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Studies on the Stereoselective Synthesis and Immunosuppressive Activity of Dihydroartemisinin-O-Glycoside Derivatives

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Abstract: Eight new dihydroartemisinin-O-glycosides were synthesized with their relative configurations were determined based on NMR spectrum. *In vitro* immunosuppressive assay showed that 10α-dihydroartemisinin-β-O-D-mannoside (**19a)** demonstrate 88% inhibition towards T cells proliferation and 98% reduction in IFN-γ levels in cell media. These results suggest that dihydroartemisinin-O-glycoside as a potential lead for further in vivo evaluation.

Keyword: Dihydroartemisinin; Glycoside; Immunosuppressive;
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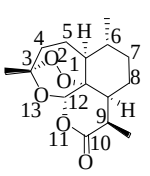
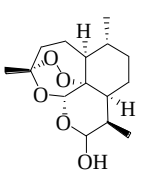
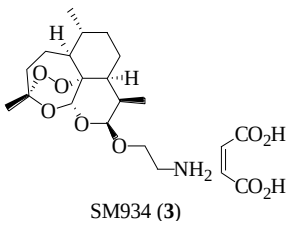
Artemisinin is a natural sesquiterpene lactone isolated from *Artemisia annua* [1] with a unique peroxobridge hemiacetal motif. Artemisinin and its derivatives are widely reported for its anti-malarial and anti-tumor activities.[2-5] In addition, artemisinin derivatives have potent immunosuppressive properties and have been reported effective for treating of autoimmune diseases including *Systemic Lupus Erythematosus* (SLE) [6], *Atopic Dermatitis* (AD) [7-8] and *Rheumatoid Arthritis* (RA).[9] Currently clinically available artemisinin derivatives drugs include anti-malarial drugs such as artesunate, artemether, arteether and dihydroartemisinin (DHA).[10] Among them, DHA demonstrated therapeutic effect in SLE patients and is currently under Phase II clinical trial (ClinicalTrials.gov Identifier: NCT03396393). The mode of action may involves an increased SIGIRR expression and a blockage of TLR4/NF-κB signal pathway. [11-12] However, a main limitation of DHA is its structural stability due to an active hemiacetal hydroxyl group. β-aminoarteether maleate (SM934, **3** , **Fig 1**) improved the stability of DHA by protecting the hemiacetal hydroxyl group. In addition, the solubility, bioavailability and immunosuppressive activity of SM 934 were also significantly improved. *In vitro* studies showed SM 934 had outstanding inhibitory effects on lymphocyte proliferation, with an IC_{50} of 1.2 μM for T lymphocytes proliferation and 2.6 μM for B lymphocytes proliferation.[13] SM934 is currently under the Phase II clinical trial for the SLE treatment in China (ClinicalTrials.gov Identifier: NCT03951259).



Artemisinin (**1**)



Dihydroartemisinin (**2**)



SM934 (**3**)

Figure 1. The structures of Artemisinin (**1**), Dihydroartemisinin (**2**) and SM934 (**3**)

Based on the safety and activity profile of DHA and SM934, it is of interest to look for other artemisinin derivatives with improved activities. From medicinal chemistry point of view, DHA contains an active hemiacetal hydroxyl group, a formation of ether or ester on this hydroxyl group is a direct choice for structural modification. For example, the well-known anti-malarial drugs, artemether and artesunate, were derived from DHA through this strategy. Another approach for DHA structure modification is to conjugate a saccharide to DHA, as it is expected to improve the water solubility and bioavailability of artemisinin. In 1992, Lin A. J. et al [14] prepared a series of dihydroartemisinin α-O-glycosides and evaluated their anti-malarial activities. (**Figure 2**.) However, the anti-malarial activity of dihydroartemisinin α-O-glycosides was not significantly improved. This result could be attributed to the difficulty for water-soluble dihydroartemisinin α-O-glycosides to penetrate the pellicle of plasmodium.

