Synthesis and Biological Properties of Polyamine Modified Flavonoids as
Hepatocellular Carcinoma Inhibitors

Qian Li,[†] Yangyang Zhai,[§] Wen Luo,^{*,§} Zixin Zhu,[§] Xin Zhang,[†] Songqiang Xie,^{*,⊥}
Chen Hong,[†] Yuxia Wang,[§] Yabin Su,[§] Jin Zhao,[†] Chaojie Wang,^{*,†}

[†] Key Lab of Natural Medicine and Immune Engineering, [§] Chemistry Department,

[⊥] Institute of Chemical Biology, Henan University, Henan, China

Abstract

A series of polyamine conjugates of flavonoids with a naphthalene motif were synthesized and evaluated for their anti-hepatocellular carcinoma properties by in vitro and in vivo assays. Compound **8a** displayed favorable selectivity between hepatocellular carcinoma cells and normal hepatocyte cells, and the combination of **8a**-Aspirin displayed additive in vitro activity toward tumor cell growth and migration. The obviously elevated therapeutic effect and improved body weight index on both H22 liver tumor growth and pulmonary metastasis animal models were also obtained by the combination trials of **8a**-Aspirin. The preliminary mechanistic research revealed that the **8a**-regulated expression of apoptosis-related proteins such as p-p38, p-JNK, p53 and Bcl-2 was further amplified by Aspirin. Therefore, the cocktail therapy with Aspirin might help exploit the potential of flavonoid-polyamine conjugates as antitumor agents.

Key words: synthesis; polyamine; flavonoids; antitumor.

1. INTRODUCTION

Flavonoids are a vast group of polyphenolic compounds which exhibit multiple health beneficial effects such as anticoagulant, cancer prevention and neuroprotective properties.¹ Many naturally occurring flavonoids have drawn a great deal of attention toward cancer therapy due to their wide safety margin.² Moreover, some of them are found to possess intriguing biological features, for instance, reversing multidrug resistance and reducing tumor cell migration,³ the major obstacles in present cancer treatment, and are thus among the attractive anti-cancer agents.⁴ Substantial studies have been conducted in search for diverse structurally-modified flavonoids with biological benefits superior to their natural counterparts.⁵ For example, LY294002 (Figure 1) entered clinical trials as potential antineoplastic agents.⁶ Meanwhile, significant advances have been made in the design of novel flavonoid derivatives focused on modulating multidrug resistance⁷ or inhibiting invasion and migration.^{8,9}

Natural polyamines play a pivotal role in eukaryotic cell growth, division, and proliferation. Both native and synthetic polyamines have been appended to diverse pharmacophores to furnish multiple biological functions, such as cancer inhibitive, neuroprotective, and antiparasitic properties.¹⁰⁻¹² F14512 (Figure 1), a promising polyamine-based antitumor agent, is currently evaluated in Phase I trials.¹³⁻¹⁵ Recently, there are gaining interests in the associations between polyamine and tumor migration.¹⁶ Meanwhile, Russo *et al* found that the natural flavonoid Quercetin inhibited the colon cancer growth partially by decreasing the endogenous synthesis of polyamines.¹⁷ Thus, the polyamine modified flavonoids, which are rarely reported to

date, may possess some intriguing antitumor properties.

Hepatocellular carcinoma (HCC) accounts for the third major cause of all cancer-associated death in the world.¹⁸ In spite of advances in early diagnose, surgical techniques and chemotherapy, the five-year survival rate for hepatocellular carcinoma patients remains unsatisfactory, mainly because of a high incidence of recurrence and metastasis after tumor excision.¹⁹ We have put efforts into the development of polyamine conjugates as HCC inhibitive agents. It is surprising that there are few instances focused on polyamine-based anti-metastatic drug design. In our previous flavonoid associated work,²⁰ 8-flavonemethyl nitrogen mustard was found to be as potent as Melphalan against cancerous K562 and B16 cells. In the present study, a flavonoid scaffold, composed of a naphthalene ring linked to the benzopyrano moiety, was coupled with diverse amines or polyamines at 8-position. And their anti-HCC properties were evaluated alone or with other agents.

2. Results and Discussion

2.1 Chemistry

For the synthesis of 8-methyl flavonoid nucleus (**3**), we adopted a route previously reported by our lab.²⁰ In Scheme 1, 2-acetyl-6-methylphenol (**1**), a starting material prepared from 2-methylphenol *via* conventional Fries rearrangement, was treated with 1-naphthaldehyde in EtOH to generate chalcone (**2**). **2** was heated in DMSO with I₂/H₂SO₄ as catalysts to furnish the 8-methyl flavone backbone (**3**).

The bromination of **3** with NBS in the presence of benzoyl peroxide (BPO) afforded the intermediate **4**.²⁰ Intermediate **5**, prepared from **4** by the conventional

Gabriel method, was reacted with chloroacetyl chloride to furnish compound **6** in a nearly 100% yield. The tandem aminations of compound **6** with diverse amine/polyamine compounds **RNH₂** (as shown in figure 2), and respective Boc removal or neutralization of formed intermediates **7a-7f** with 4M HCl afforded target compounds **8a-8f** as hydrochloride salts.²¹ The structures of all the target compounds were confirmed by ¹H NMR, ESI-MS and elemental analysis.

2.2 In vitro Structure-Activity Relation Study

On the basis of our previous findings of effective HCC growth inhibition by the polyamine conjugates,²² two HCC cell lines (HepG2, SMMC7721) were selected for the in vitro screen of novel flavonoid-polyamine conjugates. In addition, the cell selectivity between tumor and normal cells, which may result from polyamines and/or flavonoids, was assessed with one normal hepatocyte cell line (QSG7701).

The inhibitory activity of target compounds toward two tumor cells was measured by the traditional MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tests. The flavonoid Quercetin (Que), and Mitoxantrone (Mito, a clinical drug in HCC therapy), were selected as positive drugs. The concentrations of new compounds were 30 and 100 μM, respectively. The respective concentration of Mitoxantrone was 1 and 5 μM for its high potency. The in vitro structure-activity relationship of target conjugates was obtained from the analysis of biological data as illustrated in Table 1.

The elevated potency to tumor cells and cell selectivity to normal cells are expected by the introduction of polyamine chains into parent drugs. Indeed, the improved

biological properties mentioned above have been reported in the cases of F14512^{14, 15} or naphthalimide-polyamine conjugates.²² Compounds **8a-8f** generally displayed moderate cytotoxicity toward tumor cells. The compound **8d** with a synthetic homospermidine skeleton exhibited best activity against two tested liver tumor cells, which is in accordance with previous reports.²³

Taking account of the impairment on both tumorous and normal liver cells, two positive controls, Mitoxantrone and Quercetin, behaved in an opposite way. Mitoxantrone kills more normal liver QSG7701 cells than corresponding cancerous cells at the same concentration, and Quercetin showed more favorable cell selectivity. The cell type selectivity of synthesized flavonoids **8a-8d** was superior to that of Mitoxantrone. However, only compound **8a** displayed cell type specific feature resembled to that of Quercetin. A more detailed study was subsequently conducted for compound **8a**. As shown in Figure 1, the respective IC₅₀ values of **8a** for HepG2, H22 (Murine carcinoma cells used in mice models) and QSG7701 cells are 65.0μM, 32.1μM and over 100μM, respectively. It clearly demonstrated that compound **8a** exhibited a more advantageous dose-dependent inhibition against HepG2 and H22 while retaining the favorable cell selectivity with respect to QSG7701 cells.

The combination therapy has been applied widely in clinical treatment of cancer. Using two agents in combination may produce more beneficial outcomes than either intervention alone, including the potentiation of antiproliferative responses and the reduction of required doses which might attenuate the probable adverse effects. However, the joint assays are sparsely utilized in preliminary drug screen due to their

complexity. We previously found that Aspirin, a non-steroid anti-inflammatory agent, could elevate the anti-HCC activity of polyamine conjugates²⁴ Here, a comparative study was conducted to evaluate the joint effect of novel conjugates with 800 μ M Aspirin, which showed no apparent effect on cell growth alone. The addition of Aspirin upregulated the potency of compound **8a** against HepG2 (from 65.0 μ M to 59.2 μ M), and H22 (from 32.1 μ M to 21.1 μ M) (Fig.3).

