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•Review•

Potential of ginsenoside Rh_2 and its derivatives as anti-cancer agents

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[ABSTRACT] As a steroid skeleton-based saponin, ginsenoside Rh_2 (G-Rh_2) is one of the major bioactive ginsenosides from the plants of genus *Panax* L. Many studies have reported the notable pharmacological activities of G-Rh_2 such as anticancer, antiinflammatory, antiviral, antiallergic, antidiabetic, and anti-Alzheimer's activities. Numerous preclinical studies have demonstrated the great potential of G-Rh_2 in the treatment of a wide range of carcinomatous diseases *in vitro* and *in vivo*. G-Rh_2 is able to inhibit proliferation, induce apoptosis and cell cycle arrest, retard metastasis, promote differentiation, enhance chemotherapy and reverse multi-drug resistance against multiple tumor cells. The present review mainly summarizes the anticancer effects and related mechanisms of G-Rh_2 in various models as well as the recent advances in G-Rh_2 delivery systems and structural modification to ameliorate its anticancer activity and pharmacokinetics characteristics.

[KEY WORDS] Ginsenoside Rh_2; Anticancer; Mechanisms of action; Synergistic effect; Drug delivery systems
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Introduction

Cancer has seriously threatened global health. Although diagnosis and treatment of cancers have made progress, the morbidity and mortality are still substantially increased in recent years [1]. Currently, it is important to find out a novel medication with potent anti-cancer effects and little or no adverse effects. Natural products with particular pharmacological and biological activities have been one of the most important and essential sources in drug design and development for diseases including cancer [2-4].

Ginseng, a medicinal herb with widespread use in Asia and North America, has many pharmacological effects [5-8]. Ginseng contains numerous active components, including ginsenosides, polysaccharides, fatty acids, and mineral oils [9]. Among them, ginsenosides are considered as the major active ingredients of ginseng [10]. Ginsenosides exert their anticancer effects by the modulation of diverse signaling pathways, including the regulation of cell proliferation mediators, growth factors, tumor suppressors, oncogenes, cell death mediators, inflammatory response molecules, and protein kinases [11]. Categorized into protopanaxadiol, protopanaxatriol, and oleanane types according to their chemical structure, ginsenosides have shown wide anticancer properties with diverse characteristics [12, 13]. Ginsenoside Rh_2 (G-Rh_2), a ginsenoside of protopanaxadiol type, has a dammarane core similar to steroid connected to a glucose moiety and a stereocenter on C-20. Thus, it exists as two stereoisomeric forms namely 20(*S*)- and 20(*R*)-G-Rh_2 (Fig. 1). Compared with 20(*R*)-G-Rh_2, 20(*S*)-G-Rh_2 displays an evident anticancer activity both *in vitro* and *in vivo* [14, 15]. The reported studies have indicated stereoselective pharmacokinetic profiles and intestinal biotransformations of G-Rh_2 epimers. The stereoselectivity of G-Rh_2 epimers is one of the factors contributing to its poor oral absorption [16]. ZHANG *et al*. developed a stereoselective LC-MS method for the quantification of G-Rh_2 epimers and the deglycosylation metabolites [17]. G-Rh_2 exists as a trace component in the roots, leaves, and fruit of plants from genus *Panax* L. such as ginseng [18-20]. Moreover, it has been found that protopanaxadiol-type ginsenosides are

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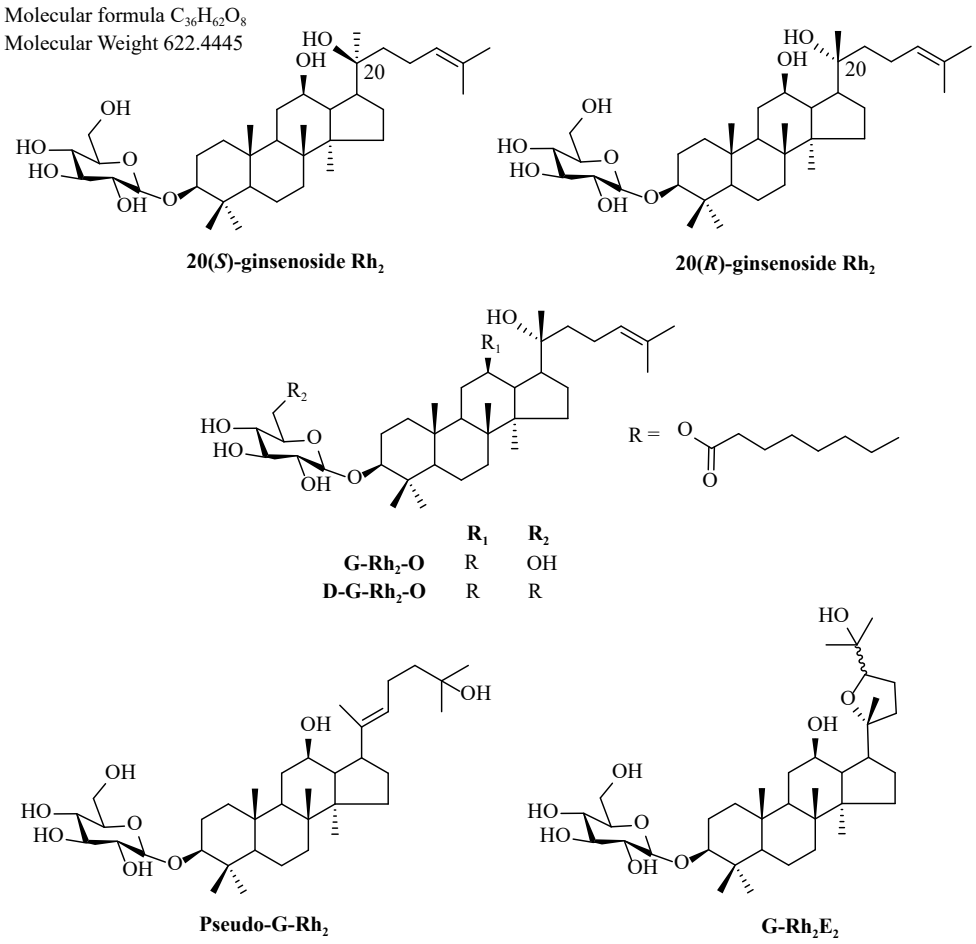

Fig. 1 The structures of the epimers of G-Rh_2 and its derivatives

mainly hydrolyzed or metabolized to G-Rh_2 in the digestive system following oral administration of ginseng extracts [21]. On the other hand, the combinative method of chemistry and biotransformation is applied in the preparation of G-Rh_2 and its derivatives. Total ginsenoside extract or single ginsenoside can be transformed into G-Rh_2 in large quantities by fungal and enzymatic biotransformation [22, 23]. The construction of various engineering bacteria of glycosidase will become one of the research fields to achieve the directional preparation of G-Rh_2 [24]. In addition, biosynthesis is also an important way to obtain G-Rh_2 [25, 26].

The diverse bioactivity of ginsenosides has made them attractive targets for biomedical purposes. A large number of researches have reported that G-Rh_2 has many remarkable biological activities and therapeutic properties such as anti cancer [27, 28] (Fig. 2), antiinflammatory [29], antiviral [30], antiallergic [31], antidiabetic [32, 33], and anti-Alzheimer's [34] activities. The most prominent effect of G-Rh_2 might be its anticancer activity. However, the anti-cancer pharmacological effects of G-Rh_2 have not been systematically reviewed yet. This article aims to summarize the anti-cancer effects and mechanisms of G-Rh_2 and its synergistic effect in combination with other anti-cancer agents (Tables 1−3). The newly developed DDS (drug delivery systems) and structural modifications of G-Rh_2 to strengthen its anticancer effects are also discussed (Tables 4 and 5).

Anti-cancer Effects of G-Rh_2

Leukemia

G-Rh_2 is active against a number of human leukemia cell lines such as KG1α, HL60, and K562 though different mechanisms. G-Rh_2 is involved in multiple processes in the death of tumor cells through different pathways. The following aspects have been observed *in vitro*: 1) G-Rh_2 is able to regulate cell cycle and induce cell cycle arrest. The arrest of the G_1/S phase by G-Rh_2 in HL-60 cells, the block of G_0/G_1 phase by G-Rh_2 in Jurkat cells, and the arrest of G_0/G_1 phase by 20(*S*)-G-Rh_2 in KG-1α and K562 cells have been reported in earlier studies [35-38]. 2) G-Rh_2 inhibits proliferation and induces autophagy and apoptosis through TNF-α (tumor necrosis factor-α) signaling pathway and the suppression of Bcl-2 (B cell lymphoma/lewkmia-2) by modulating miR-21 in HL-60 cells [39, 40], inhibits the PI3K/AKT (phosphotylinositol 3 kinase/serine threonine protein kinase) pathway [38], activates p-p38 in K562 cells [41], reduces AKT/mTOR (mammalian target of rapamycin) signaling and thereby down-regulates PGI/AMF (phosphoglucose isomerase/autocrine motility factor) in KG1α cells [42], and suppresses the

