Bioorganic & Medicinal Chemistry Letters 18 (2008) 5046–5049

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Impacts of baicalein analogs with modification of the 6th position of A ring on the activity toward NF-κB-, AP-1-, or CREB-mediated transcription

Sheng-Teng Huang [a,b], Yashang Lee [a], Elizabeth A. Gullen [a], Yung-Chi Cheng [a,*,†]

[a] Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street SHM B-226A, New Haven, CT 06510, USA
[b] Department of Chinese Medicine, Chang Gung Memorial Hospital—Kaohsiung Medical Center, Chang Gung Institute of Technology, Taiwan, ROC

ARTICLE INFO

Article history:
Received 5 July 2008
Revised 31 July 2008
Accepted 1 August 2008
Available online 6 August 2008

Keywords:
Scutellariae baicalensis Georgi
Baicalein
Structure–activity relationship
NF-κB
AP-1
CREB

ABSTRACT

The water extract of *Scutellariae baicalensis Georgi* (*S. baicalensis*) has potential anti-tumor and anti-inflammatory activities. A major flavonoid isolated from *S. baicalensis*, baicalein, was also found to have anti-tumor and anti-inflammatory activities. These biological activities could be due to their antioxidant action and/or effect on different signal transduction pathways. We investigated the effects of several baicalein analogs with a substitution of hydrogen of the hydroxyl group at the 6th position of A ring on three signal pathway mediated transcription (NF-κB, AP-1, and CREB) associated with inflammation and cancer growth. We found that the analogs with *O*-alkyl group of the different carbon chain length or *O*-benzyl activated NF-κB transcription without TNFα stimulation. Some of the analogs increased TNFα stimulated NF-κB transcription by two- to threefold. None of the analogs studied has major effect on AP-1 signal transduction with or without TPA stimulation. All of the analogs increased CREB transcription with forskolin stimulation up to twofold. However, they did not have a potent effect (less or about twofold activation) on intrinsic CREB signal transduction. The modification of baicalein at the 6th position of A ring was not correlated with change in these signal transduction pathways and cytotoxicity. Though, they are structural analogs, they are not functional analogs. Modification of baicalein at the 6th position could alter the specificity of action toward different cellular targets. Flavonoids could be chemophores in the development of drugs targeted at different signal transcriptional pathway.

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Scutellaria baicalensis Georgi (*S. baicalensis*) in combination with other herbs is commonly used for the treatment of fever, cough, inflammation, dysentery, jaundice, and hypertension in traditional Chinese medicine.[1] It is used in the treatment of allergies.[2] *S. baicalensis* contains four major bioactive flavonoid compounds: baicalin, baicalein, wogonin, and oroxylin-A that have anti-inflammation and anti-cancer effects.[3–5] Flavonoids are polyphenolic compounds that are abundant in vegetables and plants. Flavonoids are implicated to have different anti-cancer, anti-platelet, anti-ischemic, and anti-inflammatory activities.[6] These biological activities could be partly due to their antioxidant property.[6,7] Moreover, some flavonoids can regulate the expression of genes that are relevant to the synthesis or action of several pro-inflammatory mediators such as prostaglandins, reactive oxygen species, nitric oxide, and intercellular adhesion molecules.[2,6,7] Prostaglandins play an important role in inflammatory process.[8] COX-2, a key enzyme in the synthesis of prostaglandins, plays an important role in inflammatory process. The transcription complex at the COX-2 promoter requires the transcriptional co-activator p300 to bind to the cyclic-AMP response element binding protein (CREB), activator protein-1 (AP-1), and nuclear factor-kappa B (NF-κB) in controlling the initiation of transcription.[9] The interplay of NF-κB, AP-1, and CREB nuclear transcription factors in enhancing the transcription of COX-2 m-RNA is not well understood. Interleukin (IL)-1, a pro-inflammatory cytokine could induce NF-κB, AP-1, and CREB activation[10] to exert its inflammatory activity. The downstream gene activation of each pathway could differ in different tissues due to epigenomic differences. We previously synthesized a series of flavonoids from baicalein with structural variations at the 5th, 6th, and 7th position of A ring.[11] We investigated whether these structural analogs are also functional analogs with regard to their effect on different cellular transcriptional pathways. We studied the activity of baicalein analogs (A ring 6th position modified) on three signal transduction pathways (NF-κB, AP-1, and CREB) in HepG2 cells with specific response element coupled to a luciferase reporter. These transcription factors play important roles in cancer