2.3 In vitro Anti-migration Study

The spread of tumor cells from the original sites to distant tissues is one thorny issue in cancer therapy. A number of reports revealed that some natural and synthetic flavonoids are effective metastasis inhibitors.^{3, 9} We recently found the potential of polyamine conjugates in preventing the occurrence of tumor invasion in vivo.²² The traditional Transwell tests and Wound-healing assays were thus conducted to examine preliminarily the capacity of **8a** alone or with Aspirin to inhibit tumor cell invasion.²⁵ As shown in Figure 4, **8a** evidently reduced the number of HepG2 cells invasion and migration in a dose dependent manner, and the presence of Aspirin increased the inhibitive effect of **8a**.

2.4 Apoptosis

Apoptosis is an important cell death profile in which multiple signaling pathways are involved. Previous studies revealed that both polyamine conjugates and flavonoids may trigger tumor cell death via an apoptotic process.²⁶ To investigate whether novel flavonoid-polyamine conjugates induce cell apoptosis, HepG2 cells treated by compound **8a** were doubly stained by AO (Acridine Orange) with a green

fluorescence and EB (Ethidium bromide) with an orange-red fluorescence, respectively. AO can penetrate the intact cell membranes and intercalate into the nuclear DNA, which is suitable for the detection of apoptosis in the early stage. Whereas EB, excluded from the viable cells with integral membranes, is used to discern the cell necrosis and late apoptosis by intercalating into the nuclear DNA after passing through the damaged cell membrane. As shown in Figure 5A, obvious apoptosis induced by **8a** was observed in a dose-responsive manner.

To furnish more credible information about the apoptosis of HepG2 cells induced by compound **8a**, we used the high content screening (HCS) technique to detect related apoptosis indicators. Mitochondria are vital cell organelles in the process of transmitting apoptosis signals, whereas the mitochondrial membrane potential (MMP) is a critical parameter related to mitochondrial function in cells. As a MMP specific cationic fluorescent dye, Rh123 was used to measure the variation of MMP, which accumulates in healthy mitochondria with intact membrane potential and is absent in the depolarized mitochondria.²² Lysosomes participate in various cell death profiles such as apoptosis and senescence.²⁷ Thus, the lysosomal mass/pH (LYS/pH) variation was detected by a basic fluorescent probe Lyso-Tracker Red which selectively accumulates in acidic lysosomes during cytotoxicity occurs. As shown in Figure 5B, after treatment with compound **8a** for 48h, the green fluorescence of Rh123 in cytoplasm was significantly decreased in a dose dependent manner, indicating that the destroyed mitochondria lost their mitochondrial transmembrane potential. Meanwhile,

the remarkable accrue ment in the red fluorescence of cells treated with compound **8a** implied the dose-related occurrence of lysosomes impairment.

It is known that polyamine conjugates may produce reactive oxygen species (ROS).²² Although flavonoids can serve as antioxidants which suppress the production of ROS,²⁸ there are also opposing reports that flavonoids may kill cells via promoted ROS level.²⁹ The elevated ROS content could bring corresponding influences on MMP loss and LYS/pH destabilization, which generally activates the cell death. Therefore, the ROS generation was detected by the DCFH-DA (2', 7'-dichlorodihydrofluorescein diacetate) experiment. Indeed, the apparently increased ROS was observed in a dose dependent pattern as shown in Figure 5B. In general, 60 μ M **8a** and 80 μ M Quercetin displayed minor differences in their impacts on MMP, LYS/pH, and ROS. Further efforts were subsequently conducted to investigate if **8a** and Quercetin possess a similar apoptotic mechanism.

Cysteiny l aspartate specific proteinases (caspases) family is one of the most important promoters and executioners for the induction of apoptosis. Both polyamine conjugates and flavonoids can trigger apoptosis through the classical caspase-mediated pathways. The caspase 3 activation was first checked by the immunofluorescence technique, utilizing primary antibody against the cleaved portions of caspase 3. Data in Figure 5B clearly demonstrated that **8a** and Quercetin could activate caspase 3, which corroborated the speculated caspase-dependent apoptosis. Furthermore, their influences on caspases 8 and 9, the upstream proteins of caspase 3, were measured by a similar method. Quercetin activated both caspase 8

which is closely associated with extrinsic death receptor pathway, and caspase 9 which is involved in the intrinsic mitochondria pathway. **8a** had no impact on caspase 9, but activated caspase 8 (Figure 5B).

p38 is a member of the MAPKs (mitogen activated protein kinases) group which intervenes in the signaling cascades of cell proliferation and apoptosis. It is reported that p38 may function upstream of caspase 8 in the apoptotic response.^{30,31} The data in Figure 5B clearly showed that **8a** up-regulated the expression of phospho p38 (p-p38) potently, while Quercetin displayed merely moderate impact on p-p38.

To illustrate the impact of drug on H22 cells used in animal models, several apoptosis-related factors (p-p38, p-JNK, p53, and Bcl-2) in the MAPK signaling pathway were further assessed. Similarly, the protein expression of p-p38 and p-JNK (phospho Jun-amino-terminal kinase) was upregulated by **8a** in a dose dependent manner. p53 is well known to play a major role in transducing stress to the apoptotic machinery of cells, and its overexpression can result in apoptotic cell death. The upregulated protein expression of p-p38 and p-JNK generally induced the activation of p53, followed by the downregulation of Bcl-2 (B cell CLL/Lymphoma 2) family. Indeed, the alteration of protein expression of both p53 and Bcl-2 was observed as expected in the experiments. Aspirin could moderately influence these proteins, and its presence potentiated the corresponding functions of **8a**.

2.5 In vivo Tumor Growth and Metastasis Inhibition

In vivo trials, based on preliminary in vitro screens, provide more valuable messages for further drug development. A rationally designed combination therapy of

two (or more) agents at the early screening stage will aid for the finding of more promising antitumor agents. Compared to combination regimens of flavonoids with other agents, for instance, aminoflavone with Paclitaxel,³² and LY294002 with DFMO (a-difluoromethylornithine)³³, to date there are few *in vivo* evidences for joint assessments of antineoplastic polyamine conjugates. Here, both Aspirin and DFMO were chosen to potentiate the activity of **8a** in mice models.

Two tumor transplant models, one solid tumor (H22) and one H22 pulmonary metastasis tumor, were established to appraise the *in vivo* antitumor efficacy of the target compounds **8a**. It was found that animals have a better tolerance for **8a** (20 mg/Kg) than Mitoxantrone (0.4 mg/Kg) in the pre-trials for MTD (maximum tolerated dose), which is in fine agreement with their respective cell selectivity. To avoid the likelihood of detrimental side effects and facilitate the implementation of combination test, compound **8a** was injected into mice with a median dosage (10 mg/Kg, half of the MTD). However, the maximum tolerated dose of Mitoxantrone was adopted in order to reach its best biological response. The dose of Aspirin and DFMO was chosen according to previous reports.^{24, 34} The 5×10^6 H22 cells were inoculated into 80 male Kunming mice to establish the H22 cancer model, and the treatments were randomly allocated into 8 groups (n = 10 mice per group): three **8a**-containing groups, including **8a** alone, **8a** plus Aspirin, **8a** plus DFMO; four positive control groups including Aspirin, DFMO, Quercetin, Mitoxantrone, and one negative control group.

The therapeutic benefits of tested drugs for each group are shown in Table 2. Compound **8a**, DFMO and Aspirin as a single agent, displayed only moderate tumor

inhibition effects, with inhibitory ratios slightly lower than 40%. The minor up-regulated inhibition (less than 4%) was observed by the co-treatment of **8a** in conjunction with DFMO, however, the presence of Aspirin resulted in pronounced tumor regressions. The 69% inhibition rate in the group exposed to **8a**-Aspirin is almost equivalent to that of Mitoxantrone (73%), suggesting that the combination of flavonoid-polyamine conjugates with Aspirin generated better additive effects.

