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Research Article

Hybrid Molecule from Farnesylthiosalicylic Acid-diamine and Phenylpropenoic Acid as Ras-related Signaling Inhibitor with Potent Antitumor Activities

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Novel series of Farnesylthiosalicylic acid-diamine/phenylpropenoic acid hybrids were designed and synthesized. Their *in vitro* growth inhibitory assays showed that most compounds displayed strong antiproliferation activity against seven cancer cells. Especially, the new hybrid 12f, by the conjugation of 10a with ferulic acid, could selectively suppress the proliferation of tumor cells and display significantly lower toxicities to normal cells than its intermediate 10a. Furthermore, 12f dose-dependently induced SMMC-7721 cell apoptosis. Additionally, our observations demonstrated that 12f inhibited both Ras-related signaling and phosphorylated NF-κB synergistically, which may be advantageous to the strong antitumor activities of 12f. Our findings suggest that these novel hybrids may hold a great promise as therapeutic agents for the intervention of human cancers.

Key words: antitumor agents, farnesylthiosalicylic acid, hybrids, NF-κB, phenylpropenoic acid, Ras-related signaling pathway

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Cancer has become the leading cause of morbidity and mortality worldwide in 2010 (1). Ras proteins are GTPases as membrane-anchored proteins of all the superfamily members (2). With cycling between active GTP-bound state and inactive GDP-bound state associated respectively with guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (3), Ras proteins serve as molecular switches tightly regulating intracellular signal transduction pathways controlling cell proliferation, differentiation, and cell apoptosis in normal cells (4–7).

However, active GTP-bound state of Ras proteins are frequently found in human malignant tumors due to mutational activation of the Ras oncogene products (H-Ras, K-Ras, and N-Ras), and the overexpressed Ras-GTP proteins and overstimulated downstream signaling lead to the development and progression of malignancies (2,8,9). Therefore, Ras proteins and Ras-related signaling are considered as promising therapeutic targets in anticancer drug discovery (10,11). Farnesylthiosalicylic acid (FTS), a potent competitive Ras inhibitor, detaches the active Ras protein from the cell membrane, which will thereby block the initiation of downstream signaling events, resulting in the inhibition of tumor cell uncontrolled proliferation and induction of cell apoptosis (12,13). FTS has been reported to display chemopreventive activities in mouse models and clinical trials (14–17). Yet despite all that FTS displays a limited therapeutic effect (13, 18). Our previous researches reported that FTS-diamines significantly improved the antitumor activities compared with FTS, but failed to be selective to tumor cells (19). On the basic of this, searching for more potent and safer inhibitors targeting the persistently active Ras proteins and Ras-related signaling pathway will be of great significance.

Moreover, natural products play an important role in the development of anticancer drugs. Hybrids that combined with parts of natural products are promising potential leads with advantage to acquire high diversity, inherent biological activities, and improvement of therapeutic efficacy (20–22). Ferulic acid and its analog p-hydroxy-phenylpropenoic acid, two popular natural polyphenol products, widely occur in the plant kingdom and are associated with the reduction in the incidence of malignancies, including breast, colorectal, gastrointestinal, and lung cancer and display selective antiproliferative activity against some types of cancer cells (23–27). Furthermore, both of ferulic acid and p-hydroxycinnamic acid are showed to inhibit the effect of nuclear transcription factor-κB (NF-κB), which is independent of Ras signaling and plays a role in cancer promotion through the competitive inhibition of ATP binding to IKK-β (28,29). And suppression of NF-κB activity

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leads to inhibition of cellular proliferation and induction of an apoptotic response (30,31).