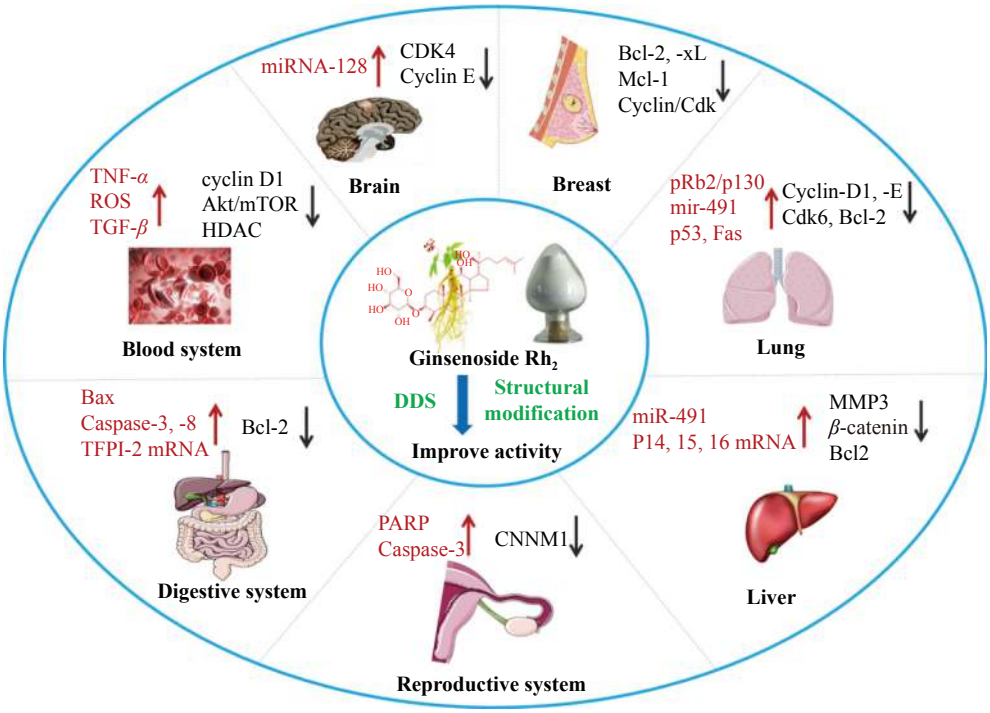

Fig. 2 Anti-cancer effects of G-Rh$_2$ in various carcinomas

Table 1 Effects of G-Rh$_2$ in various cancers *in vitro*

Cancer	Cell models	Effects	Mechanism of action	Reference
Leukemia	HL60, K562, KG1α, Reh, Jurkat, NB4	Regulate cell cycle, induce cell cycle arrest, inhibit proliferation, induce apoptosis and differentiation, autophagy	Modulation of PKC isoform levels; inhibiting HDAC1 and HDAC2, down-regulating the expression of cyclin D1 and activating p16INK4A and p21; up-regulation of TNF-α signaling (*via* suppression of caspase-3, -8, and -9); induction of miR-21; activating p-p38; inhibiting PI3K/AKT pathway; reducing AKT/mTOR signaling; promoting Nur77 translocation; reducing the expression of HDACs and increasing histone acetylation; inhibiting the Wnt/β-catenin/TCF4/CyclinD1 signaling; inducing the release of mitochondrial cytochrome c and activation of caspase-9 and -3; increasing mitochondrial ROS; up-regulation of TGF-β expression; regulating the AKT/Bax/caspase9 and TNF-α/caspase8 pathways	[14, 15], [35-50]
Lung	A549, H1299, H460, A549DDP	Regulate cell cycle, induce apoptosis, inhibit proliferation and metastasis, reverse drug-resistance	Down-regulation of cyclin-D1, cyclin-E and Cdk6, up-regulating pRb2/p130, increasing the expression of DR4; activating ATF4, CHOP, and caspase-4, -3; miRNA genes; increasing the expression of mir-491; up-regulating p53 and Fas and down-regulating Bcl-2; inhibiting Wnt and hedgehog signaling; suppressing EMT; inhibiting glycolysis through STAT3/c-Myc axis	[53-59, 62]
Liver	SK-HEP-1, SMMC-7721, HepG2, Hep3B	Induce cycle arrest, apoptosis, and differentiation, suppress proliferation and migration, autophagy	Inducing expression of p27Kip1, down-regulating cyclin E-dependent kinase activity, activation of caspase-3, cleavage of PARP; reducing telomerase activity; targeting EGFR signaling pathway by up-regulation of miR-491; accumulating ROS; activating lysosomal-mitochondrial apoptotic pathway; regulation of miR-146a-5p; changes in mRNAs in the p53 signaling pathway; decreasing β-catenin; reducing MMP3 expression through the recruitment of HDAC, inhibition of AP-1; release of mitochondrial cytochrome c, activating caspase-3 and Bax, inhibition of Bcl-2; activating CDKN2A-2B, increasing expression of P14, P15 and P16 mRNA; down-regulating Bcl2 and up-regulating Fas; over-expression of Annexin A2; activating MAPK pathway; disturbing Cdc37; inhibiting STAT3/VEGF signaling	[28], [66-79]

Continued

Cancer	Cell models	Effects	Mechanism of action	Reference
Breast	MCF-7, MDA-MB-231, MCF-7/Adr, MCF-7/Doc	Inhibit cell viability, induce cell cycle arrest and apoptosis, inhibit proliferation, reverse multidrug resistance, enhance immunogenicity	Down-regulating Bcl-2, Bcl-xL, and Mcl-1; inducing p21, p15, p27, reducing cyclin D, decreasing phosphorylation of pRb, inhibiting E2F release, down-regulating of cyclin/Cdk; suppressing the expression of C3orf67-AS1; inhibiting P-gp; maintaining the expression levels of permeability P-gp and multidrug resistance gene 1; mediating the expression of miR-222, -34a and -29a; inducing epigenetic methylation; up-regulating STXBP5-AS1; inhibiting NF-κB activation and EMT	[83-93]
Glima	C6, U87MG, U373MG, U251MG, T98MG, A172	Induce apoptosis and cell cycle arrest, inhibit invasiveness and proliferation, decrease cell viability, increase membrane potential	Production of ROS, activating caspase pathway; inhibiting MMPs; modulating AKT signaling pathway, down-regulating cyclin-dependent kinase 4 and cyclin E; up-regulating miRNA-128 expression	[99-103]
Gastric	MFC, SGC-7901SP, HCG-27	Inhibit proliferation, induces apoptosis	Ca^{2+}-dependent mitochondrial apoptosis pathway, activating JNK and caspase-3; up-regulating Bax, down-regulating Bcl-2; inducing mitochondrial damage	[105-109]
Esophageal	Eca-109	Induce apoptosis, inhibit invasion, proliferation and migration	Up-regulating caspase-3 and -8; increasing expression of TFPI-2 mRNA; inhibiting expression of p53 glycoprotein; mediating EGR1/TRL4/mTOR pathway	[109, 110, 112, 113]
Colorectal	HCT116, SW480, LoVo, LoVo/L-OHP, LoVo/5-FU, HCT-8/5-FU, SW620, Caco-2, HT-29, CoLo205	Induce apoptosis and cell death, reverse multidrug resistance, inhibit invasion and migration	Activating p53 pathway; inhibiting the phosphorylation levels of ERK1/2 and histone H3; prevention of cellular proliferation and migration, the promotion of cellular apoptosis and the alteration of drug-resistance genes; inhibiting IL-6-induced STAT3 phosphorylation and the expression of MMP-1, -2, and -9; inhibiting EMT	[27], [114-118]
Prostatic	LNCaP, PC-3, DU145	Induce cell detachment, inhibit proliferation and angiogenesis, increase membrane potential	Modulating MAPK; decreasing expression of CNNM1; suppressing microRNA-4295, activating CDKN1A	[103], [122-124]
Cervical	HeLa, C33A, HEC1A, Ishikawa	Induce cell death and apoptosis, inhibit proliferation, autophagy	Activating caspase cascade; activating ROS and Ca^{2+}-mediated c-JNK1; inhibiting EMT related proteins; Targeting VDAC1 and ETC complex III; enhancing the transcriptional level of p62	[130-137]
Ovarian	SKOV3, HRA	Inhibit proliferation, induce apoptosis	Increasing cleaved PARP and caspase-3	[139, 140]
Melanoma	B16, A375-S2	Induce cell cycle arrest, suppress cell growth	Suppressing Cdk-2 activity; apoptosis related to caspase-3 and -8 pathway	[143-145]

Table 2 Effects of G-Rh$_2$ on various cancers in mouse *in vivo*

Cancer	Mechanism of action	Reference
Leukemia	Prolong the survival time, inhibit the growth of human leukemia xenograft tumors, enhance immune system, promote intestinal microbiota homeostasis by blocking the PI3K/AKT/mTOR signaling pathway	[14, 15, 40, 51]
Lung	Inhibit lung cancer growth by inhibition of ER stress; decrease tumor volumes, activate the immune response against tumor growth, regulate fatty acid metabolism and immunity through the PI3K/AKT signaling pathway; inhibit the angiogenesis and growth of Lewis pulmonary carcinoma by reducing VEGF	[54, 60, 63]
Liver	Suppress tumor growth without causing severe side effects, induce cell apoptosis through down-regulating Bcl-2; modulate the immune response, and down-regulate VEGF	[69, 76, 81, 82]
Breast	Induce apoptosis by the transcript levels and protein expressions of an increase in Bax and Bak and a decrease in Bcl-2, -xL	[83]
Colorectal	Inhibit the growth of xenograft tumors and inhibit the phosphorylation levels of ERK1/2 and histone H3; alleviate tumor-associated depression by the decreases in the depression-associated cytokines, IL-6, -18, and TNF-α, increase survival time, inhibit metastasis of colorectal cancer	[115, 118, 120]
Prostatic	Inhibit tumor growth; increase apoptotic index, decrease tumor cell proliferation	[123, 126]
Cervical	Inhibit tumor growth	[138]
Ovarian	Do or don't inhibit tumor growth and prolong survival time	[140-142]
Melanoma	Enhance the antitumor immunological response, inhibit tumor growth, improve survival time	[146]

Table 3 Effects of G-Rh$_2$ in combination with chemotherapy agents or biophysical therapy *in vitro* and *in vivo*

