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Parallel Synthesis and Biological Evolution of Quinic Acid Derivatives as Immuno-suppressing Agents against T-cell Receptors

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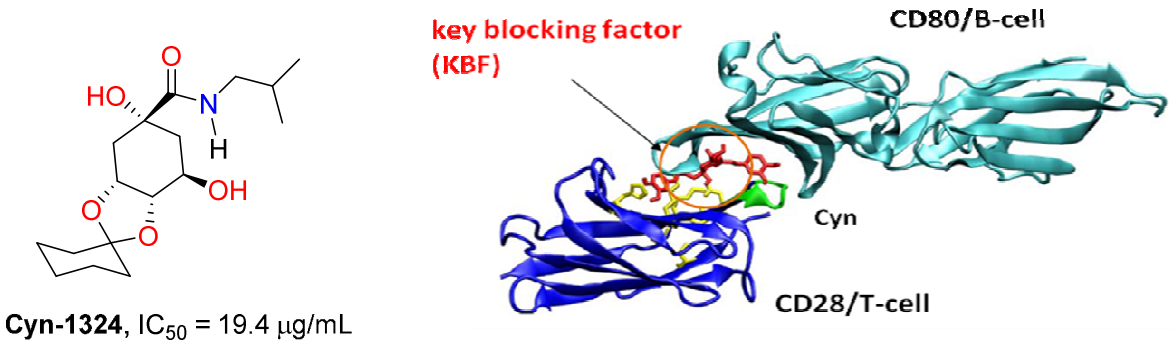
Abbreviations: APC: antigen presenting cell; Cyn: Cynarin: KBF: key blocking factor; CSA: cyclosporine A; MHC: histocompatibility complex; ORTEP: oak ridge thermal ellipsoid plot; OVA: ovalbumin; TCR: T-cell receptor; QA: quinic acid.

Graphical Abstract



Abstract

A simple protocol for the synthesis of quinic acid derivatives was established and their biological evolution against T-cells is studied. Results showed that, one of the derivatives, Cyn-1324 has low toxicity on T-cells and high effect on reducing Signal 2 of T-cell immune responses. *In vitro* binding measurements of atomic force spectroscopy further indicated that the blocking effect of Cyn-1324 between CD28 and CD80 was about 31±4 %. *In vivo* animal tests also confirmed that Cyn-1324 can reduce the allergic responses from ovalbumin-induced mice with little toxicity. Based on these observations, Cyn-1324 can be a mild immuno-suppressive candidate for future drug development.

Introduction

Immuno-response to exclude the invasion of harmful outside materials is the major defense system in human and other living creatures. Immuno-suppressive treatment for over-reacting patients to control immune responses is highly demanded as the over-reaction of immune cells cannot be self-controlled and may become a big burden for entire life. In particular, there are two signals (Signal 1 and Signal 2) produced during the conjugates of T-cells and antigen-presenting cells (APC cells such as B-cells or dendritic cells) to activate the adaptive immune responses.[1-4] The specialized formation of intercellular contact after activation of resting T-cells by APC cells is termed as immunological synapses.[5-7] More recent study by using single cell force spectroscopy showed that T-cells can be activated by dendritic cells than by B-cells based on the quantity measurement of IL-2 and IFNγ secreted from T-cells after activation.[8] Signal 1 is created as T-cell receptor (TCR) of T-cells which strongly binds with major histocompatibility complex (MHC) of APC cells. Signal 2 is a co-stimulation signal, which occurs simultaneously

with Signal 1 by two concurrent bindings: CD28 of T-cell to CD80 of APC cell (weaker binding) and CD154 of T-cell to CD40 of APC cell (stronger binding). After stimulation (Signal 1) and co-stimulation (Signal 2) bindings, the strength of these immuno-responses can be estimated by IL-2 released from activated T-cell. If Signal 2 is inhibited, the total release of IL-2 would be reduced consequently.[9] In the current work, a mild blocker for mainly blocking CD28 therefore can be found to only inhibit Signal 2 by directly blocking T-cells on their membrane surfaces.

