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Synthesis and evaluation of 1,2,4-oxadiazole derivatives as potential anti-inflammatory agents by inhibiting NF-κB signaling pathway in LPS-stimulated RAW 264.7 cells

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ABSTRACT

In this study, a series of compounds with 1,2,4-oxadiazole core was designed and synthesized for the optimization of **JC01**, an anti-inflammatory hit identified from our in-house compound library using NF-κB pathway luciferase assay and NO production assay. All the synthetic compounds **1–29** have been screened for their anti-inflammatory effects by evaluating their inhibition against LPS-induced NO release, and compound **17** exhibited the highest activity. Western blotting and immunofluorescence analysis revealed that **17** prominently inhibited LPS-induced activation of NF-κB in RAW264.7 cells and blocked the phosphorylation of p65. Consistent with these results, it was found that **17** prevented the nuclear translocation of NF-κB induced by LPS. These data highlighted **17** as a promising anti-inflammatory agent by inhibiting NF-κB activity.

Inflammation is a defensive response on exposure to harmful stimuli such as pathogens and irritants, with quintessential features like redness, swelling, pain, heat and dysfunction.[1] The pathology of inflammation is a complicated procedure by the organism to remove the injurious stimuli and to initiate the healing process of the tissues.[2] However, in certain circumstances, inflammation is also deemed to be a major risk factor for the pathogenesis of chronic diseases, including arthritis, diabetes, asthma, atherosclerosis and even cancer,[3–7] and its process involves in the activated macrophages such as RAW 264.7 cells. Macrophages play a crucial role in the innate immune response, and they protect cells from injury induced by both exogenous and endogenous factors.[8,9] The LPS-stimulated RAW264.7 cell is usually employed as a classical inflammatory cell model,[10] and the activated macrophages secrete pro-inflammatory mediators including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), metalloproteinases, nitric oxide (NO) and inducible nitric oxide synthase (iNO).[11–13] Meanwhile, the activation of nuclear factor-κB (NF-κB) signaling pathways, which have been found to play important roles in inflammatory reactions and diseases, mediates the release of pro-inflammatory factors in monocytes/macrophages. Therefore, treatments aimed at inhibiting NF-κB may have potential therapeutic advantages for inflammatory diseases.[14,15]. In past decades, non-steroidal anti-inflammatory drugs (NSAIDs) and immune drugs have been applied in some inflammatory diseases.[16,17]

To discover potential anti-inflammatory agents targeting the inhibition of NF-κB activation, an initial NF-κB inhibitor, 1,2,4-oxadiazole derivative **JC01** (Fig. 1), was screened out from our in-house compound library of 5387 compounds by using NF-κB pathway luciferase assay.[18,19] This compound exhibited an IC_{50} value of 13.83 ± 2.71 μM against TNF-α-induced NF-κB activation, which was further confirmed by western blotting and immunofluorescence analysis (Fig. S1 in Supporting Information). Then the anti-inflammatory activity of **JC01** was evaluated by testing its inhibition on the production of NO in LPS-stimulated RAW264.7 cells. NO is an important inflammatory mediator which can be produced by activated macrophages. The result from Table 1 showed that **JC01** had a suppressive effect on the production of NO in LPS-stimulated RAW264.7 cells, with an IC_{50} value of 26.96 ± 1.31 μM (data shown in Table 2). These initial results indicated that **JC01** could serve as a good starting molecule for further optimization to develop novel anti-inflammatory agents through inhibiting NF-κB activation. Herein, we report the design, synthesis, biological evaluation and mechanistic of a series of **JC01**-derived 1,2,4-oxadiazoles.

