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Rational design, synthesis and *in vitro* evaluation of novel *exo*-methylene butyrolactone salicyloylamide as NF-κB inhibitor

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ABSTRACT

(−)-Dehydroxymethylepoxyquinomicin ((−)-DHMEQ, **1**) is a specific inhibitor of NF-κB. It binds to SH group in the specific cysteine residue of NF-κB components with its epoxide moiety to inhibit DNA binding. In the present research, we have designed and synthesized an epoxide-free analog called (*S*)-β-salicyloylamino-α-exo-methylene-γ-butyrolactone (SEMBL, **3**). SEMBL inhibited DNA binding of NF-κB component p65 *in vitro*. It inhibited LPS-induced NF-κB activation, iNOS expression, and inflammatory cytokine secretions. It also inhibited NF-κB and cellular invasion in ovarian carcinoma ES-2 cells. Moreover, its stability in aqueous solution was greatly enhanced compared with (−)-DHMEQ. Thus, SEMBL has a potential to be a candidate for a new anti-inflammatory and anticancer agent.

NF-κB is a transcription factor that promotes the expression of many inflammatory cytokines, adhesion molecules, and anti-apoptosis proteins. However, its excess activation often induces inflammation and contributes to cancer progression. Thus, NF-κB is considered to be an attractive target for drug discovery. We previously designed and synthesized an NF-κB inhibitor (−)-DHMEQ (1).[1,2] It showed potent anti-inflammatory and anticancer activities in animal experiments.[3] In one hand, we clarified compound **1** directly binds to the specific cysteine residue of NF-κB components to inhibit their DNA binding activity, which consequently induces an instability and loss of importin affinity in the case of RelB.[3,4] Furthermore, our previous studies revealed that the sulfhydryl group of cysteine might make a nucleophilic attack on the epoxide of (−)-DHMEQ and form the adduct molecule with an epoxide ring-opened structure.[5] In general, however, epoxide moiety is known to be highly reactive and the molecule with an epoxide may react with various biological molecules in the body. So we searched for an epoxide-free analog considered to be more stable than (−)-DHMEQ. Sesquiterpene lactones (e.g. Helenalin **2**) are the most widely published class of natural products cited as NF-κB cysteine sulfhydryl groups of inhibitors, and their bioactivity can be explained by a Michael-type conjugate addition (e.g. compound **5**) of the nucleophilic Cys^{38} and Cys^{120} in the p65 monomer of NF-κB to one or more αβ-unsaturated carbonyl moieties of the inhibitor.[6]

Based on the experimental results above mentioned, we designed an epoxide-free analog of (−)-DHMEQ, called (*S*)-β-salicyloylamino-α-exo-methylene-γ-butyrolactone (SEMBL, **3**), in which the α-methylene-γ-butyrolactone moiety is likely able to react with the SH group in cysteine to afford compound **6** as depicted in Fig. 1.

In this study, we report an efficient and practical route for the synthesis of **3**. Additionally, the *in vitro* evaluation of **3** as NF-κB inhibitor was investigated. The target compound **3** was synthesized by condensation of β-amino-α-methylene-γ-butyrolactone **13** with salicylic acid as depicted in Scheme 1. Although compound **13** was prepared from (*S*)-tryptophan *via* ozonolysis by Maxlean et al. in 1987, their synthetic route is evidently unfit for the quantity production.[7] Thus, we developed a new synthetic approach to get compound **13** from inexpensive, commercially available *N*-*t*-Boc-l-aspartic acid β-benzyl ester **7** as depicted in Scheme 1. Reduction of compound **7** with $NaBH_4$ in the presence of isobutyl chloroformate afforded the alcohol **8**, which was subsequently