Notably, several researches reported that inhibiting Ras-related signaling pathway associated with NF-κB could lead to synergistically promoting the tumor cell apoptosis and inhibiting cell proliferation (32–34). In view of the above description, we hypothesized that the novel types of hybrids through conjugating FTS-diamine with phenylpropenoic acids could efficiently block the Ras-related signaling, selectively inhibit tumor cell proliferation and induce tumor cell apoptosis with a synergy for treatment of cancer. Therefore, twenty FTS-diamine/phenylpropenoic acid hybrids were designed and synthesized, and their *in vitro* antitumor activities, apoptotic effects, and inhibitory effect of Ras-related signaling and NF-κB were evaluated. Herein, the synthesis and biological evaluation of these compounds were reported.

Materials and Methods

Infrared (IR) spectra (KBr) were recorded on a Nicolet Impact 410 instrument (KBr pellet). ^{1}H NMR spectra were recorded with a Bruker Avance 300 MHz spectrometer at 300 K, using TMS as an internal standard, and the ^{1}H NMR spectra for part of the synthetic compounds can be found in the Appendix S1. MS spectra were recorded on a Mariner Mass Spectrum (ESI). Element analysis was performed on an Eager 300 instrument. All compounds were routinely checked by TLC and ^{1}H NMR. TLCs and preparative thin-layer chromatography were performed on silica gel GF/UV 254, and the chromatograms were conducted on silica gel (200–300 mesh; Merck, Merck & Co., Inc., Darmstadt, Germany). All solvents were reagent grade and, when necessary, were purified and dried by standards methods. Organic solutions were dried over anhydrous sodium sulfate.

Compounds **2** and **4** were commercially available. Compounds **3** and **5** were synthesized following the synthetic protocols we reported previously (19). Methods of synthesis and analytical data of the target compounds are presented in the Appendix S1.

MTT assay

Human gastric cancer cells (SGC7901), human hepatocellular carcinoma cells (SMMC-7721), human bladder carcinoma cells (EJ), human ovarian cancer cells (SKOV-3), murine hepatocarcinoma cell (H22), human lung cancer cells (H460), human pancreatic carcinoma cells (Panc-1) at 10^4 cells per well were cultured in 10% FBS DMEM in 96-well flat-bottom microplates overnight. The cells were incubated in triplicate with, or without, different concentrations of each tested compound for 48 h. During the last 4 h incubation, 30 μL of tetrazolium dye (MTT) solution (5 mg/mL) was added to each well. The resulting MTT-formazan crystals were dissolved in 150 μL DMSO, and absorbance was measured spectrophotometrically at 570 nm using an ELISA plate reader. The inhibition induced by each tested compound at the indicated concentrations was expressed as a percentage. The concentration required for 50% inhibition (IC_{50}) was calculated using the software (GraphPadPrism, GraphPad Software Version 4.03, Inc., La Jolla, CA, USA).

Flow cytometry assay of cell apoptosis

SMMC-7721 cells were cultured overnight and incubated in triplicate with the tested compound (3.0, 6.0, and 12 μM) or vehicle for 48 h. The cells were harvested and stained with FITC-Annexin V and PI (BioVision, Inc., San Francisco, CA, USA) at room temperature for 15 min. The percentage of apoptotic cells was determined by flow cytometry (Beckman Coulter, Fullerton, CA, USA) analysis.