Abbreviations: *S. baicalensis*, *Scutellariae baicalensis Georgi*; SAR, structure–activity relationship; NF-κB, nuclear factor-kappa B; AP-1, activator protein-1; CREB, cyclic-AMP response element binding protein; TNFα, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

* Corresponding author. Tel.: +1 203 785 7118; fax: +1 203 785 7129.
E-mail address: yccheng@yale.edu (Y.-C. Cheng).
† Fellow of the National Foundation for Cancer Research.

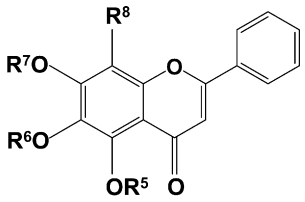
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doi:10.1016/j.bmcl.2008.08.001

and inflammatory processes. This study provides information on the mechanism of action of these compounds and their potential use in drug discovery to target on these pathways.

6-Methoxy-5,7-dihydroxyflavone (O), 6-acetoxy-5,7dihydroxyflavone (S2), 6-propyloxy-5,7dihydroxyflavone (S17), 6-benzyloxy-5,7dihydroxyflavone (S15), and 6-ethoxy-5,7dihydroxyflavone (S32) were synthesized previously in our laboratory.[11] KB (human nasopharyngeal squamous cell carcinoma) or KB/MDR (multi-drug resistant) cells were used for the growth inhibition assay. The methods for the synthesis and the activities of these flavonoids with the exception of oroxylin-A against KB and KB/MDR cells have been previously reported.[11] The potency to inhibit cell growth is presented as an IC_{50} value, which is the concentration of compound required to inhibit 50% of cell growth at 3 days of incubation with compound. The doubling time of KB and KB/MDR cells was about 20–24 h. The data presented are the mean of three independent experiments.

The assays for signal transduction-mediated transcription have been described previously.[12,13] In brief, stable cell lines (HepG2-NF-κB-luc, HepG2-CRE-luc, HepG2-AP-1-luc) were pretreated with different concentrations of compounds for 1 h and then incubated with or without stimulation by tumor necrosis factor α (TNFα), forskolin, or 12-O-tetradecanoylphorbol 13-acetate (TPA) for 4 h. The luciferase activity was measured using Promega's luciferase assay kit (Madison, WI) according to the manufacturers' instructions. We determined the concentration of compound at which activation was 50% (AC_{50}) and maximal transcriptional activity in comparison with control (Amax).

We analyzed the structure–activity relationships of baicalein analogs with modification at the 6th position of baicalein A ring (Fig. 1) to compare their cytotoxicity in KB cells and multi-drug resistant cell line (KB/MDR). KB/MDR overexpresses mdr1. KB cells were used to evaluate the susceptibility of a compound to act as a substrate or inhibitor of the P-gp 170 efflux pump. The cytotoxicity of these analogs, except that of oroxylin-A, were previously published.[11] The IC_{50} of the six baicalein analogs are shown in Figure 1. Among the analogs, S15 was the most potent against the growth of both KB and KB/MDR cells. The substitution of the hydroxyl group at position 6 of A ring to the *O*-benzyl group increased the toxicity in KB and KB/MDR cells. P-gp 170 efflux pump did not play a role in its cytotoxic action.[11] The IC_{50} of S2 in KB and KB/MDR cells were 10.5 ± 1.4 and 61.6 ± 4.8 μM, respectively. The IC_{50} of S17 in KB and KB/MDR cells were 58.9 ± 6.3 and >100 μM, respectively. This suggests that P-gp 170 is one determinant of the cytotoxicity of these two compounds. The cytotoxicity of the other four compounds (baicalein (S1), oroxylin-A (O), 6-benzyloxy-5,7-dihydroxyflavone (S15), and 6-ethoxy-5,7-dihydroxyflavone (S32)) showed no differences in KB and KB/MDR cells. This indicates that these compounds are not efficient substrates of the P-gp 170 efflux pump. However, the mechanisms responsible for the varying degrees of cytotoxicity are still not clear. Since NF-κB, AP-1, and CREB are important transcriptional processes that regulate cell growth and inflammation, we investigated if the analogs have any effect on NF-κB, AP-1, and CREB pathways.



