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Glycyrrhetinic acid derivatives containing aminophosphonate ester species as multidrug resistance reversers that block the NF-κB pathway and cell proliferation

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ABSTRACT

Novel NF-κB inhibitors based on Glycyrrhetinic acid (GA) derivatives containing aminophosphonate ester moieties were rationally designed and synthesized as well as evaluated their antitumor activities using MTT assay. Many target compounds showed potent antitumor activities against the tested human cancer cell lines including cisplatin-resistant cells, and exhibited significant inhibitory activity to the NF-κB with IC_{50} values at micromolar concentrations in A549 cells, respectively. Among them, compound **12e** possessed excellent antitumor activities against the tested human cancer cell lines and showed low cytotoxicity toward to human normal liver cells. Moreover, **12e** caused obvious loss of MMP and significantly induced ROS production, and displayed inhibition of cell migration against A549 cells *in vitro*. Importantly, **12e** arrested the cell cycle at the S phases and ultimately induced cell apoptosis in A549 cells through blockage of NF-κB signaling pathway. Our research provided an efficient strategy for targeting NF-κB antitumor drug development.

Transcription factor nuclear factor-kappa B (NF-κB) is a transcript factor discovered by Sen and co-workers in 1986, which is usually over-expressed and constitutively activated in many types of malignancies.[1-4] NF-κB, as a family of related protein hetero- or homodimers, promotes the downstream protein expression of anti-apoptosis (XIAP, Bcl-2 and Bcl-xL), proliferation (c-Myc) and invasion (MMP-2 and MMP-9), and exhibits remarkable capabilities for regulating the transcription of hundreds of target genes.[5-7] Increasing evidences indicated that the aberrant activation of NF-κB pathways allows cancer cells to escape apoptosis, invasion and metastasis, which contributes to cancer drug resistance.[6-10] Because activation of NF-κB is an essential feature of the survival of cancer cells during treatment, which results in treatment resistance, considerable researchers have focused on targeting NF-κB for cancer therapy. In facts, bortezomib (BTZ), one of the FDA approved anticancer drugs for treating multiple myeloma in the clinical, which induces apoptosis by blocking NF-κB activation.[11] This success greatly encouraged the development of NF-κB inhibitors to sensitize cancer cells of solid tumors to anticancer drugs. Thus, designing NF-κB inhibitors is a good choice to develop new cancer therapeutics and overcome drug resistance.

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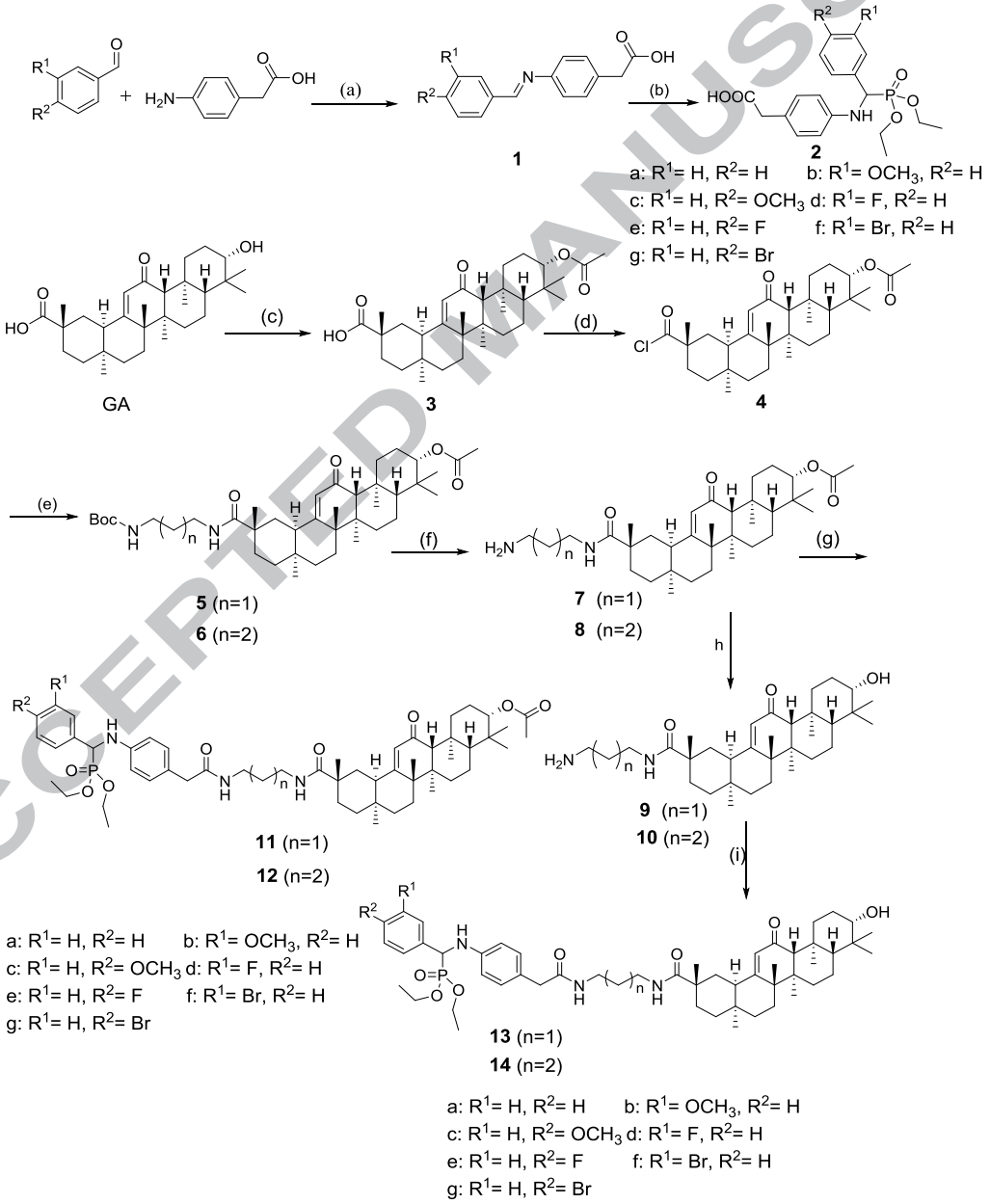
In recent years, triterpenoids are highly multifunctional and have attracted many researchers interesting as potential anticancer agents owing to their ability to interact with multiple biological targets.[12-14] Glycyrrhetinic acid (GA), also known as enoxolone, is a pentacyclic triterpenoid obtained from *Glycyrrhiza glabra*, served as one of the major effective elements of many traditional Chinese medicines. [15,16] In recent years, GA and its derivatives were reported to exhibit moderate antitumor activity against several cancer cells as well as induce cells apoptosis.[17-19] Notably, these compounds were found as potent inhibitors of NF-κB.[20] Liao and co-workers reported that aniline-derived ursolic acid (pentacyclic triterpenoids, UA) derivatives as multidrug resistance reversers by blocking NF-κB pathway.[21] Previous work has also found that the modification in position C-3 and/or C-28 of GA or UA has properties of improving the antitumor activity of anticancer drugs in various cells.[22-24] Recently, Huang and co-workers reported that asiatic acid (AA), as a pentacyclic triterpene acid isolated from *Centella asiatica*, containing α-aminophosphonates moieties at C-28 position in AA showed more potent antitumor activities against the tested human cancer cell lines compared to the parent compound AA.[25] In facts, many studies demonstrated that introduction of a phosphate ester moiety to chemotherapy drugs could significantly increase the solubility of drugs and improve transport through cellular membrane. [26] It is generally acknowledged that most of natural or synthetic aminophosphonate compounds exhibited a broad spectrum of biological activities such as antiviral, antimicrobial, antioxidant,