Literature survey demonstrates that 1,2,4-oxadiazole is a common

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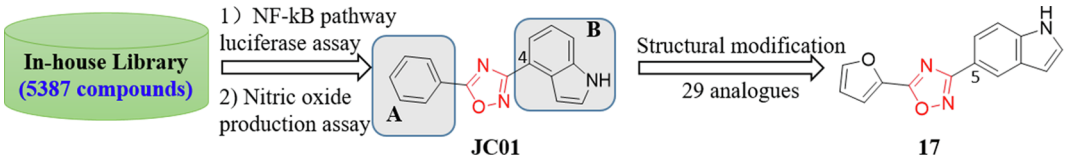


Fig. 1. The workflow of discovery of 1,2,4-oxadiazoles as anti-inflammatory agents.

heterocycle in various anti-inflammatory compounds,[20] and some indole derivatives have also been identified as NF-κB inhibitors[21,22] and anti-inflammatory agents.[23,24] Thus, both 1,2,4-oxadiazole and indole fragments were preserved in the first step modification of **JC01**, and the effect of substituting position of 1,2,4-oxadiazole on indole ring on the NO production was firstly taken into mind, which led to the preparation of analogues **1–3**. The cytotoxicity of **1–3** towards RAW264.7 cells at 20 μM were initially evaluated using MTT assay with doxorubicin as control drug. The results from Table 1 showed that only compound **3** with 1,2,4-oxadiazole on C-5 position of indole ring was non-toxic toward RAW 264.7 cells with the cell viability of 79.38%, compared with **1** and **2** (cell viability $<$ 50%). Next, the anti-inflammatory activity of **1** was determined in the model of NO production in RAW264.7 cells. The result showed that inhibition ratio of **3** (46.76%) against NO production was close to that of hit **1**. Taking these results into consideration, the C-5 substitution of 1,2,4-oxadiazole on indole was thought to be optimal.

Next, the 5-(1,2,4-oxadiazol-3-yl)-1*H*-indole core was reasonably kept, and different substituents were introduced to C-3 position of oxadiazol ring to produce 16 analogues (**4–19**). Most compounds of analogues **4–14** were toxic toward RAW264.7, and only **4**, and **13** were non-toxic and had inhibition ratio of 47.22%, and 53.94% against NO production, respectively. In order to decrease the toxicity of this series of compounds, the benzene ring was replaced by other ring systems, such as tetrahydropyran (**15**), cyclopentene (**16**), furan (**17**), cyclohexane (**18**) and cyclobutane (**19**). It was to our delight that compound **17** had little effect on the growth of RAW 264.7 at 20 μM with cell viability of 98.96%, suggesting that **17** was non-toxic towards RAW264.7 cells. Then, compound **17** showed a reduction on NO production with inhibitory ratio of 86.60% in comparison with LPS-treated group. Further, the IC_{50} value of **17** against NO production was further determined to be 12.84 ± 0.21 μM. In addition, compound **17** showed NF-κB inhibitory activity with an IC_{50} value of 1.35 ± 0.39 μM in NF-κB pathway luciferase assay (Table 2).

Alternatively, the 5-phenyl-1,2,4-oxadiazole fragment of hit **JC01** was reserved while the indole ring was replaced by various other groups, which led to the synthesis of analogues **20–29**. Except compounds **20**, **21** and **26**, the others were non-toxic towards RAW 264.7 cells and showed inhibitory activity against NO production with inhibitory ratios ranging from 34.28% to 63.58%.

The synthesis of 1,2,4-oxadiazoles **JC01** and **1–29** was achieved in one-step route summarized in Schemes 1 and 2. All of these compounds were smoothly prepared from the cyclization of substituted benzoic acids with different hydroxylcarboximidamides under microwave condition. The detailed synthetic procedure and data of ^{1}H, ^{13}C NMR and HRMS for target compounds were included in Supplementary Material.

Since the potential NF-κB inhibitor **17** showed improved activity compared with **JC01** in both LPS-induced NO production and NF-κB pathway luciferase assays, it was taken as a model compound for the follow-up tests. The further results of the MTT assay demonstrated that **17** in the concentration range from 0 to 100 μM had no cytotoxicity towards RAW 264.7cells, compared with the vehicle control (Fig. 2). Thus, **17** at specific concentration was used in the subsequent investigation.

