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Design, synthesis, and biological evaluation of chalcone oxime derivatives as potential immunosuppressive agents

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ABSTRACT

A series of deoxybenzoin oximes were recently reported as potent immunosuppressive agents by our group. In order to continue the original research for potential immunosuppressive agents with high efficacy and low toxicity, we synthesized a series of new chalcone oximes and evaluated them for their cytotoxicities and immunosuppressive activities. Among the synthesized compounds, chalcone oximes **25** and **27** exhibited lower cytotoxicities and higher inhibitory activities on anti-CD3/anti-CD28 co-stimulated lymph node cells than other compounds. Specially, compound **27** displayed 200-fold lower cytotoxicity (CC_{50} = 2174.39 μM) than cyclosporin A (CC_{50} = 10.10 μM) and showed SI value (SI = 176.69) close to cyclosporin A (SI = 154.13). Besides, the preliminary mechanism of inhibition effect of compounds **25** and **27** was also detected by flow cytometry, and the compounds exerted immunosuppressive activities via inducing the apoptosis of activated lymph node cells in a dose dependent manner. Also, the deep mechanism of apoptosis was detected by Western blot analysis.

Immunosuppressive agents (immunosuppressants) are a class of chemical or biological substances to reduce tissue damage by inhibiting cellular and humoral immune response. These agents can inhibit the body's abnormal immune response, mainly being used in organ transplant anti-rejection and treatment for autoimmune diseases such as rheumatoid arthritis, rheumatic fever, collagen diseases, lupus erythematosus and autoimmune hemolytic anemia.[1,2]

Cyclosporine A (CsA) and tacrolimus (FK506) are important therapeutic immunosuppressants. They achieve the action process by inhibiting T-lymphocyte activation, which plays an integral role in transplant rejection and autoimmune diseases.[3] CsA and FK506 both interfere with Ca^{2+}-sensitive T-cell signal transduction pathways to prevent the action of transcription factors in the lymphokine gene expression process.[4–6]

Although immunosuppressive drugs have been successfully used for organ transplantation and treatment of autoimmune diseases in clinic, their side effects cannot be neglected, such as secondary infection, inducing cancer, kidney toxicity and liver toxicity. Therefore, there is a clinical need for new therapeutic agents capable of modulating immune responses with high efficacy and low toxicity.[7–12] The effort of searching for novel classes of potential immunosuppressive compounds has never stopped.

In the search for new potential immunosuppressive agents, we turned our attention to one kind of products which could be got from nature: chalcones. Wide range of biological activities of chalcones had been reported in the literature. Using the natural chalcone structure as a core framework, large quantities of chalcone derivatives with potent antileishmanial,[13] cytotoxic,[14] antifungal[15] and anti-inflammatory[16,17] activities were synthesized. In this Letter, we also put our interest on new synthetic chalcone derivatives to explore new immunosuppressive agents with high efficacy and low toxicity.

As previously reported, many compounds with oxime groups displayed potent biological activities and low toxicities.[18,19] Some of them had been used as clinical medical agents and some oximes were reported to show immunosuppressive activities.[20] In this Letter, EN3638 (a oxime derivative of salicylic acid) in a certain dose (50, 100, or 150 mg/kg daily, 250 mg/kg three times a week, or 400 mg/kg twice a week) could suppress both clinical and histologic evidence of experimental allergic encephalomyelitis (EAE) during the course of therapy of rats.[20] Our group also had reported that a series of deoxybenzoin oximes possessed potential immunosuppressive activity.[21] In this Letter, the most active compound was **31** which exhibited immunosuppressive activity with SI >684.64 even better than CsA (SI = 235.44).

Based on our previous interesting work, we continued our research work: a series of chalcone derivatives including oxime skeletons were synthesized. The immunosuppressive activities of the synthesized compounds were evaluated with lymph node cell

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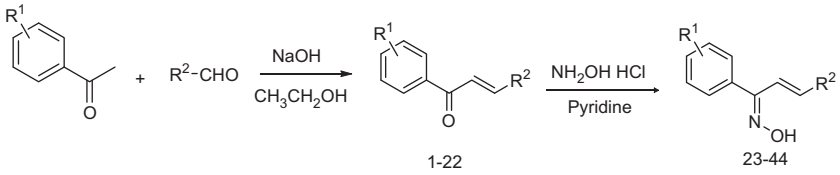
functional assay and the cytotoxicities were tested. The preliminary mechanism of some active compounds was further examined by using fluorescence activated cell sorter (FACS) and Western blot assay.