antitumor and anti-inflammatory, thus, application of these compounds is a good choice in the pharmacological and agrochemical fields.[27-30] In addition, some phosphate esters have a high affinity toward to calcium ions, thus they have been commonly applied to design targeted drugs used for bone cancer treatment.[31] Moreover, phosphonate esters can be hydrolyzed under physiological conditions, therefore, introduction of aminophosphonate esters in drugs is a promising approach to develop targeted antitumor agents.[32]

In the light of above considerations, in this paper, a series of GA derivatives containing aminophosphonate ester species were deliberately designed and obtained. These GA derivatives were expected to target tumor tissue and inhibit NF-κB, and they *in vitro* cytotoxicity against a number of tumor cell lines and a human normal liver cell line were also evaluated. In addition, the mode of action of a representative compound **12e** that blocked the TNF-α-induced NF-κB pathway was investigated.

The general procedures for the synthesis of GA contained aminophosphonate ester derivatives are shown in **Scheme 1**. Compounds **1** were prepared by the treatment of 4-aminophenylacetic acid with benzaldehyde in methanol. Compounds **1** were followed by treatment of diethyl phosphite to obtain Compounds **2**. GA was acetylated in C-3 with acetic anhydride in pyridine to obtain ester **3**, then **3** followed by its acylation with oxalyl chloride in the presence of dry dichloromethane to obtain the intermediate **4**. Compound **5** and compound **6** were achieved by the formation of amide bond between **4** and N-Boc-1, 3-propanediamine or N-Boc-1, 4-butanediamine. Compound **5** and compound **6** were taken off protecting groups by TFA to afford compound **7** and **8**. The target compounds **11a-11k** and **12a-12k** were prepared by the treatment of compound **7** and **8** with EDCI, DMAP, and compounds **2**, respectively, in DCM at room temperature. Compounds **7** and **8** were hydrolyzed by NaOH aqueous solution to afford Compounds **9** and **10**. The target compounds **13a-13k** and **14a-14k** were synthesized by the same method as compounds **11** and compounds **12**. The structures of target compounds **11a-11k**, **12a-12k**, **13a-13k** and **14a-14k** were then confirmed by ^{1}H NMR, ^{13}C NMR and high resolution mass spectrometry (HR-MS).



Scheme 1. Synthetic route for the final compounds **11a-11g**, **12a-12g**, **13a-13g** and **14a-14g** from 18β-glycyrrhetinic acid. Reagents and conditions: (a) CH_3OH, reflux, overnight; (b) diethyl phosphite, 50 °C, 2 h; (c) acetic anhydride, pyridine, 0 °C, 24 h; (d) oxalyl chloride, CH_2Cl_2, 0 °C, 12 h; (e) TEA, CH_2Cl_2, 0 °C, 2 h; (f) TFA, CH_2Cl_2, 0 °C, 1 h; (g) EDCI, DMAP, CH_2Cl_2, rt, 1 h; (h) NaOH aq., MeOH/THF, 40 °C, 5 h, 12 h; (i) EDCI, DMAP, CH_2Cl_2, rt, 1 h.