As NF-κB is an important transcription factor that regulates the expression of most pro-inflammatory cytokines such as iNOS, IL-6 and TNF-α, the NF-κB signaling pathway was then investigated as a likely target underpinning the anti-inflammatory action of **17**. Indeed, in the immunofluorescence analysis, overlaid fluorescence microscopic images of LPS-stimulated RAW 264.7 cells revealed that treatment of **17** at 20 μM impeded LPS-stimulated nuclear translocation of p65 (Fig. 3A). Then, we investigated the critical proteins of this signaling pathway by western blotting to determine the effect of **17** on the NF-κB activity, as shown in Fig. 3B. Compound **17** strongly suppressed the phosphorylation of p65 which is indicative of activation of p65 for NF-κB transcriptional activity, as well as the phosphorylation and degradation of IκB-α, in a dose-dependent manner. Collectively, the anti-inflammatory effect of compound **17** was at least in part mediated by inhibition of the NF-κB signaling pathway.

To determine the protective effect of **17** on RAW264.7 macrophage inflammatory responses induced by LPS, we analyzed the mRNA expression levels of IL-6, IL-1β and TNF-α by real-time PCR. As shown in Fig. 4A–C, the expressions of IL-6, IL-1β and TNF-α induced by LPS were significantly up-regulated at the indicated time points, whereas dose-dependent reductions in LPS stimulated IL-6, IL-1β and TNF-α mRNA expression levels were observed in macrophages after co-incubation with **17** ($p < 0.05$). Thus, **17** showed significant inhibition on IL-6, IL-1β and TNF-α mRNA expressions. At the same time, the effect of **17** pre-treatment on iNOS synthesis was also investigated. iNOS was detected on western blot of the cell lysate after exposure to LPS. As shown in Fig. 4D, the production of iNOS was up-regulated after the LPS stimulus. However, pre-treatment of the cells with **17** significantly reduced the increase in iNOS production.

In summary, twenty-nine 1,2,4-oxadiazole derivatives were designed and prepared in order to optimize the anti-inflammatory profile of hit **JC01**. The bioassay results highlighted compound **17** as the most promising anti-inflammatory agent, which could significantly inhibit NO production and NF-κB activation and had significant effects on the inhibition of pro-inflammatory mediators. The results demonstrated that **17** decreased NO and iNOS productions in RAW 264.7 macrophage cells after LPS stimulation. The anti-inflammatory activity of **17** may be mediated by the NF-κB activation mechanism in the RAW 264.7 cells. These results indicated that **17** could function as a lead compound deserving further studies for developing anti-inflammatory drugs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 1
Effects of compounds on the cell viability of RAW 264.7 for 24 h and their inhibition ratio against NO production at 20 μM.

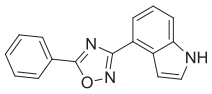
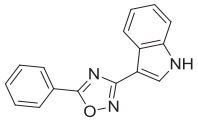
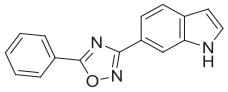
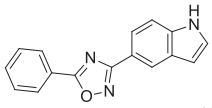
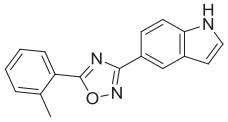
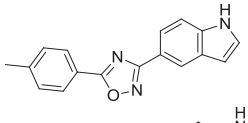
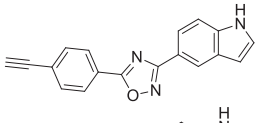
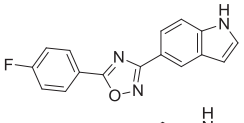
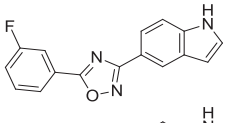
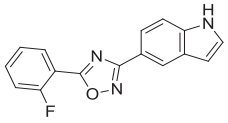
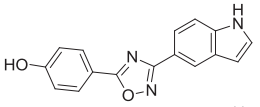
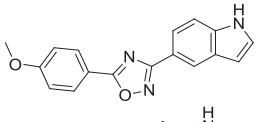
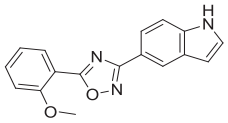
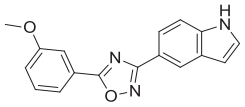
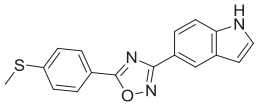
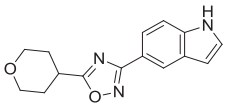
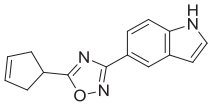
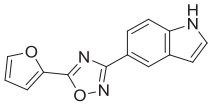
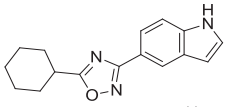
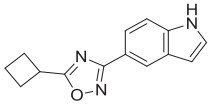
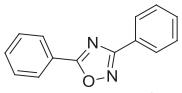
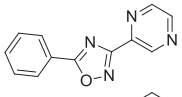
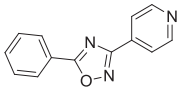
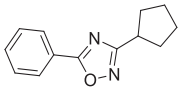
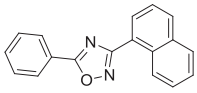
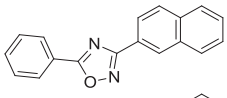
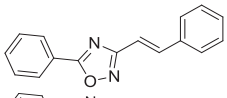
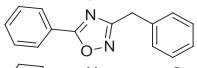
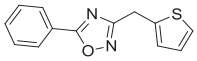
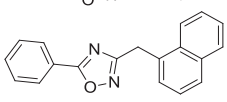
Compound	Structure	Cell viability[a]	Inhibition ratio of NO production
JC01		64.29%	45.71%
1		11.61%	-[b]
2		31.02%	–
3		79.38%	46.76%
4		85.27%	47.22%
5		51.17%	–
6		23.59%	–
7		16.75%	–
8		17.24%	–
9		26.44%	–
10		36.55%	–
11		41.31%	–
12		44.95%	–
13		88.08%	53.94%
14		22.79%	–

