DOWN	Apoptosis	[24,30,32,33,35,36,45,46,52]
Bcl-xL	DOWN	Apoptosis	[32,36]
Beclin-1	UP	Autophagy	[45,50]
Bim	UP	Apoptosis	[31,32]
Caspases	UP	Apoptosis	[23–33,39]
CDKs	DOWN	Anti-proliferation	[26,27,43,52,54,56]
Cyclins	DOWN	Anti-proliferation	[26,27,45,52–54,56]
E-cadherin	UP	Anti-metastasis and invasion	[50,73]
E2F	DOWN	Anti-proliferation	[26,54,61]
EGFR	DOWN	Anti-proliferation	[58,59,64]
ERK	DOWN	Anti-proliferation	[26,60]
Fas/FasL	UP	Apoptosis	[23,39]
HATs	UP	Anti-proliferation	[52]
HDACs	DOWN	Anti-proliferation	[52,70]
LC3-II/LC3-I	UP	Autophagy	[75]
mTOR	DOWN	Anti-proliferation	[45,49,59]
MMPs	DOWN	Anti-metastasis and invasion	[53,70–72]
PI3K	DOWN	Anti-proliferation, Anti-metastasis and invasion	[45,49,72]
PKCs	UP	Apoptosis	[29]
Rb	DOWN	Anti-proliferation	[27,54]
Snail	DOWN	Anti-metastasis and invasion	[50]
STAT3	DOWN	Anti-proliferation	[71]
TGF-β	DOWN	Anti-metastasis and invasion	[50]
TRAIL/DR4(5)	UP	Apoptosis	[23,27]
VEGF	DOWN	Anti-angiogenesis	[67–69]
Vimentin	DOWN	Anti-metastasis and invasion	[50,73]
Wnt	DOWN	Anti-proliferation	[53,73]

the plasma membrane, different cell lines could show the distinguishing sensibility of death receptors in response to external stimulus.

However, almost all GRh2 death-receptor pathway studies were only implemented as *in vitro* studies in human cancer cell lines, and a noteworthy issue that whether the death-receptor pathway is involved in GRh2-induced cancer cell death in mouse models still remains to be illuminated.

3.1.2. Mitochondrial (intrinsic) death pathway

A large number of studies have demonstrated that the mitochondrial death pathway plays a role in GRh2-induced apoptosis. When the mitochondrial death pathway is activated, cytochrome *c* can be released from mitochondrial intermembrane space, and then assemble with apoptotic protease-activating factor-1 (APAF-1) to form an apoptosome for the recruitment and autoactivation of caspase-9 [40]. Except for the proteins associated with the mitochondrial death pathway, the loss of mitochondrial membrane potential (a signal in the initial stage of the mitochondrial death pathway) has been also observed in GRh2-induced cancer cell death [24]. It is also worth mentioning that, not like the Fas/FasL pathway, the levels of mitochondrial-mediated caspase-9 expression and cytochrome c release are similar in both HeLa cells and p53-mutated SW480 cells (Fig. 1), indicating that the GRh2-induced mitochondrial death pathway is independent on p53 status in cervical cancer model [38]. Like a majority of anticancer drug, GRh2 triggers activation of the intrinsic apoptotic cascade in two possible ways (Table 2). One is that GRh2 regulates the expression of pro-apoptosis genes and pro-survival genes associated with mitochondria. The other one is that GRh2 regulates the up-steam signaling pathway of mitochondria. The possible mechanisms in the following three sections have been confirmed in the GRh2-induced mitochondrial death pathway.

3.1.2.1. Activation of caspase-3-dependent protein kinase C δ. It has been confirmed that activation of protein kinase C δ (PKCδ) could lead to translocation of PKCδ into the mitochondria followed by mitochondrial dysfunction and cytochrome *c* release [41]. Pretreatment with the specific PKCδ inhibitor rottlerin could suppress the activation of caspase-9 and caspase-3, resulting in inhibiting GRh2-induced apoptosis (Fig. 1). And it has been also proved that caspase-3 could activate PKCδ selectively in GRh2-induced apoptosis [29]. These discoveries indicate that there exists a positive feedback loop mechanism of caspase-3 and PKCδ activation in GRh2-induced apoptosis.