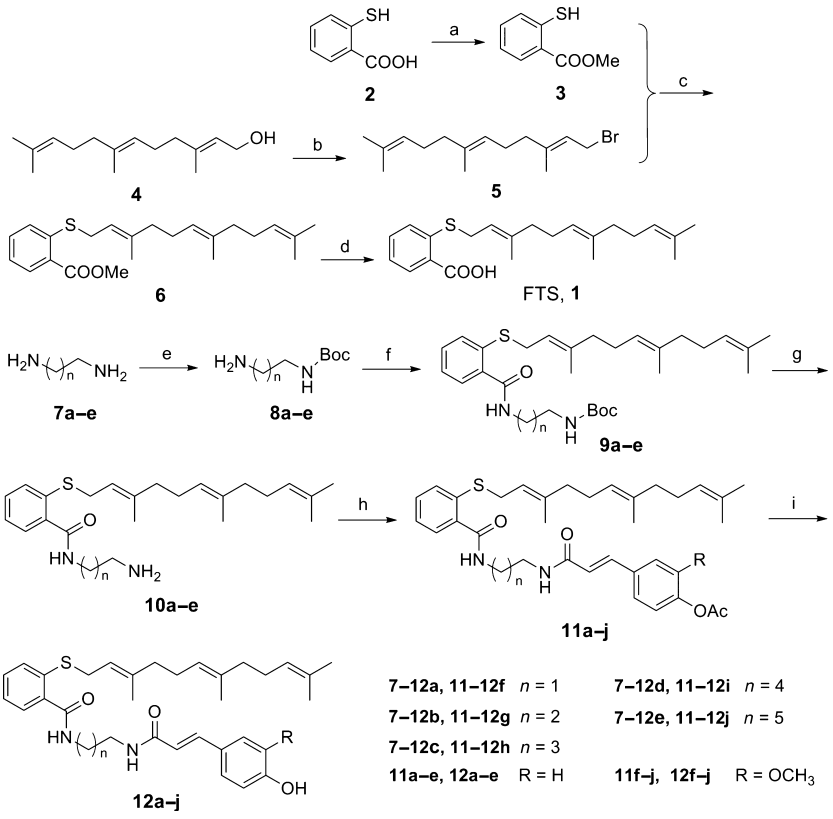
Western blot assay

The mechanisms of the cell apoptosis and the inhibitory activity of Ras-related signaling and NF-κB were determined by Western blot assay. SMMC-7721 cells at 1.5×10^5/mL were treated with 3.0, 6.0, or 12 μM **12f** or vehicle control for 8 h. After harvested and lysed, the cell lysates (50 μg/lane) were separated by SDS-PAGE (12% gel) and transferred onto nitrocellulose membranes. After blocked with 5% fat-free milk, the target proteins were probed with antiphospho-Akt (Ser473), antiphospho-ERK (Thr202/Tyr204), antiphospho-Raf (Ser259), anti-Akt, antiphospho-NF-κB p65, anti-NF-κB, and anti-β-actin antibodies (Cell Signaling, Boston, MA, USA), respectively. The bound antibodies were detected by HRP-conjugated second antibodies and visualized using the enhanced chemiluminescent reagent. The relative levels of each signaling event to control β-actin were determined by densimetric scanning.

Results and Discussion

Chemistry

The synthesis of these hybrids **11a-j** and **12a-j** were described in Scheme 1. The parent compound FTS was obtained from thiosalicylic acid **2**. Firstly, compound **2** was protected by methyl esterification with methanol and $SOCl_2$ to afford compound **3**. Next, (*E,E*)-farnesol **4** was treated with PBr_3 to give (*E,E*)-bromo-farnesyl **5**, which was then reacted with compound **3** to obtain methyl (*E,E*)-farnesylthiosalicylicate **6**. Then, the intermediate **6** was hydrolyzed with NaOH solution to gain FTS **1**. In addition, diamines **7a-e** was *N*-monoprotected with $(Boc)_2O$ to generate **8a-e**, which were later reacted with FTS to give compounds **9a-e** in the presence of ethyl chloroformate and *N*-methylmorpholine. Compounds **9a-e** were deprotected by treating with trifluoroacetic acid (TFA) to gain **10a-e**, which were subsequently treated with *p*-acetoxy-



Scheme 1: Synthetic pathway for the preparation of farnesylthiosalicylic acid-diamine/phenylpropenoic acid hybrids. *Reaction conditions and reagents*: (a) $SOCl_2$, MeOH, 0 °C-reflux, 8 h, 85%; (b) PBr_3, pyridine, n-hexane, ether, 0 °C-r.t., 4 h, 76%; (c) K_2CO_3, CH_3CN, 50 °C, 6 h; (d) 1 N NaOH, MeOH, 60 °C, 10 h, 82%; (e) $(Boc)_2O$, CH_2Cl_2, 0 °C, 2 h, r.t., overnight, 72–86%; (f) i) **1**, *N*-methylmorpholine, ethyl chloroformate, THF, 0 °C, 1 h; ii) Et_3N, THF, 0 °C, 1 h, 75–82%; (g) TFA/CH_2Cl_2 (v/v = 1:1), r.t., overnight, 88–94%; **(**h) *p*-acetoxy-phenylpropenoic acid chloride, Et_3N, THF, 0 °C, 1 h, 75–86%; (i) NaOH, MeOH, r.t., 2 h, 90–96%.