The synthesis of compounds **1**–**22** was outlined in Scheme 1. The chalcones were prepared by the Claisen–Schmidt condensation between ketones and aldehydes in the presence of potassium hydroxide in ethanol with 80–85% yield.[22]

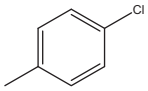
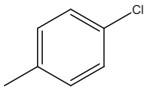
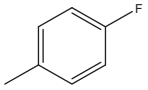
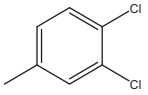
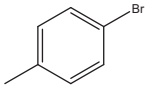
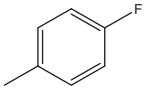
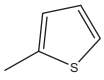
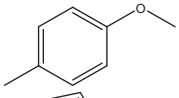
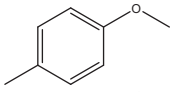
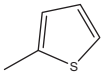
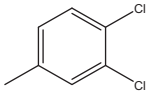
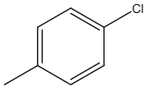
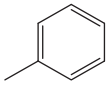
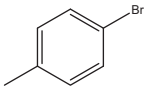
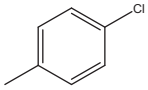
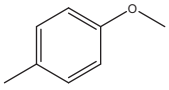
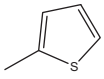
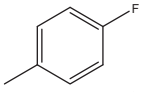
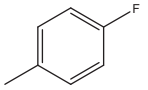
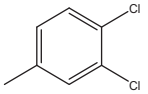
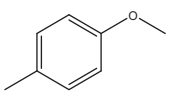
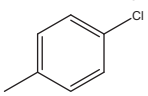
The novel oximes were synthesized by the route outlined in Scheme 1. Our interest in this area was to design and synthesize chalcone oximes for structure–activity relationship (SAR) studies in immunosuppressive activities. Thus, the treatment of chalcones **1–22** with hydroxylamine hydrochloride in pyridine afforded the oximes **23–44** with pyridine as catalyzer and alkaline environment provider. All newly synthesized oximes (Table 1) were fully characterized by spectroscopic methods and the elemental analysis. All compounds were recrystallized from ethanol. Crystals of partial compounds were obtained. The crystal structure of compound **43** was shown in Figure 1. The configuration showed that this compound could be *anti* isomer. Because all compounds were recrystallized from ethanol, it could suggest that this series of oxime compounds would exhibit *anti* configurations.

The synthesized oximes were carried out *in vitro* tests on the lymph node cells for cytotoxicities and inhibitory activities on anti-CD3/anti-CD28 co-stimulated lymph node cells. Pharmacological results of these compounds were summarized in Table 2 with



Scheme 1. Synthetic routes of chalcone oxime derivatives.

Table 1
Chemical structures of compounds **23**–**34**

Compd	R^1	R^2	Compd	R^1	R^2
23	4-OCH_3		**34**	4-Br	
24	4-OCH_3		**35**	4-Br	
25	4-OCH_3		**36**	4-Br	
26	4-OCH_3		**37**	4-Br	
27	4-OCH_3		**38**	4-Br	
28	4-OCH_3		**39**	4-CH_3	
29	4-OCH_3		**40**	4-CH_3	
30	4-Cl		**41**	4-CH_3	
31	4-Cl		**42**	4-CH_3	
32	4-Cl		**43**	4-CH_3	
33	4-Cl		**44**	4-F	

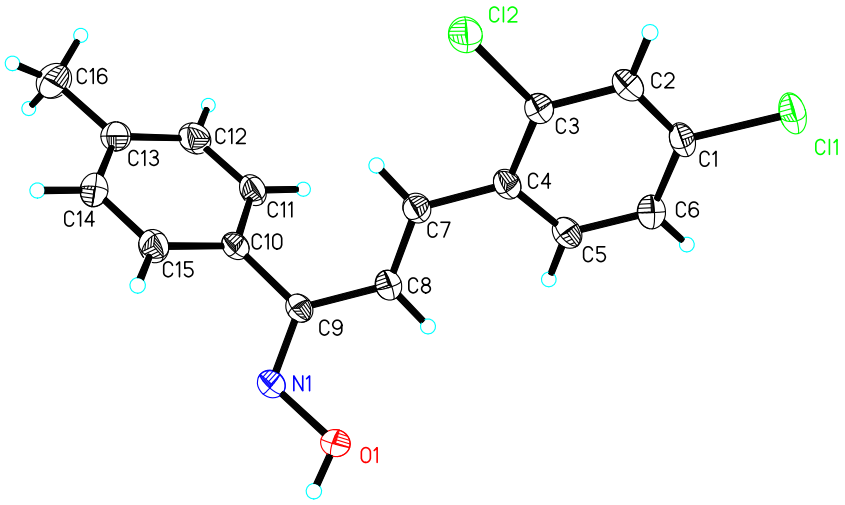


Figure 1. Crystal structure diagram of compound **43**. H atoms are shown as small spheres of arbitrary radii.