Table 1 (*continued*)

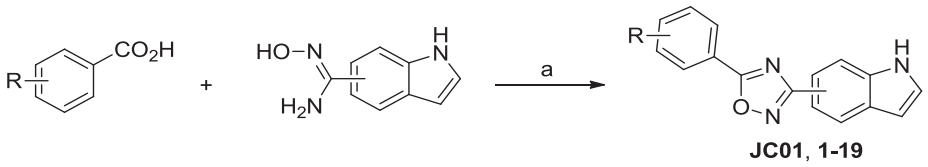
Compound	Structure	Cell viability[a]	Inhibition ratio of NO production
15		95.68%	2.75%
16		24.28%	–
17		98.96%	86.60%
18		68.81%	–
19		85.38%	57.58%
20		42.13%	–
21		46.20%	–
22		88.97%	43.13%
23		85.39%	34.28%
24		90.42%	59.89%
25		86.24%	63.58%
26		28.16%	–
27		89.59%	39.33%
28		85.17%	48.24%
29		92.81%	41.64%

[a] Cell viability was expressed as percent cell viability when compared to that of DMSO vehicle control cells (100%), and cell viability more than 75% at 20 μM was considered to be non-toxic.
[b] Not tested.

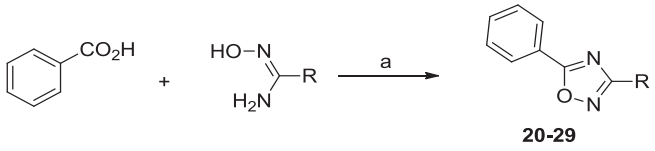
Table 2
Results of inhibition on NF-κB activation and NO production.

Compound	IC_{50} (NF-κB, μM)[a]	IC_{50} (NO production, μM)[a]
JC01	13.83 ± 2.71	26.96 ± 1.31
17	1.35 ± 0.39	12.84 ± 0.21
Bortezomib	0.03 ± 0.007	-[b]
Dexamethasone	–	4.16 ± 0.23

[a] IC_{50} value was taken as a mean from three independent experiments.
[b] Not tested.



Scheme 1. Synthesis of **JC01** and **1–19**. Reagents and conditions: (a) EDCI, HOBT, DIEA, DMF, microwave heating at 180 °C, 20 min.



Scheme 2. Synthesis of **20–29**. Reagents and conditions: (a) EDCI, HOBT, DIEA, DMF, microwave heating at 180 °C, 20 min.

