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Discovery of Novel Negletein Derivatives as Potent Anticancer Agents for Acute Myeloid Leukemia

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ABSTRACT

Baicalin and its aglycone baicalein derived from *Scutellaria baicalensis* exhibited potent anticancer effects in various types of cancer cell lines. However, the unfavorable pharmaceutical properties became the main obstacle for their potential clinical development. With the aim of development of novel anticancer agents based on the skeleton of baicalin, a series of novel negletein derivatives were designed and synthesized. Among them, compound **8** (**FZU-02,006**) with an *N*,*N*-dimethylamino ethoxyl moitey at the C6 position exhibited significant enhanced antiproliferative effect against HL-60 cells *in vitro* through regulating multi-signaling pathways. These results revealed that compound **8** with the improved aqueous solubility (as HCl salt, >1 mg/mL) and enhanced anti-leukemia potency might serve as a promising lead for further development.

Keywords: negletein derivatives, aqueous solubility, multi-signaling pathways

INTRODUCTION

Acute myeloid leukemia (AML) as one of the leading causes of cancer deaths is characterized by rapid growth of abnormal white blood cells.[1] For most of AML patients, traditional targeted anticancer therapies might eventually result in drug resistance due to compensatory cross-talk of various abnormal signal pathways.[2-4] Natural products with diverse biological characteristics[5, 6] have been explored as invaluable sources for drug design with particular effectiveness in cancerous and infectious diseases.[7-9] Flavonoids, which are ubiquitously distributed in many dietary plants, have received increasing attention because most of them display various beneficial effects on human health.[10] Previous studies have shown that many flavonoids exhibit potent anticancer activity against several human cancer cell lines.[11, 12] Representative examples are baicalin (**1**, **Figure 1**) and baicalein (**2**).[13] Accumulating evidence revealed that both of them induced growth inhibition and apoptosis in multiple hematological cancer cell lines.[13] However, like many biologically active flavonoids such as naringenin and quercetin, the poor aqueous solubility of baicalin or baicalein resulting in low *in vivo* bioavailability limits its further clinical application.[14] One potential solution to enhance aqueous solubility is introduction of polar, preferably an ionizable functionality in the unstable metabolic position.[15-17] Recent studies indicated that negletein (7-*O*-methylated baicalein derivative, **3**) displayed similar bioactivities and higher metabolic stability.[18, 19] Thus, negletein can be used as a promising starting point for further structural modification.

Previous investigations indicated that a series of baicalein derivatives (**4**-**6**, **Figure 1**) with enhanced anticancer potency were synthesized by linking various functions on the positions of 6 and 7.[20, 21] In this work, we designed and synthesized a series of negletein derivatives by incorporating nitrogen-containing fragments into the unstable metabolic region on the positions of 5 and 6. Among them, compound **8** represented as a promising lead with enhanced anti-leukemia potency and improved aqueous solubility (> 1 mg/mL).

EXPERIMENTAL SECTION

Experimental apparatus and reagents

All commercially available starting materials and solvents were reagent grade, and used without further purification. Reactions were performed under a nitrogen atmosphere in dry glassware with magnetic stirring. Preparative column chromatography was performed using silica gel 60, particle size 0.063-0.200 mm (70-230 mesh, Flash). Analytical TLC was carried out employing silica gel 60 F254 plates (Merck, Darmstadt). NMR spectra were recorded on a Bruker-400 (^{1}H, 400 MHz; ^{13}C, 101 MHz) spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with TMS as an internal reference. Chemical shifts were expressed in ppm, and *J* values were given in Hz. High-resolution mass spectra (HRMS) were obtained from Thermo Fisher Scientific Exactive Plus mass spectrometer. Purity of final compounds was determined by analytical HPLC, which was carried out on a Shimadzu HPLC system.