The *in vitro* anti-proliferative efficacy of the GA analogues **11a-11g**, **12a-12g**, **13a-13g** and **14a-14g** were evaluated by the MTT assay against HepG-2 (hepatoma), NCI-H460 (lung), MGC-803 (gastric), A549 (breast), and MG-63 (osteosarcoma) human cancer cell lines as well as human normal cell line HL-7702 (liver). GA and 10-hdroxycamptothecin (HCPT) were used as the reference compounds. As shown in Table 1, most of newly synthesized GA derivatives **11-14** showed much higher inhibitory activity than GA against the HepG-2, NCI-H460, MGC-803, A549 and MG-63 cell lines, indicating the introduction of aminophosphonate ester on GA could markedly increase of antiproliferative activity. Moreover, **12e** was the most potent compound in this series, with IC_{50} values ranging from 6.25 to 9.11 μM in the cancer cell lines, and even more potent than HCPT as well as showed lower cytotoxicity toward to human normal live cells HL-7702, respectively. Interestingly, compounds **11 (11b, 11d** and **11f)**, **12 (12b, 12d** and **12f)**, **13 (13b, 13d** and **13f)** and **14 (14b, 14d** and **14f)** with a electron donor substituent (-OCH_3) and halogen group (-F or -Br) at 3-position in benzene ring showed a reduction in anti-proliferative activity compared with **11a**, **12a**, **13a** and **14a** on HepG-2 cells, but in contrast, introduction of electron donor substituent (-OCH_3) and halogen group (-F and -Br) at 4-position in benzene ring (**11c, 11e, 11g**, **12c**, **12e**, **12g**, **13c**, **13e**, **13g**, **14c**, **14e** and **14g**) displayed a increasing of antitumor activity. The same results were also observed in NCI-H460, MGC-803, A549 and MG-63. Furthermore, **12** or **14**, with the increase of the length of the alkyl chain of the amide, showed a significant increase in antiproliferative activity compared with **11** or **13**, respectively. Interestingly, compounds **11** and **12**, an acetyl group was introduced at the 3-OH position, displayed better cytotoxicity than **13** and **14**, indicating an acetyl group introduction of the 3-OH position caused a significant increase in the antitumor activity. In shorts, these cytotoxicity screening results implied that the introduction of aminophosphonate moiety at C-28 position and an acetyl group at 3-OH in GA could obviously improve the antitumor activity.

Table 1. Effects of GA derivatives **11a-11g**, **12a-12g**, **13a-13g** and **14a-14g** against different human cancer cell lines including human normal liver cells HL-7702.

Compd.	IC_{50} (μM)[a]					
	HepG-2	NCI-H460	MGC-803	A549	MG-63	HL-7702
11a	31.63±2.21	23.25±1.91	21.03±1.68	30.28±2.25	25.29±2.29	>50
11b	38.19±2.03	31.65±1.88	36.73±2.04	39.33±2.96	39.51±1.92	>50
11c	19.31±2.16	18.77±2.04	18.23±1.25	19.28±2.12	19.13±2.08	>50
11d	37.74±1.09	29.47±1.08	27.25±0.99	33.96±1.07	>50	>50
11e	15.31±1.13	14.88±1.23	16.33±1.17	15.01±1.02	13.72±1.32	>50
11f	>50	>50	>50	>50	>50	>50
11g	23.62±1.37	17.08±1.37	29.85±1.24	20.47±1.35	21.34±0.97	>50
12a	26.28±1.54	13.23±1.37	15.24±1.70	23.08±1.29	20.16±1.64	>50
12b	35.26±2.05	27.37±1.61	32.90±1.67	27.25±2.03	36.61±2.30	>50
12c	15.35±1.25	14.12±1.15	16.33±1.18	15.25±1.07	13.93±1.29	>50
12d	21.33±1.09	28.18±2.31	21.02±1.24	26.41±1.52	22.83±1.26	>50
12e	9.11±1.23	8.28±1.07	8.73±1.09	6.25±0.95	9.01±1.01	>50
12f	48.19±2.11	47.60±2.18	41.77±2.21	39.63±2.16	>50	>50
12g	19.78±1.63	10.18±1.07	17.35±1.53	17.27±1.22	19.21±1.29	>50
13a	40.41±1.74	46.08±1.43	31.07±1.28	36.01±2.03	31.04±1.91	>50
13b	>50	>50	45.23±1.84	>50	46.55±2.13	>50
13c	36.81±1.42	29.44±1.39	25.69±1.51	23.08±1.49	23.61±1.43	>50
13d	>50	>50	>50	>50	>50	>50
13e	29.11±1.50	17.27±1.13	20.52±1.36	17.93±1.23	16.48±1.37	>50
13f	>50	>50	>50	>50	>50	>50
13g	35.63±1.27	20.27±1.82	35.72±1.89	31.93±1.67	25.13±1.81	>50
14a	37.15±0.79	30.48±1.34	36.27±1.05	33.21±1.86	27.84±2.01	>50
14b	46.39±2.01	39.25±1.73	>50	48.31±1.79	45.61±2.17	>50
14c	32.19±1.65	24.04±1.09	22.21±2.08	25.18±1.41	17.22±1.51	>50
14d	>50	>50	>50	>50	>50	>50
14e	20.21±1.56	19.37±1.39	18.31±1.66	19.76±1.33	12.39±1.19	>50
14f	>50	>50	>50	>50	>50	>50
14g	29.23±1.81	25.16±1.34	30.93±1.93	24.89±1.39	24.63±1.72	>50
GA	44.51±2.41	37.65±2.12	>50	36.15±2.38	>50	>50
HCPT	10.21±1.06	10.08±1.05	9.71 ± 1.03	9.13 ± 1.09	10.69 ± 1.15	10.23±1.19

[a] Each data represents mean ± S.D. from three different experiments performed in triplicate.