Based on preliminary in vitro anti-invasion results, the capacity of **8a** to inhibit metastasis alone or in combination with Aspirin and DFMO was appraised on the pulmonary metastasis models. As shown in Table 2, compound **8a** and Aspirin exhibited low anti-metastatic effects with the inhibition rates of about 29% and 24%, respectively. Mitoxantrone is still a more potent metastasis inhibitor (about 49%) than **8a** as a single agent. Similarly, the elevated anti-metastatic efficacy was observed in the combination regimens of **8a** with both Aspirin and DFMO. The treatment with DFMO and **8a** alleviated the tumor metastasis in mice with an inhibition rate of 48.9%, just equipotent to that of Mitoxantrone. Intriguingly, the roughly 60% inhibition rate in the animal group dealt with **8a**-Aspirin, higher than that of Mitoxantrone, indicated that these two agents acted synergistically with each other to generate potent anti-metastatic responses (Figure 7).

Unexpectedly, two mice in the group treated by **8a** died in the course of H22 tumor experiments (Table 2). We speculated that they died not from the dose toxicity of **8a**, but improper manipulation or accidents for other groups treated by the same dose of **8a** remained alive (Tables 2 and 3). According to NCI (National Cancer Institute)

criteria, a dose is regarded as toxic if the mouse body weight loss after administration is over 20% of the original body weight¹⁵. Treatment with Mitoxantrone was associated with acceptable 6.8% weight loss (Table 2). In general, mice with H22 tumor model gained more weight than those with lung metastasis model which also behaved less actively, the sign of disadvantageous impacts of tumor metastasis. Compared to the initial body weight of each group, no evident weight loss was observed in the treated mice. Notably, the presence of Aspirin improved the indexes of mouse body weight better than DFMO in all the four joint-treated groups.

3. Conclusion

A series of flavonoid-polyamine conjugates were designed and synthesized. The in vitro evaluation against HCC cell lines revealed that they generally exhibited cytotoxicity at relatively high concentration. However, additional assays against normal liver QSG7701 cells testified that compound **8a** exhibited nice cell selectivity in killing much more cancerous cells than normal ones. Based on the indicative messages from in vitro co-administrative and anti-metastatic tests, further in vivo experiments testified that Aspirin could up-regulate the moderate efficacy of **8a** at a mild dose against H22 liver cancer and pulmonary metastasis models, accompanied by favorable body weight indexes, which supported the assumption that the biological functions of designed flavonoid-polyamine conjugates can be improved efficiently. The impacts of **8a** on HepG2 cell morphology, mitochondrial transmembrane potential, and lysosomes indicated that **8a** may impair tumor cells via apoptosis. The apoptosis-related proteins (p-p38, p-JNK, p53, and Bcl-2) were further detected in

H22 cells, which supported that **8a** triggered apoptotic cell death via a different mechanism from that of Quercetin, and the presence of Aspirin could amplify the effect of **8a** on these proteins. Taken together, the parent drug in the polyamine conjugate is awarded some enticing polyamine-like biological properties. And the combination trial aimed at elevating anti-cancer responses and attenuating adverse effects may serve as an alternative approach to appraise the potential of novel polyamine conjugates as anti-tumor drug candidates.

4. Experiment section

4.1 Chemistry

All solvents and reagents were purchased from the suppliers and used without further purification. Flash column chromatography was performed with silica gel (200 - 300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd. The ^1H NMR spectra were recorded on a Bruker AV-400 model Spectrometer in D_2O , CDCl_3 , or $\text{d}_6\text{-DMSO}$. Chemical shifts for ^1H NMR spectra were reported in parts per million to TMS or residual solvent protons. MS spectra were recorded on a Shimadzu LCMS-2010A instrument with an ESI mass selective detector. The target compounds with the purity all being higher than 95% were confirmed by the combustion analysis. Elemental analyses were performed on a Gmbe VarioEL elemental instrument and were within 0.4% of the theoretical values.

4.1.1 Synthesis of intermediate 2

To a mixture of 1-naphthaldehyde (2.19 g, 14.0 mmol) and compound **1** (1.65 g,

11.0 mmol) in EtOH (20 mL) was added 50% KOH/EtOH (15 mL), the mixture was stirred at room temperature overnight. Water was added to the mixture and the hydrochloric acid was added to adjust the solution to pH=2-3. After filtration, the solid was dried to give intermediate **2**.

4.1.2 Synthesis of intermediate **3**

Intermediate **2** (3.17 g, 11.0 mmol) and H₂SO₄ (2.5 mL) were dissolved in DMSO (20 mL) and stirred at 110 °C for 20 min, and a catalytic amount of I₂ was added and heated at 110 °C for 5.5 h. After cooling down to room temperature, ice water (100 mL) was added. Then a small amount of Na₂S₂O₃ was added and stirred for 30 min to remove I₂. After filtration, the crude intermediate **3a** was purified by column chromatography with petroleum ether/acetone (8:1, v/v) as elution solvent to give the **3a** as a pale yellow solid with a yield of 70%.

8-methyl-2-(naphthalen-1-yl)-4H-chromen-4-one (3): ¹H NMR (CDCl₃, 400 MHz) δ: 8.20 (dd, J = 5.0 Hz, 1 H, H-5'), 8.16 (dd, J = 8.0 Hz, 1 H, H-4'), 8.03 (d, J = 8.2 Hz, 1 H, H-5), 7.95 (dd, J = 6.0 Hz, 1 H, H-8'), 7.79 (dd, J = 4.0 Hz, 1 H, H-2'), 7.63 - 7.51 (m, 4H, H-3, H-3', H-6', H-7'), 7.36 (t, J = 7.6 Hz, 1 H, H-6), 6.73 (s, 1H, H-3), 2.49 (s, 3H, -CH₃); ESI-MS m/z: 287.3(M+1)⁺.

4.1.5 General procedures for the synthesis of **4**

A mixture of **3** (30 mmol), NBS (5.3 g, 30 mmol), a catalytic amount of benzoyl peroxide and CCl₄ (150 mL) were reflux for 8 h under the light. The solvent was removed under reduced pressure and **4** was obtained by column chromatography with petroleum ether/EtOAc (5:1, v/v) as elution solvent. Yield: 55%.

4.1.6 Synthesis of intermediate 5

To a mixture of **4** (2.19 g, 6.0 mmol) in CH₃CN (30 mL) was added potassium phthalimide (0.88 g, 6.0 mmol), the solution was reflux for 8 h. After cooling down to room temperature, filtered and the solvent was removed under reduced pressure, the residue was purified by column chromatography on a silica gel column with petroleum ether/EtOAc (5:1, v/v) as elution solvent to give pale yellow oil.

The pale yellow oil was dissolved in EtOH (35 mL), and hydrazine hydrate (5 mL) was added, the mixture was stirred at room temperature until a great amount of precipitate was created. After removing the solvent, the residue was dissolved in CHCl₃ and washed with 10% Na₂CO₃ three times, the organic layer was dried over Na₂SO₄ and the solvent was removed, the residue was purified by column chromatography on a silica gel column with CHCl₃/CH₃OH (30:1, v/v) as elution solvent to give intermediate **5** as pale yellow oil, yield: 35%.

4.1.9 Synthesis of intermediate 6

To a mixture of **5** (0.60 g, 2.0 mmol) and anhydrous K₂CO₃ (2.0 g, 14.5 mmol) in CH₃CN (30 mL) was added chloroacetyl chloride (1.13g, 10 mmol) dropwise in ice bath, the mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was dissolved in CHCl₃, and washed with water three times. After drying over anhydrous Na₂SO₄ and evaporating the solvent to give the intermediate **6** with a crude yield of 100%, which was used for next step without further purification.