HPLC analysis conditions were as follows: Inertstil ODS2 C18 (150 mm × 4.6 mm, 5 μm) column; flow rate, 0.5 mL/min; UV detection at 210 and 254 nm; linear gradient from 30% methanol in water to 100% methanol in water in 20 min followed by 10 min of the last-named solvent. All biologically evaluated compounds are >95% pure.

General Procedure for the Synthesis of Compounds 7, 14-17

Synthesis of compound **7** was shown as an example. To a solution of **3** (511 mg, 1.80 mmol), K_2CO_3 (481 mg, 4.80 mmol) and KI (30 mg, 0.18 mmol) in MeOH (30 mL) was added 3-bromoprop-1-yne (257 mg, 2.20 mmol). The mixture was stirred at 70 ^{o}C for 3 h, and concentrated under reduced pressure. The residue was diluted with EtOAc (50 mL), washed with 1N HCl (5 mL) followed by brine (50 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash column chromatography (CH_2Cl_2) furnished the desired product **7** as a pale yellow solid (350 mg, 60%).

General Procedure for the Synthesis of Compounds 8-13

Synthesis of compound **8** was shown as an example. To a solution of **3** (57 mg, 0.20 mmol) and PPh_3 (157 mg, 0.60 mmol) in THF (2 mL) was added 2-(dimethylamino) ethanol (90 mg, 1.00 mmol) and DIAD (101 mg, 0.50 mmol). The mixture was stirred at r.t. for 2 h, and concentrated under reduced pressure. The

residue was diluted with EtOAc (30 mL), washed with brine (30 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. Purification by flash column chromatography (CH_2Cl_2:MeOH = 10/1) furnished the desired product **8** as a yellow solid (62 mg, 87%).

General Procedure for the Synthesis of Compounds 18-21

Synthesis of compound 1**8** was shown as an example. To a solution of **7** (94 mg, 0.30 mmol), sodium ascorbate (36 mg, 0.18 mmol) and copper sulfate pentahydrate (45 mg, 0.18 mmol) in DMSO/H_2O (5/1, 2 mL) was added azidobenzene (214 mg, 1.80 mmol). The mixture was stirred at r.t. for 3 h. The reaction mixture was diluted with EtOAc (20 mL) and extracted with H_2O (20 mL). The organic layer was washed with brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give the crude product. This residue was purified by silica gel chromatography (petroleum ether: EtOAc = 3/1) to provide desired product **18** (68 mg, 52%) as a yellow solid.

The detailed ^{1}H NMR, ^{13}C NMR, and HRMS data for all synthesized compounds are shown in the Supporting Information.

Cell Line and Cell Culture

The cell line HL-60 was obtained from the Cell Resource Center of Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). Cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate. Cells were maintained at 37 ^{o}C in a humidified and 5% CO_2 incubator.[22]

Cell Proliferation Assay

Cells plated in 96-well plates were treated in triplicates with negletein or negletein derivatives for 48 h at 37 ^{o}C. Cells were then incubated with 5 mg/mL MTT (Sigma) for 4 h. The supernatants were removed and cells were pulsed with 100 μL DMSO. The optical density (OD) was measured at 492/630 nm using a spectrophotometer (STAT FAX-2100). The inhibitory rate on cell proliferation was calculated as $(1\text{-}OD_{treated}/OD_{control}) \times 100\%$. The half inhibitory concentration (IC_{50}) values were obtained by the Logit method.

Mitochondrial Membrane Potential (ΔΨm) Assay

Changes of mitochondrial membrane potential (ΔΨm) was performed by JC-1 staining according to the manufacturer's instruction (Beyotime, Shanghai, China).[23] Briefly, HL-60 cells were cultured in 6-well plates and then incubated with compound

8 in fresh culture medium for 24 h. Cells were harvested and resuspened in JC-1 staining solution for 20 min at 37 ^{o}C. After washed twice with 1X assay buffer, cells were acquired by flow cytometer.