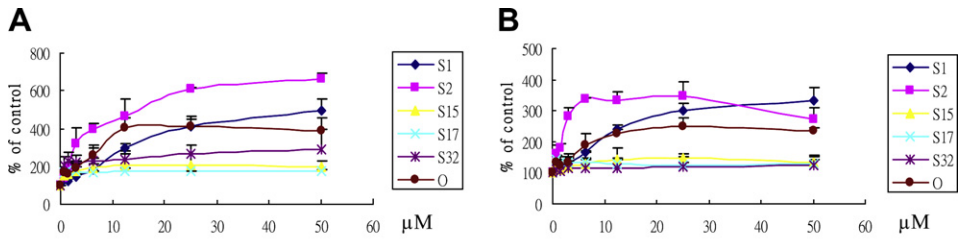
	functional group			cytotoxicity (IC_{50}, μM)	
compd	R^5	R^6	R^7	K B	KB/MDR
S1	H	H	H	62.3±3.7[a]	87.1±3.6[a]
O	H	Me	H	31.1±5.4	16.1±5.5
S32	H	Et	H	24.6±3.5[a]	17.5±5.6[a]
S17	H	Pr	H	58.9±6.3[a]	>100[a]
S15	H	Bn	H	4.3±1.6[a]	3.2±1.2[a]
S2	H	Ac	H	10.5±1.4[a]	61.6±4.8[a]

Figure 1. Chemical structures and their respective IC_{50} of baicalein analogs at the 6th position of the A ring. Values are mean ± SD of three independent experiments. [a]Published.[11]

First, we studied the effects of the baicalein analogs (Fig. 2) on NF-κB-mediated transcription with or without TNFα activation. S1, O, and S2 activated NF-κB with or without TNFα stimulation. By comparing the efficiency ($Amax/AC_{50}$), S2 is the most efficient activator of NF-κB among the three analogs with or without TNFα. In the presence of TNFα activation, we observed maximum activation by NF-κB. The mechanism of the activation through NF-κB by the three analogs may be different from that of TNFα. Moreover, there was no correlation between the potency of an analog to activate NF-κB transcription and its cytotoxicity. This suggests that the mechanism for the activation of NF-κB may not be the mechanism of their cytotoxicity. The rank order of the potency in the activation of NF-κB of the modified baicalein analogs at the 6th position of A ring was OAc > OH > OMe > OEt > OBn > OPr. The binding site for these compounds may favor a hydrophobic group but that is not a bulky group. The target site may be located downstream of the TNFα receptor. NF-κB activation is important for the initiation of inflammatory process and function of immunocytes.[5] Baicalein (S1) is known to have anti-inflammatory activity.[2,7] Our findings indicate that the anti-inflammatory action of baicalein analogs is not due to the inhibition of NF-κB transcription. It is likely due to the action of baicalein on other sites, which may play a role in inflammation. Moreover, the lack of correlation between the potency of these analogs against cell growth and their ability to activate NF-κB pathway supports the postulate that this class of compounds may have multiple sites of action.

AP-1-mediated transcription is triggered by TPA, which is a proteinkinase C activator. This signal transduction pathway involves the MAPKinase cascade. It modulates biological processes including vasodilation, inflammation, cell proliferation and differentiation, stress response, apoptosis and survival according to the cell type and stimulus.[14] Our study showed that baicalein analogs could not activate or inhibit the AP-1-mediated transcription over 50% with or without TPA stimulation (Fig. 3). Therefore, AP-1-mediated transcription may not be responsible for the anti-inflammatory or anti-cancer activity of these baicalein analogs.