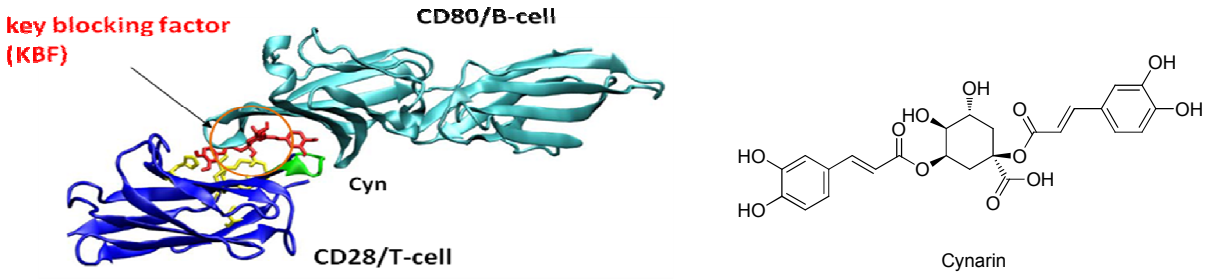


Figure 1. Computer simulation for blocking between CD28/T-cell and CD80/B-cell by Cynarin

There are many known therapeutics such as cyclosporine (CSA), cyclophosphamide, tacrolimus (FK506) and azathioprine are developed to treat immune-suppression. Although they are selective and potent to prevent the rejection of organ transplants and in diseases involving the immune system, they block both Signal 1 and Signal 2 to penetrate inside the cell and this causes severely toxic side effects. Under this consideration, development of mild immuno-suppressive agents is urgently needed which partially reduces the immune responses without rough side-effect.

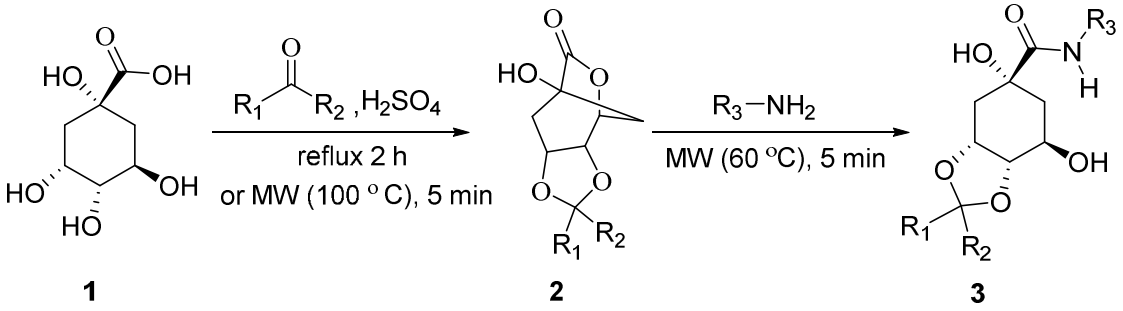
Cynarin, is a biologically active chemical constituent of *Cynara cardunculus*.[11] Earlier, we have reported that cynarin in *Echinacea purpurea* is able to block Signal 2 of T-cell activation

specifically for immunosuppression.[9-10] The blocking effect between CD28 of T-cell and CD80 of B-cell was identified by a small molecule, Cynarin (Cyn) after flowing through immobilized receptor (AFTIR). Computer simulation about this effect was displayed in Figure 1. A key blocking factor (KBF) was indicated by a red circle and a main blocking body (quinic acid-like) was assigned. Physical contact of immunological synapses mentioned above between CD28 of T-cells and CD80 of B-cells was a key point that we applied it to establish a novel drug screening method to obtain an immuno-suppressive compound. The reason is that this structural contact between CD28 and CD80 can be properly blocked by suitable molecules. Accordingly, a natural product Cynarin was found to effectively block the binding between CD28 and CD80: e.g., the immunological synapses of Signal 2 can be shut down by Cyn.[10] This blocking effect induced by Cyn on T-cells has further confirmed by using atomic force spectroscopy.[12] However, we found that the toxicity of Cyn on T-cells is observed. Our computer simulation[9] showed that the main blocking interactions between Cynarin and CD28 came from the quinic acid-like structure. Two symmetrical side chains (di-caffeoyl group) may not be essential for the effect of "blocking". Hence as a part of our ongoing research to find better immuno-suppressive agents, we synthesized a series of Cynarin derivatives by using quinic acid (QA) as a novel scaffold targeted on KBF structure. Many natural product (NP) drugs and NP-derived compounds have been found and applied in clinical trials.[13-14] The structure-based virtual screening method is one of powerful techniques to find the potential target drugs from a significant number of NP or NP-like compound libraries.[15-22] We report herein the preliminary results which indicate one of NP-derived compounds, Cyn-1324 has low toxicity and more chemical stability as compared to that of Cynarin. *In vivo* animal tests further confirmed that

Cyn-1324 has its immuno-suppressive effect on ovalbumin-induced allergic mice and therefore potentially is qualified to develop as a drug candidate in the future.

