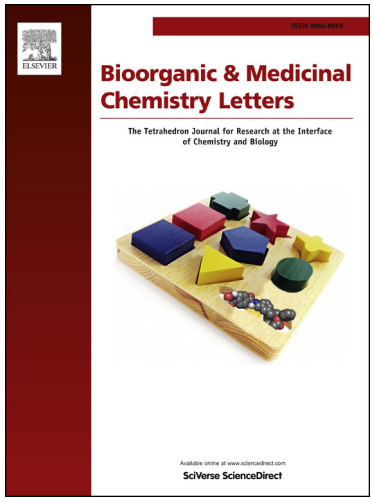


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PII: S0960-894X(15)00783-0
DOI: http://dx.doi.org/10.1016/j.bmcl.2015.07.079
Reference: BMCL 22968

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 23 April 2015
Revised Date: 4 July 2015
Accepted Date: 23 July 2015

Please cite this article as: Ku, S-K., Lee, J.H., Yuseok, O., Lee, W., Song, G-Y., Bae, J-S., Vascular barrier protective effects of 3-*N*- or 3-*O*-cinnamoyl carbazole derivatives, *Bioorganic & Medicinal Chemistry Letters* (2015), doi: http://dx.doi.org/10.1016/j.bmcl.2015.07.079

# Vascular barrier protective effects of 3-*N*- or 3-*O*-cinnamoyl carbazole derivatives

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**Abstract**

In this letter, we investigated the barrier protective effects of 3-*N*-$(MeO)_n$-cinnamoyl carbazoles (BS **1**; n = 1, BS **2**; n = 2, BS **3**; n = 3) and 3-*O*-$(MeO)_3$-cinnamoyl carbazole (BS **4**) against high-mobility group box 1 (HMGB1)-mediated vascular disruptive responses in human umbilical vein endothelial cells (HUVECs) and in mice for the first time. Data showed that BS **2**, BS **3**, and BS **4**, but not BS **1**, inhibited HMGB1-mediated vascular disruptive responses and transendothelial migration of human neutrophils to HUVECs. BS **2**, BS**3**, and BS **4** also suppressed HMGB1-induced hyperpermeability and leukocyte migration in mice. Interestingly, the barrier protective effects of BS **3** and BS **4** were better than those of BS **2**. These results suggest that the number of methoxy groups substituted on the cinnamamide or cinnamate moiety of the 9*H*-3-carbazole derivative is an important pharmacophore for the barrier protective effects of these compounds.

**Key words:** 3-*N*-cinnamoyl carbazole, 3-*O*-cinnamoyl carbazole, vascular barrier integrity, HMGB1

The vascular endothelium has diverse functions, including prevention of coagulation, regulation of vascular tonus and permeability, coordination of blood cell migration, and production of chemo-attractants [1-2]. Endothelial cells (ECs) provide a semi-selective barrier between the blood and underlying tissue interstitium, and the disruption of barrier integrity caused by cellular injury or stress induces vascular hyperpermeability, which is typically found in inflammation, tumor angiogenesis, and atherosclerosis [3]. Thus, maintaining endothelial integrity against vascular stress has emerged as an effective therapeutic strategy for the alleviation of inflammatory-associated diseases [3-4]. Among the numerous approaches used to enhance vascular integrity, inhibition of high-mobility group box 1 (HMGB1) has been recognized as a sensible protocol. HMGB1 is released into the extracellular space from necrotic cells and/or immune cells upon inflammatory stimuli. The released HMGB1 induces the expression of cell adhesion molecules and facilitates the production of an array of pro-inflammatory cytokines that mediate leukocyte adherence, increased vascular permeability, coagulation activation, and microvascular thrombosis [5-6]. Therefore, anti-HMGB1 agents capable of inhibiting HMGB1 activity may be developed into a therapeutic intervention for the treatment of vascular inflammatory disorders given the significant role of HMGB1 in rendering endothelial hyperpermeability [7-8]. In our continuing efforts to discover anti-HMGB1 agents for the intervention of pathogenic conditions associated with vascular dysfunction [9-11], we herein

elaborate the synthesis and structural elucidation of 3-*N*- and 3-*O*-$(MeO)_n$-cinnamoyl carbazoles (BS **1**–**4**). We also address whether these compounds could be developed for the treatment of vascular dysfunction by employing relevant cellular and animal models of vascular disruptive complications.

Carbazole derivatives are a significant class of heterocyclic compounds and have been reported to have various biological activities, such as anticancer, antimicrobial, antiviral, antioxidant, anti-platelet aggregation, and anti-inflammatory. In particular, we found that many compounds with a carbazole moiety exhibit anti-inflammatory activities through a variety of mechanisms, such as carprofen [12-13], 3-pyrazolyl-9-methyl carbazole [14], AF3442 [15], LCY-2CHO [16-17], and 3'-*N*-substituted carbazoles [18] (Fig. 1).