All synthesized compounds were further assayed for their inhibitory potency against the TNF-α-induced NF-κB transcriptional activity, which considered as an important therapeutic target in hematological cancer and malignancies as well as in human non-small cell lung cancer A549 cells.[33, 34] The non-small cell lung cancer A549 cells transiently cotransfected by NF-κB-Luc was used to detected

the effects of GA derivates on tumor necrosis factor-alpha (TNF-α)-induced NF-κB activation. The target compounds **11**, **12**, **13** and **14** were exposed in a concentration-dependent manner to confirm the concentration needed to inhibit 50% of TNF-α-induced NF-κB activation (IC_{50}). The results of inhibition of TNF-α-induced NF-κB activation in A549 cells by tested title compounds are depicted in Table 2. As shown in Table 2, most of title compounds showed significant inhibitory potency against NF-κB, with IC_{50} values mostly in the micromolar range. Moreover, the tested compounds displayed a significant increase in inhibition of TNF-α-induced NF-κB activation compared with corresponding parent compound GA, supporting the conclusion that introduction of aminophosphonate moiety at C-28 position in GA result to improve the anticancer activity. Interestingly, compounds **11c** (5.03±0.46 μM), **11e** (3.01±0.51 μM), **12c** (2.01±0.33 μM) and **12e** (0.97±0.21 μM), introduction of electron donor substituent (-OCH_3) or halogen group (-F) at 4-position in benzene ring and acetyl group introduction of the 3-OH position in GA, exhibited a significant increase in inhibition of TNF-α-induced NF-κB activation compared with **11a** (22.05±0.83 μM) and **12a** (13.23±0.72 μM), and the inhibitory activity was increased 4.4-fold, 7.3-fold, 6.6-fold and 13.6-fold compared with **11a** and **13a**, respectively. In contrast, introduction of electron donor substituent (-OCH_3) or halogen group (-F) at 3-position in benzene ring and deacetylation of the 3-OH position in GA, the inhibition potency was markedly decreased, such as **11a-11g** and **12a-12g** exhibited more potent inhibition of TNF-α-induced NF-κB than **13a-13g** and **14a-14g**, respectively. More importantly, compounds (such as **11a-11g** and **12a-12g**), different in the carbon chain length, exhibited different inhibition of TNF-α-induced NF-κB activation that are enhanced with an increase of the carbon chain length, consistent with the results for *in vitro* cytotoxicity assay.

Table 2. *In vitro* inhibition of TNF-α-induced NF-κB activation in A549 cells.

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
11a	22.05±0.83	**13a**	28.25±0.53
11b	>30	**13b**	N.I.
11c	5.03±0.46	**13c**	17.09±0.71
11d	>30	**13d**	N.I.
11e	3.01±0.51	**13e**	8.89±0.63
11f	>30	**13f**	N.I.
11g	28.39±0.91	**13g**	>30
12a	13.23±0.72	**14a**	25.13±0.59
12b	23.41±0.68	**14b**	29.45±0.36
12c	2.01±0.33	**14c**	13.09±0.41
12d	27.36±0.82	**14d**	>30
12e	0.97±0.21	**14e**	6.09±0.35
12f	27.05±0.62	**14f**	>30
12g	11.09±0.30	**14g**	16.29±0.85
		GA	>30

[a] N.I. = no inhibition; Results are given as the mean of at least three independent experiments with triplicates in each experiment.

In order to confirm the interactions between the most active compound **12e** and the target of interest (NF-κB), we performed molecular docking calculations on the active site of NF-κB (PDB ID: 1IKN) [35] using SYBYL-X 2.0 software and the score results are summarized in Table S1. The results of the molecular docking study are depicted in **Fig.1**, and the surflex docking score is 9.41 for **12e**, where higher score indicate greater binding affinity. It is observed that the GA moiety of compound **12e** goes deep into the pocket, and surrounded by residues ILE298, HIS193, LEU207, PR290 and ARG295, indicating that **12e** can indeed bind to the NF-κB (**Fig.1**). Notably, the binding pocket of NF-κB interacted with the aminophosphonate moiety of compound **12e** to form five hydrogen bonds through the side chains of ARG253 and ILE212, respectively, which plays an important role in binding with NF-κB. Overall, the result of the molecular docking study proves that compound **12e** is a potential inhibitor of NF-κB.

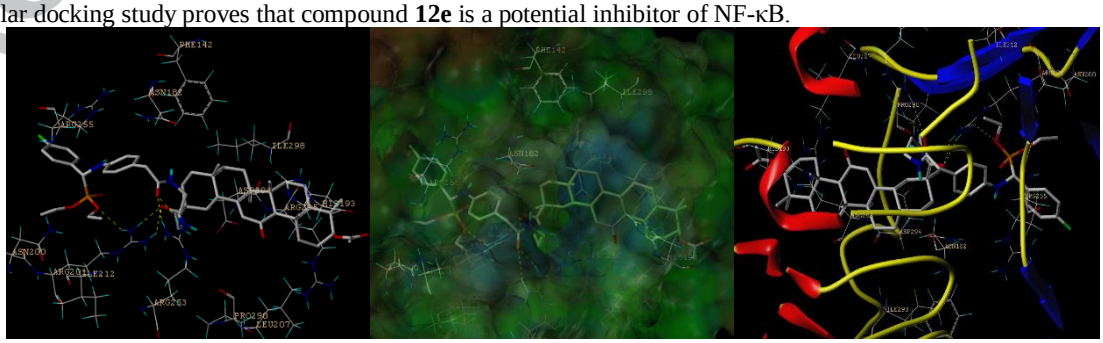


Fig. 1 Binding modes of compound **12e** in the active site of NF-κB (PDB ID: 1IKN). Ligands and the important residues for binding interactions are represented by stick and line models. The hydrogen bonds are shown as yellow dotted lines (color figure online).