Table 2
In vitro cytotoxicities[a] and inhibitory effects[b] of the synthetic oximes

Compound[c]	CC_{50}[a] (μM)		IC_{50}[b] (μM)		SI[d]
	Avg	sd	Avg	sd	
23	290.57	15.83	11.48	0.78	25.31
24	13.47	2.19	10.95	0.34	1.23
25	737.17	38.83	13.93	0.22	52.93
26	10.60	6.91	8.49	0.76	1.25
27	2174.39	334.94	12.31	1.87	176.69
28	25.01	1.26	11.18	0.43	2.24
29	28.60	5.83	11.81	0.09	2.42
30	14.08	1.70	9.15	0.24	1.54
31	11.8	1.24	9.59	0.74	1.23
32	11.49	1.65	10.64	1.73	1.08
33	24.29	0.63	5.85	0.16	4.16
34	24.74	1.09	14.72	0.03	1.68
35	<5		<5		–
36	20.26	3.13	13.26	0.77	1.53
37	23.73	1.61	<5		>4.75
38	15.35	0.45	11.15	0.03	1.38
39	21.31	0.91	11.59	0.24	1.84
40	34.17	2.56	11.61	0.12	2.94
41	117.36	1.92	6.24	0.26	18.82
42	13.21	1.20	11.25	0.64	1.17
43	14.74	0.45	6.34	0.47	2.32
44	14.83	0.31	10.88	0.09	1.36
3	93.67	5.03	25.08	0.97	3.73
5	1789.42	215.25	23.55	1.83	75.98
CsA	10.10	1.55	0.07	0.01	154.13

[a] On lymph node cells.
[b] On lymph node cells co-stimulated by anti-CD3/anti-CD28.
[c] The compounds tested for immunosuppressive activity are consistent with the description in the Experimental Section.
[d] Selectivity index: ratio of the compound concentration that decreases cell viability by 50% (CC_{50}) to the compound concentration that inhibits proliferation by 50% (IC_{50}), relative to control values.

CsA as control. The cytotoxicity of each compound, which was the concentration when cell survival rate was 50%, was expressed as CC_{50}. Immunosuppressive activity of each compound, which was the concentration when cell survival rate of anti-CD3/anti-CD28 co-stimulation of lymph node cell survival rate was 50%, was expressed as IC_{50}. Selectivity index ($SI = CC_{50}/IC_{50}$) was used to assess the compounds' biological activities in the previous papers.[21,23]

As shown in Table 2, the SI values of the compounds varied from 1.17 to 176.68. Some compounds (**25** and **27**),[25,26] especially **27** (SI = 176.69) showed good immunosuppressive activity, and **27** was even comparable to the control CsA (SI = 154.13). Clearly, the compounds were more effective when substituents were both OCH_3. This result was similar with previous study that esterification could improve activity.[23] For comparison, we also evaluated the corresponding chalcones (compound **3** and **5**) of compound **25** and **27** for their activities. As shown in Table 2, the chacones showed good immunosuppressive activities but not better than the corresponding oximes.

Table 1 summarized the substituents (R^1 and R^2) of all chalcone oximes. When R^1 groups (such as methoxy groups) were unchanged, if R^2 groups were changed (such as compounds **23**–**29**), the facts listed below were clear: (1) the SI value order was **27** (R^2 = 4-methoxyphenyl, SI = 176.69) > **25** (R^2 = 4-bromophenyl, SI = 52.93) > **23** (R^2 = 4-chlorophenyl, SI = 25.31) > **29** (R^2 = phenyl, SI = 2.42) > **28** (R^2 = 2,4-dichlorophenyl, SI = 2.23) > **26** (R^2 = 2-thiophene, SI = 1.25) > **24** (R^2 = 4-fluorophenyl, SI = 1.23). The SI value order of halogen substituents was bromine > chlorine > fluorine, which conformed to our previously reported results: bromine possessed weakest electron withdrawing capability,[21] so the compound with bromine substituent showed more inhibitory activity (SI = 52.93). (2) The activity of thiophene substituted compound **26** (SI = 1.25) to be less than the phenyl substituted compound's activity (compound **29**, SI = 2.42) showed that aromatic properties of compounds had effect on activities. The sulfur 3p orbital and carbon 2p orbital conjugated in thiophene ring. The track was not matched very well and conjugation was not very good. (3) In addition, the activity of the compound with two same substituents in two different positions such as **28** (SI = 2.23) was significantly weaker than **23** (SI = 25.31), which should be attributed to the increase amount of substituents which could improve the electron withdrawing capability.