4.1.10 Syntheses of the target compounds 8a-8f

Intermediate **6** (4.0 mmol), diverse amines RNH₂ (4.0 mmol), anhydrous K₂CO₃ (3.0 g, 21.7 mmol) was dissolved in CH₃CN (30 mL) and a catalytic amount of KI was added, the mixture was heated at 30 °C for 6 h. After filtered, the solvent was removed under reduced pressure. The obtained residue (crude **7a-7f**), purified by column chromatography with CHCl₃/CH₃OH (30:1, v/v) as elution solvent, was dissolved in CH₃CH₂OH to which 4N HCl in CH₃CH₂OH was added and then stirred at room temperature overnight until a great amount of precipitate was generated. The filtered cake was washed by anhydrous CH₃CH₂OH, dried to give the target compounds **8a-8f** as a hydrochloride salt.

2-(3-aminopropylamino)-N-((2-(naphthalen-1-yl)-4-oxo-4H-chromen-8-yl)methyl)acetamide dihydrochloride (8a). Yield 83%, white solid. ¹H NMR (400 MHz, D₂O) δ: 7.96 (d, J = 8.3 Hz, 1H, H-5'), 7.83 (t, J = 8.2 Hz, 2H, H-4'+ H-8'), 7.76 (d, J = 7.9 Hz, 1H, H-5), 7.64 (t, J = 6.2 Hz, 2H, H-2'+ H-3'), 7.47 – 7.37 (m, 4H, H-6'+ H-7'+ H-7+ H-6), 6.45 (s, 1H, H-3), 4.41 (s, 2H, 1×CH₂), 3.79 (s, 2H, 1×CH₂), 2.96 (dd, J = 15.7, 8.0 Hz, 4H, 2×CH₂), 2.04 – 1.93 (m, 2H, 1×CH₂); ESI-MS m/z: 416.5(M+1–2HCl)⁺. Anal.calcd. for C₂₅H₂₅N₃O₃·2HCl·2.5H₂O: C 56.29%, H 6.05%, N 7.88%; found C 56.24%; H 5.88%; N 7.90%.

2-(4-aminobutylamino)-N-((2-(naphthalen-1-yl)-4-oxo-4H-chromen-8-yl)methyl)acetamide dihydrochloride (8b). Yield 85%, white solid. ¹H NMR (400 MHz, D₂O) δ: 7.80 (d, J = 8.2 Hz, 1H, H-5'), 7.67 (d, J = 8.1 Hz, 2H, H-4'+ H-8'), 7.61 (d, J = 7.7 Hz, 1H, H-5), 7.54 (d, J = 7.2 Hz, 1H, H-2'), 7.48 (d, J = 7.1 Hz, 1H, H-3'), 7.30 (ddd, J = 26.1, 16.2, 7.8 Hz, 4H, H-6'+ H-7'+ H-7+ H-6), 6.24 (s, 1H, H-3), 4.27 (s, 2H,

1×CH₂), 3.75 (s, 2H, 1×CH₂), 2.94 (s, 2H, 1×CH₂), 2.86 (d, J = 6.8 Hz, 2H, 1×CH₂), 1.69 – 1.57 (m, 4H, 2×CH₂); ESI-MS:m/z: 430.5(M+1–2HCl)⁺. Anal.calcd. for C₂₆H₂₇N₃O₃·2HCl·2.6H₂O: C 56.85%, H 6.28%, N 7.65%; found C 56.66%; H 6.07%; N 7.55%.

2-(3-(4-aminobutylamino)propylamino)-N-((2-(naphthalen-1-yl)-4-oxo-4H-chromen-8-yl)methyl)acetamide trihydrochloride (8c). Yield 83%, white solid. ¹H NMR (400 MHz, D₂O) δ: 7.91 (d, J = 8.2 Hz, 1H, H-5'), 7.81 – 7.74 (m, 2H, H-4'+ H-8'), 7.70 (d, J = 8.1 Hz, 1H, H-5), 7.60 (s, 2H, H-2'+ H-3'), 7.45 – 7.31 (m, 4H, H-6'+ H-7'+ H-7+ H-6), 6.37 (s, 1H, H-3), 4.36 (s, 2H, 1×CH₂), 3.81 (s, 2H, 1×CH₂), 3.02 (ddd, J = 19.7, 15.8, 8.0 Hz, 10H, 5×CH₂), 1.80 – 1.66 (m, 6H, 3×CH₂); ESI-MS m/z: 487.6(M+1–3HCl)⁺. Anal.calcd for C₂₉H₃₄N₄O₃·3HCl·3.8H₂O: C 52.42%, H 6.77%, N 8.43%; found C 52.16%; H 6.48%; N 8.61%.

2-(4-(4-aminobutylamino)butylamino)-N-((2-(naphthalen-1-yl)-4-oxo-4H-chromen-8-yl)methyl)acetamide trihydrochloride (8d). Yield 86%, white solid. ¹H NMR (400 MHz, D₂O) δ: 7.79 (d, J = 8.2 Hz, 1H, H-5'), 7.65 (t, J = 6.9 Hz, 2H, H-4'+ H-8'), 7.59 (d, J = 7.9 Hz, 1H, H-5), 7.49 (t, J = 8.4 Hz, 2H, H-2'+ H-3'), 7.27 (dt, J = 19.2, 8.1 Hz, 4H, H-6'+ H-7'+ H-7+ H-6), 6.24 (s, 1H, H-3), 4.24 (s, 2H, 1×CH₂), 3.67 (s, 2H, 1×CH₂), 2.97 – 2.87 (m, 6H, 3×CH₂), 2.79 (s, 2H, 1×CH₂), 1.63 (s, 4H, 2×CH₂), 1.56 (s, 4H, 3×CH₂) ; ESI-MS m/z: 501.6(M+1–3HCl)⁺. Anal.calcd for C₃₀H₃₆N₄O₃·3HCl·0.8H₂O: C 57.70%, H 6.55%, N 8.97%; found C 57.68%; H 6.73%; N 8.73%.

2-(2-(dimethylamino)ethylamino)-N-((2-(naphthalen-1-yl)-4-oxo-4H-chromen-8-yl)

1)methyl)acetamide dihydrochloride (8e). Yield 82%, white solid. ^1H NMR (400 MHz, D_2O) δ : 7.85 (d, $J = 8.2$ Hz, 1H, H-5'), 7.71 (d, $J = 8.1$ Hz, 2H, H-4'+ H-8'), 7.64 (d, $J = 8.0$ Hz, 1H, H-5), 7.58 (d, $J = 7.4$ Hz, 1H, H-2'), 7.53 (d, $J = 7.2$ Hz, 1H, H-3'), 7.43 – 7.27 (m, 4H, H-6'+ H-7'+ H-7+ H-6), 6.29 (s, 1H, H-3), 4.32 (s, 2H, $1\times\text{CH}_2$), 3.93 (s, 2H, $1\times\text{CH}_2$), 3.49 (pd, $J = 8.0, 2.2$ Hz, 4H, $2\times\text{CH}_2$), 2.93 (s, 6H, $2\times\text{CH}_3$); ESI-MS m/z : 430.5($\text{M}+1-2\text{HCl}$) $^+$. Anal.calcd for $\text{C}_{26}\text{H}_{27}\text{N}_3\text{O}_3\cdot 2\text{HCl}\cdot 2.5\text{H}_2\text{O}$: C 57.04%, H 6.26%, N 7.68%; found C 57.32%; H 5.88%; N 7.57%.

2-(butylamino)-N-((2-(naphthalen-1-yl)-4-oxo-4H-chromen-8-yl)methyl)acetamide hydrochloride (8f). Yield 85%, white solid. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 8.31 – 8.25 (m, 1H, H-5'), 8.20 (d, $J = 8.2$ Hz, 1H, H-4'), 8.14 – 8.03 (m, 2H, H-8'+ H-5), 7.98 (d, $J = 7.1$ Hz, 1H, H-2'), 7.82 (d, $J = 7.3$ Hz, 1H, H-3'), 7.74 – 7.61 (m, 3H, H-6'+ H-7'+ H-7), 7.54 (t, $J = 7.7$ Hz, 1H, H-6), 6.75 (s, 1H, H-3), 4.59 (d, $J = 5.6$ Hz, 2H, $1\times\text{CH}_2$), 3.77 (s, 2H, $1\times\text{CH}_2$), 2.84 (s, 2H, $1\times\text{CH}_2$), 1.55 (dt, $J = 15.4, 7.6$ Hz, 2H, $1\times\text{CH}_2$), 1.36 – 1.17 (m, 2H, $1\times\text{CH}_2$), 0.86 (t, $J = 7.3$ Hz, 3H, $1\times\text{CH}_3$); ESI-MS m/z : 415.5($\text{M}+1-\text{HCl}$) $^+$. Anal.calcd for $\text{C}_{26}\text{H}_{26}\text{N}_2\text{O}_3\cdot\text{HCl}\cdot 0.1\text{H}_2\text{O}$: C 68.97%, H 6.06%, N 6.19%; found C 68.72%; H 5.86%; N 6.03%.