Model	Combination	Effects	Reference
U937 and K562 cells	20(*S*)-G-Rh$_2$ + blue LED	Enhancing cancer cell death through the induction of ROS, disrupted mitochondrial membrane potential and regulated apoptosis-related genes and proteins	[52]
Mice bearing A549 cells	G-Rh$_2$ + CY	Decreasing tumor volumes, reducing the side effects of CY, enhancing immune response	[63]
A549 and H1299 cells	G-Rh$_2$ + DDP	Repressing superoxide generation, PD-L1 expression, and autophagy	[65]
Mice bearing Lewis lung carcinoma cells	G-Rh$_2$ + CY	Inhibiting tumor growth, decreasing the genotoxic effects induced by cyclophosphamide	[64]
MCF-7/Adr cells	20(*S*)-G-Rh$_2$ + Adr	Enhancing cytotoxicity by down-regulating ABCB1 expression	[94]
MCF-7/MX cells	G-Rh$_2$ + MX	Enhancing cytotoxicity as the inhibitor of BCRP	[95]
MDA-MB-231 and MCF-7 cells	G-Rh$_2$ + biochanin A	Enhancing the inhibition of migration and invasion	[96]
Mice bearing MCF-7/MDR cells	G-Rh$_2$ + etoposide + coix seed oil	Suppressing tumor growth, maintaining body weight, no significant toxicity	[97]
Eca109 cells	G-Rh$_2$ + DDP	Enhancing cell apoptosis	[113]
HCT-116 cells	G-Rh$_2$ + sodium selenite	Enhancing the inhibition of cell growth, the increase of apoptosis rate, Bax/Bcl-2 ratio and caspase-3 expression, and the depletion of ROS production and autophagy, lowering systemic toxicity	[121]
LoVo/L-OHP cells	G-Rh$_2$ + L-OHP	Enhancing the expression of SMAD4, Bax and caspase-3, reducing the expression of P-gp and Bcl-2	[27]
HCT-116 cells	20(*S*)-G-Rh$_2$ + doxorubicin	Exhibiting much stronger cytotoxic activity, increasing doxorubicin sensitivity through the inhibition of STAT3 activation	[117]
LNCaP cells and mice bearing LNCaP cells	G-Rh$_2$ + paclitaxel	Inhibiting cell proliferation, decreasing tumor growth and serum prostate specific antigen	[127]
LNCaP cells and mice bearing LNCaP cells	G-Rh$_2$ + MX	No significant benefit	[127]
LNCaP and C4-2 cells	G-Rh$_2$ + calcitriol	Inhibiting proliferation, down-regulating androgen receptor expression	[128]
Mice bearing PC-3 cells	G-Rh$_2$ + Doc	Inhibiting tumor growth, decreasing cell proliferation rates	[129]
Mice bearing HRA cells	G-Rh$_2$ + DDP	Inhibiting tumor growth, prolonging survival time	[140, 141]
Mice bearing B16 cells	G-Rh$_2$ + CY	Inhibiting tumor growth, decreasing the genotoxic effects induced by cyclophosphamide	[64]

Table 4 Comparesion of G-Rh$_2$ and its derivatives on the anti-cancer effects

Action-types	Prototype			Derivatives			Reference
	G-Rh$_2$	20(*S*)-G-Rh$_2$	20(*R*)-G-Rh$_2$	G-Rh$_2$-O	D-G-Rh$_2$-O	G-Rh$_2$E2	
Cellular uptake rate in HepG2 cells (for 24 h)	63.2%	–	–	28.1%	–	–	[155]
Cytotoxicity in HepG2 cells (IC$_{50}$, μmol·L^{-1})	20.15	–	–	42.12	–	–	[155]
Cytotoxicity in QSG-7701 cells (IC$_{50}$, μmol·L^{-1})	37.3	–	–	–	80.5	–	[159]
Cell viability in LLC-1 cells (at 100 μmol·L^{-1})	–	8%	88%	–	–	25%	[162]
Cell viability in CCD-19Lu cells (at 100 μmol·L^{-1})	–	20%	95%	–	–	95%	[162]
Inhibitory rate in H22 tumor-bearing mice (10 mg·kg^{-1})	28.2%	–	–	50.6%	–	–	[82]
Inhibitory rate in H22 tumor-bearing mice (10 mg·kg^{-1})	none	–	–	none [a]	–	–	[158]
Inhibitory rate in H22 tumor-bearing mice (20 mg·kg^{-1})	53%–75%	–	–	–	52%–73%	–	[159]
Inhibitory rate in LLC-1 tumor bearing mice (80 mg·kg^{-1}, oral)	–	little or no effect	little or no effect	–	–	52.2%	[162]
Inhibitory rate in LLC-1 tumor bearing mice (20 mg·kg^{-1}, injection)	–	–	–	–	–	55.5% (20*S*) 67.3% (20*R*)	[163]

[a] The tumor sizes in the G-Rh$_2$-O and cyclophosphamide treatment groups were equivalent, smaller than that in the control group.

Table 5 G-Rh_2 delivery systems and their bioefficacy

Delivery systems	Composition	Bioefficacy	Reference
Sef-Microemulsions	SME-1: Miglyol, Tween-20, and labrasol (2 : 6 : 2, weight ratio) mixed with 40 mg DOS-1227 (purity of 76%, containing 18.8% Rh_1 and 10.1% Rh_2). SME-2: soybean, Tween-80, and glycerin (1 : 4.5 : 4.5, weight ratio) mixed with 40 mg DOS-1227 (purity of 76%, containing 18.8% Rh_1 and 10.1% Rh_2).	Increase cellar uptake, enhance transfer rate, increase distribution in lymph nodes, increase absolute bioavailability in rats	[166, 167]
	Ethyloleate-Tween 80-Tanscutal P-Rh_2 (29 : 44 : 25 : 2, weight ratio); LabrafilM 1944 CS-Tween 80-Tanscutal P-Rh_2 (29 : 39 : 30 : 2, weight ratio)	Increase solubility, increase rats intestinal absorption rate	[168]
Nanoparticles	Zinc oxide (ZnO), hyaluronic acid (HA), loading approximately 0.32 μg G-Rh_2 per 1 mg Rh_2HAZnO nanoparticles	Enhance cytotoxicity of G-Rh_2 in A549, HT29, and MCF-7 cells, increase therapeutic effects by inducing apoptosis through the generation of ROS *via* the activation of the caspase-9/p38 MAPK pathway	[169]
	Bovine serum albumin (BSA) and G-Rh_2	Exhibit good solubility and stability in an aqueous system, enhance the anti-cancer ability	[170]
	Water phase (egg yolk lecithin : PEG 2000-DSPE, 3 : 1) : oil phase (Rh_2 : PLGA, 1 : 3), 1 : 5	Show sustained release characteristics, increase the anti-proliferation and enhance the activity in combination with borneol in glioma C6 cells	[171]
	Tetraethyl orthosilicate as a silica source, 3-aminopropyltriethoxy-silane as a coupling agent, and fluorescein isothiocyanate as a dye	Show excellent biocompatibility, increase cytotoxicity in several cancer cell lines, higher amount of loading of G-Rh_2	[172]
	Hydrogen tetrachloroaurate (III) hydrate (gold salt)	Exhibit similar cytotoxicity in J774A.1 and HEK-293 cell lines at equivalent ginsenoside contents between G-Rh_2 and G-Rh_2-GNPs	[173]
	Chitosan 4 mg, 1% acetic acid 4 mL, G-Rh_2 1.2 mg, tripolyphosphate	Suppress the growth, invasion, and migration of SW480 and SW620 cells	[175]
	Pullulan polysaccharide, urocanic acid, α-lipoic acid, G-Rh_2	Increase drug release under acid environment, decrease MMP and reduce oxidative stress level with higher cytotoxicity in HepG2 cells	[174]
Nanocomposites	POL_{188} and G-Rh_2 (1 : 1, *W/W*)	Increase solubility and bioavailability, enhance cytotoxicity by the promotion of cellular uptake *via* endocytosis	[176]
Nanostructures	Porous grapheme, lysine, arginine, G-Rh_2	Enhance cytotoxic activity	[177]
Microemulsions	Etoposide 50 mg, coix seed oil 4.0 g, cremophor RH40 3.7 g, G-Rh_2 0.3 g, PEG400 1.0 g, deionized water 20 mL	Enhance cytotoxicity and reverse drug-resistance in MCF-7/MDR cells, inhibit tumor growth significantly, no significant toxicity *in vivo*	[97]
	Etoposide 4 mg, coix seed oil 400 mg, cremophor RH40 360 mg, G-Rh_2 30 mg, PEG400 120 mg, deionized water 3 mL	Prolong retention in cytoplasma and deep tumor penetration, increase the level of Th1 cytokines, no systemic toxicity	[178]
Liposomes	Rh_2-PLP: cholesterol, hydrogenated soya phosphatide (HSPC), methoxy poly(ethylene glycol)-poly(lactide) (mPEG-PLA), mannitol, G-Rh_2 Rh_2-CLP: cholesterol, hydrogenated soya phosphatide (HSPC), octadecylamine, mannitol, G-Rh_2. Rh_2-LP: cholesterol, hydrogenated soya phosphatide (HSPC), mannitol, G-Rh_2	Enhance accumulation of drug in tumor tissue, inhibit tumor growth significantly, no significant toxicity *in vivo*	[179]
	Soybean lecithin, cholesterol, glycyrrhetinic acid, DSPE-mPEG 2000, G-Rh_2	Enhance cytotoxicity	[180]
	Egg yolk lecithin 20 mg, G-Rh_2 6 mg, CH_3CH_2OH : $CHCL_3$ (1 : 1) 1 mL	Prolong drug circulation, enhance accumulation, exhibit synergistic effect, remodel tumor-associated microenvironment	[181]
Micelles	Polyethylene glycol (PEG),celastrol and G-Rh_2	Improve synergistic anti-cancer effect in A549 cells	[182]
Self-assembled micelles	G-Rh_2 17.5 mg, Solutol® HS15 140 mg, TPGS 60 mg	Increase cellular uptake and accumulation, improve solubility, enhance anti-tumor efficacy	[183]
Multicore niosomes	mPEG2000-Hz-CHEMS, mPEG2000-IS and G-Rh_2 (3 : 1 : 1, *W/W/W*); Pluronic F-68	Decrease toxicity and tumor volume	[184]
Nanoniosomes	1,2-dioleoyl-3-trimethylammonium-propane, cholesterol, Span 60, G-Rh_2	Enhance cytotoxicity, increase cellular uptake	[185]

PI3K/AKT/mTOR pathway in Jurkat cells [43]. Moreover, the induction of leukemia cell apoptosis by 20(*S*)-G-Rh_2 has been reported to involve Nur77-mediated signaling pathway in HL-60 cells [14], the inhibition of histone deacetylase HDAC1 and HDAC2 activity, the down-regulation of cyclin D1 and activation of p16INK4A and p21 in K562 cells [36], the reduction in HDACs expression and activity, the increase of histone acetylation and the regulation of key proteins by downstream signaling pathways in K562 and KG1α cells [15], the inhibition of Wnt/β-catenin/TCF4 (transcription factor 4) /cyclin D1 signaling pathway in a dose and time-dependent manner [44], the down-regulation of the transcription of β-catenin/TCF4 target genes such as cyclin D1 and c-Myc through the Wnt/β-catenin signaling pathway in KG1α cells [37], and the regulation of the AKT/Bax/caspase 9 and TNF-α (tumor necrosis factor-α) /caspase 8 pathways in NB4 cells [45] and mitochondrial pathway in K562, Reh and Jurkat cells [46-49]. In addition, the generation of mitochondrial ROS (reactive oxygen species) is aggravated when autophagy is inhibited. Mitochondrial damage is thus increased, which accelerates mitochondria-dependent apoptosis, indicating that the strategy to induce apoptosis while inhibiting autophagy can be utilized to accelerate tumor cell death [49]. 3) G-Rh_2 can induce HL-60 cells differentiation into morphologically and functionally normal granulocytes by up-regulating the expression of TGF-β (transforming growth factor-β) while the modulation of PKC (protein kinase C) isoform levels favors its differentiation [35, 50].