3.1.2.2. Selective activation or induction of BH3-only/Bcl-2 family proteins. BH3-only proteins (Bcl-2 homology domain 3 only proteins), as pro-apoptotic proteins, are sensors to apoptotic signals derived from the various extracellular stimulus and intracellular processes. When BH3-only proteins are activated, they will interact with anti- and pro-apoptotic B cell lymphoma 2 (Bcl-2) family proteins to facilitate the apoptosis process [42]. The proapoptotic Bcl-2 family proteins like Bax and Bak cause mitochondrial outer membrane permeabilization and activate the mitochondrial death pathway. Conversely, anti-apoptotic Bcl-2 family proteins including Bcl-2, Bcl-xl, and Mcl-1 can inhibit the activation of pro-apoptotic Bcl-2 family proteins [43]. Accordingly, the activation of BH3-only proteins or Bcl-2 family proteins in response to GRh2-induced initiation of the mitochondrial death pathway has been investigated. Bax, Bcl-xl, and Bcl-2 have been irrefutably implicated in the GRh2-induced mitochondrial death pathway, the activation of Bax, and the inhibition of Bcl-xl and Bcl-2 have been observed in various cancer cell lines with GRh2-treatment (Fig. 1) [32,35,36]. Concerning the BH3-only proteins, Bim, Bid and Bad have been proved to be activated during GRh2-induced apoptosis [29,32].

How GRh2 regulates BH3-only or Bcl-2 family proteins is a major confusion remaining to be specifically explained. There have been two possible models being proposed: regulation of ROS and Akt. Destabilized by ROS, the lysosomal membrane can become permeabilized, and cathepsin B can be released in hepatoma HepG2 cells treated with GRh2, which contributes to the cleavage and activation of Bid (Fig. 1) [44]. In human epidermoid carcinoma A431 cells, GRh2 could down-regulate Akt activation by inducing the internalization of lipids rafts and caveolae [31]. And the Akt activation is responsible for the inhibition of the interaction between Bad and Bcl-2 family proteins (Fig. 1) [31,45]. Additionally, overexpression of voltage-dependent anion channel 1 also made contributions to the translocation of Bcl-2 and Bax into mitochondria [46].

Taken together, these studies illustrated the mechanisms of BH3-only and Bcl-2 family proteins in activating the mitochondrial death pathway in cancer cell lines with GRh2 treatment.

3.1.2.3. Regulation of ROS activity. Reactive oxygen species (ROS) can be detected after treatment with GRh2, and treatment with antioxidant N-acetyl-L-cysteine can alleviate GRh2-induced apoptosis. Moreover, the increase in ROS level contributes to the depletion of mitochondrial membrane potential and lysosomal membrane permeabilization (Fig. 1) [24,47]. Additionally, previous studies have shown that GRh2-induced ROS could initiate endoplasmic reticulum stress, leading to the activation of the caspase cascade (Fig. 1) [25]. However, the issue of how ROS level was elevated in GRh2-induced apoptosis currently remains unresolved. Interestingly, ROS functions as double-edged swords in GRh2-induced cancer cell death. GRh2-induced ROS can activate the NF-κB pathway, and treatment with NF-κB pathway inhibitor PS-1145 promote the GRh2-induced cell death, which indicates that the ROS-induced NF-κB pathway suppresses the GRh2-induced apoptosis (Fig. 1) [36]. Even though a large number of studies have demonstrated that cellular ROS was associated with the activation of various cytoplasmic signaling pathways which have been proved to be involved in GRh2-induced apoptosis such as protein kinase C (PKC), c-Jun N-terminal kinase (JNK), p38 kinase (p38MAPK) and PI3K/Akt pathways [48]. There is no sufficient evidence proving that it is ROS that directly mediates these pathways in the GRh2-induced apoptosis model.

3.1.3. Other pathways associated

As mentioned above, GRh2-induced ROS can activate NF-κB to suppress apoptosis. Fortunately, GRh2 has found another way to suppress the activation of the NF-κB pathway. Once activated by Annexin A2, NF-κB could promote the expression of anti-apoptosis genes like c-IAP1, c-IPA2, X-IAP, and Survivin. GRh2 is found to bind with Annexin A2, and then inhibit the interaction between Annexin A2 and NF-κB, consequently, down-regulates NF-κB activity (Fig. 1) [37]. In human lung adenocarcinoma A549 cells, GRh2 can induce JNK activation which contributes to the increases in expression and activity of downstream targeted genes like AP-1 and promotes cell apoptosis [26]. However, how GRh2 activated the JNK pathway remains to be explored. In addition, it is worth noticing that the regulation of gut microbiota and the immune system by GRh2 might be also associated with GRh2-induced apoptosis of cancer cells [49].