CREB is essential for both basal and induced COX-2 transcription which is regulated through the binding CREB, NF-κB, and AP-1 proteins.[15] COX-2 plays a key role in the synthesis of inflammatory mediators and it is a target for developing anti-inflammatory drugs. In addition, the cAMP/CREB signaling pathway has been implicated in the regulation of a wide range of biological functions such as growth factor-dependent cell proliferation and survival, glucose homeostasis and inflammation.[16] We examined the effects of baicalein analogs on CREB-mediated transcriptional activity with or without forskolin stimulation (Fig. 4). None of the compounds inhibited or activated intrinsic CREB mediated transcription over two-fold. However, the compounds enhanced forskolin (an adenyl cyclase activator) stimulation of CREB-mediated transcription. Baicalein (S1) and O demonstrated 2.2- and 2.61-fold activation, respectively, whereas S32 showed 14.5-fold activation with forskolin treatment. The rank order of enhancement of forskolin activation of CREB signal pathway by modified baicalein at the 6th position of A ring was OEt > OPr = OBn > OAc > OMe = OH (Fig. 4). The structure–activity relationship was different from that

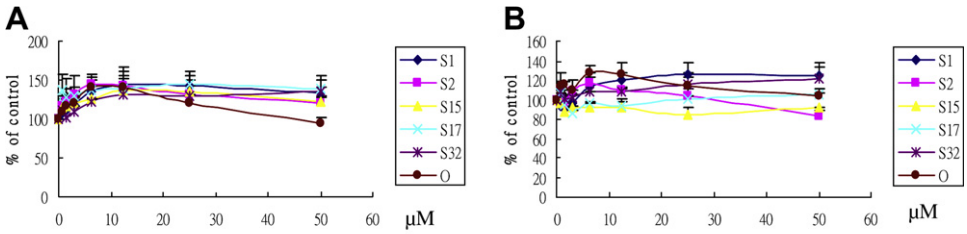


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	- TNF α		+TNF α *	
compd	Amax (fold)	AC_{50} (μM)	Amax (fold)	AC_{50} (μM)
S1	4.92±0.61	8.3±0.7	3.35±0.39	8.6±0.5
O	4.12±0.50	4.8±0.6	2.50±0.26	5.0±0.7
S32	2.92±1.02	0.7±0.3	1.27±0.27	ND
S17	1.81±0.11	ND	1.36±0.13	ND
S15	2.08±0.57	0.5±0.1	1.47±0.15	ND
S2	6.62±0.28	2.2±0.1	3.47±0.45	2.2±0.1

* TNF α (+) could stimulate 4.5±0.5 fold

Figure 2. Effect of baicalein analogs on the transcriptional activity of NF-κB. HepG2 cells stably transfected with NF-κB treated with baicalein analogs followed by activation without (A) or with (B) TNFα treatment. The data were presented as % of control, where the control was the luciferase activity without or with TNFα stimulated cells in the absence of drugs. (C) Represented the Amax (fold) and AC_{50} (μM) of baicalein analogs of NF-κB-mediated transcription in HepG2 cells. Amax stands for maximal transcriptional activity in comparison with control. AC_{50} is the concentration (μM) that leads to 50% of maximal transcriptional activity. All values represent the mean ± SD of at least three independent experiments. ND represents no detection. TNFα (+) was added to give maximal activation, which is 4.5 ± 0.5 fold above intrinsic activity.



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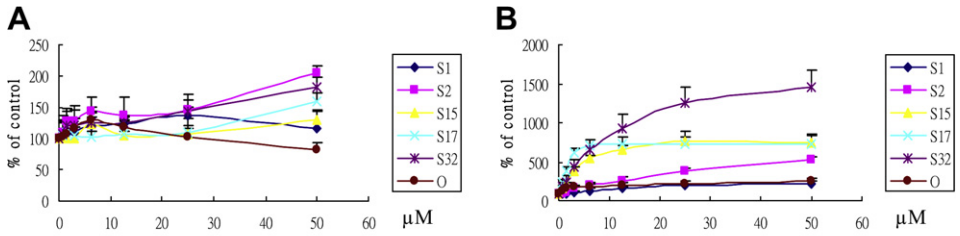
	- TPA		+TPA*	
compd	Amax (fold)	AC_{50} (μM)	Amax (fold)	AC_{50} (μM)
S1	1.44±0.18	ND	1.26±0.1	ND
O	1.41±0.14	ND	1.27±0.07	ND
S32	1.35±0.2	ND	1.21±0.13	ND
S17	1.43±0.17	ND	1.05±0.01	ND
S15	1.36±0.14	ND	0.85±0.06	ND
S2	1.44±0.09	ND	1.18±0.11	ND