Results and Discussion

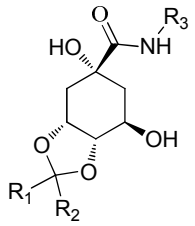
Chemistry: Synthesis of quinic acid (QA) derivatives The synthesis of quinic acid derivatives was accomplished in straightforward by two steps: lactonisation/ketalisation followed by aminolysis as shown in Scheme 1. Earlier, modifications at C-1/C-5 hydroxyl groups and formation of macrocycles between carboxylate and C-3 hydroxyl group are reported.[23-27] Stable quinic acid derivatives can be synthesized by converting *cis* C-3/C-4 hydroxyl groups into a ketal *via* a reaction with a ketone using strong acid catalyst. In addition, cytotoxicity of quinic acid may be reduced if its C-1 carboxyl group is modified to other functional groups.



Scheme1. Synthesis of quinic acid analogues **3**

Lactoniazation and ketalization of quinic acid **1** was carried out in the presence of catalytic amount of sulfuric acid in acetone to provide the acetal lactone intermediates **2** in a single step. Under the reflux conditions, the reaction took 2 h to complete conversion with the yield of 85 %. Alternatively, use of microwave irradiation in a closed vessel system at 100 °C dramatically reduced the reaction time to only 5 min with the maximum yield of 91 %. The next transformation is the aminolysis of lactone intermediates **2** with various amines. The reaction

was accomplished under microwave irradiation at 100 °C for 5 min to obtain various quinic acid derivatives.



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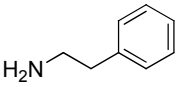
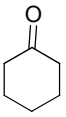
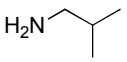
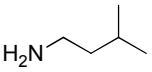
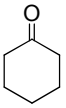
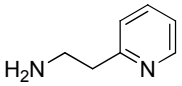
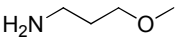
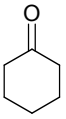
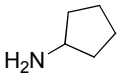
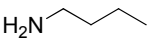
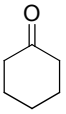
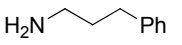
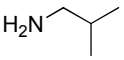
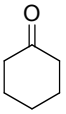
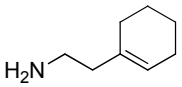
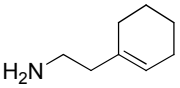
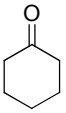
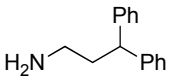
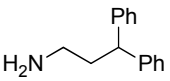
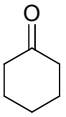
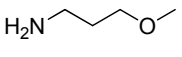
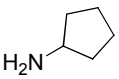
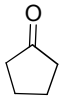
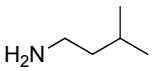
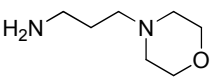
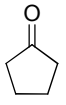
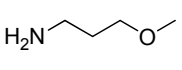
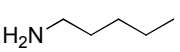
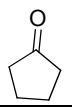
3a			76%	**3k**			93%
3b			81%	**3l**			72%
3c			86%	**3m**			85%
3d			93%	**3n**			84%
3e			91%	**3o**			86%
3f			88%	**3p**			88%
3g			90%	**3q**			89%
3h			87%	**3r**			80%
3i			68%	**3s**			82%
3j			92%	**3t**			75%

Table 1. Synthesis of quinic acid analogs **3**

Such aminolysis of lactones is required 18 h to complete conversion under the conventional refluxing condition. Furthermore, the aminolysis was performed with various amines to have different electronic nature to give a variety of quinic acid analogs as shown in Table 1. All the amines gave satisfactory yields under similar reaction conditions. The structure of compound **3k** is also confirmed by X-ray crystallography [28] as shown in Figure 2.