3.2. Anti-proliferation

The growth of the tumor can also be suppressed by GRh2 (Fig. 2). In uterine leiomyoma cells, GRh2 can induce the activation

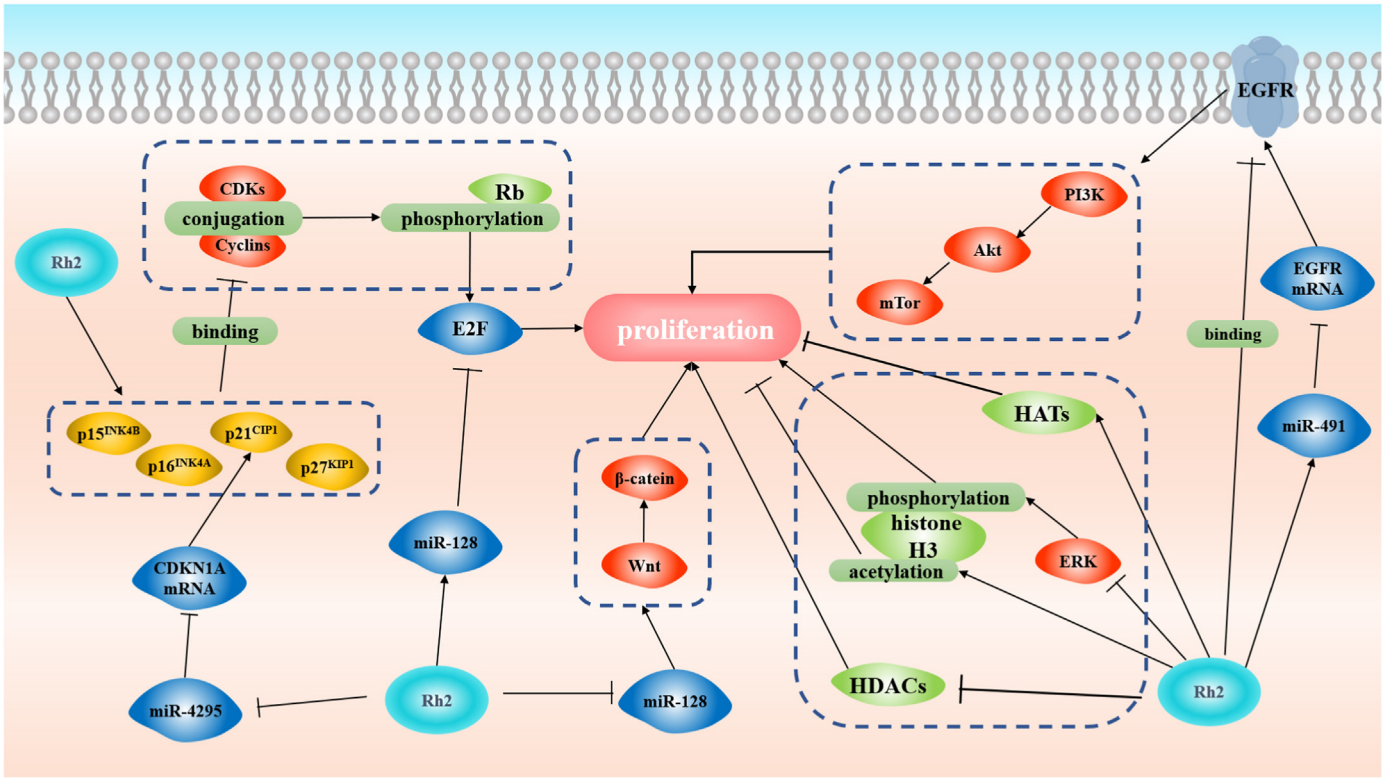


Fig. 2. Molecular mechanisms of GRh2-induced anti-proliferation.

of p38 MAPK and inhibition of c-Src, which both suppress the phosphorylation of estrogen receptor alpha (a proliferative effects initiator) [50]. Moreover, GRh2-induced anti-proliferation of cancer cells was found mainly associated with the cell-cycle arrest at the G0/G1 and G1/S boundary. A large number of studies demonstrate that the retinoblastoma tumor suppressor protein (Rb), epidermal growth factor (EGF), microRNA (miR), and histone are all involved in GRh2-inhibited proliferation (Table 2).

3.2.1. Regulation of Rb-related pathway

Cyclin-dependent kinases (CDKs) and their cyclin partners (Cyclins) are critical regulatory factors in cell proliferation. Cyclin D–CDK4/6 and Cyclin E–CDK2 complexes can activate the Rb phosphorylation which enables the E2F transcription factors to initiate transcription of genes promoting the cell cycle progression [51]. A large number of studies have proved that GRh2 could suppress the phosphorylation of Rb and the levels of CDKs, Cyclins, and E2F transcription factors in cancer cells [26,27,52–56]. GRh2 can up-regulate the levels of cyclin-dependent kinase inhibitors (including $p15^{INK4B}$, $p16^{INK4A}$, $p21^{CIP1}$, and $p27^{KIP1}$) which bind to CDKs or CDK-cyclin complexes and inhibit the kinase activity of CDKs [27,52–56]. Consequently, the phosphorylation of Rb and activation of E2F transcription factors are inhibited.