phenylpropenoic acid chloride prepared from their respective acids with $SOCl_2$ to yield target compounds **11a-j**. And **11a-j** was then hydrolyzed to gain the other series of target compounds **12a-j**. The target products were purified by column chromatography, and their structures were characterized by IR, ^{1}H NMR, MS, and elemental analyses (see Appendix S1).

Biological evaluation

To preliminarily screen out active molecules, the antiproliferation activities of target compounds **11a-j** and **12a-j** against Panc-1 (human pancreatic carcinoma cells), SMMC-7721 (human hepatocellular carcinoma cells) and SKOV-3 (human ovarian cancer cells) were evaluated *in vitro* by MTT assay with FTS and sorafenib as positive controls. The three tested cell lines were incubated with each of the target compound at a concentration of 25 μM, and their tumor inhibiting rates were described in Figure 1. Most compounds exhibited strong growth inhibitory activities with the maximum inhibiting rate of 99%, which was higher than that of FTS with near 40% on each cell line. Especially, significant antiproliferative effects of **11a**, **11f-g**, **12a-b**, and **12f-h** could be observed to possess comparably with or more potent than that of sorafenib, and these compounds were selected for further and broader investigation of the inhibitory activity against human tumor cells.

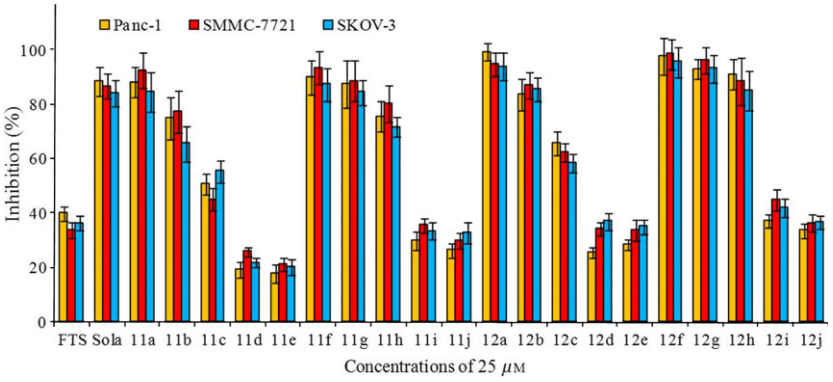


Figure 1: Inhibition of cell proliferation (%) of target compounds in Panc-1, SMMC-7721, and SKOV-3 cell lines after incubation for 48 h at a concentration of 25 μM. Data are expressed as means ± SD of each compound from three separate measurements.

Table 1: The IC_{50} values of **11a**, **11j**, **11g**, **12a**, **12b**, and **12f-h** against seven cancer cell lines