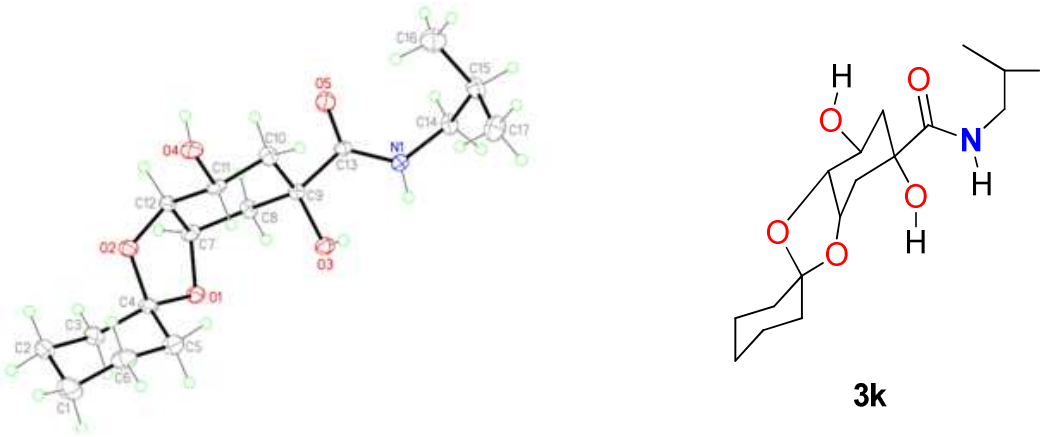


Figure 2. ORTEP representation of compound **3k** (Cyn-1324).

Biology: To investigate the potential biological applications of quinic acid analogues obtained by this synthetic protocol, the analogues of preliminary test for their cytotoxicity were performed. The results demonstrated that Cyn-1324 (**3k)** could effectively inhibit the proliferation of T-cell receptors. The efficacy is comparable with that of Cynarin, which served as a control study.

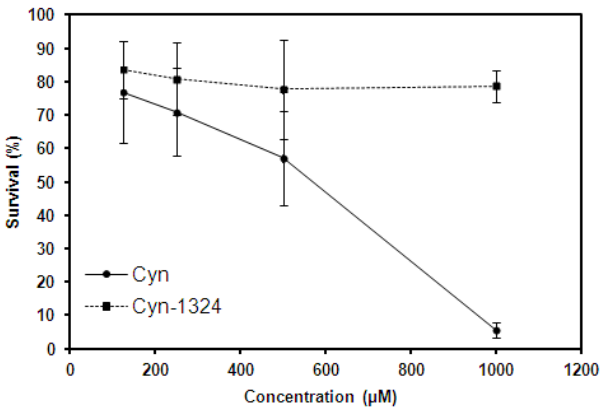


Figure 3. Cytotoxicity tests on T-cells

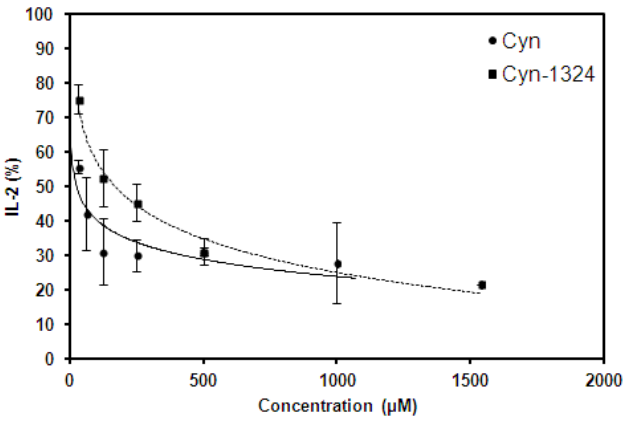


Figure 4. Efficacy tests on T-cells

The percentage cell survival and efficacy of Cyn and Cyn-1324 against T-cells were investigated. The relationship between cell survival (T-cells) *vs.* concentration of Cyn and Cyn-1324 is shown in Figure 3. The results implied that Cyn-1324 has low toxicity on T-cells up to 1,000 μM when compared to that of Cyn. At high concentration (1,000 μM), Cyn became very toxic whereas cell survival rate was less than 5 %. Similar results were observed with the efficacy test against T-cells and were shown in Figure 4. For efficacy test (blocking CD28 on T-cells with the results of reducing IL-2 release), both Cyn and Cyn-1324 showed to reduce IL-2 production. The reduction rates were similar at higher concentration for both compounds. To identify the blocking ability of Cyn-1324 on CD28 of T-cell, a real binding measurement was complete by using atomic force spectroscopy (AFM). Comparison of unbinding force distribution between CD28 and CD80 without and with the addition of Cyn-1324 was shown in Figure 5a and 5b, respectively. A larger part distribution of higher unbinding forces was observed without interruption by Cyn-1324 whereas a larger part distribution of lower unbinding forces was observed with the addition of Cyn-1324. The average unbinding force of CD28/CD80 was about 41.9 (±5.3) pN. However, the average unbinding force was reduced to about 29.1(±3.3) pN after block by Cyn-1324. Thus the "blocking effect" was observed about 31±4 %

as compared with Cynarin of 25±7 % . (be = bf (CD28/CD80)-bf(CD28/Cyn-1324/CD80)/bf(CD28/CD80; be = blocking effect; bf = binding force) These experiments were also done by investigating the blocking effect between CD154 and CD40 and found that less than 5 % was observed.