3.2.2. Inhibition of EGF-induced proliferation

EGF receptors (EGFR) are overexpressed in human carcinomas, which results in an ungovernable clinical behavior [57]. As a result, it becomes a rational way for anticancer treatment to block or abolish the functions of EGFR. The activation of EGFR is inhibited in response to GRh2-treatment in various cell lines [58–60]. GRh2 can bind to EGFR, thereby, depleting the sensitivity of EGFR to EGF, and inhibiting following PI3k/Akt/mTor signaling cascades (Fig. 2) [59]. However, GRh2 is unlikely to restrain the activity of EGF directly.

3.2.3. Regulation of miRs

According to all the studies to date, GRh2 can regulate the expression of more than 30 miRs [61], and some of them have been proved to be involved in the regulation of Rb-related pathways and suppression of EGFR. Concerning E2F transcription factors, GRh2 can suppress E2F3a via up-regulating the expression of miR-128 (Fig. 2) [62]. MiR-4295 can bind to 3′-UTR of CDKN1A mRNA (encoding $p21^{CIP1}$), then promote phosphorylation of Rb, which has been found to be inhibited by GRh2 treatment (Fig. 2) [63]. Up-regulation of miR-491 is another way for GRh2 to suppress EGFR. The miR-491 can suppress EGFR protein translation through binding to 3′-UTR of EGFR mRNA (Fig. 2) [64,65]. Other pathways that have been directly implicated in GRh2-regulated miR expression include the Wnt/β-catenin signaling pathway which promotes cell proliferation. The Wnt/β-catenin signaling pathway is inhibited in response to GRh2 treatment: GRh2 can suppress the miR-31 expression, whereas miR-31 overexpression makes contributions to the activation of Wnt/β-catein signaling pathway (Fig. 2) [53]. Moreover, a recent research revealed that long non-coding RNA (like LNRNA C3orf67-AS1) also participated in GRh2-exerted anti-proliferation [39].

3.2.4. Modification of histone

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) act as regulators of gene expression during cell proliferation by modifying chromatin. Through regulating HDACs and HATs, GRh2 can break the balance between acetylation and deacetylation of histone, leading to cell-cycle arrest in the cancer model (Fig. 2) [52]. A study by Liu et al showed that HDAC activity was decreased while HAT activity was increased in human leukemia K562 and KG1-a cells treated with GRh2 [28]. To be specific, GRh2 decreased the expression of HDAC1, 2 and 6, and promoted acetylation of histone H3 both *in vivo* and *in vitro* [28]. On the contrary, the

phosphorylation of H3 was inhibited by GRh2 in HCT116 colon cancer cells due to the inhibition of ERK2 phosphorylation and PDZ-binding kinase/T-LAK cell-originated protein kinase pathway (Fig. 2) [36]. However, how GRh2-induced modifications of histone regulated the cell cycle remained to be unclear until Li et al discovered the relationship between H3K27me^3 modification and cell proliferation [55]. GRh2 could decrease expression of H3k27me^3 and EZH2 (a histone methyltransferase of H3K27me^3), and inhibit their recruitment in p14, p15, and p16 genes promoter regions, and consequently, promote mRNA expression of the CDKN2A-2B gene cluster (encoding CDKs inhibitors) [55]. These findings revealed that GRh2 might function as a histone deacetylases inhibitor. In theory, histone deacetylases inhibitor could also induce apoptosis [66], but there is no sufficient evidence to support that the GRh2-induced apoptosis is associated with HDACs (Fig. 1).

3.3. Tumor angiogenesis, metastasis, and invasion

GRh2 has anti-angiogenic, anti-metastatic, and anti-invasive activities that play critical roles in the inhibition of tumor development and progression (Fig. 3 & Table 2). Anti-angiogenesis blocks the ways for the tumor to obtain sufficient nutrient supply, which inhibits tumor metastasis. The anti-angiogenic effect of GRh2 is achieved by inhibiting the expression of vascular endothelial growth factor (VEGF) which can promote tumor outgrowth and invasion. To be specific, GRh2-induced up-regulation of cyclin and CBS domain divalent metal cation transport mediator 1, the increase in miR-497 level and the reversion from the phenotype of M2 macrophages to M1 subtype all contribute to inhibition of VEGF (Fig. 3) [67–69].