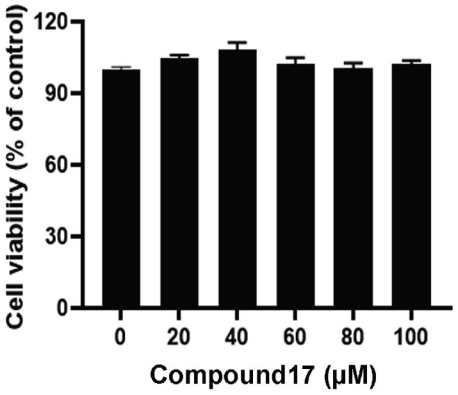


Fig. 2. Effect of **17** on viability of RAW264.7 cells analyzed by MTT method.

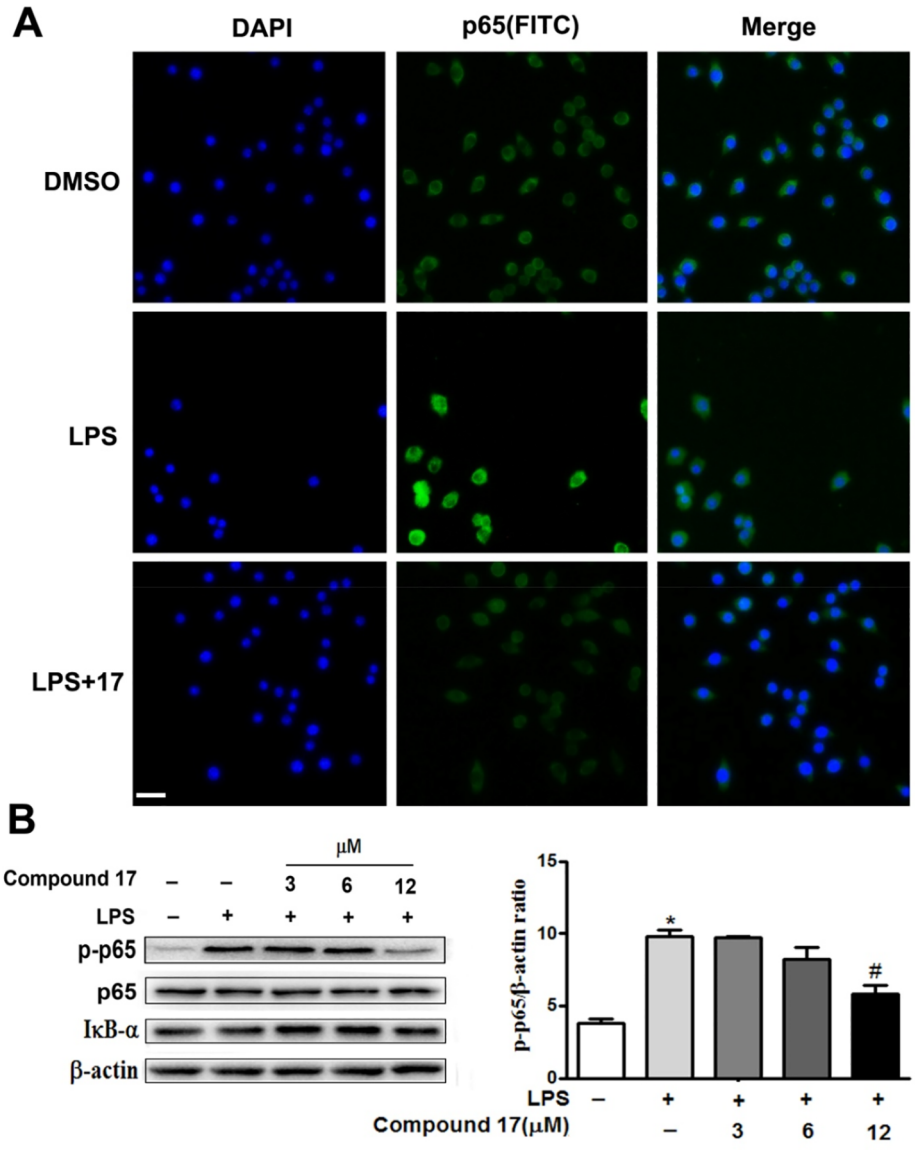


Fig. 3. Inhibition of LPS-stimulated inflammatory response by **17** through suppression of NF-kB pathway. (A) Immunofluorescence staining of p65 in RAW264.7 cells after treatment with **17** for 1 h, followed by 24 h of stimulation of LPS (1 μg/mL) or vehicle. Scale bar, 50 μm; representative images from three individual experiments with similar results shown. (B) RAW264.7 cells were treated with various concentrations of **17** for 45 min; phospho-p65, p65, IκBα and β-Actin were detected by Western blot. Similar results were obtained in three independent experiments. The results were expressed as mean ± SD (n = 3). *p < 0.05 vs. control group; #p < 0.05 vs LPS-induced group.