	In vitro inhibition of cancer cells proliferation (IC_{50}[a], μM)						
Compound	SGC-7901	SMMC-7721	EJ	SKOV-3	H22	H460	Panc-1
FTS	41.3 ± 5.56	69.7 ± 3.85	47.6 ± 3.28	51.2 ± 5.06	56.3 ± 4.28	49.2 ± 4.47	53.6 ± 5.77
Sorafenib	11.5 ± 2.71	18.7 ± 2.65	22.9 ± 3.53	9.25 ± 2.15	13.8 ± 1.43	10.8 ± 2.36	12.3 ± 1.46
11a	15.7 ± 1.34	12.2 ± 1.56	13.9 ± 1.72	17.9 ± 1.65	15.2 ± 1.36	12.6 ± 1.39	14.0 ± 1.31
11f	10.7 ± 1.23	11.8 ± 1.26	11.9 ± 1.10	9.84 ± 1.31	8.93 ± 1.03	10.4 ± 1.18	10.3 ± 1.26
11g	17.7 ± 2.10	15.5 ± 1.39	18.6 ± 1.46	15.0 ± 1.89	14.9 ± 1.60	13.6 ± 1.52	12.5 ± 1.33
12a	13.8 ± 1.81	8.93 ± 0.96	10.4 ± 1.18	7.93 ± 1.12	9.83 ± 1.16	10.1 ± 0.87	6.85 ± 1.17
12b	14.0 ± 1.62	13.9 ± 0.91	17.7 ± 1.85	20.6 ± 2.51	19.3 ± 1.77	15.6 ± 2.04	14.3 ± 1.10
12f	8.11 ± 0.90	5.22 ± 0.79	5.94 ± 1.03	6.53 ± 0.64	6.09 ± 0.58	7.87 ± 0.90	5.57 ± 0.72
12g	10.1 ± 1.13	5.89 ± 0.72	7.44 ± 1.22	7.65 ± 1.01	6.58 ± 0.87	8.29 ± 1.01	7.42 ± 0.89
12h	13.1 ± 1.23	9.82 ± 1.08	11.7 ± 1.37	13.6 ± 1.20	12.8 ± 1.04	10.5 ± 1.07	9.06 ± 1.18

[a]The antiproliferation activities of individual compounds against each of the cancer cell lines were determined by the MTT assay and expressed as the IC_{50} (a dose achieved 50% inhibition in the growth of cancer cells cultured). Data are expressed as mean ± SD from three independent experiments.

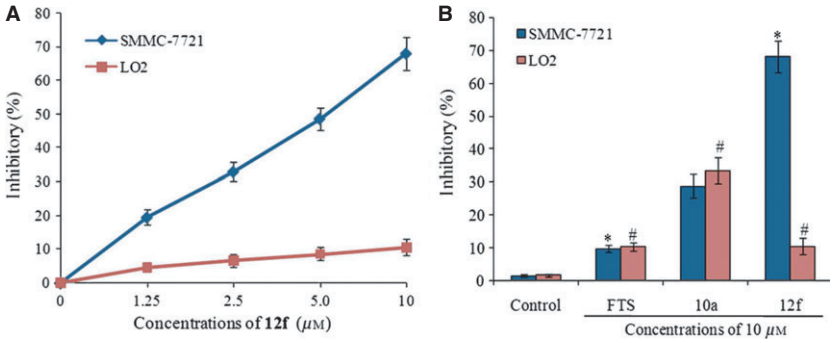


Figure 2: (A) Inhibitory effects of **12f** on the proliferation of SMMC-7721 and LO2 cells. SMMC-7721 and LO2 cells were incubated with the indicated concentrations of **12f** for 48 h. Cell proliferation was assessed using the MTT assay. Data are means ± SD of the inhibition (%) from three independent experiments. (B) Inhibitory activity of **10a**, **12f,** and farnesylthiosalicylic acid against SMMC-7721 cells. SMMC-7721 cells were incubated with the indicated compounds at 10 μM for 48 h, and cell proliferation was assessed by the MTT assay. Data are means ± SD of the inhibition (%) from three independent experiments. *p < 0.01 versus control of SMMC-7721, #p < 0.01 versus control of LO2.