When R^2 groups were remained and R_1 groups were changed, such as compound **23**, **30**, **34**, **39** and **44**. Their activity was not very different from each other. The order was: **23** (R^1 = 4-methoxyl, SI = 2.23) > **39** (R^1 = 4- methyl, SI = 1.84) > **34** (R^1 = 4-bromine, SI = 1.68) > **30** (R^1 = 4-chlorine, SI = 1.53) > **44** (R^1 = 4-fluorine, SI = 1.36). This order was similar with the order when R^1 groups were remained and R^2 groups were changed. Methoxyl and methyl groups were both electron donating groups, however, because the former group's electron donating capability was weaker than the latter, the latter inhibited stronger activity. The activity could be greatly improved when R_1 and R_2 were both electron donating groups.

In general, the most potential compound was **27** (SI = 176.68) owning two methoxy groups compared with the other oximes, even close to CsA (SI = 154.13). Through SAR analysis, it could be seen that electron withdrawing ability and the aromatic property

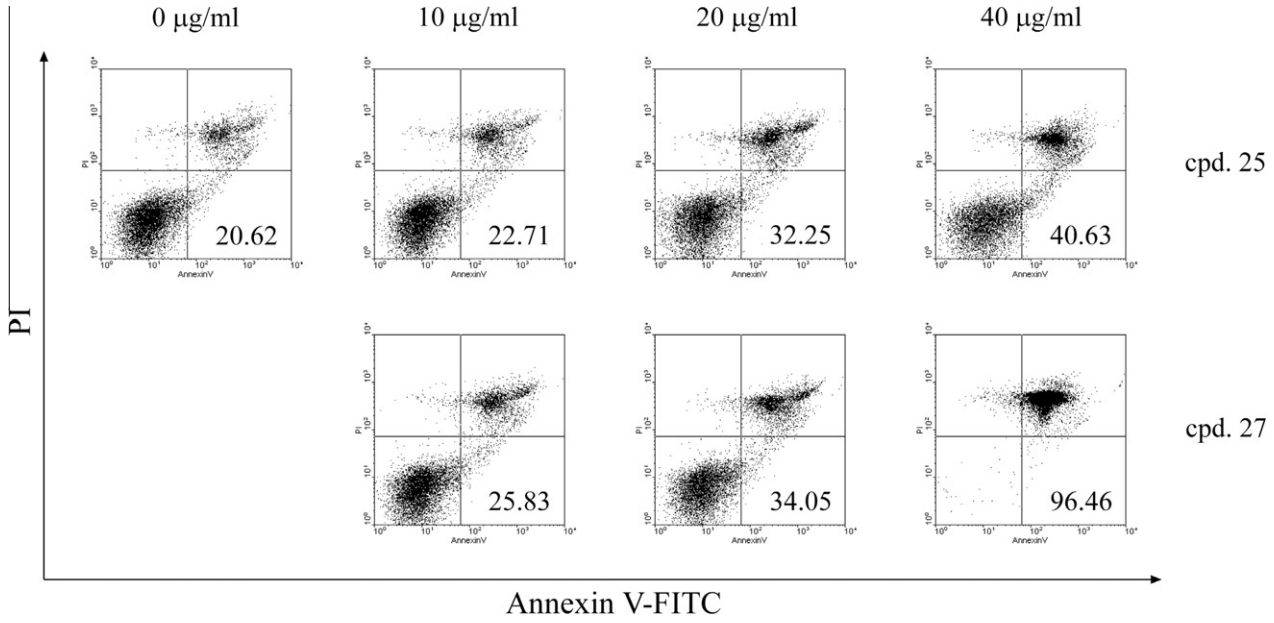


Figure 2. Lymph node cells isolated from naïve mice were cultured with anti-CD3/anti-CD28 and various concentrations of **25** and **27** for 24 h. Cells were stained by Annexin V–FITC /PI and apoptosis was analyzed by flow cytometry.

might be important factors to determine the activity of these compounds. This might provide direction to search for more active and promising treatment agent in the future.