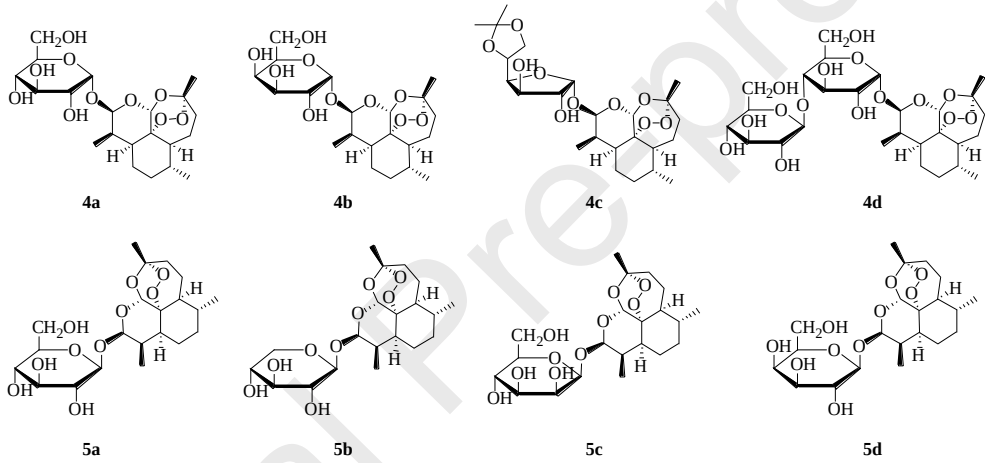


Figure 2. Dihydroartemisinin α-O-glycosides reported [14]

The glycosylation reaction used involves the hemiacetal hydroxyl group of the sugar nuclephilically attacking the oxonium ion **6** (**Figure 3**.) obtained from the elimination of the hemiacetal hydroxyl of dihydroartemisinin. Due to steric hindrance of artemisinin's peroxyl bridge and the 6α-methyl, the nucleophiles attack predominantly form the β-orientated acetal bond.

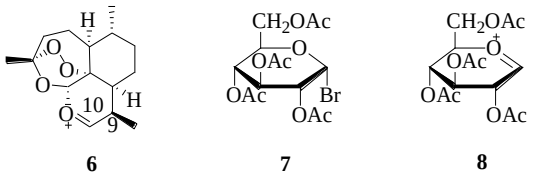


Figure 3. The key reactive intermediates of glycosylation reaction.

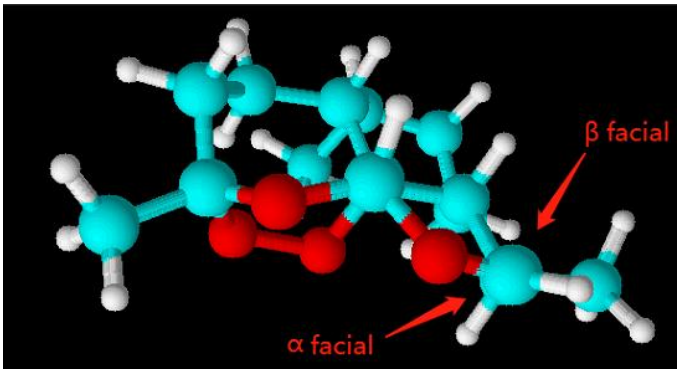
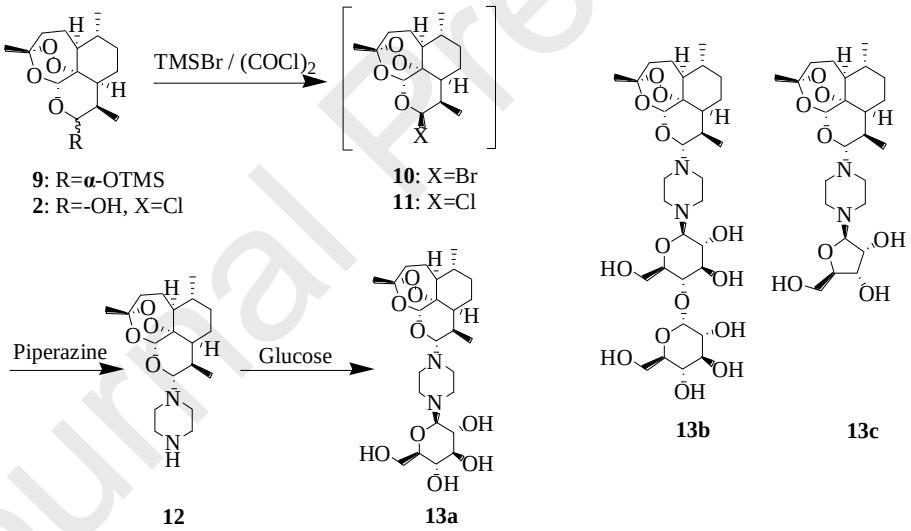


Figure 4. The illustration of α/β facial nuclephilic attack to 10-position of **6**