In addition to the experiments *in vitro*, several *in vivo* studies have been performed in the acute leukemia model to evaluate the antitumor activity of G-Rh_2. The development of human leukemia xenograft tumors was inhibited by the administration of 20 mg·kg^{-1} of 20(*S*)-G-Rh_2 in NOD/SCID (nonobese diabetic/severe combined immunodeficiency) mice carrying HL-60 and Jurkat cells, and naked mice carrying K562 cells [14, 15, 43]. Moreover, the anticancer mechanisms of 20(*S*)-G-Rh_2 involves enhancing immune system and promoting intestinal microbiota homeostasis by blocking the PI3K/AKT/mTOR signaling pathway in T-cell acute lymphoblastic leukemia mice bearing Jurkat cells [51].

ZHUANG *et al*. reported that 20(*S*)-G-Rh_2 combined with blue LED therapy had a stronger death-promoting effect on K562 cells compared with the same treatments given separately [52]. The combined treatment resulted in a reinforced mutual interaction between apoptosis and autophagy, leading to the enhanced cell death in cancer tissues.

Lung cancer

Many researches have focused on exploring the anti-proliferative effects of G-Rh_2 in lung cancer cell lines. A549 cells treated with G-Rh_2 resulted in G_1 phase growth arrest and then advancement to apoptosis. The effects on cell cycle regulatory proteins include the down-regulation of cyclin-E, cyclin-D1, and Cdk6, and the up-regulation of pRb2/p130, while a rise in DR4 (death receptor 4) expression acted as key factors in G-Rh_2 induced apoptosis [53]. G-Rh_2-induced apoptosis *via* DR4 and caspase-dependent pathway is shown in Fig. 3. It shows that G-Rh_2 induces the apoptosis of A549 cells through a DR4-dependent but Bcl-2-independent activation of a caspase-8/caspase-3 pathway. G-Rh_2 can induce ROS mediated ER (endoplasmic reticulum) stress-dependent cell apoptosis, thereby inhibiting the proliferation of H1299 cells in a dose-dependent manner, which involves the activation of CHOP (CCAAT/enhancer-binding protein homologous protein) caspase 4 [54]. Furthermore, ZHANG *et al*. reported that G-Rh_2 affected A549 cell proliferation significantly through the inhibition of Wnt and hedgehog signaling by the phosphorylation of α-catenin at S641 site [55]. G-Rh_2 also inhibited proliferation and metastasis by promoting apoptosis and suppressing EMT (epithelial-mesenchymal transition) in A549 and H460 cells, and it is more noteworthy that G-Rh_2 exerted a glycolysis inhibition effect through the STAT3 (signal transducer and activator of transcription 3)/c-Myc axis [56]. Being able to stimulate G_1 phase arrest in A549 cells and inhibit cell proliferation, 20(*S*)-G-Rh_2 has more significant anticancer effects compared with 20(*R*)-G-Rh_2 [57]. AN *et al*. reported that miRNAs played a vital role in the G-Rh_2-mediated anti-cancer effect in A549 cells by a number of target genes regulating chromatic modification, apoptosis, angiogenesis, differentiation and cell proliferation [58].

Hypoxia-induced migration can be due to increases of HIF-1α (hypoxia inducible factor-1α) and its target genes MMP-1 (matrix metalloproteinase), MMP-9, and EGFR (epidermal growth factor receptor) expressions in NSCLC (non-small cell lung cancer). In hypoxic tumor microenvironment, G-Rh_2 exhibited anti-metastasis activity in A549 and H1299 cells by increasing mir-491 expression [59]. Furthermore, the intraperitoneal injection of 0.3−3.0 mg·kg^{-1} G-Rh_2 inhibited angiogenesis and growth of lewis pulmonary carcinoma to a significant level by reducing protein expression of VEGF (vascular endothelial growth factor) in mice [60].

G-Rh_2 also exhibits its anti-cancer effects by reversing MDR (multidrug resistance). A study found that the toxic concentration of 10 μmol·L^{-1} G-Rh_2 reversed the resistance of A549DDP cells to DDP (cisplatin), and the reverse resistance was 3.5 times. The tumor apoptotic rate in DDP + G-Rh_2 group differed significantly from the control group ($P < 0.01$) [61]. Moreover, G-Rh_2 promoted the apoptosis and arrest at the G_0/G_1 phase of A549/DDP cells by up-regulating p53 and Fas and down-regulating Bcl-2 [62].

In addition, G-Rh_2 intensified the anti-tumor effect and diminished the adverse effects of CY (cyclophosphamide) in NSCLC mice, which involves the ability of G-Rh_2 to regulate fatty acid metabolism to improve immune deficiency by targeting the PI3K/AKT pathway [63]. The combination of G-Rh_2 and CY notably curbed tumor growth in C57BL/6 mice with Lewis lung carcinoma cells, as well as decreased the CY induced genotoxic effects such as DNA damage and micronuclei formation [64]. In another study, G-Rh_2 enhanced the anti-cancer effects of DDP in A549 and H1299 cells by repressing superoxide generation, PD-L1 (programmed death-ligand 1) expression and autophagy [65].

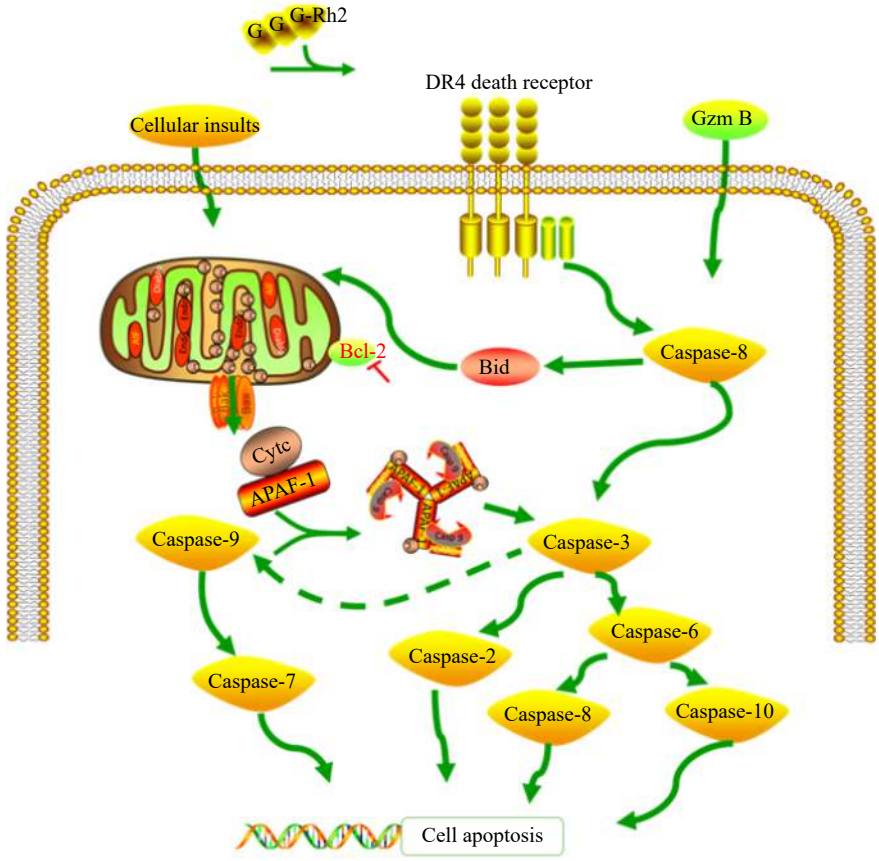

Fig. 3 Schematic representation of G-Rh$_2$ in the routes to caspase activation within the cell apoptosis

Liver cancer

G-Rh$_2$ has exhibited potent anti-cancer activity against different hepatoma cell lines. In SK-HEP-1 cells, G-Rh$_2$ selectively induced p27Kip1 protein expression to down-regulate cyclin E-dependent kinase activity, arrested the cell cycle at the G_1/S transition phase and promoted apoptosis by Bcl-2-insensitive activation of caspase-3 as well as proteolytic cleavage of PARP (poly ADP-ribose polymerase) [66, 67]. In SMMC-7721 cells, G-Rh$_2$ arrested cell cycle at the G_1/G_0 phase, induced cell differentiation, and reduced telomerase activity by affecting transcription levels of human telomerase reverse transcriptase [68]. It also targeted the EGFR signaling pathway in SMMC-7721 cells by the up-regulation of miR-491, showing the effects of decreasing tumor cell proliferation and cell viability [69]. G-Rh$_2$ induced apoptosis in HepG2 cells either by accumulating ROS and activating the lysosomal-mitochondrial apoptotic pathway involving the release of Cat B (cathepsin B) [70] or by the regulation of miR-146a-5p expression [71]. G-Rh$_2$ also inhibited proliferation in a time and dose-dependent manner, and the anti-proliferation effect was related to changes in mRNAs in the p53 signaling pathway [28], or by *β*-catenin signaling through coordinated autophagy [72]. G-Rh$_2$ inhibited the migratory ability of HepG2 cells by reducing the MMP-3 expression through the recruitment of HDAC and the inhibition of AP-1 (activator protein 1) [73]. As an HDAC1 inhibitor, G-Rh$_2$ increased histone acetylation, activated the MAPK (mitogen-activated protein kinase) signaling pathway, down-regulated the expression levels of HIF, promoted apoptosis and inhibited tumor angiogenesis in HepG2 cells [74]. In Hep3B cells, G-Rh$_2$ has been found to induce apoptosis and stimulate the release of mitochondrial cytochrome c, inhibit Bcl-2, activate caspase-3 and Bax, and produce intracellular ROS through mitochondrial signaling pathways [75].