In our previous study, we knew that synthesized oximes posed their immunosuppressive effect through cell apoptosis. Caspase 3 and poly(ADP-ribose) in polymerse (PARP) beared important parts in this process. Caspase was considered to be the central executor of apoptosis process to activate and degrade its substrate to cause terminal effect incidents by the complex multi-factor and multi-channel interaction. This activation could cause biochemical changes of characteristic morphology to complete the process of apoptosis. Also, PARP was a genomic testing and DNA repair enzyme, degraded by the caspase-3 and other cysteine protease and the most characteristic protease substrate solution.[24]

As the most active agents, compounds **25** and **27** had been under systematic investigations in vitro experiments. We detected the mechanism of proliferation inhibition by FACS, and found that these compounds could induce the apoptosis of activated lymph node cells in a dose-dependent manner. As shown in Figure 2, lymph node cells co-stimulated with anti-CD3/anti-CD28 were treated with 10, 20 and 40 μg/mL of **25** or **27** for 24 h. The compound increased the percentage of apoptosis detected by Annexin V-FITC/PI staining in a dose-dependent manner. The result indicated that compound **25** and **27** induced apoptosis of anti-CD3/anti-CD28 stimulated lymph node cells and compound **27** displayed more outstanding activity.

To examine the status of the caspase 3 protein, we performed Western blot analysis by using an anti-cleaved caspase-3 antibody, which recognized p17 cleaved caspase-3. The p17 cleavage product of the lymph node cells treated with **25** or **27** appeared a significant increase in a dose-dependent manner (Fig. 3). This result confirmed the promotion function of compounds **25** and **27** on the cell apoptosis.

In conclusion, a series of new chalcone oximes were synthesized and their immunosuppressive activities and cytotoxicities were evaluated. Most chalcone oximes derivatives displayed potent immunosuppressive activities. Compound **27** exhibited significant immunosuppressive activity with SI = 176.69, even comparable to CsA (SI = 154.13). In general, electron donating substituents at both of the two rings were favorable for the immunosuppressive

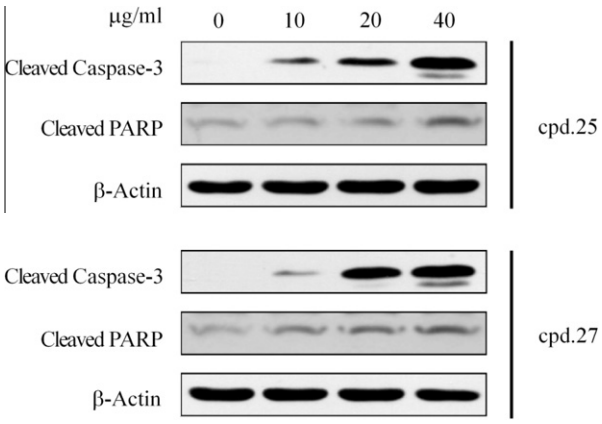


Figure 3. Protein levels of cleaved caspase-3 and PARP examined by Western blotting. Data were representative of three independent experiments.

activities of the synthesized oximes. The immunosuppression process was achieved through the promotion of apoptosis of the lymph node cells, by increasing the cleaved caspase-3 and the cleaved PARP. All the results outlined the potential of compound **27** for further exploitation as immunosuppressant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.080.

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25. *Physical and spectroscopic data for compound* ***25***: white crystal, yield 65%; R_f = 0.30 (PE/EtOAC 2:1); mp 137 °C. ^{1}H NMR (500 Hz, $CDCl_3$): δ 3.86 (s, 3H); 6.74 (d, J = 16.45 Hz, 1H); 6.96 (d, J = 8.7 Hz, 2H); 7.20–7.26 (m, 1H); 7.41–7.45 (m, 4H), 7.63 (d, J = 16.5 Hz, 2H). ESI-MS: 333.19 ($C_{16}H_{15}BrNO_2$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{14}BrNO_2$: C, 57.85; H, 4.25; N, 4.22. Found: C, 57.82; H, 4.23; N, 4.23.
26. *Physical and spectroscopic data for compound* ***27***: white crystal, yield 65%; R_f = 0.72 (PE/EtOAC 2:1); mp 131–134 °C. ^{1}H NMR (500 Hz, $CDCl_3$): δ 3.81 (s, 6H); 6.78 (d, J = 16.47 Hz, 1H); 6.88–6.94 (m, 3H); 6.96-7.04 (m, 2H); 7.27 (s, 1H); 7.47 (d, J = 8.58, 2H); 7.51–7.62 (m, 1H); 7.70–7.88 (m, 1H). ESI-MS: 284.12($C_{17}H_{18}NO_3$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{17}NO_3$: C, 72.07; H, 6.05; N, 4.94. Found: C, 72.10; H, 6.04; N, 4.93.