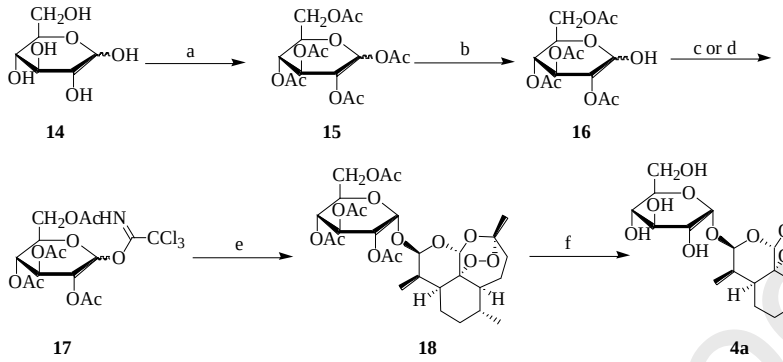
Ren Y.R. et al.[15] use a slightly different strategy to synthesize the dihydroartemisinin-β-O glycosides. Dihydroartemisinin was used as a nucleophile to attack bromineated saccharide (**7**). Since the dihydroartemisinin is a mixture of 10α-(OH) dihydroartemisinin and 10β-(OH) dihydroartemisinin, theoretically the final product should be a mixture of diastereomers. However, only 10β-dihydroartemisinin-O-glycosides were isolated according to the literature. It is suspected to be attributed to the fact that 10α-dihydroartemisinin has higher tendency to eliminate and form artemisitene.



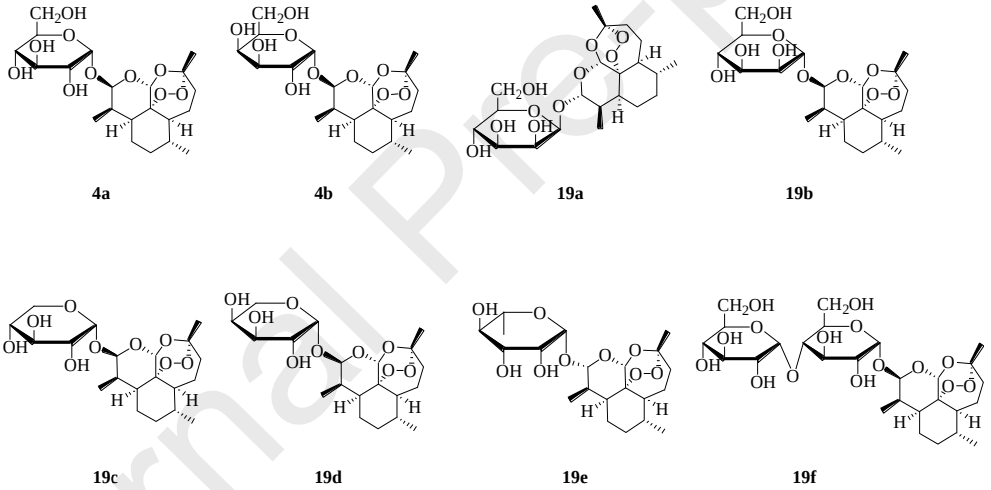
Scheme 1. Synthesis of N-glycosylated DHA piperazine derivatives

In order to obtain 10α-dihydroartemisinin-O-glycoside, Nicoletta Basilico et al.[16] used piperazine as a nucleophile to react with 10β-halogenated dihydroartemisinin, and obtained 10α-piperazine substituted dihydroartemisinin derivative (**12**). The subsequent aminoglycosidation reaction yielded three novel 10α-N-dihydroartemisinin-N-glycoside piperazine derivatives (**Scheme 1**. **13a**-**13c**). These three compounds showed good anti-malarial and anti-cancer activities. However, due to lack of comparison data, it still is difficult to conclude which configuration of dihydroartemisinin-O-glycoside has better biological activities.

As described above, a numbers of dihydroartemisinin-O-glycosides were synthesized with their anti-malarial and anti-cancer activities reported. However, the immunosuppressive activity of dihydroartemisinin-O-glycoside were not yet explored. Herein, we report the synthesis of eight dihydroartemisinin-O-glycoside derivatives and their immunosuppressive activities.



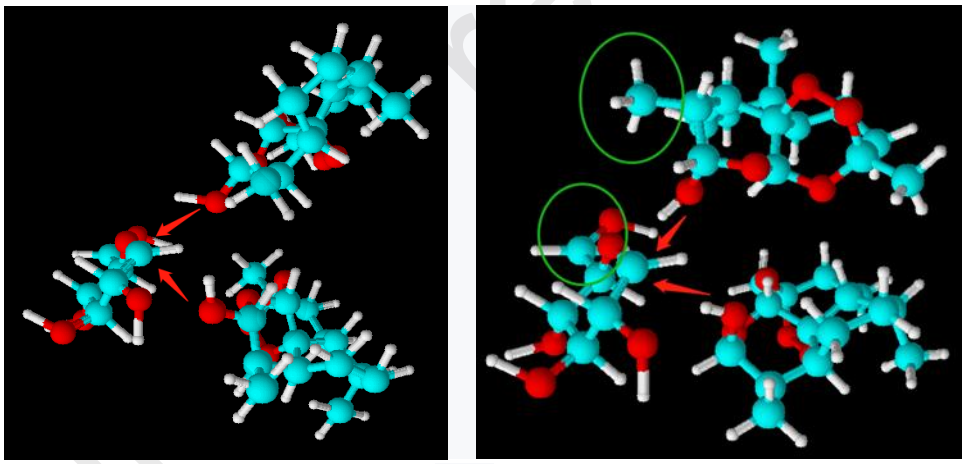
(a) Ac_2O/NaOAc, 130^{o}C. (b) Hydrazine acetate, DMF, r.t. (c) $CNCCl_3$, DBU, DCM, 0 ^{o}C
(d) $CNCCl_3$, K_2CO_3, DCM, r.t. (e) $BF_3.Et_2O$, DHA, DCE, -20 ^{o}C. (f) K_2CO_3, MeOH, r.t.