The suppression of matrix metalloproteinases (MMPs) is the key to the GRh2-induced anti-metastatic effect. Four different ways have been reported to inhibit the expression of MMPs with GRh2 treatment. GRh2 can increase HDAC4 level and lead to the recruitment of HDAC4 to the MMP-3 promoter site and the binding site of proximal analyze activator protein 1, which inhibits the transcription of MMP-3 (Fig. 3) [70]. MicroRNAs are also involved in GRh2-induced inhibition of MMPs. The overexpression of miR-31 increased MMP-2 and MMP-9 levels, consequently reversed the anti-metastatic effect of GRh2 [53]. As mentioned above, GRh2-induced ROS can activate the NF-κB pathway, and then the IL-6 level was up-regulated. IL-6 can promote the expression of MMPs via activating the STAT3 signaling pathway, and GRh2 can reverse the overexpression of MMPs by inhibiting the interaction between IL-6 and its receptor (Fig. 3) [71]. Moreover, the MMP-13 was found inhibited owing to the suppression of the PI3k/Akt pathway induced by GRh2 in glioblastoma multiforme [72]. The increase in E-cadherin level and the decrease in epithelial-mesenchymal transition (EMT)-related proteins like vimentin, TGF-β, and Snail were observed under GRh2 treatment (Fig. 3), which indicated that EMT-mediated tumor metastasis was inhibited by GRh2 [73]. However, the mechanism underlying GRh2-inhibited EMT remains to be elusive.

3.4. GRh2-induced autophagy

The GRh2-induced autophagy has been associated with an increased expression level of autophagy-related protein (Table 2). During the vesicle nucleation stage of autophagy, Beclin-1 can scaffold class III PI3K complex [74], and GRh2 treatment can increase the Beclin-1 level in various cell lines [75,76]. LC3-II, the conjugation of LC3-I and PE, is an autophagosome marker that is mediated by ATG7 [77]. The higher ATG7 level and LC3-II/LC3-I ratio can be observed in cells with GRh2 treatment than in control groups (Fig. 3) [75,76]. Taken together, these studies provide pieces of evidences supporting the role of GRh2 in inducing autophagy