20(*S*)-G-Rh$_2$ suppressed cell proliferation and migration by targeting EZH2 (enhancer of zeste homologue 2) to activate CDKN2A-2B gene transcription and increase expression of P14, P15, and P16 mRNA genes in HepG2 and Hep3B cells [76]. CHEN *et al*. found that 20(*S*)-G-Rh$_2$ exerted the anti-tumor effects by targeting HSP90A (heat shock protein 90 alpha) and consequently disturbing the HSP90A-Cdc37 (cycle control protein 37) chaperone system in HepG2 cells [77]. 20(*S*)-G-Rh$_2$ also showed more potent anti-cancer effects through autophagy than their 20(*R*)-forms *via* calcium-dependent apoptosis in HepG2 cells [78]. Furthermore, WANG *et al*. identified Annexin A2 as a potential target for the anti-cancer effects of 20(*S*)-G-Rh$_2$, as its overexpression resulted in promoting apoptosis in HepG2 cells [79]. Further studies showed that 20(*S*)-G-Rh$_2$ was a potent inhibitor of STAT3, and the anti-cancer activity was manifested through the inhibition of STAT3/VEGF signaling by targeting Annexin A2 [80].

The anti-tumor effect of G-Rh$_2$ *in vivo* has also been re-

ported. In H22-tumor bearing mice, both 20(*S*)- and 20(*R*)-G-Rh_2 with 6 mg·kg^{-1} caused suppression in tumor growth without causing severe adverse effects and induced cell apoptosis through down-regulating Bcl-2 [81]. Another study reported that 10 mg·kg^{-1} G-Rh_2 inhibited tumor growth by inducing apoptosis of tumor cells, down-regulating VEGF expression, and regulating immune response [82]. A concentration of 1 mg·kg^{-1} body weight of G-Rh_2 significantly inhibited the growth of tumors in NOD/SCID mice carrying SMMC-7721 cells[69]. 20(*S*)-G-Rh_2 significantly suppressed tumor development as well as angiogenesis in nude mice carrying HepG2 cells [76].

Breast cancer

G-Rh_2 is active against human breast cancer cell lines. Through mitochondria-mediated apoptosis and down-regulation of Bcl-xL, Bcl-2, and Mcl-1, G-Rh_2 inhibited the viability of both MDA-MB-231 and MCF-7 cells in a concentration-dependent manner [83]. It also induced G_1 arrest by inducing p21, p15, and p27, reducing cyclin D, decreasing pRb phosphorylation and inhibiting E2F release, which is involved in the down-regulation of cyclin/Cdk complex kinase activity [84, 85]. G-Rh_2 also exhibited an anti-proliferative effect by suppressing the expression of non-coding RNA C3orf67-AS1 in MCF-7 cells [86]. Moreover, G-Rh_2 has shown a potent effect of reversing multidrug resistance. In MCF-7/Adr cells, 20(*S*)-G-Rh_2 increased the amount and rate of Adr (adriamycin) seeping into cellular/subcellular compartments by inhibiting P-gp (P-glycoprotein) activity, and in turn augmenting the apoptosis induced by Adr [87, 88]. G-Rh_2 has the ability to reverse multidrug resistance in MCF-7/Adr cells without changing the levels of expression of P-gp and multidrug resistance gene 1 [89]. It can mediate the expression of miRNAs (miR-222, miR-29a, and miR-34a) to reduce drug-resistance in MCF-7/Doc (docetaxel) and MCF-7/Adr cells, cutting off the drug-resistance process [90]. In addition, in MCF-7 cells, G-Rh_2 can stimulate epigenetic methylation changes in genes designated for immune response and tumorigenesis, hence enhancing immunogenicity and inhibiting their growth [91], and upregulate long noncoding RNA STXBP5-AS1 to sponge microRNA-4425 in suppressing cell proliferation [92]. Notably, 20(*S*)-G-Rh_2 exerted anti-cancer effects by the inhibition of NF-κB activation and related EMT *via* targeting Annexin A2 in MDA-MB-231 cells [93].

G-Rh_2 also exerted anti-tumor effect in *in vivo* xenografts. Oral gavage of 5 mg·kg^{-1} G-Rh_2 in mice bearing MDA-MB-231 cells presented significant apoptosis caused by a rise in transcript levels and protein expressions of Bak and Bax and a decrease in that of Bcl-xL and Bcl-2 [83].

The cytotoxicity of Adr was synergistically affected by 20(*S*)-G-Rh_2 through down-regulating the expression of Adr-induced ATP-binding cassette B1 in MCF-7/Adr cells *via* MAPK/NF-κB pathway [94]. G-Rh_2 acted as the inhibitor of breast cancer resistance protein and significantly enhanced the MX (mitoxantrone) cytotoxicity to MCF-7/MX cells in human breast carcinoma [95]. The combination of G-Rh_2 and biochanin A enhanced the inhibition of migration and invasion against proliferation in MCF-7 cells and MDA-MB-231 cells [96]. Furthermore, QU *et al*. reported that G-Rh_2 exhibited a synergistic anti-tumor effect with etoposide and coix seed oil on mice xenograft models of drug-resistant breast tumors (bearing MCF-7/MDR) tissue [97].

Glioma

G-Rh_2 induced apoptosis in C6Bu-1 cells through the members of Bcl-2 family [98], and regulated cell death in C6 cells through ROS generation and Bcl-xL-independent activation of the caspase pathway [99]. Glioma invasion progresses through the multiple interplay of many MMP molecules, and the broad-spectrum inhibition by G-Rh_2 suppressed the invasiveness of glioma cells by the inhibition of MMPs through NF-κB and AP-1 in U87MG and U373MG cells [100]. G-Rh_2 also inhibited proliferation and arrest cell cycle by modulating the AKT signaling pathway in A172 cells, and the cycle arrest took place alongside the down-regulation of cyclin-dependent kinase 4 and cyclin E [101]. In human glioma neurosphere cultures that possessed features of glioma "stem-like" cells, the anti-proliferative effect of G-Rh_2 was mediated partly *via* up-regulation in the expression of miRNA-128 in U251, T98MG, and A172 cells [102]. Lee *et al*. found that the anti-cancer effect of G-Rh_2 in C6 cells was related to an increase in membrane potential in a Na^+ independent manner [103]. Furthermore, both 20(*S*)-G-Rh_2 and G-Rh_2 can cause a dose- and time-dependent decrease in the viability of glioma cells [100, 104].

Gastric cancer

In vitro, G-Rh_2 induced apoptosis in MFC cells possibly through Ca^{2+}-dependent mitochondrial apoptosis pathway and by activating the JNK transduction pathway and expression of caspase-3, and the anti-proliferation effect was in a dose-and-time-dependent manner [105, 106]. As a subtype of cells, SP (side population) has a faster proliferation rate, so SP may play a crucial role in tumor progression. 20(*S*)-G-Rh_2 caused a gradual inhibition in the proliferation of SGC-7901 SP cells through G_1/G_0 phase arrest, followed by the apoptosis involving an up-regulation of Bax and a simultaneous down-regulation of Bcl-2 [107]. G-Rh_2 could inhibited the proliferation in HCG-27 cells by inducing mitochondria damage, dysfunction of lysosomes, and blockade of autophagy flux [108].

Esophageal cancer

G-Rh_2 exhibited anti-cancer effects through inducing apoptosis and inhibiting invasion and proliferation in Eca-109 cells. The cell apoptosis was possibly caused by up-regulating the expressions of caspase-3 and -8 and the inhibition of invasion was related to an increased expression of TFPI-2 (tissue factor pathway inhibitor-2) mRNA. G-Rh_2 has an effect on the expression of cell cycle components (cyclinE, CDK2 and p21WAF1) to inhibit Eca-109 cell proliferation. The suppression of proliferation involves the cell cycle arrest at the G_0/G_1 phase and the cell differentiation to normal [109-111]. G-Rh_2 exhibited the inhibition of cell proliferation, migration and EMT of Eca-109 cells by mediating

EGR1/TRL4/mTOR (early growth response protein 1/Toll like receptor 4) signaling pathway [112]. G-Rh_2 synergized with DDP in the apoptosis of Eca109 cells, and the mechanism was related to inhibiting p53 glycoprotein expression [113].

Colorectal cancer

G-Rh_2 induced apoptosis and paraptosis-like cell death by activating the p53 pathway in HCT116 and SW480 cells [114]. The plausible molecular mechanisms regulating the anti-cancer effects of G-Rh_2 was elucidated in HCT116 cells, indicating that G-Rh_2 can directly bind with PBK/TOPK (PDZ-binding kinase/T-LAK cell-originated protein kinase) and inhibit PBK/TOPK activity, thus inducing cell death by suppressing the degree of phosphorylation of ERK1/2 (extracellular regulated protein kinases) and histone H3 [115]. By altering the drug resistance gene, preventing cellular proliferation and migration, and simultaneously promoting apoptosis, G-Rh_2 can effectively reverse drug-resistance in human colorectal carcinoma drug-resistance cells [27, 116].

HAN *et al*. found that, compared with 20(*R*)-G-Rh_2, only 20(*S*)-G-Rh_2 inhibited cancer cell invasion, which was acted by inhibiting IL-6-induced STAT3 phosphorylation and the expression of MMP-1, -2, and -9 in HCT116 and SW620 cells [117]. Meanwhile, 20(*S*)-G-Rh_2 inhibited the migratory and invasive abilities of SW480 and CoLo205 cells by the down-expression of MMP-2 and -9, and regulated cell metastasis by affecting EMT [118]. 20(*S*)-G-Rh_2 also inhibited cell growth by arresting the cell cycle S phase in Caco-2 and HT-29 cells [119].

In *in vivo* studies, 10 $mg \cdot kg^{-1}$ of G-Rh_2 inhibited the growth of HCT116 tumors xenograft and supressed the phosphorylation levels of ERK1/2 and histone H3 in nude mice [115]. Furthermore, G-Rh_2 alleviated tumor-associated depression in NOD/SCID mice carrying Caco-2 cells at the dose of 5 $mg \cdot kg^{-1}$ through the decreases in the depression-associated cytokines, TNF-α, IL-6 (interleukin 6), and IL-18, significantly increasing survival time [120]. In nude mice bearing CoLo205 cells, 20(*S*)-G-Rh_2 significantly inhibited lymph node metastasis and tumour growth without affecting other normal organs [118].