4.2 Biological Materials and Methods

4.2.1. Cell Lines and MTT Assessments.

Human hepatocellular carcinoma cell line HepG2, SMMC7721 and Human normal liver cells QSG7701 cell lines were purchased from Shanghai Institute for Biological Science, Chinese Academy of Science (Shanghai, China). Mice hepatic cancer H22 cells were extracted from mice H22 ascites carcinoma and propagated for three

generations before formal experiments. Cells were cultured in RPMI1640 supplemented with 10% FBS and penicillin (100 units/ml)-streptomycin (100 ug/ml). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ atmosphere. The growth inhibition of tested compounds was evaluated in HepG2, SMMC7721 and QSG7701 cells by the conversion of MTT to a purple formazan precipitate as previously described. Cells were seeded in 96 well plates (6×10^3 cells/well), cultured for 24 h to obtain a confluent monolayer and then treated with different concentrations of tested compounds for 48 h. The inhibition rate was calculated from plotted results using untreated cells as 100%.

4.2.2 Anti-proliferative Activity in the Presence of Aspirin.

HepG2, H22cells or QSG7701 cells were seeded in 96 well plates (6×10^3 cells/well), cultured for 24 h to obtain a confluent monolayer and then treated with different concentrations of test compounds with or without 800 μ M Aspirin for 48 h.

4.2.3 In Vitro Migration Assay.

The inhibitory effect of compound **8a** on HepG2 cells migration was assayed using 24-well Transwell cell culture chambers (6.5 mm diameter, 8.0 μ m pore size, polycarbonate membrane, Corning) which precoated 100 μ L Matrigel (Matrigel basement membrane matrix, Corning, USA). HepG2 cells (2×10^5) were suspended in RPMI 1640 supplemented with 0.1% FBS and added to the upper chamber. The lower chamber contained 500 μ L of RPMI 1640 with 10% FBS. After the addition of different concentrations of compound **8a** or 80 μ M Quercetin, and 24 h of incubation at 37°C in a 5% CO₂ incubator, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The cells on the lower surface of the membrane were stained with 0.1% crystal violet after fixation with methanol. After

photographed under the microscope, the stains were eluted with 33% glacial acetic acid and the absorbance of the cell lysate was measured at 570 nm by microplate reader.

4.2.4. Cellular Apoptotic Evaluation.

HepG2 cells were seeded in 96 well plates (6×10^3 cells/well), cultured for 24 h to obtain a confluent monolayer and then treated with various concentrations of tested compounds for 48 h. Cells were incubated with Acridine Orange (50 μ M)/Ethidium Bromide (50 μ M) in the dark for 30 min., then washed with PBS to remove unbound dyes. Images were obtained on the High Content Screening (HCS, ArrayScan) reader using Target Activation BioApplication software.

4.2.5 ROS, MMP and LYS/pH Staining Experiments.

HepG2 cells were seeded in 96 well plates (6×10^3 cells/well), cultured for 24 h to obtain a confluent monolayer, and then treated with various concentrations of tested compounds for 48 h. Cells were incubated with Rh123 (0.25 nM), or DCFH-DA (10 μ M), or Lyso-Tracker Red (50 nM) in the dark at 37 °C for 30 min. for the measurement of mitochondrial membrane potential (MMP), or Reactive Oxygen Species (ROS) or lysosomal mass/pH staining, respectively. After being washed with PBS to remove excess dyes, cells were further stained with the nuclear dye Hoechst 33342 (10 μ M) in the dark at 37 °C for 20 min.. Images were acquired on the ArrayScan HCS reader using Target Activation BioApplication software.

4.2.6 Immunofluorescence Staining for Caspases and p-p38.

After treatment with compound **8a**, HepG2 cells were fixed in 4% paraformaldehyde

for 30 min., rinsed with PBS, permeablized in 0.1% Triton X-100 for 5 min., blocked in PBS containing 5% BSA (30 min. at 37 °C), and labeled with primary antibody: Caspase 3, Caspase 8, Caspase 9, p-p38 (overnight at 4°C). Fitc-conjugated secondary antibody was incubated for 2 h at 37 °C. Nuclear counterstaining was performed using Hoechst 33342 (10 µM) for 20 min at dark. Fluorescence was quantitated using HCS.

4.2.7 Immunofluorescence Staining for p53, p-p38, p-JNK, Bcl-2.

After treatment with compound **8a**, H22 cells were harvested to 2 ml centrifuge tubes and fixed in 4% paraformaldehyde for 30 min., rinsed with PBS, permeablized in 0.1% Triton X-100 for 5 min., blocked in PBS containing 5% BSA (30 min. at 37 °C), and labeled with primary antibody: p53, p-p38, p-JNK, or Bcl-2 (overnight at 4°C). Fitc-conjugated secondary antibody was incubated for 2 h at 37 °C. Fluorescence was measured with flow cytometry.

4.2.8 Subcutaneous xenograft of H22 Cells in Kunming Mice.

All mice care and experimentation conformed to the Guide for the Care and Use of Laboratory Animals published by Henan University. For solid tumor development, 100 male Kunming mice (Laboratory Animal Center of Henan, Zhengzhou) aged 5 weeks (weighing 18–22 g) were injected subcutaneously with 2×10^6 H22 cells. On the eighth day after inoculation, the mice were randomly divided into ten groups and then were administered by caudal vein injection with **8a** with or without DFMO or Asp (n=10) for 7 consecutive days (Note: Asp and DFMO were injected intraperitoneally). Mitoxantrone and normal saline were chosen as positive and

negative control, respectively. On day 16, the mice were killed by ether anesthesia, and solid tumors were removed and weighed. The inhibitory rate was calculated as follows: inhibitory rate (%) = $[(A-B)/A] \times 100$, where A was the mean tumor weight of the negative control group and B was that of the drug treated or positive group. The mouse body weight change was calculated as follows: weight change (%) = $[(A-B)/B] \times 100$, where A was the mean terminal body weight of each group and B was the mean initial body weight of the same group.

4.2.9 H22 Cells Lung Metastasis Models.

H22 cells (5×10^6 cells per mouse) for Kunming mice were injected iv through the tail vein for tumor passive metastasis. To ensure all mice bore actively growing lung tumors before the drug treatment, pulmonary metastasis was allowed to develop for 10 days. On day 11, 80 Kunming mice were randomly divided into ten groups. Mice were injected intravenously with corresponding compounds, and normal saline (negative control) (n = 8) for 7 consecutive days. On day 18, mice were killed by ether anesthesia and lungs were removed and then fixed in Bouin's fluid. After the lungs were fixed, lung metastases nodes were numbered. The inhibitory rate was calculated as follows: inhibitory rate (%) = $[(A-B)/A] \times 100$, where A was the mean metastases nodes of the negative control group and B was that of the drug treated or positive group. The mouse body weight change was calculated as mentioned above.

4.2.10 Data Analysis.

All data are presented as the mean $\pm$ SD and analyzed using Student's t test or analysis of variance (ANOVA) followed by q test: compared with control, (*) $p <$

0.05, (**) $p < 0.01$ as significant.

■ AUTHOR INFORMATION

*Corresponding Author.