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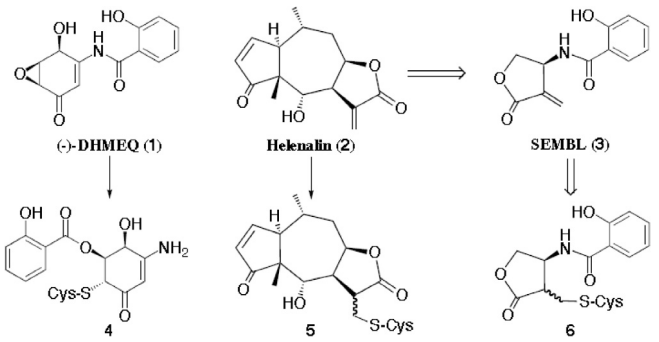
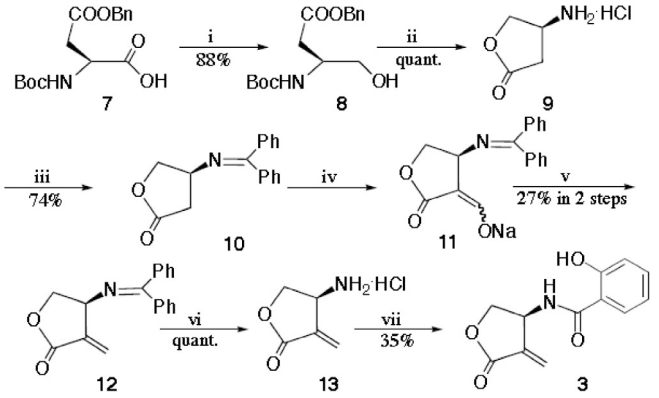


Fig. 1. Molecular design of epoxide-free analog of (−)-DHMEQ **(1)**. Epoxide moiety has been replaced by the exomethylene carbonyl moiety in SEMBL **(3)**. Compound **3** may react with cysteine SH to give the adduct molecule **6**



Scheme 1. *Reagents and conditions*: (i) *N*-methylmorpholine, *i*-$BuCO_2Cl$, $NaBH_4$, MeOH/THF −10 °C to 0 °C; (ii) 2N HCl/Et_2O; (iii) Ph_2C=NH, CH_2Cl_2; (iv) NaH, HCO_2Et, Et_2O: (v) (HCHO)n, THF, reflux, 3hr; (vi) 1 N HCl, Et_2O; (vii) HOBt(leq.), EDCI (2 eq.), salicylic acid(1 eq.), *i*-Pr_2NEt(3 eq.), DMF

treated with 2N-HCl in Et_2O to give the amino-lactone **9**. After the protection of amino group of **9** with benzophenone imine, the imine-lactone **10** was reacted with ethyl formate followed by paraformaldehyde in order to obtain α-methylene-γ-butyrolactone **12** according to the protocol of Reid et al., in a 27% yield in 2 steps.[8] Next, deprotection of the amino group of **12** with 1N-HCl afforded β-amino-α-methylene-γ-butyrolactone **13** in a quantitative yield. Finally, compound **13** was condensed with salicylic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and *N*,*N*-diisopropylethylamine in DMF to give the target compound SEMBL **3** in 35% yield.

Next, we evaluated the biological activity of SEMBL. Firstly, we examined the inhibitory activity of SEMBL on *in vitro* NF-κB binding to κB DNA. Mouse monocytic leukemia RAW264.7 cells were incubated with LPS for 2 h to accumulate NF-κB in the nucleus. Then, the nuclear extract was prepared and used for NF-κB. As shown in Fig. 2A, the addition of LPS led to the increase of nuclear NF-κB. SEMBL was added directly to the nuclear extract, and it inhibited the p65 binding to κB DNA at 3–10 μg/ml. The inhibitory activity was similar to that of (−)-DHMEQ (Fig. 2A). The p65-κB DNA binding was assessed by the TransAM NF-κB p65 transcription factor assay kit (Active Motif, Tokyo, Japan). Previously, DHMEQ was reported to have anti-inflammatory effects in macrophage cells.[9] Hence, we studied the cellular anti-inflammatory effect of SEMBL in RAW264.7 cells. SEMBL did not show any cellular toxicity at 1 μg/ml (Fig. 2B). LPS induced accumulation of p65 in 2 h in cultured RAW264.7 cells. SEMBL or (−)-DHMEQ was added to the cells 30 min before LPS. SEMBL inhibited the activation of NF-κB, and the activity was stronger than (−)-DHMEQ (Fig. 2C). LPS induces NF-κB-dependent inducible NO synthase (iNOS) in macrophages to produce NO. As shown in Fig. 2D, LPS induced NO production in RAW264.7 cells, which was inhibited by SEMBL dose-dependently at 0.03–1 μg/ml without any toxicity. The inhibitory activity was again stronger than that of (−)-DHMEQ. Consistent with this result, SEMBL inhibited the LPS-induced expression of iNOS mRNA in PCR analysis at 0.1–1 μg/ml (Fig. 2E). LPS induces NF-κB-dependent expressions and secretions of many inflammatory cytokines including interleukin-6 (IL-6), IL-1β, and TNF-α in macrophages. As shown in Fig. 2F, SEMBL inhibited LPS-induced IL-6, IL-1β, and TNF-α secretions. Thus, SEMBL showed *in vitro* anti-inflammatory activity in macrophage-like RAW264.7 cells.

