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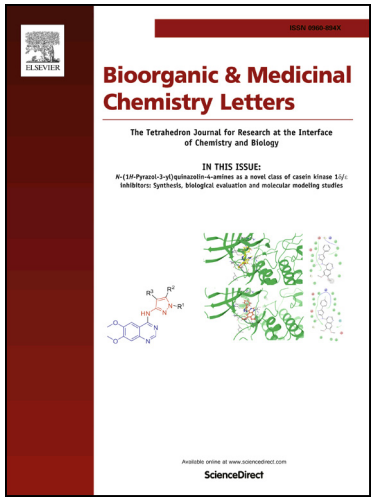

Design and synthesis of pregnenolone/2-cyanoacryloyl conjugates with dual NF-κB inhibitory and anti-proliferative activities

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Graphical Abstract

Design and synthesis of pregnenolone/2-cyanoacryloyl conjugates with dual NF-κB inhibitory and anti-proliferative activities

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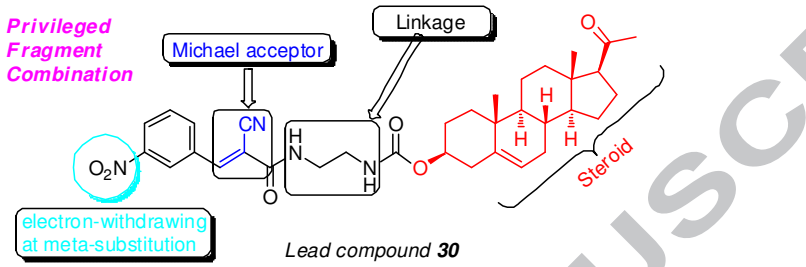

1) NF-κB inhibitory activity with IC_{50} = 2.5 μM
2) Cytotoxicity against MCF-7, A549, H157, and HL-60 cell lines with IC_{50} = 6.5~36.2 μM
3) Effect of electron-withdrawing group at meta-substitution increased their activity

Design and synthesis of pregnenolone/2-cyanoacryloyl conjugates with dual NF-κB inhibitory and anti-proliferative activities

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Abstract: Twenty-five novel pregnenolone/2-cyanoacryloyl conjugates (**6-30**) were designed and prepared, with the aim of developing novel anticancer drugs with dual NF-κB inhibitory and anti-proliferative activities. Compounds **22** and **27-30** showed inhibition against TNF-α-induced NF-κB activation in luciferase assay, which was confirmed by Western blotting. Among them, compound **30** showed potent NF-κB inhibitory activity (IC_{50} = 2.5 μM) and anti-proliferative against MCF-7, A549, H157, and HL-60 cell lines (IC_{50} = 6.5~36.2 μM). The present study indicated that pregnenolone/2-cyanoacryloyl conjugate I can server as a novel scaffold for developing NF-κB inhibitors and anti-proliferative agents in cancer chemotherapy.

Keywords: Pregnenolone, NF-κB, 2-Cyanoacryloyl, Michael acceptor, Anti-proliferative activity.

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Cancer is a group of diseases characterized by uncontrolled cell growth, which has became a major public health concern over the last several decades.[1] The unmber of new diagnosed cancer cases reached nearly 14.5 million in 2014 and is expected to rise by about 30% in the next decade.[2] The abnormal cell growth can be a result of gene mutations induced by DNA damage or aberrant activation of cell signaling pathways, such as hormones cytokines and chemokines.[3-5] Among the factors involved in cancer, the nuclear factor-κB (NF-κB) as a ubiquitous eukaryotic transcription factor plays an important role in regulating the expression of more than 150 genes associated with inflammation, immunity, and cell growth.[6] In particular, the aberrant activation of NF-κB has been frequently observed in various types of human cancers, and suppression of NF-κB can limit the proliferation of cancer cells.[7,8] Therefore, NF-κB has been pointed as a therapeutical target in cancer, and inhibitors of NF-κB function could be developed into new anticancer drugs or leads.[9-11]