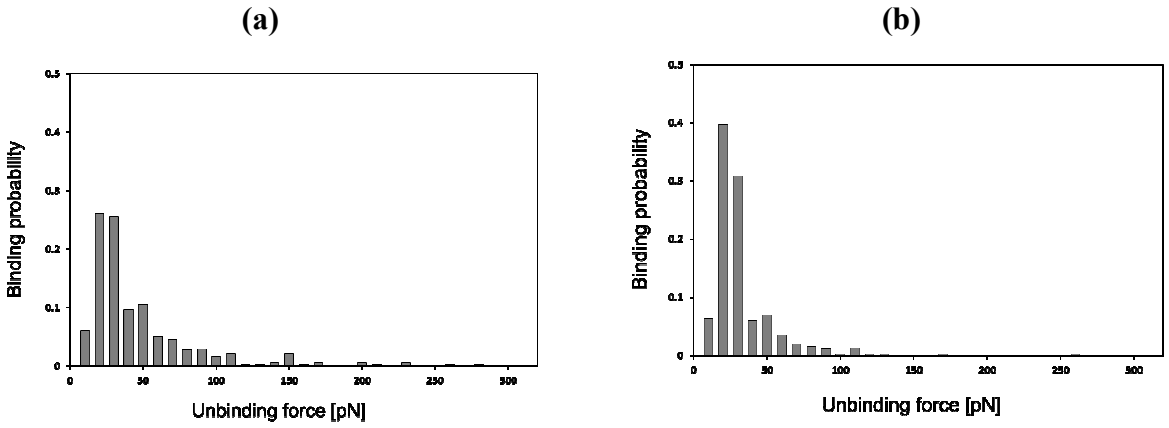


Figure 5. *In vitro* blocking effect is tested by AFM. (a) Unbinding forces distribution diagram between CD28 and CD80 (41.9 pN in average); (b) Unbinding forces distribution diagram between CD28 and CD80 after addition of Cyn-1324 (29.1 pN in average). The distribution of higher unbinding forces is reduced with the addition of Cyn-1324. The loading rate and contact time of AFM were 1.44×10^4 pN/s and 0.5 s, respectively.

Immuno-suppressive effect on mice was done by using ovalbumin (OVA) as an immunization inducer and cyclosporine A (CSA) as a reference drug. For their efficacy investigation (testing quantity change of IgG and IgE), mice were divided by four groups (5 mice/per each group): OVA/Cyn-1324 (n_1 group); OVA/CSA (n_2 group); OVA only (n_3 group) and PBS buffer only (n_4 group). Blood samples were collected and both quantities of IgG/IgE were measured. Results showed that IgG were reduced about 30% for n_1 group (OVA/Cyn-1324) and 45% for n_2 group (OVA/CSA) as compared with n_3 group (OVA only) at 14^{th} day.