Because of the major role of the transcription factor NF-κB in tumorigenesis and survival [36, 37], it was supposed that compound **12e** might mediate its anticancer effects through modulating the NF-κB activation pathway. Thus, we next detected the effect of **12e** on constitutive NF-κB activation in A549 cells. As expected, results of ELISA-based DNA binding assay obviously indicated that **12e** clearly suppressed TNF-α-induced NF-κB activation after cells treated with **12e** at the tested concentrations (5, 10 and 20 μM) (**Fig. 2**).

Interestingly, compound **12e** decreased the NF-κB DNA binding ability by nearly 2.3-fold than that of TNF-α, and these results suggested that **12e** can significantly modulate constitutive NF-κB activation in A549 cells.

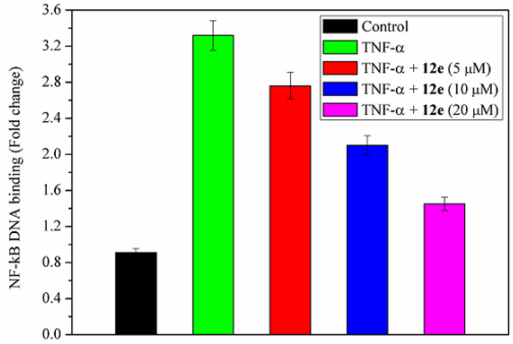


Fig. 2. ELISA based DNA binding assay to evaluate NF-κB DNA binding ability following compound **12e** treatment. The data is expressed as mean ± SD, compared with the untreated control, and TNF-α treated control ($p < 0.05$). The experiments were performed three times, and the results of the representative experiments are shown.

In recent years, several studies have shown that pentacyclic triterpenoids effectively suppressed NF-κB translocation to the nucleus by inhibition the activity of IκB kinase, which is known to be involved in the process of the activation of NF-κB.[38, 39] Thus, in order to determine the effect of the highly potent compound **12e** on the NF-κB pathway, the relative levels of NF-κB, IκBα and phosphorylated IKKβ were investigated by Western blotting assay. As shown in **Fig. 3**, consistent with expectations, phosphorylated IκBα was significantly inhibited in a concentration-dependent manner and the IκBα protein was observed to be slightly suppressed after cells treated with compound **12e** at the indicated concentration. Meanwhile, as illustrated in **Fig. 3**, incubation of the cells with compound **12e** for 24 h obviously suppressed the phosphorylation of IKKα/β. In summary, these results further suggested that compound **12e** suppressed NF-κB activation, mainly through inhibition of IκBα phosphorylation mediated by the IKKα/β phosphorylation.

Drug resistance has seriously limited the efficacy of cisplatin in clinical applications for treatment of solid tumors.[31] In terms of the strong inhibiting abilities on the tested cancer cell lines, compounds **11e** and **12e** were further evaluated against cisplatin-resistant SK-OV-3/CDDP and cisplatin sensitive SK-OV-3 cancer cells. As shown in Table 3, the IC_{50} values of cisplatin against SK-OV-3/CDDP was increased to 27.62±1.41 μM, whiles the cytotoxicity of title compounds **11e** and **12e** against SK-OV-3 and SGC-7901/CDDP cancer cells were quite similar, indicating that GA derivates **11e** and **12e** may have the potential to overcome cisplatin resistance. Importantly, compound **12e** is very active in cisplatin-resistant SK-OV-3 cancer cells with a resistance factor value of 0.92.

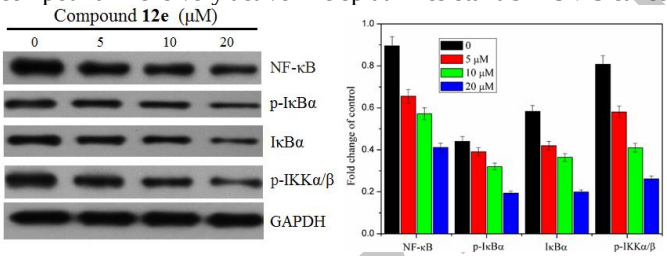


Fig. 3. A549 cells were incubated with compound **12e** at the tested concentrations (5, 10 and 20 μM) for 24h. The expressions of NF-κB, p-IκBα, IκBα and p-IKKα/β were determined by western blotting assay. GAPDH was used as internal control. The experiments were performed three times, and the results of the representative experiments are shown.

Table 3. *In vitro* growth inhibitory effect of **11e** and **12e** on cisplatin-resistant cancer cells.

Compd.	IC_{50} (μM) [b]		
	SK-OV-3	SK-OV-3/CDDP	resistant factor [c]
11e	10.11±1.03	13.36±1.13	1.32
12e	9.75±1.09	8.94±1.05	0.92
CDDP [a]	4.81±0.31	27.62±1.41	5.74
GA	45.62±0.38	49.36±2.35	1.08

[a] Cisplatin, [b] Each data represents mean ± S.D. from three different experiments performed in triplicate, [c] The values express the ratio between IC_{50} determined in resistant and nonresistant cell lines.

In order to investigate whether the observed cytotoxicity of compound **12e** is owing to induction of apoptosis, therefore, an annexin V-FITC/PI dual staining assay was measured after treatment of A549 cells. Q1, Q2, Q3, and Q4 represented four different cell states: necrotic cells, late apoptotic or necrotic cells, early apoptotic cells and living cells, respectively. As depicted in **Fig. 4**, after 24 h of treatment, significant apoptotic effects were observed for **12e** in A549 cells. The analysis of the apoptotic effects in **Fig. 4** obviously showed a concentration-dependent manner, especially for early apoptosis. At the maximal tested concentration, **12e** (20 μM) caused early and late apoptosis in 19.5% and 39.7% of A549 cells, respectively. In shorts, these results clearly indicated that **12e** significantly induced apoptosis in A549 cancer cells.