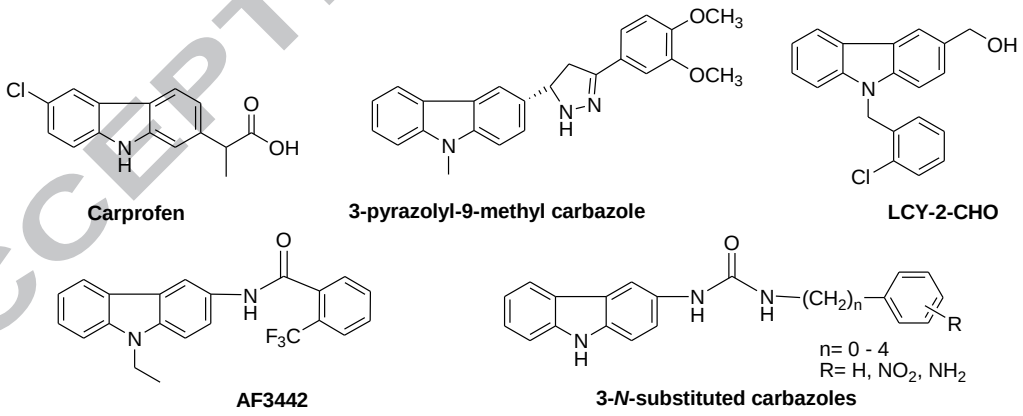


**Figure 1**. Anti-inflammatory active carbazole compounds

In a previous study, we identified that 3'-*N*-substituted carbazole derivatives with a urea linkage introduced could be new candidate drugs for severe vascular inflammatory diseases,

such as sepsis. Meanwhile, it has been found that some compounds with the cinnamoyl moiety possess anti-inflammatory effects, such as tranilast [19], avenanthramide [20], piperlongumine [21], and lupeol cinnamate [22] (Fig. 2).

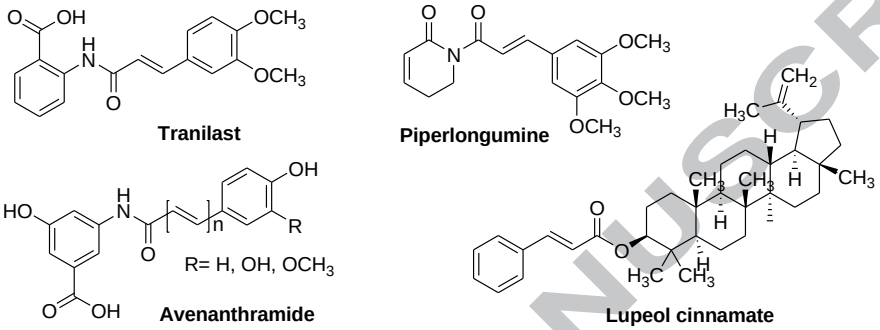


**Figure 2**. Anti-inflammatory active compounds with a cinnamoyl moiety

Therefore, the combination of a carbazole moiety and cinnamoyl moiety are expected have a synergistic effect on anti-inflammatory activity (Fig. 3).

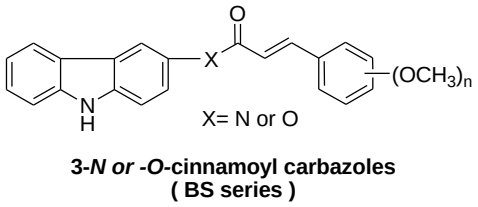


**Figure 3**. Combination of a carbazole moiety and cinnamoyl moiety

In this study, we designed and synthesized novel 3-*N*- or -*O*-cinnamoyl carbazole derivatives (BS **1**–**4**), and introduced a cinnamamide and cinnamate linkage in carbazole's framework, instead of a urea linkage. Moreover, the effects of BS **1–4** on vascular barrier

integrity in both cellular systems and animal models have not yet been elucidated. The objective of the present study was to determine the vascular barrier protective effect of BS **1–4** on ECs and mice.

To determine the effects of synthesized BS **1**–**4** (supplementary data) on the HMGB1-mediated hyperpermeability primary HUVECs, the flux of albumin in a dual-chamber system was monitored as described previously [23]. HUVECs [23-26] were treated with each compound for 6 h after the addition of HMGB1 (1 μg/mL). The results show that BS **2**–**4** at 10 μM induced the down-regulation of HMGB1-mediated membrane disruption and the vascular protective effects of BS **3** and BS **4** were better than that of BS **2** (Table 1).