For C.W. Tel: 86-13619810550. Fax: 86-378-2864665. E-mail: wcjsxq@henu.edu.cn;

For S.X. Tel: 86-13938637212. E-mail: kfxsq@yahoo.com.cn; For W.L. Tel: 86-15225477220. E-mail: luowen83@henu.edu.cn,

Author Contributions

‡ These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGEMENT

This work was supported by National Science Foundation of China (No. 21172053, 21302041).

REFERENCES

1. (a) M. Correia-da-Silva, E. Sousa, B. Duarte, F. Marques, F. Carvalho, L.M. Cunha-Ribeiro, M.M. Pinto, Flavonoids with an oligopolysulfated moiety: a new class of anticoagulant Agents, *J. Med. Chem.* 54 (2011) 95–106.
(b) C.S. Yang, X. Wang, G. Lu, S.C. Picinich, Cancer prevention by tea: animal studies, molecular mechanisms and human relevance, *Nat. Rev. Cancer.* 9 (2009) 429–439.
2. W.M. Alarif, A. Abdel-Lateff, A.M. Al-Abd, S.A. Basaif, F.A. Badria, M. Shams, S.E. Alarif, Selective cytotoxic effects on human breast carcinoma of new methoxylated flavonoids from *Euryops arabicus* grown in Saudi Arabia, *Eur. J. Med. Chem.* 66 (2013) 204–210.
3. (a) J.M. Pavese, R.L. Farmer, R.C. Bergan, Inhibition of cancer cell invasion and metastasis by genistein, *Cancer Metastasis Rev.* 29 (2010) 465–482.
(b) C.J. Weng, G.C. Yen, Flavonoids, a ubiquitous dietary phenolic subclass, exert extensive in vitro anti-invasive and in vivo anti-metastatic activities, *Cancer Metastasis Rev.* 31 (2012) 323–351.

- (c) C. Danciu, F. Borcan, F. Bojin, I. Zupko, C. Dehelean, Effect of the isoflavone genistein on tumor size, metastasis potential and melanization in a B16 mouse model of murine melanoma, *Nat. Prod. Commun.* 8 (2013) 343–346.
4. B.N. Singh, S. Shankar, R.K. Srivastava, Green tea catechin, epigallocatechin-3- gallate (EGCG): mechanisms, perspectives and clinical applications, *Biochem. Pharmacol.* 82 (2011) 1807–1821.
5. (a) A. Kralj, M.T. Nguyen, N. Tschammer, N. Ocampo, Q. Gesiotto, M.R. Heinrich, O. Phanstiel, Development of flavonoid-based inverse agonists of the key signaling receptor US28 of human cytomegalovirus, *J Med Chem.* 56 (2013) 5019–5032.
- (b) S. Kim, L. Li, Z. Maliga, Q. Yin, H. Wu, T.J. Mitchison, Anticancer flavonoids are mouse-selective STING agonists, *ACS Chem. Biol.* 8 (2013) 1396–1401.
- (c) H.M. Fekrazad, C.F. Verschraegen, M. Royce, H.O. Smith, L.F. Chyi, I. Rabinowitz, A phase I study of flavopiridol in combination with gemcitabine and irinotecan in patients with metastatic cancer, *Am. J. Clin. Oncol.* 33 (2010) 393–397.
6. (a) C.J. Vlahos, W.F. Matter, K.Y. Hui, R.F. Brown, A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), *J. Biol. Chem.* 269 (1994) 5241–5248.
- (b) G.A. Morales, J.R. Garlich, J. Su, X. Peng, J. Newblom, K. Weber, D.L. Durden, Synthesis and cancer stem cell-based activity of substituted 5-morpholino-7H-thieno[3,2-b]pyran-7-ones designed as next generation PI3K inhibitors, *J. Med. Chem.* 56 (2013) 1922–1939.
7. K.F. Chan, I.K. Wong, J.W. Kan, C.S. Yan, L.M. Chow, T.H. Chan, Amine linked flavonoid dimers as modulators for P-glycoprotein-based multidrug resistance: structure–activity relationship and mechanism of modulation, *J. Med. Chem.* 55 (2012) 1999–2014.
8. N. Yao, C.Y. Wu, K. Motonishi, H.J. Kung, K.S. Lam, Novel flavonoids with antiproliferative activities against breast cancer cells, *J Med Chem.* 54 (2011) 4339–4349.
9. (a) B.M. Ku, H.W. Ryu, Y.K. Lee, J. Ryu, J.Y. Jeong, J. Choi, H.J. Cho, K.H. Park, S.S. Kang, 4'-Acetoamido-4-hydroxychalcone, a chalcone derivative, inhibits glioma growth and invasion through regulation of the tropomyosin 1 gene, *Biochem. Biophys. Res. Commun.* 402 (2010) 525–530.
- (b) F. Li, C. Li, H. Zhang, Z. Lu, Z. Li, Q. You, N. Lu, Q. Guo, VI-14, a novel flavonoid derivative, inhibits migration and invasion of human breast cancer cells, *Toxicol. Appl. Pharmacol.* 261 (2012) 217–226.
10. M.L. Bolognesi, N. Calonghi, C. Mangano, L. Masotti, C. Melchiorre, Parallel synthesis and cytotoxicity evaluation of a polyamine-quinone conjugates library, *J. Med. Chem.* 51 (2008) 5463–5467.
11. (a) G.E. Magoulas, O.N. Kostopoulou, T. Garnelis, C.M. Athanassopoulos, G.G. Kournoutou, M. Leotsinidis, G.P. Dinos, D. Papaioannou, D.L. Kalpaxis, Synthesis and antimicrobial activity of chloramphenicol-polyamine conjugates, *Bioorg. Med. Chem.* 23 (2015) 3163–3174.
- (b) D. Vourtsis, M. Lamprou, E. Sadikoglou, A. Giannou, O. Theodorakopoulou, E. Sarrou, G.E. Magoulas, S.E. Bariamis, C.M. Athanassopoulos, D. Drainas, D. Papaioannou, E. Papadimitriou, Effect of an all-trans-retinoic acid conjugate with spermine on viability of human prostate cancer and endothelial cells in vitro and angiogenesis in vivo, *Eur. J. Pharmacol.* 698 (2013) 122–130.
12. F. Lizzi, G. Veronesi, F. Belluti, C. Bergamini, A. López-Sánchez, M. Kaiser, R. Brun, R.L. Krauth-Siegel, D.G. Hall, L. Rivas, M.L. Bolognesi, Conjugation of quinones with natural

polyamines: toward an expanded antitrypanosomatid profile, *J. Med. Chem.* 55 (2012) 10490–10500.

13. G. Palermo, E. Minniti, M.L. Greco, L. Riccardi, E. Simoni, M. Convertino, C. Marchetti, M. Rosini, C. Sissi, A. Minarini, V.M. De, An optimized polyamine moiety boosts the potency of human type II topoisomerase poisons as quantified by comparative analysis centered on the clinical candidate F14512, *Chem Commun.* 51 (2015) 14310–14313.

14. A. Kruczynski, I. Vandenberghe, A. Pillon, S. Pesenl, L. Goetsch, J.M. Barret, Y. Guminski, A. Le Pape, T. Imbert, C. Bailly, N. Guilbaud, Preclinical activity of F14512, designed to target tumors expressing an active polyamine transport system, *Invest. New. Drugs* 29 (2011) 9–21.

15. J.M. Barret, A. Kruczynski, S. Vispe, J.P. Annereau, V. Brel, Y. Guminski, J.G. Delcros, A. Lansiaux, N. Guilbaud, T. Imbert, C. Bailly, F14512, a potent antitumor agent targeting topoisomerase II vectored into cancer cells via the polyamine transport system, *Cancer Res.* 68 (2008) 9845–9853.

16. (a) S. Tsujinaka, K. Soda, Y. Kano, F. Konishi, Spermine accelerates hypoxia-initiated cancer cell migration, *Int. J. Oncol.* 38 (2011) 305–312.

(b) D.L. Koomoa, D. Geerts, I. Lange, J. Koster, A.E. Pegg, D.J. Feith, A.S. Bachmann, DFMO/eflornithine inhibits migration and invasion downstream of MYCN and involves p27 Kip1 activity in neuroblastoma, *Int. J. Oncol.* 42 (2013) 1219–1228.