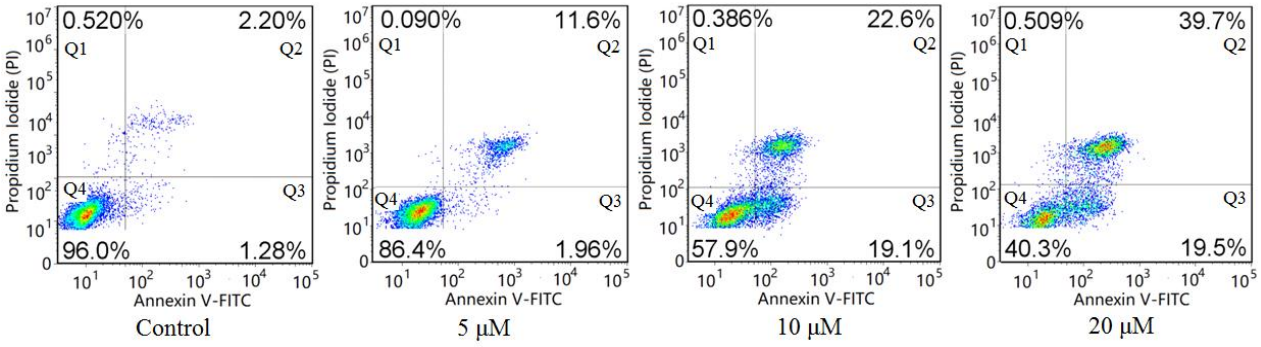


Fig. 4. Compound **12e** induced the apoptosis of A549 cells. Cells were treated with **12e** at the tested concentrations (5, 10 and 20 μM) for 24. Then, the cells were harvested, stained with Annexin V-FITC and PI, and analyzed by flow cytometry, respectively. The experiments were performed three times, and the results of the representative experiments are shown.

It is well-known that the cell cycle is divided into G1, G2, S, and M stages. In order to further investigate that the inhibition of cancer cell growth by the **GA** derivatives was caused by cell cycle arrest, untreated cells were used as a negative control and the A549 cells treated with compound **12e** (5, 10 and 20 μM) for 24 h, respectively, and the cell cycle distribution was detected by flow cytometry analysis after staining of DNA with propidium iodide (PI). As shown in **Fig. 5**, G1 period cells gradually decreased, but in contrast, S phase cells gradually increased compared with the control cells, S population of 38.76% was observed in control cells, while 40.09% (5 μM), 43.60% (10 μM) and 54.32% (20 μM) were also observed in compound **12e** group compared with the control cells, respectively. In short, these results indicated that compound **12e** arrested the cell cycle of A549 cells at the S stage.

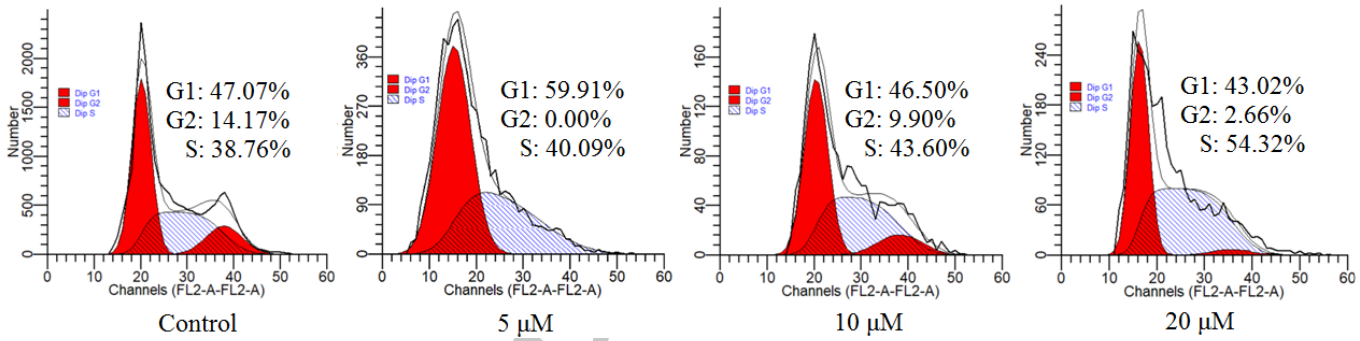


Fig. 5. Cell cycle arrest effect of compound **12e**. A549 cells treated with **12e** at the tested concentrations (5, 10 and 20 μM) for 24 h. The cells were trypsinized, harvested and washed three times with ice-PBS for PI-stained DNA content detected by flow cytometry. The experiments were performed three times, and the results of the representative experiments are shown.

The ability of compound **12e** to induce apoptosis was further investigated by analyzing the nuclear morphology of the exposed A549 cells using Hoechst 33258 staining. Nuclei of cells were treated with compound **12e** for 24 h and then stained by membrane permeable blue Hoechst 33258 to evaluate apoptosis morphologically. As shown in **Fig. 6**, in the control cells, nuclei of the A549 cancer cells appeared as regular round contours, and the control group A549 cells with smaller nuclei and also condensed chromatin were rarely observed, but in contrast, after cells treated with 5, 10 and 20 μM of compound **12e**, nuclei morphology of the cells slightly changed, and much more nuclei of the A549 cells appeared hyper condensed (brightly stained). In facts, the observation results further indicated that compound **12e** remarkably induced apoptosis in A549 cells, respectively.