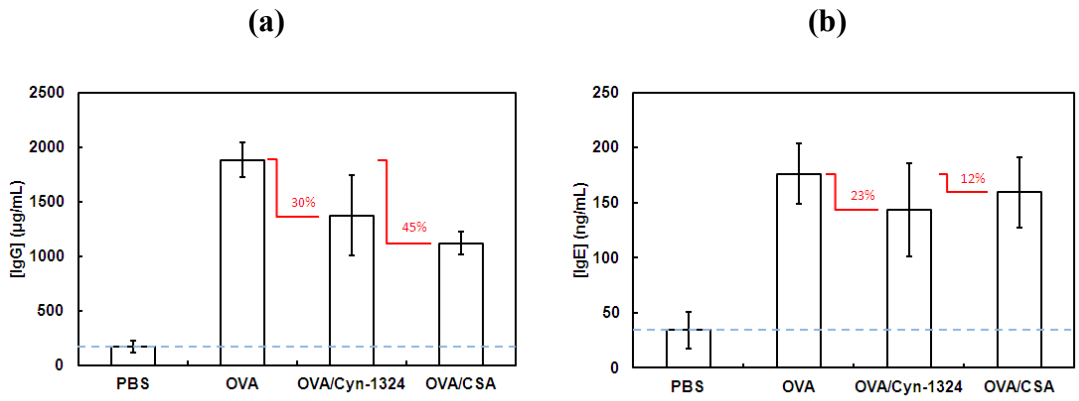


Figure 6. Animal model test of immuno-suppressive effect induced by Cyn-1324 and CSA. Both IgG and IgE were used to test Cyn-1324-treated and CSA-treated ovalbumin (OVA)-sensitized mice. Mice were sensitized by intraperitoneal injections for n1 (PBS buffer only), n2 (OVA), n3 (OVA+Cyn-1324) and n4 (OVA+CSA) groups. Blood was obtained from tail vein. Serum samples were analyzed by mouse-IgG and IgE ELISA test. (a) IgG reduction; (b) IgE reduction. Results shown here were taken at day-14. Beyond day-14, the similar results were obtained.

Oppositely, about 23 % (n_1 group) and 12 % (n_2 group) on the reduction of IgE was observed as shown in Figure 6. This implied that Cyn-1324 might not be a better candidate to reduce IgG, but is stronger to reduce IgE as compared with cyclosporine A. There are four types of symptoms to cause hypersensitivity reactions: (a) type I (anaphylactic or immediate-type) reaction; (b) type II (cytotoxic) reaction; (c) type III (immune complex) reaction; (c) type IV (cell-mediated or delayed-type) reaction. Two main factors of immune responses, IgE and IgG are related to type I and type IV, respectively. Results of over-reacting behavior will increase the production of both IgE and IgG. For suppressing type I symptom (reducing IgE production), it is done by blocking T-cells from binding with APC cells.