17. M. Linsalata, A. Orlando, C. Messa, M.G. Refolo, F. Russo, Quercetin inhibits human DLD-1 colon cancer cell growth and polyamine biosynthesis, *Anticancer. Res.* 30 (2010) 3501–3507.

18. J. Ferlay, H.R. Shin, F. Bray, D. Forman, C. Mathers, D.M. Parkin, Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008, *Int. J. Cancer.* 127 (2010), 2893–2917.

19. (a) N. Grabinski, F. Ewald, B.T. Hofmann, K. Stauffer, U. Schumacher, B. Nashan, M. Jücker, Combined targeting of AKT and mTOR synergistically inhibits proliferation of hepatocellular carcinoma cells, *Mol. Cancer.* 11 (2012) 85.

(b) Y. Yang, H. Nagano, H. Ota, O. Morimoto, M. Nakamura, H. Wada, T. Noda, B. Damdinsuren, S. Marubashi, A. Miyamoto, Y. Takeda, K. Dono, K. Umeshita, S. Nakamori, K. Wakasa, M. Sakon, M. Monden, Patterns and clinicopathologic features of extrahepatic recurrence of hepatocellular carcinoma after curative resection, *Surgery.* 141 (2007) 196–202.

20. (a) J. Zhao, Y. Liu, M.H. Zheng, S.Q. Xie, C.J. Wang, Synthesis and biological activity of 8-substituted flavonoids, *Chin. J. Org. Chem.* 28 (2008) 84–88.

(b) W. Luo, Y.B. Su, C. Hong, R.G. Tian, L.P. Su, Y.Q. Wang, Y. Li, J.J. Yue, C.J. Wang, Design, synthesis and evaluation of novel 4-dimethylamine flavonoid derivatives as potential multi-functional anti-Alzheimer agents, *Bioorg. Med. Chem.* 21 (2013) 7275–7282.

21. Z.Y. Tian, S.Q. Xie, Y.W. Du, Y.F. Ma, J. Zhao, W.Y. Gao, C.J. Wang, Synthesis, cytotoxicity and apoptosis of naphthalimide polyamine conjugates as antitumor agents, *Eur. J. Med. Chem.* 44 (2009) 393–399.

22. Y.X. Wang, X.B. Zhang, J. Zhao, S.Q. Xie, C.J. Wang, Nonhematotoxic naphthalene diimide modified by polyamine: synthesis and biological evaluation, *J. Med. Chem.* 55 (2012) 3502–3512.

23. N. Kaur, J.G. Delcros, J. Imran, A. Khaled, M. Chehtane, N. Tschammer, B. Martin, O. Phanstiel, 4th. A comparison of chloroambucil- and xylene-containing polyamines leads to improved ligands for accessing the polyamine transport system, *J. Med. Chem.* 51 (2008) 1393–1401.

24. (a) S.Q. Xie, J.H. Wang, Y.H. Zhang, C.J. Wang, Antitumor conjugates with polyamine vectors and their molecular mechanisms, *Expert Opin. Drug. Deliv.* 7 (2010) 1049–1061.
- (b) S.Q. Xie, L.L. Zhang, T. Yang, Y. Ma, Y.H. Zhang, Q. Li, J.H. Wang, J. Zhao, C.J. Wang, Acetylsalicylic acid strengthens the effects of ANISpm against hepatocellular carcinoma and its molecular mechanism, *Yao Xue Xue Bao.* 46 (2011) 1045–1050.
25. M. Ogasawara, T. Matsunaga, H. Suzuki, Differential effects of antioxidants on the in vitro invasion, growth and lung metastasis of murine colon cancer cells, *Biol. Pharm. Bull.* 30 (2007) 200–204.
26. (a) C. Ballot, M. Jendoubi, J. Kluza, A. Jonneaux, W. Laine, P. Formstecher, C. Bailly, P. Marchetti, Regulation by survivin of cancer cell death induced by F14512, a polyamine-containing inhibitor of DNA topoisomerase II, *Apoptosis.* 17 (2012) 364–376.
- (b) J. Jakubowicz-Gil, E. Langner, D. Bądziul, I. Wertel, W. Rzeski, Apoptosis induction in human glioblastoma multiforme T98G cells upon temozolomide and quercetin treatment, *Tumour Biol.* 34 (2013) 2367–2378.
27. (a) A.M. Ellegaard, L. Groth-Pedersen, V. Oorschot, J. Klumperman, T. Kirkegaard, J. Nylandsted, M. Jäätelä, Sunitinib and SU11652 Inhibit Acid Sphingomyelinase, Destabilize Lysosomes, and Inhibit Multidrug Resistance, *Mol. Cancer Ther.* 12 (2013) 2018–2030.
- (b) Z. Chen, X. Liang, H. Zhang, H. Xie, J. Liu, Y. Xu, W. Zhu, Y. Wang, X. Wang, S. Tan, D. Kuang, X. Qian, A new class of naphthalimide-based antitumor agents that inhibit topoisomerase II and induce lysosomal membrane permeabilization and apoptosis, *J. Med. Chem.* 53 (2010) 2589–2600.
28. D.E. Lee, M.Y. Chung, T.G. Lim, W.B. Huh, H.J. Lee, K.W. Lee, Quercetin suppresses intracellular ROS formation, MMP activation, and cell motility in human fibrosarcoma cells, *J. Food Sci.* 78 (2013) 1464–1469.
29. (a) F. Fonseca-Silva, J.D. Inacio, M.M. Canto-Cavaleiro, E.E. Almeida-Amaral, Reactive oxygen species production by quercetin causes the death of *Leishmania amazonensis* intracellular amastigotes, *J. Nat. Prod.* 76 (2013) 1505–1508.
- (b) N. Miyoshi, K. Naniwa, T. Yamada, T. Osawa, Y. Nakamura, Dietary flavonoid apigenin is a potential inducer of intracellular oxidative stress: the role in the interruptive apoptotic signal, *Arch. biochem. Biophys.* 466 (2007) 274–282.
30. D. Huang, Y. Ding, W.M. Luo, S. Bender, C.N. Qian, E. Kort, Z.F. Zhang, K. VandenBeldt, N.S. Duesbery, J.H. Resau, B.T. The, Inhibition of MAPK kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis in vivo, *Cancer Res.* 68 (2008) 81–88.
31. B.M. Kim, H.W. Chung, Desferrioxamine (DFX) induces apoptosis through the p38-caspase8-Bid-Bax pathway in PHA-stimulated human lymphocytes, *Toxicol. Appl. Pharmacol.* 228 (2008) 24–31.
32. K.E. Reinicke, M.J. Kufel, M.P. Goetz, M.M. Ames, Synergistic interactions between aminoflavone, paclitaxel and camptothecin in human breast cancer cells, *Cancer Chemoth. Pharm.* 66 (2010) 575–583.
33. D.L. Koomoa, L.P. Yco, T. Borsics, C.J. Wallick, A.S. Bachmann, Ornithine decarboxylase inhibition by alpha-difluoromethylornithine activates opposing signaling pathways via phosphorylation of both Akt/protein kinase B and p27Kip1 in neuroblastoma, *Cancer Res.* 68 (2008) 9825–9831.

34. (a). A. Bilska-Wilkosz, M. Ochenduska, M. Iciek, M. Sokołowska-Jeżewicz, B. Wiliński, M. Góralska, Z. Srebro, L. Włodek, Effects of acetylsalicylic acid on the levels of sulfane sulfur and non-protein sulfhydryl groups in mouse tissues, *Pharmacol. Rep.* 65 (2013) 173–178.
- (b). M. Li, Q. Li, Y.H. Zhang, Z.Y. Tian, H.X. Ma, J. Zhao, S.Q. Xie, C.J. Wang, Antitumor effects and preliminary systemic toxicity of ANISpmin vivo and in vitro, *Anti-Cancer Drugs*. 24 (2013) 32–42.