Apoptosis Assay

Briefly, 1.0 × 10^{5}/mL of HL-60 cells in RPMI 1640 medium with 10% fetal bovine serum (FBS) were plated in 6-well plates. After 24 h of incubation with negletein or negletein derivatives, cells were harvested and washed with PBS, and then stained with Annexin V-FITC/7-AAD (Becton-Dickinson, NJ, USA) according to the manufacturer's instructions. The early apoptotic cells were quantified by flow cytometry.

Western Blotting

HL-60 cells were exposed to compound **8** at different concentrations for 24 h. Total protein was extracted and western blotting was performed as described previously.[24] Primary antibodies against human caspase-9, caspase-3, poly(ADP-ribose) polymerase (PARP), p-Akt (Ser473), p-p70S6K (Thr389) were obtained from Cell Signaling Technology (Beverly, MA, USA). β-Actin and Bcl-2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Statistical Analysis

Statistical significance was determined using student *t*-test. *Represents a p value less than 0.05.

RESULTS AND DISCUSSION

Design

Extensive metabolic investigations revealed that baicalin with two hydroxyl groups at the positions of C-5/C-6 and *O*-glucoside group at C-7 position did not possess favorable metabolic stability. By contrast, negletein with methyl group at C7-position displayed significantly enhanced metabolic stability.[13] The skeleton of negletein with two hydroxyl groups at 5, 6-position was allowed to improve the solubility via facile introduction of functional fragments. Previous studies showed that introduction of nitrogen-containing moiety at C-6 position based on the scaffold of **4** afforded compound **6** may improve druglike properties and antiproliferative activities.[21] In addition, triazoles have been proved to possess various pharmacological properties including anti-infective, anticancer, antiviral, and anti-hypertensive activities.[25-27] Based on the above mentioned results and our initial studies, we directed our chemical modification on C-5 and C-6 positions through introduction of different kinds of moieties including various nitrogen-containing or triazole moieties (**Figure 2**).

Chemistry

The synthetic routes to new negletein derivatives reported in this work are outlined in **Scheme 1**. Negletein (**3**) was synthesized from baicalin (**1**) via our developed simple two-steps/one-pot strategy.[28] Esterification of **1** catalyzed by catalytic H_2SO_4 in methanol followed by treatment with excess $NaBH_4$ afforded **3** in 51% yield. Compounds **8-13** were synthesized from negletein via Mitsunobu coupling with corresponding alcohols. Compounds **14-17** were obtained through nucleophilic substitution of various organobromo compounds with negletein. Treatment of negletein with 3-bromoprop-1-yne provided the key intermediate **7** in 60% yield. The 1,2,3-triazol derivatives **18-21** were easily prepared by click reaction of **7** with various aryl azides[29] in the presence of copper sulfate and sodium ascorbate.

Biology

The calculated lipophilicity (cLogP) and topological polar surface area (TPSA) values of synthesized negletein derivatives are listed in **Table 1**. The result suggests that most of these new compounds are conformed to the criteria of Lipinski's "Rule of Five" and may have good physicochemical properties. To explore the meaningful SAR studies and examine how the substitutions on the key moieties affect biological activities of newly synthesized negletein derivatives, the antiproliferative potency of these compounds was evaluated on the proliferation of human promyelocytic leukemia (HL-60) cells by using standard MTT assay. The activity is expressed as the

concentration of drug inhibiting 50% cell growth (IC_{50}) and the data are presented in **Tables 1**.

Screening of 15 negletein derivatives showed that compound **8** had the strongest antiproliferative activity with an IC_{50} value of 7.24 μM against HL-60 cells (**Table 1**). Compounds **9** and **12** showed a slightly enhanced potency, indicating that appropriate modifications on C-6 position might improve the antiproliferative activity. Compounds **13** and **14** did not show any antiproliferative effect even at 40 μM, indicating that introduction of moieties into both C-5 and C-6 positions resulted in a significant loss of antiproliferative activity. It was disappointed to find that 1,2,3-triazol derivatives **18-21** were inactive with a dramatic loss of antiproliferative activity. Through our preliminary SAR studies, compound **8** with excellent aqueous solubility (as HCl salt, aqueous solubility >1 mg/mL) exhibited promising antiproliferative effect in a dose-dependent manner (**Figure 3**), and was subjected to further biological characterization.