G-Rh_2 and sodium selenite combination enhanced cell growth inhibition, increased apoptosis rate, Bax/Bcl-2 ratio, caspase-3 expression, and the depletion of ROS production and autophagy, resulting in a synergistic anti-cancer effect and contributing to lower systemic toxicity in HCT-116 cells [121]. Similarly, G-Rh_2 and L-OHP (oxaliplatin) treatment significantly enhanced the expression of SMAD4 (SMAD family member 4), caspase 3, and Bax and reduced P-gp and Bcl-2 expression in LoVo/L-OHP cells [27]. Moreover, the combination of 20(*S*)-G-Rh_2 and doxorubicin exhibitd a stronger cytotoxic activity and enhanced doxorubicin sensitivity by inhibiting STAT3 activation and inducing apoptosis in HCT-116 cells [117].

Prostatic cancer

G-Rh_2 can induce cell detachment and inhibit PC3 and LNCaP cells from proliferating by modulating MAPK [122]. By inhibiting angiogenesis *via* decreasing the expression of the CNNM1 gene, G-Rh_2 inhibited cell growth of LNCaP, PC3, and DU145 [123]. In another study, GAO and ZHENG reported the growth inhibition of PC3 and DU145 cells (but not altered cell apoptosis) by G-Rh_2 through the suppression of microRNA-4295 that activated CDKN1A (cyclin-dependent kinase inhibitor 1A) [124]. G-Rh_2 also increased membrane potential in PC3 cells in a G-protein-coupled receptor independent manner [103]. As in other cancer cell types, only 20(*S*)-G-Rh_2 showed proliferation inhibition in *in vitro* models, which were verified in androgen-dependent LNCaP cells as well as androgen-independent PC-3 and DU-145 cells [125].

G-Rh_2 can significantly inhibit the volume of tumor tissue in the dorsum of nude mice bearing cancer cells [123], and it has been proved to prevent PC-3 tumor progression, increase the apoptotic index and reduce the tumor cell proliferation in nude mice [126].

Paclitaxel combined with G-Rh_2 had a synergistic effect on growth inhibition in LNCaP cells and resulted in a notable decrease in the growth of tumor and serum prostate-specific antigen *in vitro* and *in vivo*. However, no significant benefit was observed in the combination of G-Rh_2 and MX [127]. The combination of G-Rh_2 and calcitriol in LNCaP and C4-2 cells exhibited significant proliferation inhibitory effect, and the synergism was related to the downregulation of androgen receptor expression [128]. Meanwhile, the combination of G-Rh_2 and Doc in nude mice bearing PC-3 cells could inhibit tumor growth (15% smaller), lower apoptotic indices and induce significant tumor regression, causing a decrease in the rate of cell proliferation as opposed to a rise in the extent of apoptosis [129].

Cervical cancer

G-Rh_2 induced cell death by activation of caspase cascade and inhibit proliferation [130] and induced apoptosis and mitochondrial depolarization by ROS and Ca^{2+}-mediated JNK1 (c-Jun NH_2-terminal kinase 1) activation in HeLa cells [131]. G-Rh_2 induced apoptosis of HeLa cells by targeting the gene of VDAC1 (voltage-dependent anion channel 1) [132]. HeLa cells were more sensitive to G-Rh_2 than C33A cells with regard to MMP and ATP generation, and G-Rh_2 induced mitochondrial ROS production and promoted cell apoptosis by targeting the ETC (electron transport chain) complex III [133]. G-Rh_2 enhanced the transcriptional level of p62 protein, and the silencing of p62 had no significant effect on autophagy induced by G-Rh_2, which indicated that combining G-Rh_2 with inhibition of p62 might improve the treatment of cervical cancer [134]. Notably, G-Rh_2 did not affect apoptosis in cancer cells cultured in normal medium, but markedly enhanced apoptosis under serum deprivation conditions in Hela and C-33A cells by caspase-independent mechanism *via* autophagy [135]. Using HEC1A and Ishikawa cell lines, KIM *et al*. found that G-Rh_2 caused a significant dose-dependent suppression of cell proliferation and induced apoptosis through EMT related proteins inhibition, such as E-cadherin, vimentin, TGF-β and Snail [136]. Moreover, 20(*S*)-G-

Rh_2 also induced apoptosis and enhanced cell autophagy through inhibiting AMPK/mTOR (adenosine 5'-monophosphate (AMP)-activated protein kinase) pathway in Hela cells [137].

In vivo, oral gavage of G-Rh_2 at 10 mg·kg^{-1} significantly inhibited the growth of tumor in the U14 cervical cancer-bearing mouse, and the effect of 20(*S*)-G-Rh_2 was stronger than that of 20(*R*)-G-Rh_2 [138].

Ovarian cancer

G-Rh_2 inhibited proliferation while simultaneously inducing apoptosis in SKOV3 cells, which were related to the increase in the levels of cleaved PARP and caspase-3. Additionally, the migration assays showed that G-Rh_2 inhibited the cell mobility of SKOV3 cells. The EMT was regulated by transcription factors, including Snail, Twist, and ZEB [139]. A virtually similar anti-proliferative effect was also found in HRA cells [140].

The growth of HRA cells transplanted in nude mice can not be inhibited by G-Rh_2 [140, 141]. However, TODE *et al*. found that G-Rh_2 can inhibit tumor growth and prolong survival time [142]. So it will require further efficacy evaluation of the treatment with G-Rh_2 on ovarian tumors.

The combination of G-Rh_2 and DDP can significantly inhibit tumor growth and prolong survival time compared with DDP or G-Rh_2 alone [140, 141].

Melanoma

G-Rh_2 induced G_1 phase arrest in B16 cells by suppressing Cdk-2 activity [143, 144], which induced apoptosis to inhibit the growth of A375-S2 cells that was dependent in part on caspase-3 and -8 pathway [145].

G-Rh_2 can enhance T-lymphocyte infiltration in tumor and trigger cytotoxicity in spleen lymphocytes. *In vivo*, G-Rh_2 treatment enhanced the anti-tumor immunological response, suppressed tumor enlargement, and improved the survival span of C57BL6 mice bearing B16-F10 cells [146].

The combination of G-Rh_2 and CY significantly inhibited tumor growth in C57BL/6 mice bearing B16 cells. It also decreased the genotoxic effects including micronuclei formation and DNA damage induced by CY [64].

Other cancers

G-Rh_2 has been shown to exert a number of anti-cancer effects in other models. Autophagy not only results in cell growth arrest, but also might result in cell death. G-Rh_2 has been found to inhibit skin squamous cell carcinoma A431 cells in a dose-dependent way. The inhibition possibly occured by reducing the number of Lgr5-positive cancer stem cells *via* the interaction between autophagy and *β*-catenin signaling [147]. By activating caspase-1 and -3 and up-regulating Bax, G-Rh_2 induced the apoptosis of human neuroblastoma SK-N-BE(2) cells [148]. NR2F2 (Nuclear receptor subfamily 2 group F member 2) can reduce cell apoptosis but promote autophagy and DNA damage repair, and the effect of NR2F2 can be attenuated by the downregulation of LAMP2 (lysosomal associated membrane protein 2). The treatment with G-Rh_2 contributed to cell apoptosis and autophagy in vestibular schwannoma cells by inducing and suppressing DNA damage *via* the down-regulation of NR2F2/LAMP2 [149]. G-Rh_2 functioned in cell proliferation, apoptosis and autophagy in human retinoblastoma RBL-13 and Y79 cells through miR-638-mediated up-regulation of p53 and inactivation of the PI3K/AKT/mTOR pathway [150]. In human pancreatic cancer cell line Bxpc-3 and oral cancer cell lines YD10B and Ca9-22, G-Rh_2 inhibited proliferation, migration, and invasion and induce apoptosis [151, 152], and the anti-cancer activity in oral cancer was presented by inhibiting the Src/Raf/ERK signaling pathway [152]. G-Rh_2 induced apoptosis by promoting MAPK signaling pathway and inhibiting PI3K/AKT/mTOR and NF-*κ*B signaling pathway in U20S cells [153]. Similarly, G-Rh_2 showed potential anti-cancer activity in suppressing metastatic osteosarcoma in an MAPK and PI3K/AKT/mTOR-dependent manner in 143B and MG63 cells [154].

It can be concluded from above that the anti-cancer mechanism of G-Rh_2 mainly involves inhibition of tumor cell growth, induction of cell cycle arrest and apoptosis, suppression of angiogenesis, inhibition of invasion, enhancement of immunity, and reversal of drug resistance. These mechanisms have neen observed in different types of cancer and are summarized as follows: (1) The effects of G-Rh_2 on cancer cell cycle regulatory proteins includes the down-regulation of cyclin-E and cyclin-D1, which acts as the key factors in G-Rh_2 induced apoptosis. (2) G-Rh_2 induces apoptosis and inhibits the growth of cancer cells *via* caspase-dependent pathway involving caspase-1, -3, -4, -8 and -9. (3) G-Rh_2 inhibits proliferation and induces autophagy and apoptosis by targeting the PI3K/AKT/mTOR pathway. (4) G-Rh_2 induces ROS mediated ER stress-dependent cell apoptosis, thus inhibiting the proliferation of cancer cells. (5) G-Rh_2 also promotes the apoptosis of cancer cells by regulating Bax and Bcl-2 pathway. (6) G-Rh_2 exhibits the inhibition of metastasis by suppressing EMT on cancer cells. Moreover, whether there are other signal pathways involved in the anti-cancer effect of G-Rh_2 remains to be further studied. The beneficial effects of G-Rh_2 on anti-cancer treatments, such as improving the survival span and diminishing the adverse effects of chemotherapy, still need more attention and in-depth study.

Anti-cancer Effects of G-Rh_2 Derivatives

Great efforts have been made to find reasonable ways to enhance anti-cancer potency of G-Rh_2 while reducing its toxicity to normal cells. Moreover, being a hydrophilic agent, G-Rh_2 is not solubilized easily and is difficult to be transported through the membrane bilayers composed of phospholipids, resulting in lower membrane permeability [155]. To improve these defects, some studies have been made to modify the structure of G-Rh_2, and the available compounds are shown in Fig. 1.