List of figures

Figure 1. Chemical Structures of LY294002 and F14512

Figure 2. Library of the amine/polyamine motifs

Figure 3. The proliferation inhibition of **8a** for HepG2, H22, and QSG7701 cells assayed by MTT after 48 hr. with or without Aspirin. Each sample is the mean of three independent experiments ($n = 3$, $\bar{x} \pm s$).

Figure 4. (A) Inhibition of HepG₂ cells migration of **8a** by Transwell assays; (B) Inhibition of the HepG₂ cells invasion of **8a** by Wound-healing assays at different concentrations (50, 60, 70 μM) after 48 hr treatment. Each sample is the mean of three independent experiments ($n = 3$, $\bar{x} \pm s$ * $P < 0.05$, ** $P < 0.01$)

Figure 5. (A) **8a** induced apoptotic cells at different concentrations (50, 60, 70 μM) assessed by HCS using AO/EB staining after 48 hr treatment in HepG₂ cells. (B) Impacts of **8a** at different concentrations (50, 60, 70 μM) on caspases 3, 8, 9, p-p38 by immunofluorescence assays after 48 hr treatment in HepG₂ cells. Quercetin (80 μM) served as the positive control. Each sample is the mean of three independent experiments ($n = 3$, $\bar{x} \pm s$ * $P < 0.05$, ** $P < 0.01$)

Figure 6. Impacts of **8a** at different concentrations with or without ASP (800 μ M) on p53, p-p38, p-JNK and Bcl-2 detected by FCM after 48 hr treatment in H22 cells. ($n = 3$, $\bar{x} \pm s$, $*P < 0.05$, $**P < 0.01$ versus control; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ versus 8a with ASP)

Figure 7. Images of in vivo metastasis inhibition by **8a** with or without Aspirin. Que served as the positive control.

Figure 1. Chemical Structures of LY294002 and F14512

Figure 3. The proliferation inhibition of **8a** for HepG2, H22 and QSG7701 cells assayed by MTT after 48 hr. with or without Aspirin. Each sample is the mean of three independent experiments ($n = 3$, $\bar{x} \pm s$).

Figure 4. (A) Inhibition of HepG₂ cells migration of **8a** by Transwell assays, and (B) Inhibition of the HepG₂ cells invasion of **8a** by Wound-healing assays at different concentrations (50, 60, 70 μM) after 48 hr treatment. Each sample is the mean of three independent experiments ($n = 3$, $\bar{x} \pm s$ * $P < 0.05$, ** $P < 0.01$)

Figure 5. (A) **8a** induced apoptotic cells at different concentrations (50, 60, 70 µM) assessed by HCS using AO/EB staining after 48 hr treatment in HepG₂ cells. (B) Impacts of **8a** at different concentrations (50, 60, 70 µM) on MMP, ROS, LYS/pH, detected by HCS using Rh123, DCFH-DA, and Lyso-Tracker Red staining respectively after 48 hr treatment in HepG₂ cells. (C) Impacts of **8a** at different concentrations (50, 60, 70 µM) on caspases 3, 8, 9, p-p38 by immunofluorescence assays after 48 hr treatment in HepG₂ cells. Quercetin (80 µM) served as the positive control. Each sample is the mean of three independent experiments ($n = 3$, $\bar{x} \pm s$ * $P < 0.05$, ** $P < 0.01$)

Figure 6. Impacts of **8a** at different concentrations with or without ASP (800 μM) on p53, p-p38, p-JNK and Bcl-2 detected by FCM after 48 hr treatment in H22 cells. (n = 3, $\bar{x} \pm s$, * $P < 0.05$, ** $P < 0.01$ versus control; Δ $P < 0.05$, $\Delta\Delta$ $P < 0.01$ versus **8a** with ASP)

Figure 7. Images of in vivo metastasis inhibition by **8a** with or without Aspirin. Que and Mito served as the positive control.

Scheme 1. Synthesis of flavonoid derivatives. Reagents and conditions: (a) 1-naphthaldehyde, KOH, EtOH, rt, 12 h; (b) I_2 , H_2SO_4 , DMSO, 110 °C, 5.5 h; (c) NBS, BPO, CCl_4 , $h\nu$, reflux, 8 h, 55%. (d) phthalimide potassium, CH_3COCH_3 , reflux, 8 h; (e) hydrazine hydrate, overnight, 35% for two steps; (f) chloroacetyl chloride, K_2CO_3 , CH_3CN , rt, overnight, 100%; (g) RNH_2 , K_2CO_3 , KI, CH_3CN , 30 °C, overnight, 45%; (h) 4M HCl, EtOH, 0 °C, overnight, 80%.

Table 1. The In Vitro Inhibitory Rates of Novel Conjugates Towards One Normal Liver Cell Line and Two Liver Tumor Cell Lines ^a

No.	R-, n	SMMC 7721 (%)		HepG2 (%)		QSG 7701(%)	
		30 μ M	100 μ M	30 μ M	100 μ M	30 μ M	100 μ M
8a	(CH ₂) ₃ NH ₂ , 2	5.1	87.1	7.9	84.2	<5	41.8
8b	(CH ₂) ₄ NH ₂ , 2	<5	39.8	<5	16.1	<5	10.6
8c	(CH ₂) ₃ NH(CH ₂) ₄ NH ₂ , 3	7.6	32.5	<5	28.9	<5	22.4
8d	(CH ₂) ₄ NH(CH ₂) ₄ NH ₂ , 3	70.2	85.5	62.0	75.9	46.7	74.4
8e	(CH ₂) ₂ N(CH ₃) ₂ , 2	<5	16.6	<5	7.5	<5	17.9
8f	(CH ₂) ₂ CH ₃ , 1	14.6	28.4	6.0	21.6	19.6	41.7
Que ^b		29.5	62.9	33.2	56.3	<5	36.0
Mito ^c		20.5 ^d	62.2 ^e	41.2 ^d	78.6 ^e	75.81 ^d	82.5 ^e

^aThe inhibitory rates were determined at two different concentrations. Each sample is the mean of three independent experiments. ^bQue. Quercetin;. Mito^c Mitoxantrone. ^dThe inhibitory rates were determined at the concentration of 1 μ M. ^eThe inhibitory rates were determined at the concentration of 5 μ M.

Table 2. In Vivo H22 Tumor Growth Inhibitions by **8a** with or without Aspirin or DFMO

Groups	Dose (mg/Kg)	Tumor Weight (g)	Inhibition Rate (%)	Weight Change ^a (%)	No. Dead
control		2.14±0.51		21.0	0/10
8a	10	1.30±0.29	39.2	4.0	2/10
8a+DFMO	10+200	1.22±0.82	43.0	16.7	0/10
8a+Asp	10+10	0.66±0.39	69.2	19.4	0/10
DFMO	200	1.31±0.33	38.8	16.2	0/10
Asp	10	1.37±0.60	36.0	16.7	0/10
QUE ^b	100	1.30±0.25	39.7	6.3	0/10
Mito ^c	0.4	0.57±0.27	73.4	-6.6	0/10

^aPositive value: mouse body weight gain; negative value: mouse body weight loss. ^bQue.

Quercetin. Mito^c Mitoxantrone

Table 3. In Vivo Lung Metastasis Inhibitions by **8a** with or without Aspirin or DFMO

Groups	Dose (mg/Kg)	Metastatic Focus (No.)	Inhibition Rate (%)	Weight Change ^a (%)	No. Dead
control		57.3±14.6		5.2	0/8
8a	10	40.5±13.5	29.3	3.7	0/8
8a+DFMO	10+200	29.3±12.7	48.9	1.2	0/8
8a+Asp	10+10	23.0±7.1	59.9	4.3	0/8
DFMO	200	35.1±12.0	38.7	-0.3	0/8
Asp	10	43.6±13.0	23.9	1.2	0/8
QUE ^b	100	37.8±7.2	34.1	2.1	0/8
Mito ^c	0.4	29.1±7.3	49.2	-0.8	0/8

^aPositive value: mouse body weight gain; negative value: mouse body weight loss. ^bQue.

Quercetin. Mito^c Mitoxantrone

Table of Contents Graphic