To determine whether compound **8** induces cell apoptosis in HL-60 cells, we next used Annexin V-PE/7-AAD staining assay to investigate the role of compound **8** on the induction of apoptosis. The result showed that compound **8** did induce cell apoptosis in a dose-dependent manner (**Figure 4**). Apoptosis induces loss of membrane asymmetry resulting in alterations in mitochondrial membrane potential (ΔΨm). The ΔΨm is obviously changed when treated with 10 μM of compound **8** for

24 h as determined by flow cytometry analysis, further indicating that compound **8** induced HL-60 cells apoptosis **(Figure 5)**.

To better understand the antiproliferative mechanism of compound **8**, we evaluated its effect on the cell cycle distribution of HL-60 cells. The cells were treated with compound **8** for 24 h, stained with propidium iodide (PI) and analyzed by flow cytometry. As shown in **Figure 6**, the incubation of HL-60 cells with 10 μM of compound **8** for 24 h obviously increased the percentage of cells at G0/G1 phase (from 54.7% to 72.7%). Meanwhile, at the same condition, the percentage of cells in S phase was obviously decreased (from 28.0% to 13.7%). These results revealed that compound **8** arrested the cell cycle at the G0/G1 stage, leading to the inhibition of cell proliferation.

<Insert Figure 6 here>

To gain deeper insight into the mechanism of compound **8**, we also investigated its effect on the some key signal pathways by using western blot analysis. HL-60 cells were treated with different concentrations of compound **8** for 24 h. Apoptosis as a cell death program is regulated by a family of caspases. We found that treatment of HL-60 cells with compound **8** resulted in the cleaved caspase-9 and proteolytic cleavage of pro-caspase 3 to its active form (**Figure 7**). Meanwhile, an up-regulation of cleaved poly (ADP-ribose) polymerase (PARP) was also observed. Activation of

caspase-3 resulted in the cleaved PARP, yielding an 85 kDa fragment (cleaved PARP) which is a marker of cells undergoing apoptosis.[30] These results suggested that compound **8** might induce cell death through caspase-dependent apoptosis.

<Insert Figure 7 here>

Constitutive Akt activation plays an important role in the progression of AML patients. Akt activation indirectly stimulates the transcription of anti-apoptotic genes including Bcl-2.[24, 31] Our results showed that compound **8** suppressed the activation of Akt in a dose-dependent way and the phosphorylation of p70S6K was also down-regulated. Accordingly, the expression of anti-apoptotic protein Bcl-2 was also blocked. Akt activation promoted cell cycle transition from the G1 to S phase via phosphorylation of the downstream targets p70S6K. The above results revealed that compound **8** arrested cell cycle at the G1 phase and reduced S phase cell population.

CONCLUSION

In conclusion, this SAR studies indicated that introduction of triazol groups at C-6 position resulted in loss of anticancer activities and C-5 hydroxyl moiety is essential for potency. Compound **8** (**FZU-02,006**) with an *N*,*N*-dimethylamino ethoxyl moitey at C-6 position exhibited enhanced anti-leukemia potency and improved excellent aqueous solubility (>1 mg/mL). Further work demonstrated that this compound might activate caspase-dependent apoptotic pathway and inhibit Akt signaling pathway.

ACKNOWLEDGEMENTS

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ASSOCIATED CONTENT

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

^{1}H NMR, and ^{13}C NMR data and spectra of all synthesized compounds (PDF)

CONFLICTS OF INTEREST

The authors declare no competing financial interest.

THE LIST OF FIGURE LEGENDS

Figure 1. Chemical structures of baicalin (**1**), baicalein (**2**), negletein (**3**) and the reported improved anticancer flavonoids (**4**-**6**) based on the skeleton of baicalein.