One strategy is to synthesize esterification products (Fig. 4). G-Rh_2-O, the octyl ester derivative of C-12 hydroxyl, exhibited inhibitory effects against cell proliferation through apoptosis by disrupting mitochondrial membrane stability, mod-

ulating Bcl-2 group proteins, and activating caspase cascades, which was mediated by intracellular ROS overproduction, and also through inducing cell cycle arrest at G_1 phase by inactivation of AKT and phosphorylation of p38 MAPK [156]. The apoptosis in HepG2 cell induced by G-Rh_2-O and G-Rh_2 involved comparable mechanisms, but the anti-tumor activity of G-Rh_2-O is better than that of G-Rh_2 due to its increased cellular uptake (Table 4) [155]. A study on H22 tumor-bearing mice showed that tumor growth was inhibited by both G-Rh_2 and G-Rh_2-O through immune response modulation and down-regulation of VEGF expression, which promoted tumor cell apoptosis, while G-Rh_2-O had higher inhibitory rates and less toxicity than G-Rh_2 [82, 157]. Further studies revealed that G-Rh_2-O treatment enhanced the proliferative capacity and cytotoxicity of splenic lymphocytes in H22 tumor-bearing mice, and the effects were partially mediated by Tlr4 [158]. The dioctanoyl ester derivative of C-12 and C-6 hydroxyl (D-G-Rh_2-O) had a significantly decreased toxicity to human hepatocyte cell line QSG-7701 *in vitro* but an unattenuated antitumor activity *in vivo* [159]. Based on the aforementioned, the esterification strategy is a reliable method to synthesize G-Rh_2 derivatives with lessened side effects and enhanced anti-cancer activity, which will be possibly developed into more probable candidates for anti-tumor drugs.

Another reported strategy was the selective modification of the C-17 side chain (Fig. 4). The derivative pseudo-G-Rh_2 was achieved by three steps: acetylation, elimination–addition, and saponification. Compared with G-Rh_2, pseudo-G-Rh_2 greatly enhanced the anti-proliferative effect on eight different human tumor cell lines (SGC, A549, HT1080, Hela, A375, K562, HL-60, and MCF-7 cell lines) [160]. Furthermore, pseudo-G-Rh_2 was able to induce mitochondrial apoptosis in A549 cells which was related to excessive activation of the Ras/Raf/ERK/p53 pathway [161]. G-Rh_2E2, modified from the no cytotoxic activity form of 20(*R/S*)-G-Rh_2, had a potent anti-cancer activity observed both *in vitro* and *in vivo* with significantly reduced toxicity on normal cells and mice. The lower dose intraperitoneal injection achieved almost the equivalent therapeutic effect of higher dose oral administration, and 20(*R*)-Rh_2E2 was better than 20(*S*)-Rh_2E2 (Table 4). Experiments involving proteomic profiling revealed the inhibition of ATP production in cancer cells by G-Rh_2E2 in particular *via* the down-regulation of metabolic enzymes involved in *β*-oxidation of fatty acid, glycolysis, and citric acid cycle, leading to S-phase cell cycle arrest and specific cytotoxicity in cancer cells, which indicates that 20(*R/S*)-Rh_2E2 isomers can be safe anti-tumor metabolic suppressors [162, 163].

Delivery Systems of G-Rh_2

As stated above, G-Rh_2 has exhibited a broad range of anti-cancer effects in many preclinical studies. However, since G-Rh_2 is less water-soluble and has a significant ATP-binding cassette transporters-mediated efflux, its clinical use and true exhibition of its biological activity are limited [164]. A number of previous studies have reported that the oral bioavailability of G-Rh_2 is 5% in rats and 16% in dogs [165], and formulating a suitable G-Rh_2 delivery systems can improve bioavailability, enhance accumulation at tumor sites, and increase drug-loading and bioefficacy, so it has rather broad application prospects (Table 5).

G-Rh_2 based self-microemulsions (SME) DDS have been developed to efficiently deliver G-Rh_2 in combination with CYP450 and P-gp inhibitory excipients. Two SMEs, SME-1 and SME-2 with an average size of about 65 nm were prepared to evaluate the improved bioavailability of G-Rh_2 [166, 167]. After analyses from multiple aspects, the findings are as follows. SME-1 and SME-2 respectively showed a cellular uptake of G-Rh_2 1.79 and 1.82 folds higher than that of the free drug; the permeability of G-Rh_2 to Caco-2 cell monolayer in SME-1 was 1.48 times and that in SME-2 was 1.36 (1.48 and 1.36 being values of P_{app}) times greater than that of free drug, and the everted rat gut sac results showed a significantly enhanced permeability with improvement in transfer rates by 3.56 and 1.97 times for SME-1 and SME-2 of G-Rh_2; exceptionally high concentrations of G-Rh_2 was found in lymph nodes following 15 and 60 min of administration of SME-1 and SME-2 compared with that of free drug. Based on the prepared SMEDDS, the absolute oral bioavailability of G-Rh_2 in rats increased from 15.02% (that of the free drug) to 48.69% in SME-1 whereas 41.73% in SME-2 for G-Rh_2 [166]. Similarly, SUN *et al*. also developed two SMEDDS to test the solubility and absorption of G-Rh_2 in the small intestine of rats. Optimized SMEDDS contained transcutol P to increase drug-loading up to 48.5 and 38.9 mg·g^{-1} in two formulations, respectively, and the intestinal absorption rate increased to 56.8% compared with that of free drug (33.6%

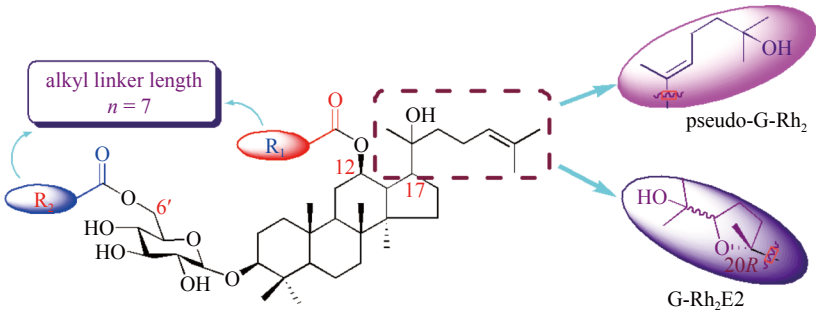

Fig. 4 The strategies of structural modification of G-Rh_2

only) after administration 2 h [168].

It has been proved that the novel Rh_2HAZnO nanoparticles exhibited stronger anti-cancer effects at 10 μg·mL^{-1} in A549, HT29, and MCF-7 cells, and in particular, the cell viability of MCF-7 cells was less than 30% of G-Rh_2, and the investigation into the mechanism showed that the anti-cancer activity of Rh_2HAZnO nanoparticles might be linked to the generation of ROS *via* the caspase-9/p38 MAPK pathway which automatically resulted in inducing apoptosis [169]. BSA-G-Rh_2 (bovine serum albumin) nanoparticles were prepared by desolvation at room temperature, and the synthesized BSA-G-Rh_2 nanoparticles exhibited good aqueous solubility and stability for up to one month without precipitation. The anticancer ability of G-Rh_2 in A549 lung cancer and HT29 colon cancer cells was sufficiently enhanced by BSA compared with free G-Rh_2 [170]. Lipid-based nanoemulsion particles were also developed, from which 52.4% of G-Rh_2 was released in about 24−96 h, showing obviously sustained-release characteristics. In a 48 h cytotoxicity study, G-Rh_2 nanoparticles were found to significantly enhance the anti-proliferation effect in glioma C6 cells with an IC_{50} value of 11.46 μmol·L^{-1}, in comparison with an IC_{50} value of 19.13 μmol·L^{-1} for free G-Rh_2. Moreover, a combination of G-Rh_2 nanoparticles and borneol had a synergistic effect in a concentration-dependent manner with an IC_{50} value of 8.04 μmol·L^{-1} [171]. The carrier system for G-Rh_2 delivery based on 200 nm mesoporous silica nanoparticles can enhance the efficacy, as evidenced by the high amount of loading and the increase of cytotoxicity against A549 lung cancer cells, HaCaT skin cells, HT-29 colon cancer cell and HepG2 liver carcinoma cells lines in mesoporous silica nanoparticles-G-Rh_2-fluorescein isothiocyanate test, and the quite promising effect was realized in A549 cells with the toxicity at 1 μmol·L^{-1} [172]. One-pot synthesis of GNPs (gold nanoparticles) with G-Rh_2 was carried out and the cytotoxicity was evaluated *in vitro*. G-Rh_2-GNPs loading efficiency was determined to be 54.91%. G-Rh_2 and G-Rh_2-GNPs exhibited similar cytotoxicity in human kidney epithelial HEK-293 and mouse macrophage J774A. 1 cell lines at equivalent ginsenoside contents, revealing that interaction with GNPs does not lower the systemic toxicity of G-Rh_2 to normal cells [173]. A novel G-Rh_2 nanoparticles with pH/reduction dual response which was encapsulated by *α*-lipoic acid and N-urocanyl pullulan had a higher cytotoxicity than free Rh_2 in HepG2 cells, and the oxidative stress played a key role in cell apoptosis by mitochondrial pathway [174]. G-Rh_2 chitosan tripolyphosphate nanoparticles suppressed the growth, invasion, as well as migration of colon cancer cells and accelerated their apoptosis more significantly than free Rh_2 *via* upregulating miR-491 in SW480 and SW620 cells [175].

G-Rh_2 nanocomposites prepared using aerosol solvent extraction system technique exhibited a remarkably good dissolution profile in water and good dispersibility in aqueous phase. *In vitro* anticancer assay on human tongue squamous cell carcinoma cell line SCC-15, G-Rh_2 nanocomposites-induced cytotoxicity was predominantly governed by concentration changes and was shown to rise with the increase in G-Rh_2 concentration. It is clear that the reduction in the particle size of G-Rh_2 nanocomposites resulted in enhanced cytotoxicity due to the promotion of cellular uptake *via* endocytosis [176].