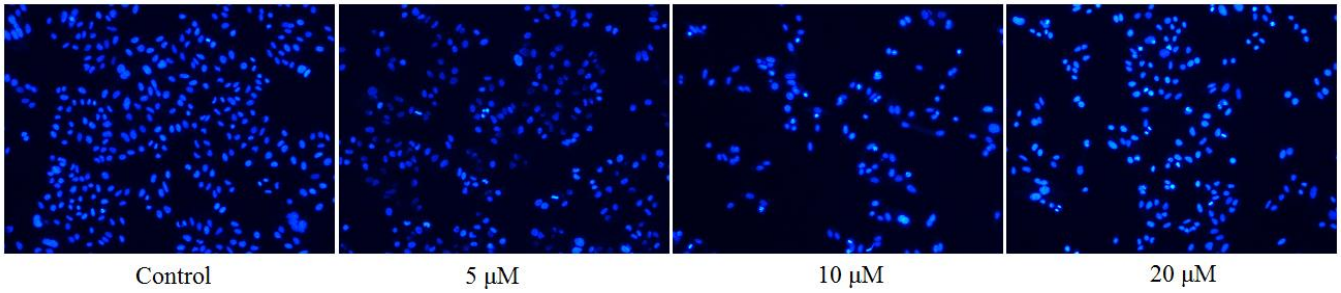


Fig. 6. Morphological changes in the nuclei (typical of apoptosis) of A549 cells treated with compound **12e** at the tested concentrations (5, 10 and 20 μM) for 24 h, respectively. Then, the cells were harvested, and stained by Hoechst 33258. The experiments were performed three times, and the results of the representative experiments are shown.

Increasing evidence has indicated that the metastatic cancer cells exhibited great capability of migration and invasion.[40, 41] Migration plays an important role in later period of cancer progression. Thus, transwell migration assays were conducted to evaluate if GA derivative **12e** could prevent the migration of human lung cancer cells. As shown in **Fig. 7**, the presence of **12e** obviously attenuated the migration of A549 cells in a concentration-dependent manner. In short, these results indicated that **12e** markedly inhibited migration of human lung cells in a concentration-dependent manner.

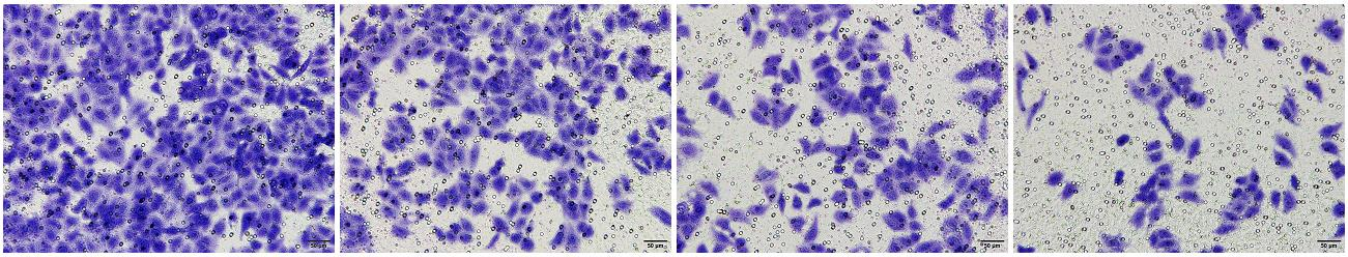


Fig. 7. Migration inhibition of A549 cells after incubation with the tested compound **12e** at the tested concentrations (5, 10 and 20 μM) for 24 h, respectively by transwell assay. The experiments were performed three times, and the results of the representative experiments are shown.

Changes in mitochondrial membrane potential (MMP) have been shown to play an important role in both extrinsic and intrinsic apoptosis.[6, 30] In order to further investigate whether apoptosis induced by compound **12e** was related to mitochondrial dysfunction, we therefore decided to examine the influence of **12e** on the MMP of A549 cells using the fluorescent probe JC-1 in this study. As shown in **Fig. 8**, compared with control group cells, treatment with A549 cells of compound **12e** caused obvious loss of MMP as presented by the increase of J-monomers (exhibiting green fluorescence and showing depolarized mitochondria) and concurrent decrease of Jaggregates (exhibiting red fluorescence and showing hyperpolarized mitochondria).

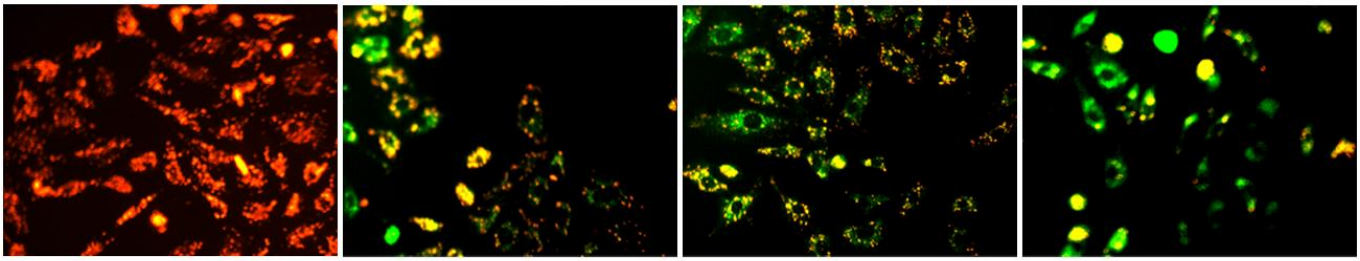


Fig. 8. Compound **12e** decreased the MMP of A549 cells. The A549 cells were treated with **12e** at the tested concentrations (5, 10 and 20 μM) for 24 h followed by incubation with the fluorescence probe JC-1 for 30 min. Then, the cells were visualized by fluorescence microscope. The experiments were performed three times, and the results of the representative experiments are shown.