Figure 2. Drug design strategy for new negletein derivatives.

Figure 3. Effects of compound **8** on HL-60 cell proliferation after 48 h co-incubation.

Figure 4. The apoptotic cell percentages in HL-60 cells after incubation with compound **8** at different concentrations for 24 h.

Figure 5. Changes of mitochondria membrane potential of HL-60 cells after treated with compound **8** for 24 h.

Figure 6. Cell cycle analysis of HL-60 cells treated with compound **8** for 24 h. After treatment with compound **8** for 24 h, the harvested cells were stained with PI and analyzed by flow cytometry.

Figure 7. Western blot analysis of biochemical markers for apoptosis induction and inhibition by compound **8** in HL-60 cell line. Cells were treated with compound **8** at different concentrations. Levels of procaspase-3, cleaved Caspase-9, PARP, Bcl-2, p-P70S6K, and p-Akt were probed by specific antibodies. Actin was used as the loading control.

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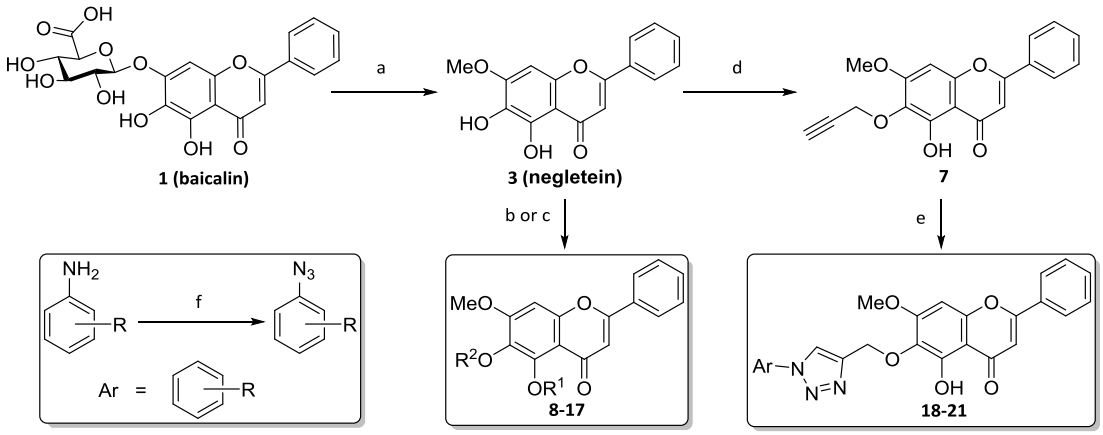
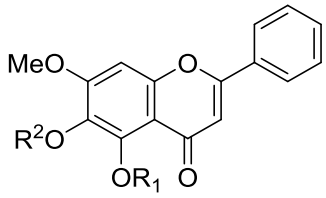
Scheme 1. Synthesis of negletein derivatives

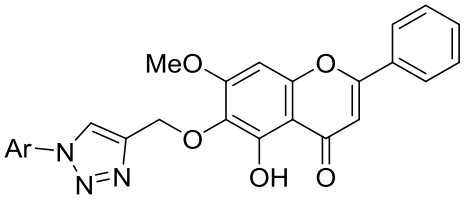


Reagents and conditions: (a) cat. H_2SO_4, MeOH, reflux, 2 h; $NaBH_4$, r.t., 48 h, 51%; (b) corresponding alcohols, DIAD, PPh_3, r.t., 2 h, 9-87%; (c) organobromo compounds, cat. KI, K_2CO_3, MeOH, 70 ^oC, 3 h, 17-60%; (d) 3-bromoprop-1-yne, cat. KI, K_2CO_3, MeOH, 70 ^oC, 3 h, 60%; (e) N_3-Ar, cat. $CuSO_4 \cdot 5H_2O$, sodium ascorbate, DMF/H_2O (9/1), r.t., 3 h, 23-66%; (f) 1N HCl, $NaNO_2$, NaN_3, H_2O, 0-5 ^oC, 1 h, 60-95%.