**Table 1.** Effects of different BS compounds on HMGB1-mediated hyperpermeability in HUVECs

| Compound | Dose | ELISA $OD_{650}$ | Inhibition (%) |
|---|---|---|---|
| DMSO | | 0.116 ± 0.021 | |
| HMGB1 | 1 μg/mL | 0.531 ± 0.039 | |
| BS **1** | 10 μM | 0.512 ± 0.019 | 3.6 |
| BS **2** | 10 μM | 0.313 ± 0.023* | 42.9 |
| BS **3** | 10 μM | 0.168 ± 0.021* | 68.4 |
| BS **4** | 10 μM | 0.158 ± 0.031* | 70.2 |

Each entry represents the mean ± SEM of three different experiments. * $p < 0.05$ significantly

different from HMGB1.

DMSO: dimethyl sulfoxide; HMGB1: high-mobility group box 1; HUVECs: human umbilical vein endothelial cells; SEM: standard error of the mean.

The *in vivo* effects of BS **2**–**4** on vascular permeability [27] were assessed in order to corroborate the *in vitro* results. Thus, as can be seen in Table 2, BS **2**–**4** at 10 μM induced marked inhibition of the peritoneal leakage of dye induced by HMGB1. Because the average circulating blood volume for mice is 72 mL/kg [28], the average weight of the mice used was 27 g, and the average blood volume was 2 mL. The amount of BS **1** (342.4 μg/kg), BS **2** (372.4 μg/kg), BS **3** (402.4 μg/kg), and BS **4** (403.4 μg/kg) injected yielded a maximum concentration of 10 μM in the peripheral blood.

**Table 2.** Effects of different BS compounds on HMGB1-mediated hyperpermeability in mice

| Compound | Dose | μg/mouse (n=5) | Inhibition (%) |
|---|---|---|---|
| DMSO | | 0.35 ± 0.05 | |
| HMGB1 | 100.0 μg/kg | 5.72 ± 0.29 | |
| BS **1** | 342.4 μg/kg | 5.64 ± 0.35 | 1.4 |
| BS **2** | 372.4 μg/kg | 4.22 ± 0.42* | 26.2 |
| BS **3** | 402.4μg/kg | 2.43 ± 0.21* | 57.5 |
| BS **4** | 403.4 μg/kg | 2.35 ± 0.22* | 58.9 |

The amount of BS 1 (342.4 μg/kg), BS 2 (372.4 μg/kg), BS 3 (402.4 μg/kg), and BS 4 (403.4 μg/kg) injected yielded a concentration of 10 μM in the peripheral blood.

Each entry represents the mean ± SEM of three different experiments. * $p < 0.05$ significantly different from HMGB1.

DMSO: dimethyl sulfoxide; HMGB1: high-mobility group box 1; SEM: standard error of the mean.

To exclude the possibility that the inhibition of permeability was due to cytotoxicity caused by each compound, cellular viability assays [27] were performed in HUVECs treated with each compound for 24 h. At the concentrations used (up to 20 μM), BS **1**–**4** did not affect cell viability (data not shown).

The migration of leukocytes to ECs is an important step in the HMGB1-mediated inflammatory response [29]. We conducted studies to determine whether BS **2**–**4** could block the migration of human neutrophils [30-31] to HMGB1-stimulated HUVECs as described previously [23]. We demonstrated that BS **2**–**4** at 10 μM effectively inhibited the migration of human neutrophils to HMGB1-stimulated ECs (Table 3).

**Table 3.** Effects of different BS compounds on HMGB1-mediated neutrophil migration in HUVECs

| Compound | Dose | Migration Index | Inhibition (%) |
|---|---|---|---|
| DMSO | | 35.3 ± 2.7 | |

| | | | |
|---|---|---|---|
| HMGB1 | 1 μg/mL | 188.2 ± 13.7 | |
| BS **1** | 10 μM | 179.2 ± 14.5 | 4.8 |
| BS **2** | 10 μM | 106.4 ± 9.4* | 43.5 |
| BS **3** | 10 μM | 71.6 ± 6.1* | 62.0 |
| BS **4** | 10 μM | 65.2 ± 5.1* | 65.4 |

Each entry represents the mean ± SEM of three different experiments. * $p < 0.05$ significantly different from HMGB1.

DMSO: dimethyl sulfoxide; HMGB1: high-mobility group box 1; HUVECs: human umbilical vein endothelial cells; SEM: standard error of the mean.

To confirm this effect *in vivo*, HMGB1-induced leukocyte migration in mice was examined. HMGB1 significantly stimulated leukocyte migration into the peritoneal cavity of mice and BS **2**–**4** at 10 μM significantly decreased leukocyte counts (Table 4).

