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Synthesis and biological evaluation of novel spin labeled 18β-glycyrrhetinic acid derivatives

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ABSTRACT

Eighteen novel spin-labeled 18β-glycyrrhetinic acid (GA) derivatives were designed, synthesized, and evaluated for cytotoxicity against four human tumor cell lines (A-549, DU-145, KB and KBvin). Most of the derivatives showed more significant cytotoxicity than that of the parent compound GA. The best compound, **6j**, with a tryptophan amino moiety and piperidine nitroxyl radical showed GI_{50} values of 13.7–15.0 μM, and was fivefold more potent than GA. In a mechanism of action study, compound **7a** was confirmed as a 20S proteasome inhibitor in both in vitro and cell-based assays. These findings support further optimization efforts based on 18β-GA as a lead compound to develop potential anticancer drug candidates.

Cancer is currently one of the most severe health problems worldwide. Because natural products exhibit significant biological properties and often have a broad safety window during administration, the development of natural product-derived derivatives to treat various types of cancer has received increasing interest.[1–4]

Glycyrrhetinic acid (**1**, GA), an active triterpenoid metabolite of glycyrrhizin present abundantly in licorice roots, shows diverse biological activities, including antiviral, anti-allergic, anti-inflammatory, and anti-ulcer effects.[5,6] In addition, GA and its derivatives also display promising antitumor activities, such as inhibition of tumorigenesis and induction of apoptosis in various cancer cells, including hepatoma, leukemia, breast cancer, and gastric cancer cells, at high concentrations. It was discovered that GA acts directly on mitochondria to induce apoptosis through increased mitochondrial swelling, loss of mitochondrial membrane potential, and release of cytochrome *c*.[7–10] GA was also reported to potentiate the apoptotic effects of trichostatin A, a histone deacetylase inhibitor, in ovarian cancer cells by increasing the activation of the caspase-8-dependent pathway, as well as the activation of the mitochondria-mediated cell death.[11] GA was found to be a proteasome inhibitor, and other studies have revealed that GA and its derivatives can target peroxysome proliferator-activated receptors (PPARs) and act on the tumor cell environment, angiogenesis, inflammation, and immune cell functions.[12]

However, because GA's cytotoxicity is usually only weak to moderate, many researchers have tried to enhance the potency of GA by various derivatizations.[8–10,13,14] In our recent search for potential antitumor drugs derived from natural products, we found that incorporation of a stable nitroxyl radical into antitumor molecules could increase their activity and decrease their toxicity compared with parent compounds, as well as impart some lipophilicity. In addition, reports have shown that conjugation of amino acids and natural products can provide improved bioactivity;[15,16] an approach that was also successful in our prior studies where amino acid moieties effectively enhanced the potency of antitumor molecules.[17,18]

The above information together with our previous studies of GA derivatives as potential anticancer compounds[19,20] prompted us to introduce a nitroxyl functionality and amino acid segments into the design of novel spin labeled GA derivatives, in order to improve inhibitory effects against human tumor cell lines. All newly synthesized compounds (**4a–c**, **6a–j** and **7a–e**) were tested for cytotoxic activity against a panel of human tumor cell lines, including A549 (non-small cell lung cancer), DU145 (prostate cancer cell line), KB (nasopharyngeal carcinoma) and KB-VIN (MDR KB subline selected using vincristine). We report herein the design, synthesis, and in vitro cytotoxicity screening of these novel spin-labeled GA derivatives, as well as a mechanistic investigation of proteasome inhibitory effect.

Scheme 1 outlines the synthetic route to compounds **4a–c**. Compound **1** was first reacted with acetic anhydride in pyridine to convert into its 3-*O*-acetate **2**, which was then treated with

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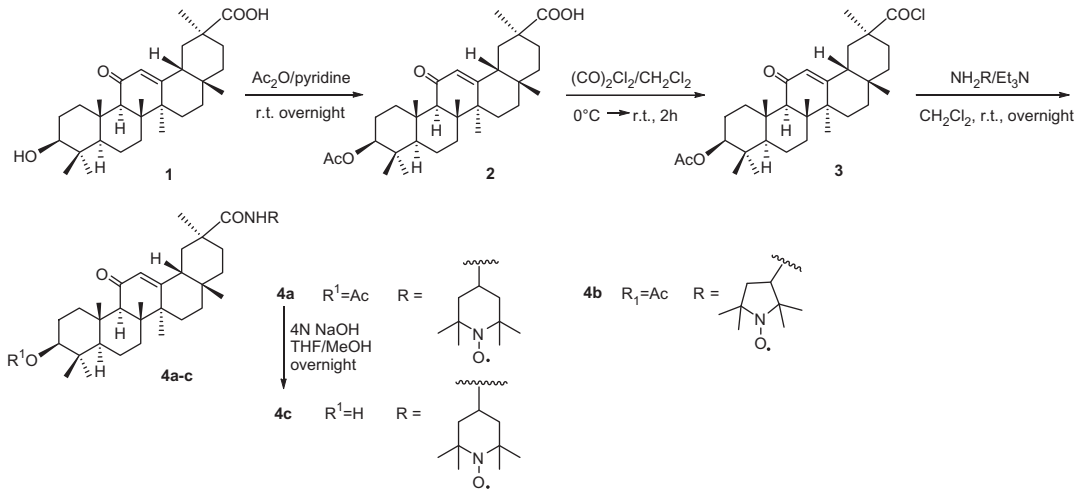