It is generally believed that the Michael acceptors in bioactive compounds can form adducts with reactive thiol groups of proteins to induce protein modification and misfolding, which might be responsible for their various biological effects, such as anti-proliferative activity.[12-14] As a Michael acceptor, 2-cyanoacryloyl moiety has been extensively applied in the design of anticancer drugs. For example, tyrphostin AG490 (**1a**, Fig. 1) is the first small molecular Jak2 inhibitor that is clinically used as an anticancer agent and is also effective in various models of inflammatory and autoimmune diseases;[15] indole/2-cyanoacryloyl hybrid **1b** was reported to show anti-proliferative activity against a range of cancer cells;[16] CDDO-Me (**1c**) is a semi-synthetic triterpenoid acting as an inhibitor of NF-κB pathway, which was shown to be a drug candidate for treating cancer, chronic kidney disease, and other diseases.[17]

Pregnenolone (**2a**, Fig. 1) is an important naturally occurring endogenous steroid and known as a precursor to most of steroid hormones like estrogen, progesterone, testosterone, and glucocorticoids.[18] The unique structural features and the broad-spectrum bioactivites of **2a** make it to be a promising drug leads, attracting

numerous interest in structural modification from the fielf of medicinal chemistry.[19-21] As part of our project to develop new drugs derived from pregnenolone,[22,23] a new NF-κB inhibitor, derivative **2b** (Fig. 1) with an IC_{50} value of 12.2 μM, was recently screened out from our in-house compound library using NF-κB pathway luciferase assay. In anti-proliferation assay, **2b** did not show significant activity against A549 and MCF7 cancer cell lines.

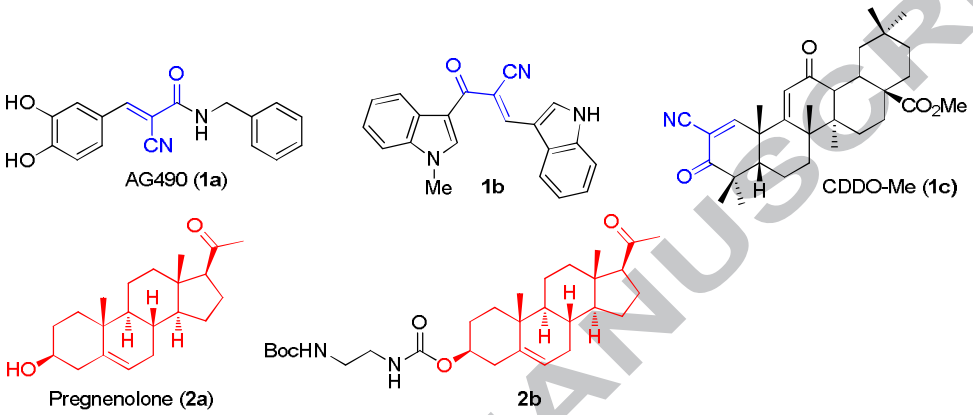


Figure 1. 2-cyanoacryloyl derivatives **1a-1c** and steroids **2a** and **2b**

In our continuous effort to discover anticancer drugs with dual NF-κB inhibitory and anti-proliferative activities, a privileged fragment combination (PFC) strategy was recently employed to further modify the structure of **2b**. Since pregnenolone is a privileged molecular skeletion and 2-cyanoacryloyl moiety as Micheal acceptor is a very useful moiety in the design of anticancer drugs, both pharmacophores was combined to generate conjugates **I**, as shown in Fig. 2. In addition, the ethanediamine linker was preserved since numerous bioactive compounds or drugs contain the *N*-CH_2-CH_2-*N* moiety.[24] Herein, we reported the synthesis of pregnenolone/2-cyanoacryloyl conjugate **I**, and the biological evaluation for their dual NF-κB inhibitory and anti-proliferative activities.