A series of G-Rh_2 nanostructures including Graphene oxide-Rh_2, Graphene-Arginine-Rh_2, and Graphene-Lysine-Rh_2 were synthesized to evaluate their bioefficacy. In comparison with their cytotoxic activity against normal cell lines (primarily human mesenchymal stem cells), these nanostructures were more active against cancer cell lines. The IC_{50} values were higher than 100 μg·mL^{-1}, and the highest toxicity of nanostructures was observed in Gr-Lys-Rh_2 on human melanoma A375 cell lines [177].

For the treatment of drug-resistant breast cancer, a multicomponent MEs (microemulsion) containing G-Rh_2 (G) coix seed oil (C) and etoposide (E) with an average particle size of 46.3 nm was developed by air-drying [97]. It was found that ECG-MEs showed the strongest cytotoxicity toward MCF-7/MDR cells with an IC_{50} value of 3.3 μg·mL^{-1}, 10.8 times, and 7.8 times lower than that of etoposide and EC-MEs, respectively. This suggested that the presence of G-Rh_2 in the MEs contributes to a stronger anti-proliferative effect by inhibiting the P-gp-mediated efflux of etoposide from ECG-MEs. In the studies on MCF-7/MDR tumor xenograft mice, a tumor growth inhibition rate of 70.7% was observed in ECG-MEs compared with 36.1% in EC-MEs, indicating that in the inhibition of first-staged P-gp, sequential drug release played an important role. Consequently, more therapeutics got accumulated in the MDR cancer cells for overcoming the resistance and enhancing the antitumor efficacy. Furthermore, the safety evaluation of ECG-MEs showed that no obvious solid lesion or abnormality was seen in the major organs including heart, lung liver, kidney, and spleen of mice. The use of MEs as the delivery vehicles for ECG can be an efficient and safe oral anti-cancer drug delivery system for MDR breast cancer therapy [97]. Later, an optimum ECG-MEs targetting lung cancer was prepared [178]. For the deep tumor penetration, 3% of G-Rh_2 not only maintains a stable nanostructure for ECG-MEs but also favors deep penetration due to its small size. In cytotoxicity study, 95.4% of A549 cells upon treatment with ECG-MEs for 5 h were induced to apoptosis, which displayed an overwhelming advantage of ECG-MEs over EC-MEs and etoposide. In *in vivo* anti-tumor assay, an ECG mixture was found to have a significantly weaker anti-tumor capability than that of ECG-MEs. This indicated that a rational combination and a stable MEs formation are the two crucial factors governing enhanced *in vivo* anti-lung cancer efficacy. In the A549 cells and xenograft tumors, ECG-MEs exhibited synergistical anti-lung cancer effect which can be ascribed to the deep tumor penetration, extended retention in the cytoplasm, and an increase of Th1 cytokines [178].

G-Rh_2 was also loaded in liposomes for the evaluation of

liver cancer therapy. Three types of liposomes of Rh_2-PLP, Rh_2-CLP, and Rh_2-LP, with a mean particle size of 80−125 nm, were prepared by freeze-drying. G-Rh_2 was found to be efficiently pack into the liposomes with an encapsulation efficiency value higher than 90% in each case. The small particle size range and the high encapsulation efficiency of G-Rh_2-loaded liposomes facilitated drug accumulation in tumor tissue. The anti-cancer efficacy assessments in HepG2-xenografted mice indicated that the maximum inhibitory effect on the growth of tumors without any significant toxicity was presented by Rh_2-PLP, followed by Rh_2-CLP, Rh_2-LP, and free Rh_2 [179]. In another study, WANG *et al*. found that both G-Rh_2 liposomes and their surface-modified forms with glycyrrhetinic acid increased the cytotoxicities in SMMC-7721 cells by 0.88 and 0.52 fold compared with free G-Rh_2 [180]. G-Rh_2 liposomes accumulated effectively at the tumor site in 4T1 breast carcinoma xenografted mice model as compared with free Rh_2, and it synergically enhanced the efficacy of paclitaxel by remodeling tumor-associated microenvironment and stimulating the immune system [181].

Polymeric micelles based on polyethylene glycol (PEG) were also used for celastrol and G-Rh_2 delivery (celastrol-PEG-G-Rh_2) with 85.2% encapsulation efficiency, and this method was capable of forming micelles for endo/lysosomal delivery, hence enabling synergistic treatment of lung cancer [182]. A self-assembled micelles of G-Rh_2 could not only increase the cell uptake, but also transport more drug to tumor sites, and the anti-tumor effect of G-Rh_2 could be effectively improved *in vitro* and *in vivo* [183].

Multicore niosomes were prepared for G-Rh_2 delivery through a novel double pH-sensitive mixed micellar system with 91.5% encapsulation efficiency. The G-Rh_2-multicore niosomes were able to release G-Rh_2 encapsulated within them at an accelerated rate under lower pH conditions, maintain stability, and have a decreased toxicity compared with G-Rh_2-pH-sensitive mixed micelles in MCF-7 cells. Furthermore, it suppressed tumor growth most efficiently in hepatoma-bearing BALB/c mice [184]. G-Rh_2 loaded nanoniosome increased inhibition of PC-3 cells in a dose-dependent manner, and the cellular uptake was improved with the addition of 1,2-dioleoyl-3-trimethylammonium-propane to the niosomal formulation [185].

Clinical Trials of G-Rh_2

A number of clinical trials on G-Rh_2 in cancer patients have been carried out (www.chictr.org.cn and www.clinicaltrials.gov), but there are only a few published results in the literature presently. In the first study, 68 patients presenting stage IIB-III cervical cancer were treated with either G-Rh_2 + chemoradiotherapy (34 cases) or chemoradiotherapy alone (34 cases) [186]. Jinxing Capsule was given twice a day (approximately equivalent to G-Rh_2 81 mg·d^{-1}) for three months. This study showed that there were no notable variations ($P > 0.05$) in the 3-year survival rates (73.53% *vs* 61.76%), local recurrence rate (5.88% *vs* 8.82%), and distant metastasis rate (2.94% *vs* 8.82%) between the two groups. However, patients in the G-Rh_2 + chemoradiotherapy group had lower incidence rates of bone marrow suppression and gastrointestinal reactions than the chemoradiotherapy group ($P < 0.05$). This suggests that combining G-Rh_2 with chemoradiotherapy would reduce the adverse effects related to chemoradiotherapy [186].

In another study, 123 patients with advanced non-keratinized undifferentiated nasopharyngeal carcinoma (stage III-IVa) were divided into two groups receiving G-Rh_2 + chemoradiotherapy (63 cases) or chemoradiotherapy alone (60 cases) [187]. G-Rh_2 was administered as Jinxing Capsule twice a day (approximately equivalent to G-Rh_2 81 mg·d^{-1}) for six weeks. The outcomes of the study revealed that the two-year survival rates were improved in the case of G-Rh_2 + chemoradiotherapy compared with chemoradiotherapy alone (90.48% *vs* 76.67%; $P < 0.05$). In addition, patients who received G-Rh_2 had a higher objective remission rate and a lower incidence of adverse effects. This study indicated that the clinical efficacy and safety were improved by the combination of G-Rh_2 and chemoradiotherapy for the treatment of nasopharyngeal carcinoma [187].

In the third study, a group of 99 diseased individuals diagnosed with advanced NSCLC (stage III−IV) were randomized into two groups to receive chemotherapy (DDP + pemetrexide) alone (58 cases) or chemotherapy in combination with G-Rh_2 (41 cases) (the same dosage as mentioned above) for 10 days [188]. The results showed that patients who received G-Rh_2 had a higher objective remission rate and clinical benefit rate ($P < 0.05$), and an improved function of the immune system evidenced by the enhanced activity of $CD4^+$ T cells and the normal ratio of $CD4^+/CD8^+$ T cells. No significant differences, however, were observed in the adverse effects such as nausea, vomiting, anemia, and liver and kidney dysfunction between the two groups, but the administration of G-Rh_2 decreased the rates of thrombocytopenia and leucopenia ($P < 0.05$). In addition, the improvement of the down-regulation of CEA, CA125, and CYFRA21-1 tumor markers was also observed in the group administered with G-Rh_2. G-Rh_2 exhibited good short-term efficacy, protected immune function, and decreased the toxic reaction of chemotherapy drugs for the NSCLC therapy [188].

Conclusion and Perspectives

The immense potential of G-Rh_2 in the treatment and prevention of various carcinomas has been confirmed by a number of studies. This article provided comprehensive reviews of the preclinical studies of the anti-cancer effects of G-Rh_2. G-Rh_2 acts as an effective anti-cancer agent by regulating cell cycle, inhibiting proliferation and invasion, inducing apoptosis and differentiation, and reversing multidrug resistance in various cancer models. G-Rh_2 also has enhanced potential against several cancers and lower toxicity when delivered with many other anti-cancer drugs. Additionally, a number of researches have shown a significant impact of G-

Rh_2 on inducing autophagy to prevent cancer cell growth. Therefore, G-Rh_2 can be a potential preventive and therapeutic drug for carcinomatous diseases.

Due to the challenges of poor water solubility and bioavailability of G-Rh_2, several G-Rh_2 delivery systems have been designed to lead to better outcomes. However, to evaluate the effectiveness of such systems as potential drug delivery systems, further experiments and clinical trials are needed. Besides, the use of G-Rh_2 alone as the drug to design the delivery systems is still rare. Meanwhile, synthesized G-Rh_2 derivatives have been proved to attenuate the side effects while enhancing the anti-cancer activity *in vitro* and *in vivo*. The drug delivery systems as well as semi-synthetic modifications are valuable in providing unique ideas and new directions to develop anti-cancer drugs like G-Rh_2.

It is noteworthy that most studies investigating the anti-cancer efficacy of G-Rh_2 are based on cell lines and animal models. Relatively few clinical trials have been conducted on G-Rh_2 even though the therapeutic effects of G-Rh_2 have been widely known for the management of a wide range of carcinomas when used alone or in combination with other therapeutic drugs. Therefore, long-term and well-controlled clinical trials of G-Rh_2 are necessary to be performed. Moreover, stereoisomerically pure 20(*S*)-G-Rh_2 or 20(*R*)-G-Rh_2 should be differentiated and compared comprehensively in the studies in terms of their pharmacokinetic profiles, molecular and cellular mechanisms of action and toxicity in animal models and humans in the future. Therefore, the preclinical experiments in various species are highly recommended to continue investigating the promising anti-cancer activity of G-Rh_2 and to promote translation of research from bench to bedside.

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