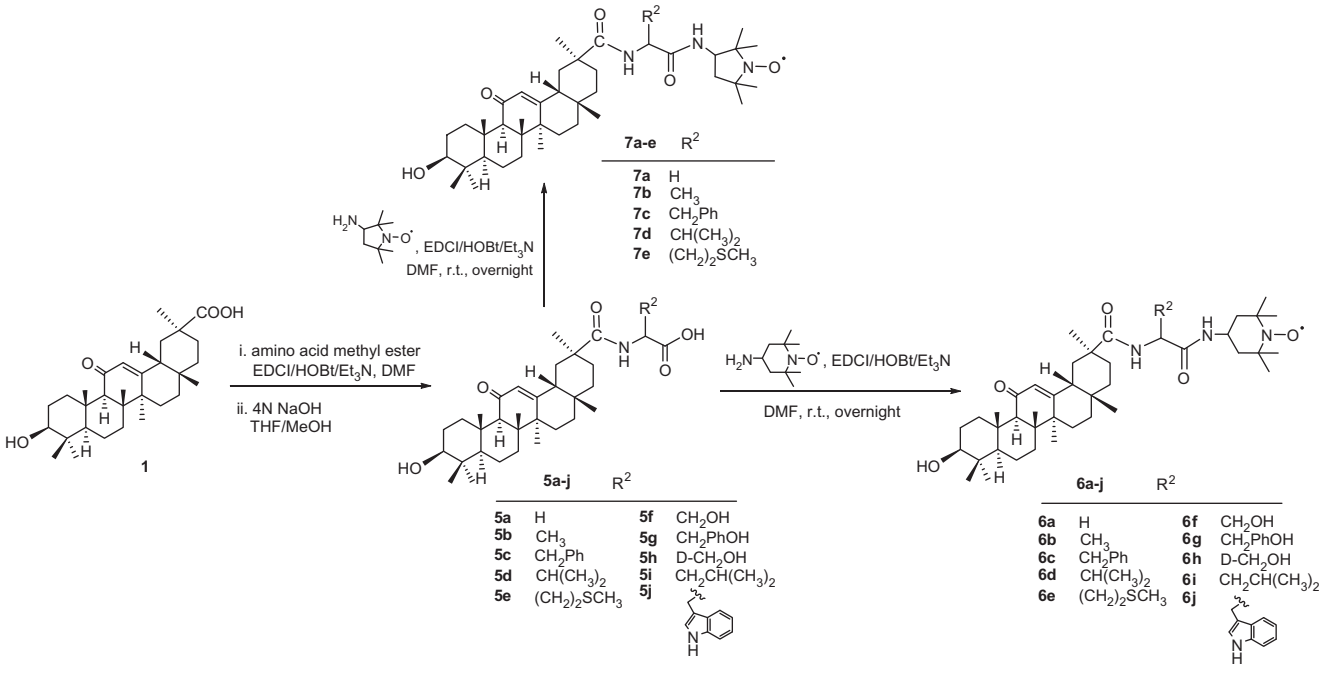
Scheme 1. Syntheses of compounds **4a–c.**

oxalyl chloride to give the 30-acyl chloride **3**. This key intermediate was then condensed with the appropriate piperidine (pyrroline) nitroxyl amines in the presence of triethylamine at room temperature overnight to give amides **4a–b**. Saponification of the C-3 acetyl protection of compound **4a** using 4 N NaOH in MeOH and THF mixed solvent yielded the corresponding compound **4c**.