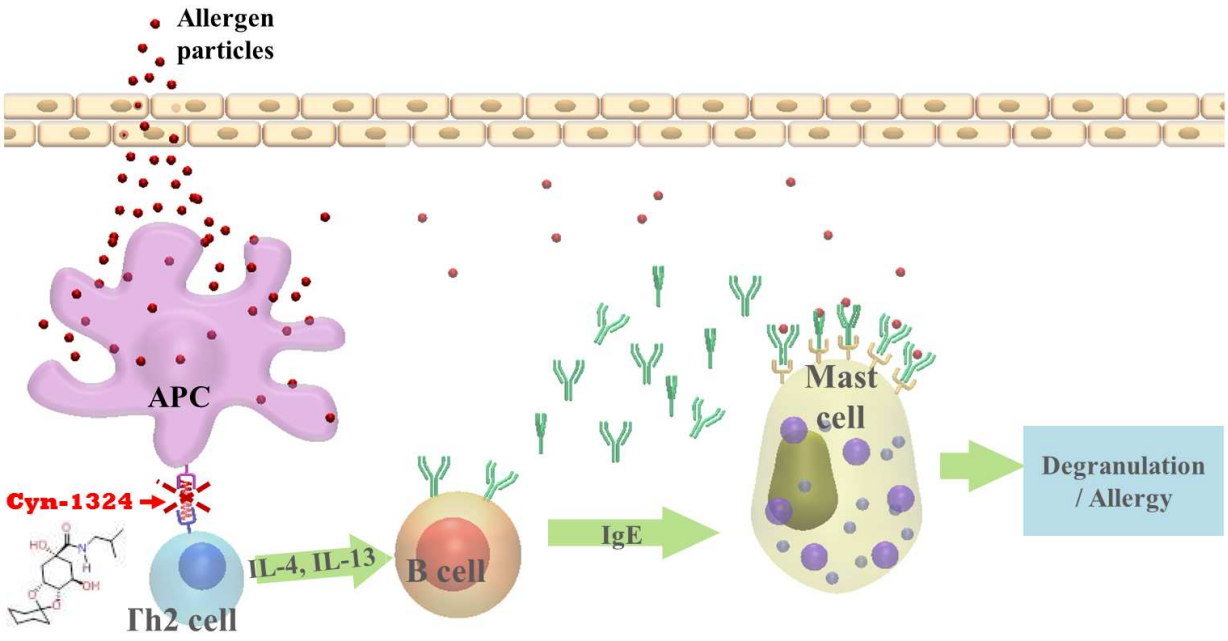


Figure 7. Inhibition of type I hypersensitivity reaction by Cyn-1324

However, the reducing strategy should be only "mild" since one would expect to bring the immune responses back to normal, but not completely suppress it. Our current results showed that Cyn-1324 could reduce IgE to certain extent (23 % in average) when compared with 12 % (in average) using cyclosporine A. This means that Cyn-1324 could be a potential candidate for the treatment of the anaphylactic immune disease (type I) better than that of cyclosporine A. More experiments will be investigated its pharmacokinetics in animals to classify the efficacy with time-releasing so that immediate responses of type I disease can be dose-controlled. Possible mechanisms of Type I immune response blocked by Cyn-1324 is shown in Figure **7**. In future, clinically curing strategy (suppressing the allergic reaction) is that the extra production of IgE from B-cells due to the incoming allergen particles will be partially ceased by in taking Cyn-1324 which will block the attachment of T-cell to B-cell. Without activation of B-cell, IgE is not

produced. Future experiments such as PK (pharmaco-kinetics) and ADME (adsorption/distribution/metabolism/excretion) will be done to further support the above arguments. Therefore, the mechanism of action (MoA) of Cyn-1324 on immune system will be understood. This compound has major valuable benefits containing the low cost preparation and facile synthetic strategy with high yield. Its low toxicity may further lead it as a potential drug candidate to cure allergic type I disease in the future.

Conclusion

In conclusion, we have synthesized a series of quinic acid derivatives for their potential application as immune-suppressive agents against T-cell receptors. Among these compounds, we found that compound **3k** (Cyn 1324) shows similar efficacy with lower toxicity as compared to Cynarin. The mechanism of action of Cyn-1324 on immune system is identified. Because of easy synthesis and low toxicity of Cyn 1324, it may further develop to a possible candidate to eliminate allergic type I disease in the future.

Experimental Section

General procedure for the synthesis of (3a*R*,5*R*,7*R*,7a*S*)-5,7-dihydroxy-2,2-dimethyl-*N*-(2-phenylethyl)hexahydro-1,3-benzodioxole-5-carboxamide (3a).

To a solution of compound **1** (0.1 g, 0.52 mmol) in acetone (10 mL) was added conc.H_2SO_4 (2 drops) and the reaction mixture was allowed to stir at reflux for 2 hours. After the completion of the reaction, the solvent was evaporated, diluted with ethyl acetate (15 mL), washed with water (2 x 30 mL) followed by brine solution (20 mL). The obtained crude compound **2a** (0.1 g, 91 %) was pure enough to proceed the next step. To a solution of compound **2a** (0.1g, 0.46 mmol) in dichloromethane (5 mL) was added triethyl amine (0.07 g, 0.7 mmol) followed by 2-

phenylethanamine (0.083 g, 0.7 mmol) and the reaction mixture was subjected to microwave irradiation for 5 minutes at 60° C. After the completion of the reaction, the solvent was evaporated, diluted with ethyl acetate (25 mL), washed with water (2x 50 mL) and brine solution (30 mL). The crude product was purified by flash chromatography using 5 % methanol/dichloromethane to afford the pure product **3a** (0.2 g, 76 %).

Spectral data

(3a*R*,5*R*,7*R*,7a*S*)-5,7-dihydroxy-2,2-dimethyl-*N*-(2-phenylethyl)hexahydro-1,3-benzodioxole-5-carboxamide (3a)

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33 – 7.28 (m, 5H), 7.04 (s, 1H), 4.86 (s, 1H), 4.51 (m, 1H), 4.12 (m, 1H), 3.81 (m, 1H), 3.52 (dd, J = 9.2, 6.8 Hz, 2H), 3.39 (s, 1H), 2.90 – 2.73 (m, 1H), 2.81 (m, 1H), 2.37 (m, 1H), 2.05 – 1.88 (m, 2H), 1.48 (s, 3H), 1.33 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.6, 138.5, 128.8, 128.7, 126.7, 108.7, 76.1, 72.9, 72.1, 65.9, 40.5, 37.0, 35.6, 34.4, 27.1, 24.4; MS (EI) 335.2; HRMS (EI) calcd. for $C_{18}H_{25}NO_5$ 335.1733; found 335.1729; IR (cm^{-1}, neat) 3359, 1644.