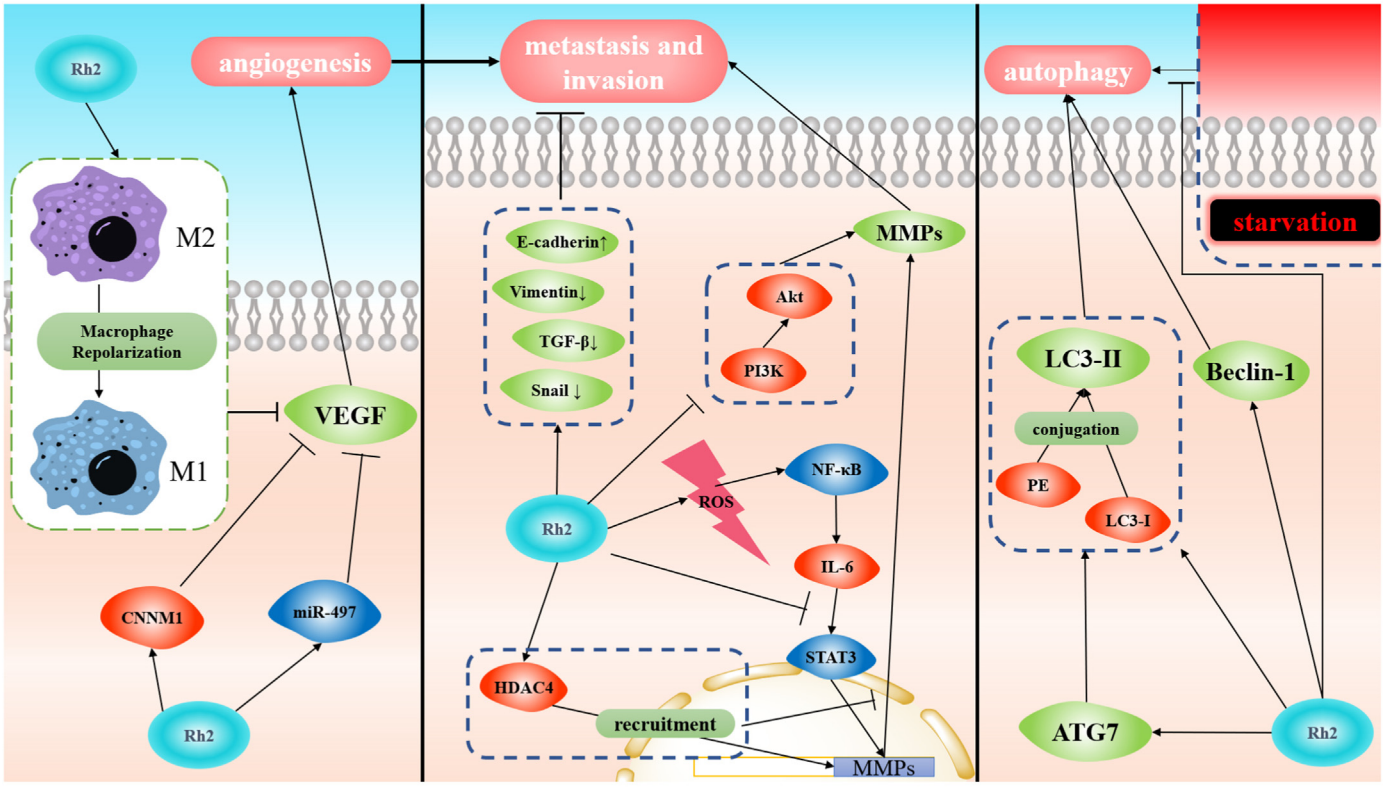


Fig. 3. Molecular mechanisms of GRh2-induced angiogenesis, autophagy, anti-metastasis, and anti-invasion.

through regulating of the expression of autophagy-related proteins. Interestingly, GRh2 was found to inhibit autophagy caused by starvation (free-serum cultivation), and starvation-induced autophagy could suppress the apoptosis of cancer cells (Fig. 3) [78].

4. Combining GRh2 with other drugs

GRh2 has shown its potential to be a single-anticancer agent. However, accumulated empirical experiments revealed that chemotherapeutic combination could achieve synergistic effects or attenuate drug toxicity.

4.1. Synergistic effects

4.1.1. With chemotherapeutic drugs

Synergistic death of various cancer cells could be observed following treatment with GRh2 in a combination of chemotherapeutics. Initially, GRh2 was found to yield synergistic activity in lowing cell viability when combined with paclitaxel, mitoxantrone, or docetaxel [79,80]. Then studies on the molecular level revealed that BH3-only/Bcl-2 family proteins and caspase family proteins played important roles in the synergistic effect.

In the human colorectal carcinoma cells model, GRh2 and sodium selenite could increase the Bax/Bcl2 ratio and the caspase-3 expression synergistically [81]. Given that both regorafenib and GRh2 can inhibit VEGF or VEGF receptors, they could function synergistically when downregulating survivin and overexpressing caspase-3, thereby, exhibited a synergistic inhibitory effect on HepG2 cells [82]. GRh2 could synergize with SMI-4a in increasing the LC3-II expression and caspase 3/caspase 7 activity in melanoma cells [83].

4.1.2. With phytochemicals

The treatment with a combination of GRh2 and other phytochemicals (which have not been utilized in clinic) could also cause the synergistic death of cancer cells. Li et al extracted and purified corilagin from longan, and found that the combination of GRh2 and corilagin exerted synergistic cytotoxicity on SKOv3ip and Hey cells [84]. Li et al found that the combination treatment with GRh2 and betulinic acid could increase the expression of cleaved caspase-8, cleaved caspase-9, tBid, and the release of cytochrome *c* in Hela cells [85]. GRh2 could also synergize with its derivative − protopanaxadiol on antiproliferative activity in human breast cancer cells, and the phosphorylation of BAD, p53 and p38 protein has been proved to contribute to this synergistic effect [86].

GRh2 has already been proved to function synergistically with chemotherapeutic drugs or other phytochemicals. However, in many instances, the mechanisms of drug combinations involving GRh2 were still hypotheses, and the related experiments still could not elucidate why the combination could function synergistically.

4.2. Reversal effect on drug resistance

During chemotherapy, the acquisition of drug resistance is the major reason for the decline in therapeutic efficacy. Fortunately, previous reports proved that GRh2 could downregulate the expression of drug-resistance genes and proteins (eg. MRP1, MDR1, LRP, and GST) in 5-FU-resistant colorectal carcinoma cells, consequently, enhance the cytotoxicity of 5-FU to 5-FU-resistant colorectal carcinoma cells [87]. Drug efflux is the major mechanism for the acquisition of drug resistance. The over-expression of P-gp accounts for the efflux, and the GRh2 could inhibit P-gp via regulating the pentose phosphate pathway and redox balance [88]. Consequently, GRh2 could enhance the accumulation of adriamycin in nuclei, mitochondria, and cytosol in the adriamycin-resistant MCF-7 cells [89].

4.3. Decreasing side effects of chemotherapy

Highly proliferated cells would be inhibited or eliminated during chemotherapy, however, the benign cells, tissue, and organ could be impaired at the same time. Normal cellular senescence is the main side effect of chemotherapy due to DNA impairment. Previous reports indicated that GRh2 could inhibit the DNA damage induced by cyclophosphamide [90]. Subsequently, GRh2 was proved to inhibit cellular senescence-associated migration and invasion of human breast cells [91]. These effects could be explained by Hou et al who found GRh2 could suppress the phosphorylation of MEK1, MAPK p38, STAT3, and NF-κB p65 in senescent breast cells induced by doxorubicin [92]. Hou et al also found that GRh2 could regulate the mitochondrial dynamic, eliminate ROS level, promote mitophagy, and subsequently inhibit senescence-associated secretory phenotype in senescent breast cells [91].