Next, MTT assay employing SGC7901 (human gastric cancer cells), SMMC-7721 (human hepatocellular carcinoma cells), EJ (human bladder carcinoma cells), H22 (murine hepatocarcinoma cell), SKOV-3 (human ovarian cancer cells), H460 (human lung cancer cells), and Panc-1 (human pancreatic carcinoma cells) incubated with **11a**, **11f-g**, **12a-b**, and **12f-h** was carried out using FTS and sorafenib, a well-known Ras-related signal inhibitor, as positive controls. The values of half-inhibitory concentration (IC_{50}) about selected active compounds were measured and presented in Table 1. The results indicated that each of them displayed clearly increased antitumor activities with IC_{50} values in a low micromolar range compared with FTS and even had a comparable to or lower IC_{50} values than sorafenib did. Notably, **12f** showed the strongest antitumor activities with IC_{50} value range of 5.22–8.11 μM, which were 5–13-fold less than those of FTS (IC_{50} = 41.3–69.7 μM). Inspired by the prominent antitumor activities of **12f** *in vitro*, an insight into whether **12f** possessed strong cytotoxicity to normal cell would be required.

Given the strong growth inhibitory activity of **12f** *in vitro*, the selectivity profile was investigated by examining the inhibitory effects of **12f** on the growth of SMMC-7721 cells and LO2 cells (human hepatocellular normal cells), which were dosed with **12f** at increasing concentrations. A dose-response curve was presented in Figure 2A. As a result, no apparent growth inhibitory activity was observed on LO2 cells; however, **12f** displayed obvious antiproliferation activity on SMMC-7721 cells in a dose-dependent manner *in vitro*, which demonstrated that **12f** could selectively suppress the proliferation of tumor cells. As compound **10a** was the intermediate of **12f** (Scheme 1), the inhibitory activity of **12f**, FTS, and **10a** at a concentration of 10 μM or the vehicle control against SMMC-7721 cells and LO2 cells were also evaluated for 48 h. Figure 2B showed that treatment with FTS could cause a nearly decreased proliferation function of both SMMC-7721 and LO2 cells, which were similar to those of the cells treated with vehicle. Furthermore, treatment with **10a** retained a certain degree of inhibitory potency