**Table 4** Effects of different BS compounds on HMGB1-mediated leukocyte migration in mice

| Compound | Dose | x $10^6$ | Inhibition (%) |
|---|---|---|---|
| DMSO | | 1.45 ± 0.13 | |
| HMGB1 | 100.0 μg/kg | 7.35 ± 0.72 | |
| BS **1** | 342.4 μg/kg | 7.29 ± 0.65 | 0.8 |
| BS **2** | 372.4 μg/kg | 4.48 ± 0.35* | 39.0 |
| BS **3** | 402.4μg/kg | 2.92 ± 0.21* | 60.3 |

| | | | |
|---|---|---|---|
| BS **4** | 403.4 μg/kg | 2.85 ± 0.25* | 61.2 |

The amount of BS 1 (342.4 μg/kg), BS 2 (372.4 μg/kg), BS 3 (402.4 μg/kg), and BS 4 (403.4 μg/kg) injected yielded a concentration of 10 μM in the peripheral blood.

Each entry represents the mean ± SEM of three different experiments. * $p < 0.05$ significantly different from HMGB1.

DMSO: dimethyl sulfoxide; HMGB1: high-mobility group box 1; HUVECs: human umbilical vein endothelial cells; SEM: standard error of the mean.

In addition, the anti-inflammatory effects of BS **3** and BS **4** were better than that of BS **2** (Tables 2–4). The structure–activity relationship (SAR) of 3-cinnamoyl carbazole derivatives (BS **1**–**4**) revealed that the more methoxy groups present on the cinnamoyl moiety, the more potent was the anti-inflammatory activity, regardless of whether cinnamamide or cinnamate linkage was used.

The present work aimed to study the vascular barrier integrity protection offered by the three new structurally similar compounds, BS **2**–**4**. Maintenance of vascular barrier integrity is emerging as an important therapeutic strategy. Along with involvement in the progression of sepsis, HMGB1 has also been implicated as a proinflammatory cytokine and a proangiogenic factor in the sustained proinflammatory responses observed in severe vascular inflammatory diseases, such as sepsis or septic shock [32]. The results of this study suggest that the anti-septic

effects of the three new structurally similar compounds, BS **2**–**4**, occur through inhibition of HMGB1-mediated inflammatory responses.

**Acknowledgements**

This study was supported by the National Research Foundation of Korea (NRF) funded by the Korea government [MSIP] (Grant Nos. 2012R1A5A2A42671316) and by the Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815).

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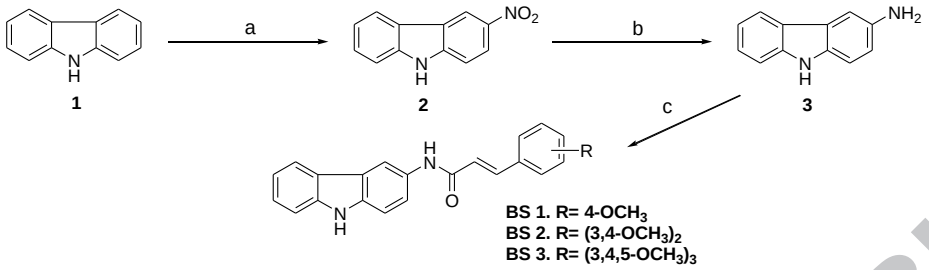
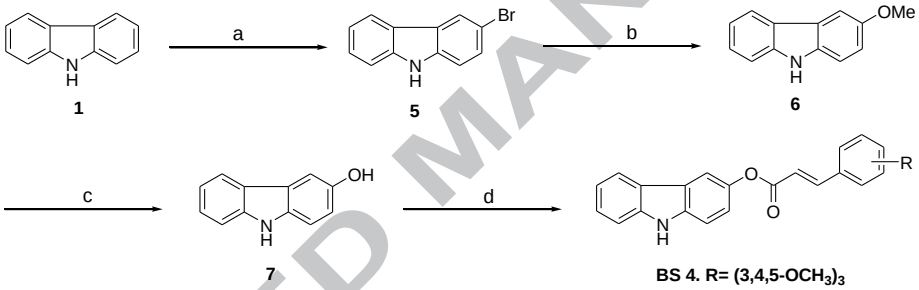



**Scheme 1**. Reagents and conditions: (a) $HNO_3$, water, RT → 80°C, 3 h, (b) Pd/C 10%, ethanol, 3 h, (c) EDC, 4-DMAP, substituted cinnamic acid, THF anhydrous, RT, 24 h




**Scheme 2**. Reagents and conditions: (a) NBS, THF, RT, 5 h, (b) CuI, DMF, MeONa/MeOH, 120°C, 20 h, (c) $BBr_3$ 1 M solution, dry MC, RT, 3 h, (d) EDC, 4-DMAP, substituted cinnamic acid, THF anhydrous, 24 h.

Graphical abstract