5. Discussion and future perspectives

GRh2 is a traditional anticancer phytochemical that induce tumor cell death, differentiation, and cell-cycle arrest. These effects attribute to a large number of molecular events (eg. the activation of death-receptor, mitophagy, oxidative stress, and the secretion of cytokine) and participation of various molecules (including proteins and RNAs). Moreover, GRh2 could synergize with other anticancer agents, decrease the side effects of chemotherapy, and reverse the drug resistance.

When GRh2 exerts its anticancer effects, lipid rafts disruption and ROS generation seem to play a significant role. This could be its unique mechanism when compared with other phytochemicals like polyphenols. The structure of GRh2 might be the reason. It is acknowledged that cholesterol could induce apoptosis and autophagy through generating ROS [93]. And GRh2 (a protopanaxadiols type ginsenoside a protopanaxadiols type ginsenoside which has no C-6 substituents) possesses a similar structure with cholesterol (Fig. 4), thereby, might exert similar biological activity (eg. generating ROS). Meanwhile, with a similar structure, GRh2 shows a good affinity to cholesterol which is an important substance to constitute lipid rafts in the plasma membrane. Therefore, GRh2 could insert into lipid rafts and interact with cholesterol, consequently, disrupt the internalization of lipid rafts [94]. This reminds us that GRh2 might exert better functions if the structure of GRh2 could be modified to possess better compatibility with the plasma membrane. And the function of GRh2 might be extended based on the biological effects of cholesterol, for example, GRh2 might regulate the lipid metabolism in the cancer cell.

Synergistic effects between drugs are usually attributed to that 1) they can interact with the same target and the interaction of one drug with the target can promote the interaction of other drugs with the target, or 2) they are regulated in different pathways, but these pathways can interact with each other. The current studies mainly focus on the typical pathways GRh2 and other drugs both regulate. However, considering the chemotherapeutic agents are well-acknowledged to regulate the typical anticancer pathways, it is more worthwhile to study other associated pathways (eg. metabolism-associated pathways) GRh2 might regulate during exerting synergistic effects.

The side effects of drugs were mainly due to their non-organ-specific or non-tissue-specific. The biological processes in benign cells would be disrupted. GRh2 and its derivative have been proved that they could modulate abnormal biological processes into normal levels (eg. reversing the immunosuppression) [95].