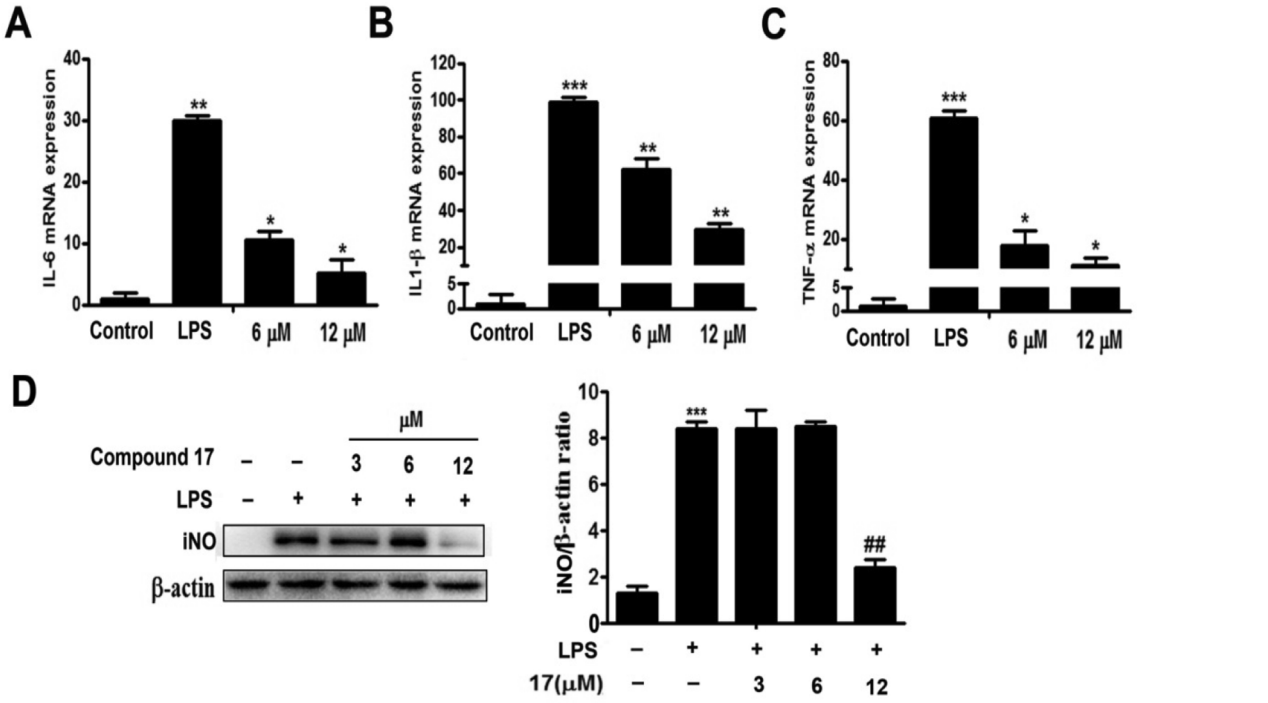


Fig. 4. (A, B and C) Effects of **17** on IL-6(A), IL-1β (B) and TNFα (C) mRNA expressions in LPS-stimulated RAW264.7 macrophages measured by qRT-PCR. RAW264.7 cells were co-treated with different concentrations of **17** and LPS (1 μg/mL) for 24 h. Graphs represent relative expression values, the ratio of different gene to reference gene. Data are expressed as mean ± SD (n = 3); compared with control group, *p < 0.05, **p < 0.01, ***p < 0.001. (D) Western blotting analysis of iNOS in RAW264.7 cells after LPS stimulation. Data are expressed as mean ± SD (n = 3); compared with control, ***p < 0.001; compared with group LPS, ##p < 0.01.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127373.

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