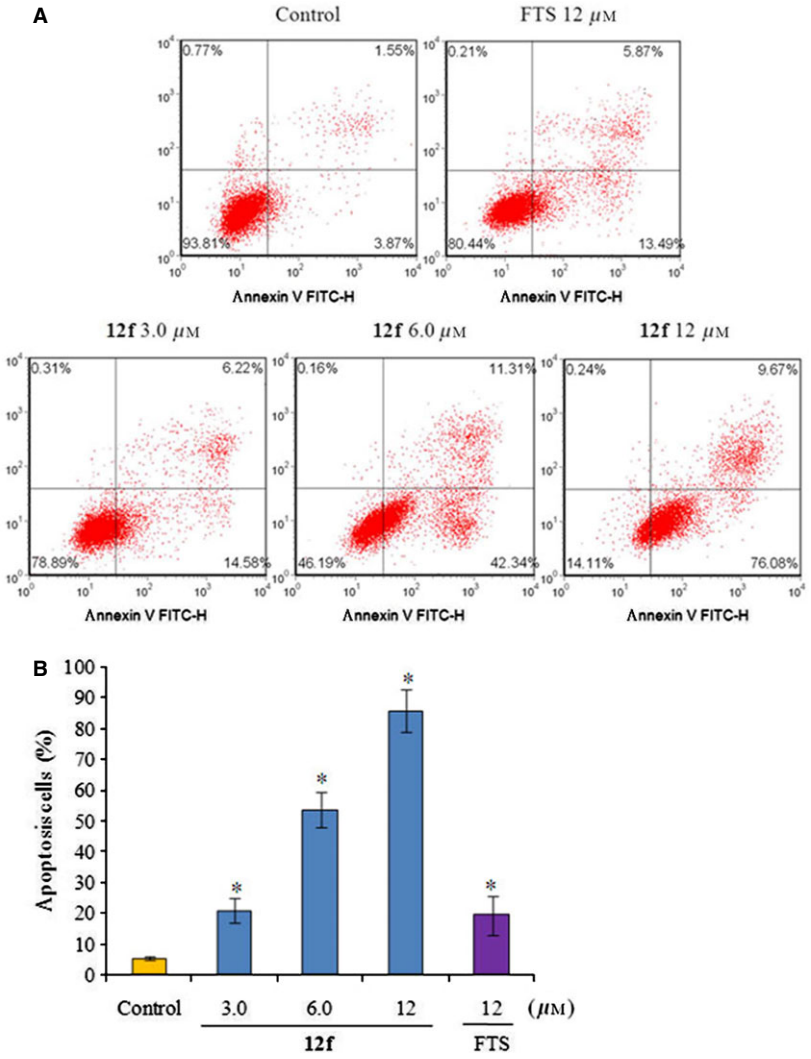


Figure 3: Compound **12f** induced SMMC-7721 cell apoptosis *in vitro*. SMMC-7721 cells were incubated with the indicated concentrations of **12f** and 12 μM farnesylthiosalicylic acid for 48 h, and the cells were stained with FITC-Annexin V and PI, followed by flow cytometry analysis. (A) Flow cytometry analysis. (B) Quantitative analysis of apoptotic cells. Data are expressed as means ± SD of the percentages of apoptotic cells from three independent experiments. *$p < 0.01$ versus control.

but was significantly less potent than **12f** against SMMC-7721 cells and had no selectivity to tumor cells. More noteworthy aspect, the new hybrid **12f**, after the conjugation of **10a** with ferulic acid, not only had higher inhibitory effects on the tumor cell proliferation, but also significantly reduced molecule toxicity compared with **10a,** which had high toxicities to normal cells. These observations indicated that **12f** was believed to be a safer and more potent antitumor agent, which may be worthy of further research.

To determine whether the hybrids' antiproliferation activities were attributed to their pro-apoptotic role in tumor cells, the apoptosis rates of SMMC-7721 cells incubated with different concentrations of **12f** (3, 6, or 12 μM), FTS (12 μM), or vehicle alone were determined by FITC-Annexin V/PI staining and flow cytometry. As described in Figure 3, treatment with **12f** at 3.0 μM showed an apoptosis effect on SMMC-7721 cells that induced 20.80% of cell apoptosis compared with the control (5.42%). Furthermore, with the dose of **12f** gradually increased, apoptotic SMMC-7721 cells exhibited an accumulation that were 53.65% for 6.0 μM and 85.75% for 12 μM, which were in sharp contrast to those induced by FTS at high concentration (only 19.36% for 12 μM). The data demonstrated that **12f** could result in an occurrence of SMMC-7721 cell apoptosis in a dose-dependent manner and have an enhancement of apoptosis inducing effect in SMMC-7721 cells compared with FTS.

To get insight into the mechanisms underlying the anticancer activity of these FTS-diamine/phenyl-propenoic acid hybrids, we examined the inhibitory effects of the active compound **12f** on the expression of Ras-related pathway and NF-κB proteins. The levels of phospho-Raf, phospho-ERK, phospho-Akt, Akt, as well as phosphor-NF-κB, NF-κB were determined in SMMC-7721 cells by immunoblotting assays, and using β-actin as the control. As shown in Figure 4, the levels of phospho-Raf, phospho-ERK1/2, phospho-Akt were obviously