Ovarian carcinoma often metastasizes in the lung, liver and peritoneal cavity.[10] We then evaluated the cellular anti-metastasis activity of SEMBL using ovarian clear cell carcinoma ES-2 cells. Using the nuclear fraction from ES-2 cells, SEMBL inhibited the NF-κB-DNA binding *in vitro* (Fig. 3A). Fig. 3B shows the effect of SEMBL on cell viability. SEMBL inhibited the constitutively activated NF-κB activity when added to the cells without any toxicity. The inhibitory activity was significantly stronger than that of DHMEQ as shown in Fig. 3C. We then studied the effect of SEMBL on cellular migration. It inhibited the cellular migration when monitored with a Radius 96-well cell migration assay (Cell Biolabs, Inc., CA), as shown in Fig. 3D. It also inhibited the cellular invasion monitored by a Matrigel chamber assay (Discovery Labware, Inc., MA) at lower concentrations and the inhibitory activity is stronger than DHMEQ (Fig. 3E). As the mechanism of inhibition, Matrix metalloproteinase-2 (MMP-2) expression was inhibited by SEMBL (Fig. 3F and G). Thus, SEMBL inhibited cellular migration and invasion without toxicity in ovarian clear cell carcinoma ES-2 cells.

We then compared the stability of SEMBL and (−)-DHMEQ in phosphate-buffered saline (PBS). Each chemical was dissolved and incubated in PBS at 100 μg/ml for 0, 30 min, 1 h, 2 h, 6 h, 12 h at 37 °C. We carried out HPLC analysis of SEMBL and DHMEQ after incubation in PBS. The results showed that more SEMBL than DHMEQ remained intact at 30 min-12 h of incubation (Fig. 4A). We also evaluated the inhibitory activity on NO production. Each compound was incubated in PBS again at 100 μg/ml. Then, the inhibitory activity of the solution was tested for LPS-induced NO production in RAW264.7 cells. Fig. 4B shows the amount of NO production. (−)-DHMEQ was quickly inactivated in PBS at 30 min-12 h of incubation. In comparison to (−)-DHMEQ, SEMBL in PBS showed stronger inhibitory activity at 30 min-12 h of incubation.

The presence of a conjugated exomethylene group was shown to be significant in eliciting NF-κB inhibition in sesquiterpene lactones.[12–14] In this study, we employed an exomethylene carbonyl moiety for the substitution of epoxide but also tried to maintain the structure of (−)-DHMEQ as much as possible, since the hydroxyl group at the 2-position of the benzamide ring system was found to be essential for the inhibitory activity.[15] We could successfully synthesize an epoxide free DHMEQ analog, SEMBL, with a 35% yield starting from **13**. This synthesis route is more attainable than the previous one reported by Maclean et al. because the starting compound, *N*-*t*-Boc-l-aspartic acid β-benzyl ester, has a simpler structure and is more commercially available. Biological evaluation demonstrated that this new analog, SEMBL, is able to inhibit NF-κB-DNA binding as (−)-DHMEQ, and it also inhibits NF-κB-dependent inflammatory cytokine secretions. It also inhibited cellular metastatic activity in ovarian carcinoma cells. SEMBL is likely to bind to Cys38 as (−)-DHMEQ, since it directly inhibits p65-DNA binding (Figs. 2A, 3A). Reaction of SEMBL with protected