* TPA (+) could stimulate 2±0.5 fold

Figure 3. Effect of baicalein analogs on the transcriptional activity of AP-1. HepG2 cells stably transfected with AP-1 treated with baicalein analogs followed by activation without (A) or with (B) TPA treatment. The data were presented as% of control, where the control was the luciferase activity without or with TPA stimulated cells in the absence of drugs. (C) Represented the Amax (fold) and AC_{50} (μM) of baicalein analogs of AP-1-mediated transcription in HepG2 cells. Amax stands for maximal transcriptional activity in comparison with control. AC_{50} is the concentration (μM) that leads to 50% of maximal transcriptional activity. All values represent the mean ± SD of at least three independent experiments. ND represents no detection. TPA (+) was added to give maximal activation, which is 2 ± 0.5 fold above the intrinsic activity.

of NF-κB-mediated transcription or cytotoxicity. The rank order of the efficiency of these analogs estimated by $Amax/AC_{50}$ was OPr > OEt = OBn > OMe > OAc > OH. Forskolin can increase the intracellular levels of cAMP, which could activate PKA.[17] The activated PKA could enter nucleus to phosphorylate CREB at serine 133.[17] It was reported that the formation of phosphorylated CRE-binding protein can induce expression of IL-10 in monocytes in down-regulation of inflammation caused by NF-κB.[18] The analogs studied enhanced CREB activation by forskolin. The anti-inflammatory activity of baicalein could be partly due to its action and/or its metabolite, such as oroxylin-A, in activating CREB-mediated transcription. S17 and S32 may have better anti-inflammatory activity; this needs further exploration.

Although these compounds are close structural analogs, baicalein analogs modified at the 6th position of the A ring exhibited different cell cytotoxicities and effects on the signal transduction pathway of NF-κB, AP-1, and CREB. Baicalein analogs may mimic the TNFα effect to activate intrinsic level of NF-κB-mediated transcription. They also enhanced NF-κB- and CREB-mediated transcription with TNFα and forskolin, respectively. However, they did not enhance AP-1-mediated transcription with TPA. Though these structural analogs differ at only one position, having different



C

	- Forskolin		+Forskolin*	
compd	Amax (fold)	AC_{50} (μM)	Amax (fold)	AC_{50} (μM)
S1	1.35±0.14	ND	2.20±0.32	13.5±3.2
O	1.28±0.22	ND	2.61±0.3	2.2±0.6
S32	1.81±0.17	ND	14.5±2.25	8.2±1.1
S17	1.59±0.14	ND	7.35±0.85	1.7±0.2
S15	1.31±0.15	ND	7.69±1.19	4.3±1.6
S2	2.04±0.11	38.3±1.3	5.21±0.44	13.9±1.7

* Forskolin (+) could stimulate 4±0.5 fold

Figure 4. Effect of baicalein analogs on the transcriptional activity of CREB. HepG2 cells stably transfected with CREB treated with baicalein analogs followed by activation without (A) or with (B) forskolin treatment. The data were presented as% of control, where the control was the luciferase activity without or with forskolin stimulated cells in the absence of drugs. (C) Represented the Amax (fold) and AC_{50} (μM) of baicalein analogs of CREB-mediated transcription in HepG2 cells. Amax stands for maximal transcriptional activity in comparison with control. AC_{50} is the concentration (μM) that leads to 50% of maximal transcriptional activity. All values represent the mean ± SD of at least three independent experiments. ND represents no detection. Forskolin (+) was added to give maximal activation, which is 4 ± 0.5 fold above the intrinsic activity.

O-alkyl, *O*-acyl, and *O*-benzyl groups, their effect on the three pathways and cell growth are quite different. Flavonoid modification appears to be a possible approach for developing drugs with unique activities.

Acknowledgments

The authors thank Dr. T.J. Lee from Taiwan for providing oroxylin-A. We thank Dr. Elijah Paintsil and Scott Bussom for the critical reading of the manuscript. Dr. Sheng-Teng Huang was supported by Chang Gung Memorial Hospital Grant. This investigation was supported by Grant CA 063477 from the National Cancer Institute and Y.C. Cheng is a fellow of National Foundation for Cancer Research.

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