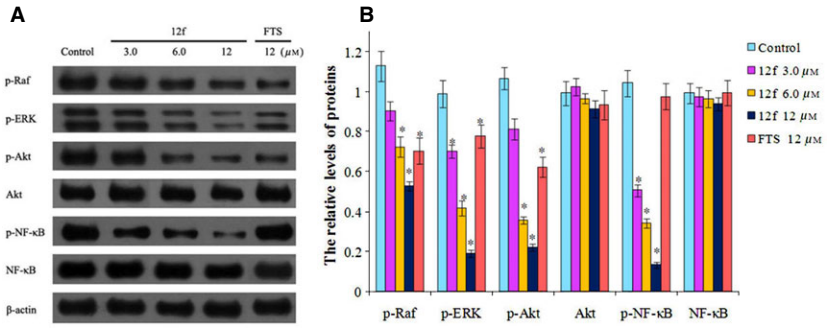


Figure 4: Immunoblot analysis of the expression and phosphorylation of the Ras-related signal events *in vitro*. (A) SMMC-7721 cells were treated with vehicle (control), different doses of **12f**, or farnesylthiosalicylic acid were homogenized, and their lysates were subjected to immunoblot analysis using antiphospho-Raf (Ser259), antiphospho-ERK1/2 (Thr202/Tyr204), anti-Akt, antiphospho-Akt (Ser473), antiphoshpo-NF-κB p65, anti-NF-κB, and anti-β-actin antibodies, respectively. β-Actin was used as the control. (B) Quantitative analysis. The relative levels of each signaling event to control β-actin were determined by densimetric scanning. The data are expressed as means ± SEM of three duplicate experiments. *$p < 0.01$ versus control.

reduced under the treatment of **12f** (3.0, 6.0, or 12 μM), whereas no significant change in the expression of Akt was found, indicating that our designed hybrid could inhibit the Ras downstream signaling in a dose-dependent manner which might attribute to the impact of the FTS fragment in **12f**. Moreover, the suppression effect of **12f** (dose of 12 μM) on Ras-related signaling was more remarkable than that of FTS at the same concentration. On the other hand, it is necessary and interesting to probe further into the level of phosphor-NF-κB expression, and the result highlighted a concentration-dependent inhibition of the phosphor-NF-κB. In contrast, there was no phosphor-NF-κB inhibiting effect of FTS at the same dose (12 μM). These results gave an evidence at the molecular levels that **12f** could not only block Ras-related signaling, but also have an inhibition on phosphorylated NF-κB simultaneously, which may synergistically contribute to the enhanced activities of **12f** in the tumor growth inhibition and induction of cell apoptosis.

Structure–activity relationships analysis

Structure–activity relationships (SARs) revealed that an improvement of antitumor activities was observed for FTS-diamine/phenylpropenoic acid hybrids compared with FTS. Among these hybrids, FTS-diamine/ferulic acid hybrids showed a relatively stronger growth inhibitory activity against tumor cells than FTS-diamine/p-hydroxy-cinnamic acid hybrids did, such as **11f-j**, **12f-j** *versus* **11a-e**, **12a-e**, indicating that ferulic acid fragment exhibited more helpful effect than *p*-hydroxy-cinnamic acid did on enhancing antitumor activities. Furthermore, after the removal of acetyl group on **11a-j**, the expose of phenolic hydroxyl group on **12a-j** led to a significant potentiation of antitumor activities. One plausible explanation may be that **12a-j** with more hydrogen bonds would be more active to bind the galectin site of farnesyl-Ras. In addition, with an introduction of hydroxylphenylpropenoic acid, hybrid molecules not only exerted more sensitive to tumor cells than FTS-diamines, but also evidently reduced normal cell toxicities. Moreover, FTS/hydroxylcinnamic acid hybrids, compounds with relatively shorter diamine linker displayed more potent, for instance, **12f** exhibited optimal antitumor activities. However, the precise mechanisms underlying the SAR of these hybrids remain further investigation.

Conclusions

Summarily, a series of FTS/hydroxylcinnamic acid hybrids was designed and synthesized and their *in vitro* antitumor activities were evaluated. Most of them displayed strong antiproliferation activities against seven cancer cells *in vitro*. Especially, novel hybrid **12f**, with the strongest *in vitro* antitumor activities (IC_{50} = 5.22–8.11 μM), exerted selectively grow inhibitory activity against tumor cells, greatly improving the sensitive to tumor cells compared with FTS and **10a** *in vitro*. Moreover, **12f** could dose-dependently induce cancer cell apoptosis superior to FTS. Interestingly, **12f** exhibited a simultaneous inhibition to Ras-related signaling and phosphorylated NF-κB, which may synergistically contribute to the significant antitumor activities and apoptosis inducing effects.

Acknowledgments

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Conflict of Interests

All authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Synthesis and analytical data of the target compounds.