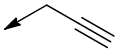
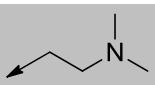
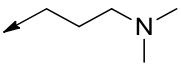
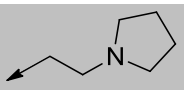
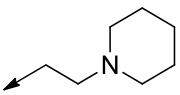
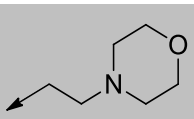
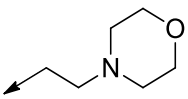
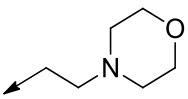
Table 1. SARs of 5- and 6-Substituted Negletein Derivatives

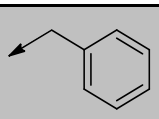
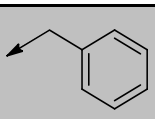
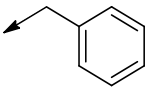
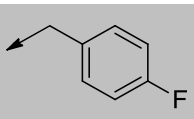
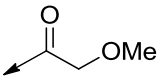
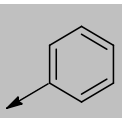
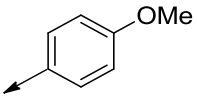
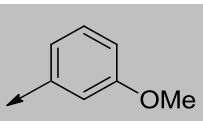
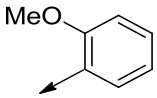


7-17



18-21[a]

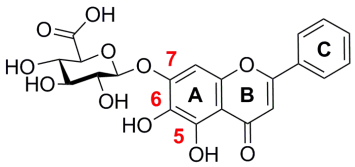
No	R^1	R^2	TPSA[b]	cLogP[c]	IC_{50} (μM)
					HL-60
1			187.1	1.27	304.8 ± 2.4
2			90.9	3.19	234.6 ± 1.7
3			79.9	2.76	192.7 ± 1.9
4			79.9	4.77	13.8 ± 0.4
7	H		68.9	3.12	>40
8	H		72.1	2.79	7.24 ± 0.15
9	H		72.1	3.05	39.9 ± 0.9
10	H		72.1	3.23	>40
11	H		72.1	3.62	>40
12	H		81.4	2.42	34.3 ± 0.8
13			82.9	2.20	>40

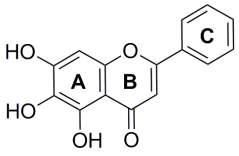
14			57.9	4.31	>40
15	H		68.9	4.47	>40
16	H		68.9	5.79	>40
17	H		95.2	2.70	>40
18[a]	H		99.62	3.74	>40
19[a]	H		108.9	4.14	>40
20[a]	H		108.9	4.14	>40
21[a]	H		108.9	4.14	>40
		Doxorubicin (ADR)			0.26 ± 0.01
		Emodin			24.3 ± 0.7

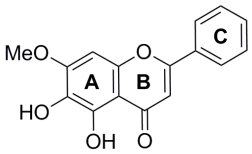
[a] The chemical structures of negletein derivatives were varied at aromatic group (Ar).

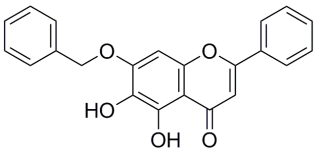
[b] TPSA: http://www.molinspiration.com/cgi-bin/properties.

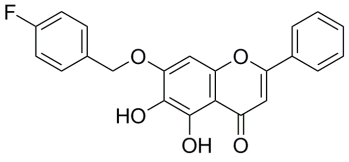
[c] Average cLogP: http://146.107.217.178/lab/alogps/start.html.











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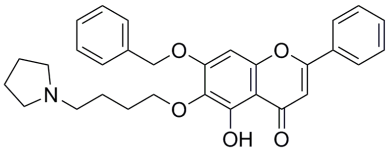

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