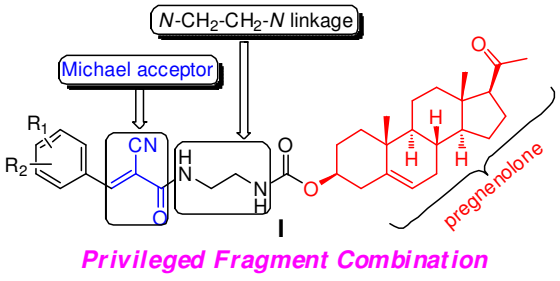
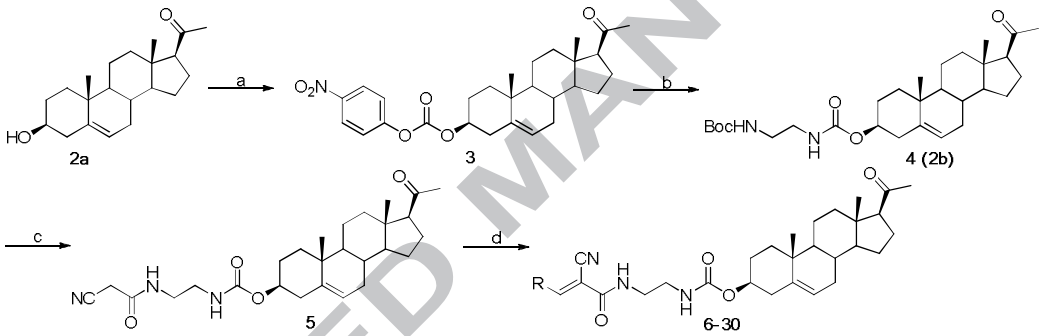


Figure 2. Proposed novel pregnenolone/2-cyanoacryloyl conjugate **I**

The synthesis for target compounds **6-30** is depicted in Scheme 1. Briefly, pregnenolone (**1a**) was reacted with *p*-nitrophenyl chloroformatein dichloromethane using pyridine as the base to obtain the intermediate **3**, which was then reacted with *N*-Boc-ethylenediamine in the presence of Et_3N to yield compound **4**.[25] *N*-Boc protection in **4** was removed by treating with TFA followed by coupling with cyanoacetic acid in the presence of EDCI and HOBT to give amide **5**.[26,27] Knoevenagel condensation reaction of **5** with various substituted benzaldehydes catalyzed by piperidine[28] finally afforded pregnenolone/2-cyanoacryloyl conjugates **6-30**. The general procedures and spectrascopic data of all synthetic compounds were described in Supplementary data.

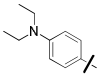


Scheme 1. Synthesis of **6-30**. Reagents and conditions: (a) *p*-nitrophenyl chloroformate, pyridine, CH_2Cl_2, rt, overnight, 79%; (b) *N*-Boc-ethylenediamine, Et_3N, CH_2Cl_2, rt, overnight, 84%; (c) i. trifluoroacetic acid, CH_2Cl_2, rt, overnight; ii. cyanoacetic acid, EDCI, HOBT, DIEPA, CH_2Cl_2, 36 h, 84%; (d) substituted benzaldehyde, piperidine, EtOH, 19%-76%.

Table 1

NF-κB inhibitory activity and cytoxicity of analogs **4-30** against HEK293/NF-κB cells.

No.	R	% Inhibition on NF-κB at 20 μg/mL	Viability at 20 μg/mL (%)	IC_{50}[b] (NF-κB, μM)
4		76.6	85.5	12.2±0.2
5		74.4	87.9	12.4±0.1
6		46.7		
7		44.9		

8		22.3		
9		22.2		
10		17.8		
11		59.3		
12		34.6		
13		14.5		
14		5.8		
15		4.2		
16		22.6		
17		5.0		
18		2.1		
19		1.2		
20		0.5		
21		43.1		
22		64.2	60.8	7.1±0.5
23		18.8		
24		46.4		
25		15.9		
26		30.9		
27		64.4	64.4	7.5±1.2
28		81.4	59.6	3.3±0.1

29		78.3	74.5	10.3±0.2
30		83.9	54.7	2.5±0.1
PS-341	-			0.030±0.007

[a] IC_{50} values are taken as a mean from three independent experiments.