Many studies indicated that mitochondria are the main organelles that produce reactive oxygen species (ROS), and ROS has been found to have a double sword role in the cytotoxicity in many solid tumors.[6, 30] Thus, intracellular ROS production by compound **12e** was further measured by the fluorescent probe DCF-DA (2', 7'-Dichlorofluorescein Diacetate). No significant green fluorescence was observed in the control group cells, but in contrast, **12e** significantly induced ROS production at the indicated concentrations as reflected by the increase green fluorescence in cytoplasm (**Fig. 9**).

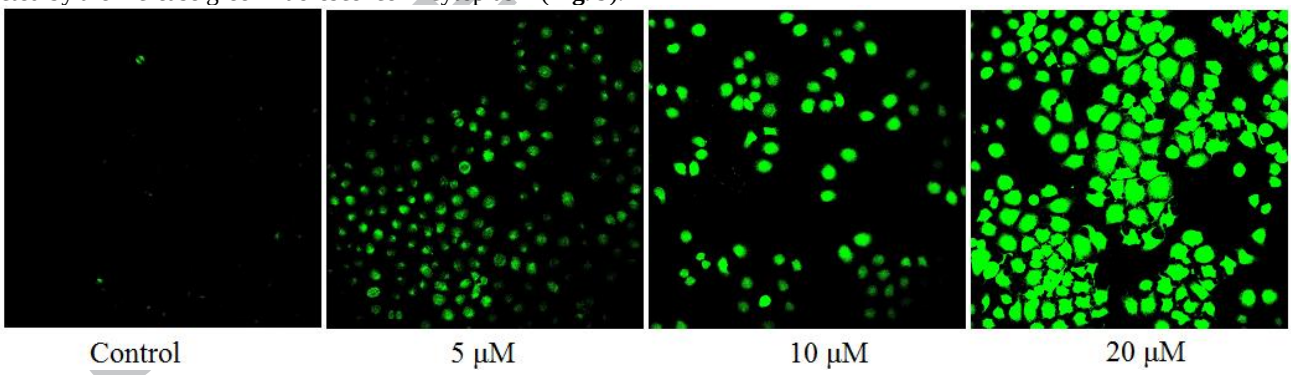


Fig. 9. Intracellular production of ROS by compound **12e** following a 24 h incubation visualized by fluorescence microscope. The experiments were performed three times, and the results of the representative experiments are shown.

In summary, a novel series of GA derivatives containing aminophosphonate ester species were rationally designed and synthesized and exhibited potent *in vitro* antitumor activities. Many compounds showed better antitumor activities than that of parent compound GA, and some target compounds displayed better inhibitory activity than the commercial anticancer drug HCPT. Interestingly, the selected target compounds significantly reversed multidrug resistance in cisplatin-resistant cancer cells. It is noted that compound **12e** (IC_{50} = 6.25 ± 0.95 μM) displayed the best antitumor activity against the A549 cells and showed more potent inhibitory activity than that of HCPT (9.13 ± 1.09 μM), and indicated low cytotoxicity toward to human normal liver cells HL-7702, respectively. In addition, a docking study of the most active compound **12e** revealed key interactions between **12e** and the active site of NF-κB in which the aminophosphonate ester moiety at the C-28 position and ester group at the C-3 position was important for increasing the antitumor activity. Further biological studies indicated that compound **12e** significantly caused cell cycle arrest at S phase and induced apoptosis in A549 cells by blockage of NF-κB signaling pathway. Importantly, compound **12e** caused obvious loss of MMP and effectively induced ROS production, and exhibited inhibition of cell migration against A549 cells *in vitro*. Consequently, GA derivatives containing an aminophosphonate ester moiety at the C-28 position were able to effectively improve the anticancer activity and potentially provide anticancer agents with bone-targeting ability. In short, this rational design offers a significant potential for the discovery of a new class of NF-κB inhibitors with the ability to suppress migration and induce apoptosis in the A549 cells.

Acknowledgments

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Graphical abstract

Glycyrrhetinic acid derivatives containing aminophosphonate ester species as multidrug resistance reversers that block the NF-κB pathway and cell proliferation

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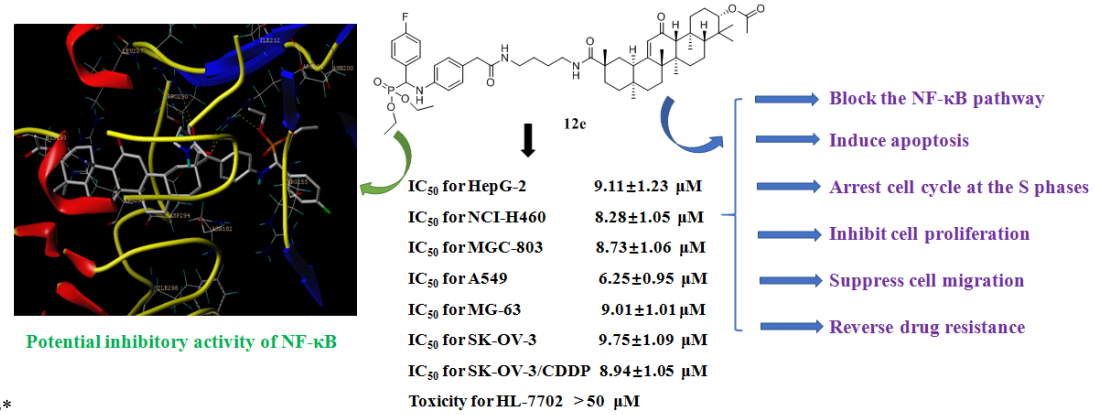

Highlights

- Representative compound **12e** showed significant inhibitory activity against NF-κB.
- Molecular docking study indicated that **12e** strongly binds to the active site of NF-κB.
- **12e** exhibited stronger antitumor activity and lower toxicity than HCPT.
- **12e** effectively induced apoptosis and arrested cell cycle at S stage in A549 cells.