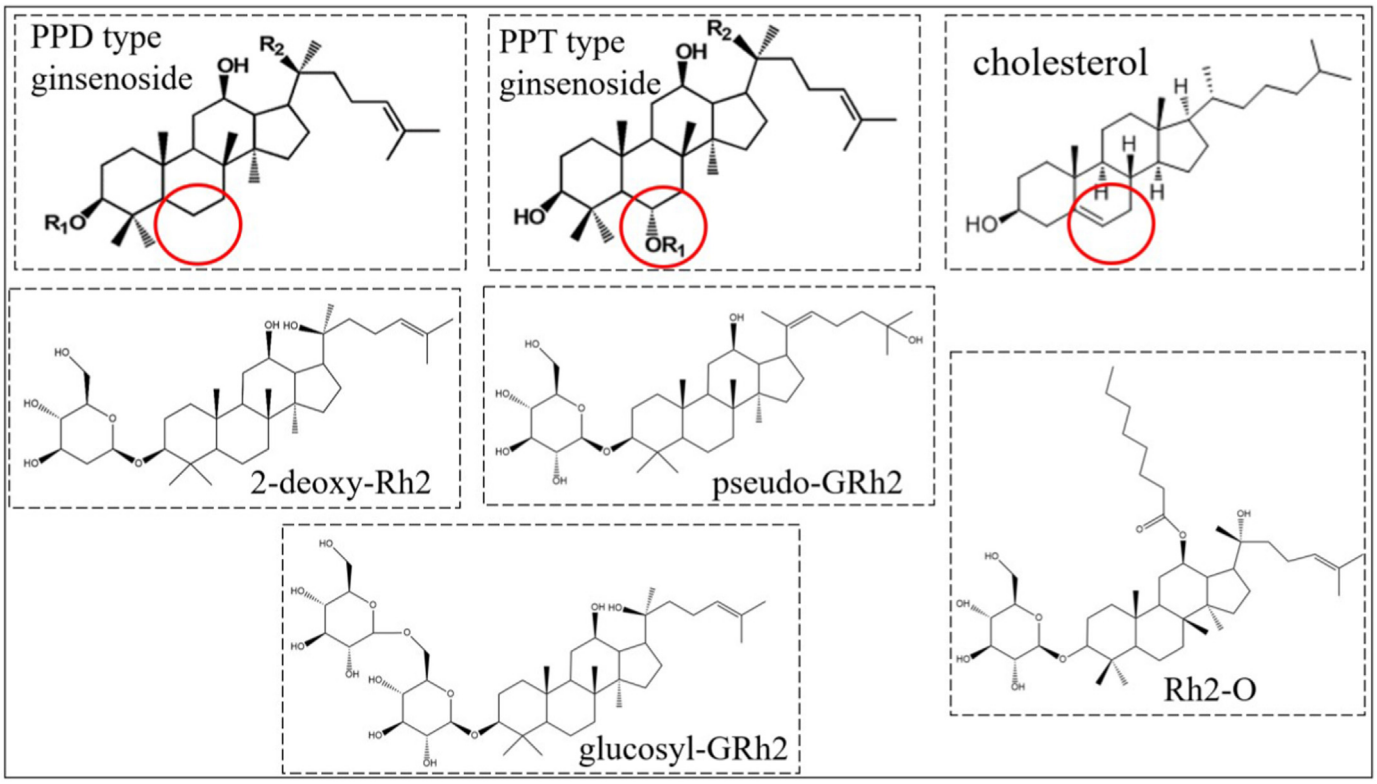


Fig. 4. The structures of cholesterol, ginsenoside and their derivative.

Therefore, the use of GRh2 in adjuvant treatment or polypharmacy would be gradually accepted.

Obviously, there are still several aspects limiting the use of GRh2:

1) There are few clinical trial data that could prove the anti-cancer effects of GRh2. The studies on GRh2 were still in the pre-clinical stage (cell model and mouse model). Considering GRh2 could synergize with many anticancer agents, it would be promising to use GRh2 as an adjuvant agent during cancer therapy if the clinical trial could provide enough evidence for the effectiveness and the safety of GRh2.
2) The aqueous solubility and the bioavailability of GRh2 are relatively low, which limits its function in the human body. According to Lipinski's rule of five, the logP of the compounds should be lower than 5 (from 0-3, to be specific) to obtain high oral bioavailability. However, the logP of GRh2 is 5.62, so the structure of GRh2 needs to be modified. Esterification might be an effective way to increase the lipophilic solubility of GRh2 by inserting a carbon chain [96], thereby increasing the oral bioavailability of GRh2. Zhang et al [97] synthesized octyl ester derivative of ginsenoside Rh2 (Rh2–O) with ethyl acetate and GRh2 (Fig. 4). And the Rh2–O was found to possess higher absorption rate than GRh2 in both Caco2 and HepG2 cell models [97,98]. And then the Rh2–O has been proved to exert better anti-hepatoma activity than GRh2 *in vitro* and *in vivo* [98]. And other derivatives of GRh2 have also been proved to improve the anticancer activity of GRh2. Qian et al synthesized b-D-Glucopyranoside,(3b,12b,20E)-12,25-dihydroxydammar-20(22)-en-3-yl (pseudo-GRh2) (Fig. 4) and the pseudo-GRh2 exerted better anticancer activity than GRh2 in various cell lines [99]. The modification of the sugar moieties (eg. decreasing the number of hydroxyls) is another way to increase the lipophicity. Gao et al [93] replaced the glucose moiety of GRh2 by 2-deoxy-glucose (Fig. 4), and found this modification increased the toxicities of GRh2 to various cancer cell lines. However, considering that the glycosyl addition decrease the lipophicity, the glucosyl-GRh2 exerted poorer anticancer activity than GRh2 [100].
3) the mechanisms of GRh2 exerting the anti-cancer effect have not been completely elucidated yet. There were amounts of typical cell signaling pathways (eg. PI3K/Akt/p-mTOR pathway and ERK/JNK pathway) that participated in GRh2-induced anticancer progress. However, how GRh2 triggered these pathways remain unclear. In general, drugs can bind to target proteins in the cytomembrane or cytosol to activate the signaling cascade. Nevertheless, the target of GRh2 for anticancer has not been discovered adequately, only a few proteins (eg. Annexin A2 and EGFR) have been proved to be the binding target of GRh2. The transcriptomics and proteomics which the current studies on GRh2 lacked might help to discover some novel targets. With these problems solved, the use of GRh2 for adjuvant therapy in cancer might have a bright future. For example, GRh2 could be used as an anti-angiogenesis agent to combine with immune checkpoint blockade for cancer treatment. And as a P-gp inhibitor, GRh2 could be used to improve the absorption of other oral drugs (not only for chemotherapeutics). Moreover, even if GRh2 could not be applicated in clinic at present, it could still be added as a functional phytochemical into the dietary intervention for cancer patients.

Declaration of competing interest

The author declare no conflict of interest.

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