The NF-κB inhibitory activity of target compounds **6-30** and their synthetic intermediates **4** and **5** was evaluated in TNF-α-stimulated HEK293/NF-κB cells, according to a previously reported luciferase assay.[29] TNF-α (tumor necrosis factor alpha) is a known inducer of NF-κB activity,[30] and PS-341 (bortezomib) as known NF-κB inhibitor[31] was used as reference compound. In brief, The HEK293/NF-κB cells were inoculated into 96-well plates. After 24 h, the cells were treated with TNF-α and then incubated with tested compounds. Signal strength of luciferase was detected by EnVision. The results for these tests were summarized as percentage of inhibition or IC_{50} values in Table 1.

At first, the inhibitory activity of compounds against NF-κB was tested at the concentration of 20 μg/mL (equal to 42 ~ 31 μM for each different compound). The percentage inhibition of synthetic intermediates **4** and **5** was 76.6% and 74.4%. For most target compounds, especially those (**6-20**) with electron-donating substituents in benzene moiety, showed very weak activity (<50%) against NF-κB at the concentration of 20 μg/mL, while **22** and **27-30** possessing electron-withdrawing groups (such as F, CN, and NO_2) displayed significant inhibitory effect on NF-κB at the same tested concentration. These results indicated that electron-withdrawing effect of substituent in benzene ring plays an important role in their bioactivity. The cytoxicity of selected compounds including **4**, **5**, **22** and **27-30** was evaluated at 20 μg/mL. From the results, these compounds did not show significant cytotoxicity against HEK293/NF-κB cells at the tested concentration.

Then, the IC_{50} values of **4**, **5**, **22** and **27-30** for NF-κB inhibition were further tested. The synthetic intermediates **4** and **5** showed NF-κB inhibitory activity with IC_{50} values ~12 μM. Among these compounds, **28** and **30** showed the strongest inhibition against NF-κB (IC_{50} ~ 3 μM) with around 2- and 4-fold increased activity

compared with **27** and **29**, respectively, indicating that *meta*-substitution of electron-withdrawing group was superior to its *para*-substitution. A similar result was also observed by comparing the activity of compounds **21-26**. In addition, it was obvious that the inhibitory activity increased as the effect of electron-withdrawing substituents increased (**30**, NO_2 > **28**, CN> **22**, F > **24**, Cl >**26**, Br).

To further confirm these compounds do inhibit NF-kB in cell, the inhibitory effects of compound **29** on NF-κB pathway activation was examined by Western blotting.[32] This compound was selected to be tested due to it showing the lowest cytotoxicity. The result from Fig. 3 indicates the inhibitory effect of 10 μM **29** was obvious.

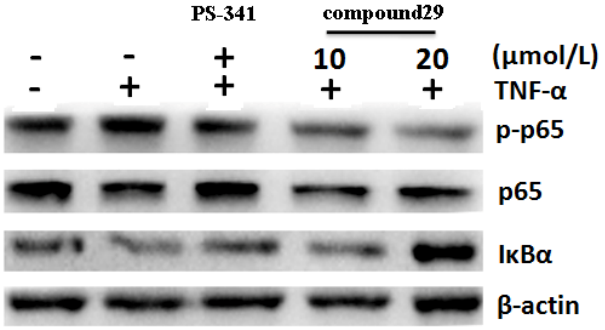


Figure 3. Western blotting analysis of the key proteins in the NF-κB pathway after compound **29** treatment for 4 h.

The introduction of 2-cyanoacryloyl moiety was expected to increase anti-proliferative activity, thus these above bioactive compounds were furthermore selected to be tested for anti-proliferative activity against a panel of cancer cell lines, including MCF-7 (human breast adenocarcinoma), A549 (human lung carcinoma), H157 (human oral squamous cell carcinoma), HepG2 (human hepatocellular carcinoma), and HL-60 (human acute promyelocytic leukemia) using the MTT method[33] with doxorubicin as positive control. The results are shown in Table 2.