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References

1. O. Acuto and F. Michel. *Nat. Rev. Immunol.,* 2003, **3**, 939.
2. J. P. Allison. *Curr. Opin. Immunol.,* 1994, **6,** 414.
3. C. H. June, J. A. Ledbetter, P. S. Linskley and C. B. Thompson. *Immunol. Today*. 1990, **11**, 211.
4. A. H. Sharpe and G. J. Freeman. *Nat. Rev. Immunol.,* 2002, **2**, 116.
5. C. R. Monks, B. A. Freiberg, H. Kupfer, N. Sciaky and A. Kupfer. *Nature.,* **1998**, *395*, 82.
6. J. B. Huppa and M. M. Davis. *Nat. Rev. Immunol*., 2003, **3**, 973.
7. D. R. Fooksman, S. Vardhana, V. G. Shamis, J. Liese, D. Blair, J. Waite, C. Sacristán, G. Victoria, A. Z. Zhorov and M. L. Dustin. *Annu. Rev. Immunol*., 2010, **28**, 79.
8. T. S. Lim, J. K. H. Goh, A. Mortellaro, C. T. Lim, G. J. Hammerling and P. R. Castagnoli. *Plos ONE.,* 2012, **7**, 45185.
9. G. C. Dong, P. H. Chuang, K. C. Chang, P. S. Jan, P. I. Huang, H. B. Wu, M. Yi, H. X. Zhou and H. M. Chen. *Pharma. Res.,* 2009, **26**, 375.
10. G. C. Dong, P. H. Chuang, M. D. Forrest, Y. C. Lin and H. M. Chen. *J. Med. Chem.,* 2006, **49**, 1845.
11. L. Panizzi and M. L. Scarpati. *Nature*., 1954, **174**, 1062.
12. F. S. Kao, W. Ger, Y. R. Pan, H. C. Yu, R. Q. Hsu and H. M. Chen. *Biotech. Bioeng.,* 2012, **109**, 660.
13. G. M. Cragg and D. J. Newman. *Biochim Biophys Acta*., 2013, **1830**, 3670.
14. M. S. Butler, A. A. Robertson and M. A. Cooper. *Nat Prof Rep*. 2014, **31**, 1612.
15. D. S. H. Chan, H. M. Lee, F. Yang, C. M. Che, C. C. L. Wong, R. Abagyan, C. H. Leung and D. L. Ma. *Angew. Chem. Int. Ed.,* 2010, **49**, 2860.

16. C. H. Leung, D. S. H. Chan, H. Yang, R. Abagyan, M. Y. Lee, G. Y. Zhu, W. F. Fong and D. L. Ma. *Chem. Commun.,* 2011, **47**, 2511.

17. D. L. Ma, D. S. H. Chan and C. H. Leung. *Chem Sci.,* 2011, **2**, 1656.

18. C. H. Leung, D. S. H. Chan, Y. W. Li, W. F. Fong and D. L. Ma. *BMC Phar Toxi.,* 2013, **14**, 3.

19. H. J. Zhong, L. J. Liu, C. M. Chong, L. Lu, M. Wang, D. S. H. Chan, P. W. H. Chan, S. M. Y. Lee, D. L. Ma and C. H. Leung. *Plos One., **9***, 92905.

20. L. J. Liu, K. H. Leung, D. S. H. Chan, Y. T. Wang, D. L. Ma and C. H. Leung. *Cell Death Disease.,* 2014, **5**, 1293.

21. D. L. Ma, D. S. Chan, G. Wei, H. J. Zhong, H. Yang, L. T. Leung, E. A. Gullen, P. Chiu, Y. C. Cheng and C. H. Leung. *Chem. Commun.,* 2014, **50**, 13885.

22. D. L. Ma and C. H. Leung. *Austin J. Bioorg. & Org. Chem.* 2014, **1**, 2.

23. N. Kalia, W. S. Somers, B. E. Thomas, P. Thakker, K. Janz, S. DeBernardo, S. Tam, W. J. Moore, R. Yang, W. Wrona, P. W. Bedard, D. Crommie, J. C. Jr. Keith, D. H. H. Tsao, J. C. Alvarez, H. Ni, E. Marchese, J. T. Patton, J. L. Magnani and R. T. Camphausen. *J. Med. Chem.,* 2005, **48***,* 4346.

24. B. Belhu, B. B. Metaferia, L. Chen, H. K. Baker, X. Y. Huang and Bewley. C. A. *J. Am. Chem. Soc.,* 2007, **129**, 2434.

25. C. R. Yates, D. D. Miller and K. E. Thompson. US 2009054015, 2009.

26. C. R. Yates, K. Zeng, and D. D. Miller. WO 200906400, 2009.

27. C. R. Yates, D. D. Miller, W. Gaber, K. E. Thompson, C. Wilson and K. Zeng. US 2010132504, 2010.

28. CCDC 1058469 contains the supplementary crystallographic data for the compound **3k**. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.