The syntheses of compounds **6a–j** and **7a–e** were conducted according to the procedures in Scheme 2. Compound **1** was reacted with appropriate amino acid methyl esters in the presence of HOBt/EDCI/Et_3N in DMF at room temperature overnight, which afforded the corresponding intermediate amides. Subsequently, the saponification of the methyl ester of the intermediate amides was carried out with 4 N NaOH in MeOH/THF which gave the corresponding carboxylic acid derivatives **5a–j.** Reaction of **5a–j** with piperidine nitroxyl amine in the presence of HOBt/EDCI in DMF led to target compounds **6a–j** in good yields. Compounds **7a–e** were obtained with similar methods using pyrroline nitroxyl amine under HOBt/EDCI/Et_3N in yields of 67% to 82%.

Target compounds **4a–c**, **6a–j** and **7a–e** were evaluated for cytotoxic activity against four human tumor cell lines, A549, DU-145, KB and KBvin, using a sulforhodamine B colorimetric (SRB) assay in parallel with **1**.[21] The results are summarized in Table 1. Generally, the introduction of a piperidine or pyrroline nitroxyl radical into the C-30 side chains of **1** increased the cytotoxic effects of **1** by 2- to 4-fold. Compound **4a**, with a C-3 acetyl moiety, exhibited slightly improved antitumor activity compared with **4c**, with a free C-3 hydroxy group. The best compound in this series, **4a**, showed cytotoxicity against four human tumor cell lines with GI_{50} values of 14.6–18.7 μM, which were fourfold better than those of **1** (GI_{50}: 61.2–64.9 μM). This finding indicated that both the 3-*O*-acetyl group and 30-nitroxyl radical group might be important for increasing the tumor cell growth inhibition of GA derivatives.

Regarding the influences of the nitroxyl radical functionality and amino acid segments, it was first obvious that intermediates **5a–j** with only various free amino acids at C-30 showed no significant cytotoxicity (GI_{50} >70 μM). However, incorporation of a



Scheme 2. Syntheses of compounds **6a–j** and **7a–e.**

Table 1
Growth inhibition of spin labeled 18β-GA derivatives against four human tumor cell lines[a]

Entry	GI_{50} (μM)			
	A549	DU145	KB	KBvin
4a	18.7 ± 1.21	14.9 ± 0.768	14.6 ± 0.415	15.2 ± 0.235
4b	27.3 ± 2.29	32.5 ± 2.46	22.1 ± 0.500	20.6 ± 0.886
4c	24.8 ± 1.90	24.6 ± 1.02	20.6 ± 0.606	19.2 ± 0.887
6a	>70	>70	>70	>70
6b	>70	>70	>70	>70
6c	19.4 ± 0.909	19.3 ± 0.292	14.6 ± 0.448	14.9 ± 0.471
6d	34.2 ± 1.88	28.9 ± 0.921	17.5 ± 0.927	18.6 ± 0.931
6e	23.3 ± 0.304	21.7 ± 0.402	16.9 ± 0.501	19.2 ± 0.497
6f	44.0 ± 0.057	45.5 ± 0.666	39.9 ± 0.618	47.6 ± 1.06
6g	18.3 ± 0.373	17.4 ± 0.619	15.3 ± 0.469	19.5 ± 1.33
6h	>70	>70	>70	>70
6i	19.6 ± 1.60	22.0 ± 0.546	16.0 ± 0.368	17.0 ± 0.377
6j	15.0 ± 0.689	15.0 ± 0.363	14.2 ± 0.670	13.7 ± 1.25
7a	46.7 ± 1.90	46.2 ± 0.697	45.5 ± 1.04	46.9 ± 0.230
7b	46.1 ± 0.653	45.2 ± 1.27	41.3 ± 0.346	44.2 ± 0.280
7c	19.0 ± 1.13	22.5 ± 0.606	17.8 ± 0.193	16.6 ± 0.591
7d	34.5 ± 0.187	39.5 ± 1.05	30.7 ± 0.480	27.3 ± 0.338
7e	41.5 ± 1.83	43.2 ± 1.61	38.4 ± 1.15	38.5 ± 0.956
1	61.2 ± 2.33	64.9 ± 0.505	61.2 ± 0.118	62.3 ± 1.41

[a] The results are averaged from three independent experiments. Compounds **5a–5g** did not show significant inhibition (GI_{50} >70 μM).