Table 2

In vitro anti-proliferative activity of **4**, **5**, **22**, and **27-30** presented as IC_{50}[a] (μM)

No.	R	MCF-7	A549	H157	HepG2	HL-60
4		>50[b]	>50	>50	>50	>50
5		>50	>50	>50	>50	>50
22		6.1±0.2	>50	17.9±0.8	>50	45.6±2.2

27		>50	>50	24.5±0.6	>50	>100
28		8.8±0.8	30.9±0.5	21.3±1.4	>50	15.6±1.7
29		>50	>50	>50	>50	10.1±0.1
30		6.5±0.1	36.2±0.5	30.6±3.2	>50	11.2±1.3
Doxorubicin		4.4±0.5	11.0±0.2	0.61±0.02	1.0±0.1	0.91±0.14

[a] IC_{50} value was taken as a mean from three independent experiments.

[b] IC_{50} value higher than 50 μM was considered inactive.

From the results, both intermediates **4** and **5** lacking the 2-cyanoacryloyl moiety were inactive against all the tested cancer cell lines. Compared with **27** and **29** with weak anti-proliferative activity, compounds **28** and **30** showed potent activity against most tested cancer cell lines, including MCF-7, A549, H157, and HL-60 with IC_{50} values ranging from 6.5 to 36.2 μM. These results confirmed the vital importance of the *meta*-substitution of electron-withdrawing group in benzene ring for their activity. However, their anti-proliferative activity seems not directly rely on NF-κB inhibitory activities.

In conclusion, a series of pregnenolone/2-cyanoacryloyl conjugates were synthesized and evaluated for their NF-κB inhibitory activity and *in vitro* anti-proliferative against different cancer cell lines. Several compounds showed significant inhibitory activity on TNF-α-induced NF-κB activation and exhibited potent anti-proliferative against MCF-7, A549, H157, and HL-60 cell lines. The preliminary SAR studies suggested that the electron-withdrawing effect of substituent in this series of conjuates plays an important role in their bioactivity. More studied was needed to clearify their anticancer mechanism, and further inversitgation on the effect of diamine linkage and heterocycles, instead of benzene, on their bioactivity are in progress and will be reported in due course.

Acknowledgements

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Supplementary data

Supplementary data related to this article can be found, in the online version, at doi:#.

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29. The luciferase assay was carried out according to Li's protocol (Acta Pharmacol. Sin. **2013**, *34*, 939-950.). In detail, HEK293/NF-κB cells, which are HEK293 cells stably transfected with an NF-κB-responsive luciferase reporter plasmid, were generated as follows. A luciferase reporter plasmid containing an NF-κB binding site and pcDNA3.1 were co-transfected into HEK293 cells using Lipofectamine 2000 at a concentration of 10:1 (Invitrogen, Carlsbad, CA, USA). Stable recombinant cells were

selected for resistance to 1 mg/mL G418 and for a strong luciferase signal. The cells were inoculated into 96-well plates (25 μL). After 24 h, the cells were treated with TNF-α (0.2 μg/mL, 25 μL) and then incubated with 2 μL of each test compound at nine different concentrations (0.78-20 μM) for 6 h. A 25 μL amount of complexed liquid was removed from the 96-well plates and then incubated in a dark place with 25 μL of zymolyte for 30 min. Signal strength of luciferase was detected by EnVision microplate reader, and their activity was evaluated by IC_{50} value.

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32 Western blotting analysis: anti-p65, anti-phospho-p65 (Ser536), anti-IκBα(L35A5) were purchased from Cell Signaling Technology (Boston, Massachusetts, USA). All antibodies were used as recommended by the manufacturers. Cell lysates were subjected to electrophoresis on 10% SDS-PAGE gels and blotted with the indicated antibodies. Relative expression was determined by densitometric comparison with an internal control such as β-actin, and the treatment with compounds **29** or PS-341 at varying concentrations was estimated from IC_{50} value in luciferase assay.

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Legends

Fig. 1. 2-cyanoacryloyl derivatives **1a-1c** and steroids **2a** and **2b**

Fig. 2. Proposed novel pregnenolone/2-cyanoacryloyl conjugate **I**.

Fig. 3. Western blotting analysis

Scheme 1. Synthesis of **6-30**.

Supporting Information.