Table 2
Inhibition of the chymotrypsin-like activity of 20S proteasome

Compound	**7a**	**1**	Lactacystin
IC_{50} (μM)	16.9	22.3	11.4

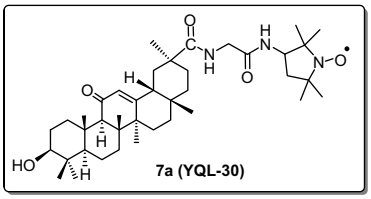
piperidine (**6a–j**) or pyrroline (**7a–e**) nitroxyl radical at the terminus of the C-30 side chains potentiated the cytotoxic effects against the tested human tumor cell lines. The difference in the GI_{50} values paralleled the size and lipophilic character of the substituents at the α-carbon of the C-30 amino acid chain in these derivatives. Similar results were seen in our previous study.[17,18] Specifically, glycine (**6a** and **7a**) and alanine (**6b** and **7b**) amino substituents showed little to weak potentiation of the cytotoxicity, while compounds **6c** and **7c** with a phenylalanine amino moiety showed significant growth inhibitory activity with GI_{50} values of 14.6–19.4 and 16.6–22.5 μM, respectively. Tyrosine (**6g**) and leucine (**6i**) also increased the cytotoxicity, with GI_{50} values of 15.3–19.5 and 16.0–22.0 μM, respectively. The best compound **6j** with a tryptophan amino moiety and piperidine nitroxyl radical exhibited GI_{50} values of 13.7–15.0 μM, and thus, was fivefold more potent than the parent compound **1**.

To investigate whether the chiral center introduced in the amino acids has any influence on the cytotoxic activity, compound **6f** containing an L-serine side chain and its corresponding D-serine diastereomer **6h** were prepared. The L-serine substituted **6f** was more active than the D-serine **6h**, which showed no significant cytotoxicity, suggesting that the stereochemistry in the amino acid side chain might be important for the derivatives' growth inhibition effects.

Proteasome hydrolyzes various unwanted cell cycle regulators, transcription factors, and antigenic proteins. It is a promising target for the development of therapeutic treatments for various disorders, such as neurodegenerative diseases, cancers, and inflammatory diseases. Because proteasome is so critically related to the development of numerous major human diseases, various proteasome inhibitors have been designed and synthesized,[22–24] resulting in novel classes of anticancer drugs or clinical trial candidates, such as bortezomib and salinosporamide A. In our previous studies, we reported that many triterpenoids, such as betulinic, oleanolic, ursolic, moronic, and glycyrrhetinic acids, as well as their derivatives, can regulate proteasome activities.[20,25] Among these compounds, **1** and its derivatives are interesting structural phenotypes for the identification of novel anti-proliferative agents and 20S proteasome inhibitors.

Therefore, in the current study, we also evaluated the proteasome regulating activity of newly synthesized spin-labeled GA derivative **7a**, using in vitro 20S proteasome and cell-based assays in HeLa Ub^{G76V}-GFP cells,[26] with lactacystin as positive control. Compound **7a** was selected due to its availability in quantity, and should represent the mechanism of other novel spin-labeled GA derivatives due to their structural similarity. In the in vitro assay for inhibition against the chymotrypsin-like activity of 20S proteasome, **7a** inhibited proteasome activity with an IC_{50} of 16.9 μM, slightly better than that of the parent compound **1** (Table 2). This effect was confirmed in the cell-based luminescent proteasome inhibition assay using HeLa Ub^{G76V}-GFP cells, which light up when proteasome is inhibited (Fig. 1).

In conclusion, three series of novel spin-labeled derivatives of 18β-GA were designed and synthesized, and the in vitro cytotoxicity was evaluated against four human tumor cell lines using an SRB assay. Most of the new compounds with nitroxyl radical functionalities displayed better activity compared with **1**. Among the new derivatives, **6j** with a tryptophan amino moiety and piperidine nitroxyl radical showed the greatest cytotoxicity, five-fold more potent than **1**. These results suggested that the incorporation of



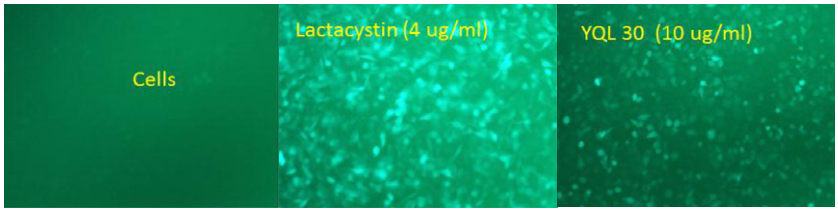


Figure 1. The cell-based proteasome inhibition assay. HeLa Ub^{G76V}-GFP cells light up when the proteasome is inhibited. (Left: HeLa Ub^{G76V}-GFP without compounds).

nitroxyl functionality and amino acid segments into GA is important in increasing the parent compound's cytotoxicity. In the mechanistic investigation, it was found that **7a** showed proteasome inhibition activity in both in vitro and cell-based assays. Although the anti-proteasome activity is in a concentration range similar to that of the anti-proliferative inhibitory activity against cancer cells, further studies are required to determine whether the proteasome is the major anticancer target of **7a**. Further optimization efforts based on GA are ongoing.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.041.

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