Scheme 2. Synthesis of DHA-glycosides

As illustrated in **Scheme 2,** pentacetyl D-glucose (**15**) was used as starting material and Schmidt reaction[17] was used as the key step in our synthesis. After selectively removing 1-acetyl at room temperature, the crude product **16** reacted with trichloroacetonitrile to form an active intermediate trichloroacetimidate ester **17.** Intermediate **17** underwent glucosidation reaction with dihydroartemisinin catalyzed by $BF_3{\cdot}Et_2O$. The 10β-dihydroartemisinin-O-tetraacetyl glucoside **18** was obtained. Hydrolyzation of the acetyl groups on **18** with K_2CO_3 in methanol, yielded the 10β-dihydroartemisinin-O-glucoside (**4a**) in 6.4% overall yield in four steps. In the synthesis, **17** was converted into an active intermediate oxonium ion **8**, then the hemiacetal hydroxyl group of dihydroartemisinin nucleophilic attacking oxonium ion **8** to form corresponding glycoside. Since the hemiacetal hydroxyl group of 10α-dihydroartemisinin can easily undergo elimination reaction in acidic condition, the stereochemistry of the product is dependent on the relative speed of

gluosidation and elimination reaction. If the glycosidation speed is faster than the elimination, the main product will be the mixture of 10β-dihydroartemisinin-O-glycoside and 10α-dihydroartemisinin-O-glycoside. If the glycosidation speed is slower than the elimination, the main product will be the 10β-dihydroartemisinin glycoside since the 10α-dihydroartemisinin was consumed. Due to the planar nature of the oxonium ion, as illustrated in **Figure 5**, the nucleophilic attack of 10β-dihydroartemisinin hemiacetal hydroxyl group to the oxonium ion **8** is in favor from the less steric hindrance below the Harworth structure of the saccharide to form the 10β-dihydroartemisinin-O-glycoside , and the two biggest groups on the saccharide moiety of the product, 6-hydroxymethlene group and the 1-dihydroartemisinin were in equatorial conformation. (**Figure 5**), In contrary, the 10α-dihydroartemisinin hemiacetal hydroxyl group tend to attack the oxonium ion **8** from above the Harworth structure of the saccharide to form the 10α-dihydroartemisinin-O-glycoside (**Figure 6**). Hence, total eight dihydroartemisinin-O-glycosides were synthesized, namely, 10β-dihydroartemisinin-α-O-D-glucoside (**4a**); 10β-dihydroartemisinin-α-O-D-galactoside (**4b**); 10α-dihydroartemisinin-β-O-D-mannoside (**19a)**; 10β-dihydroartemisinin-α-D-mannoside (**19b**); 10β-dihydroartemisinin-α-D-xyloside (**19c**); 10β-dihydroartemisinin-β-L-arabinoside (**19d**) ; 10α-dihydroartemisinin-α-L-rhamnoside (**19e**) ; 10β-dihydroartemisinin-α-D-maltoside (**19f**).



Glucosidation of 10α-Dihydroartemisinin (left), Glucosidation of 10β-Dihydroartemisinin(Right)

Figure 5. The illustration of stereoselectivity of glucosidation of dihydroartemisinin

Among them, **4a**, **4b**, **19c-19f** were obtained as the major product. In mannose case, the ortho effect of axial orientated 2-acetoxyl increased the reactivity of the oxonium ion, the speed of glycosidation reaction was faster than the elimination reaction, hence a mixture of 10α-dihydroartemisinin-β-O-D-mannoside and 10β-dihydroartemisinin-α-O-D-mannoside was obtained. Since 10α-hydroxyl-dihydroartemisinin is in favor of attacking the oxonium ion from above the Harworth structure while 10β-hydroxyl-dihydroartemisinin tend to approach the oxonium ion from below the Harworth structure. Therefore, **19a** and **19b** were obtained at a ratio of approximately 1:1.

The relative configuration of compound **19a** and compound **19b** were determined by NMR spectroscopic method (**Figure 6**). The chemical shift of H-1 on glucose of **19a** was δ 5.03 ppm ($J_{1\text{-}2}$=1.6 Hz) and H-10 on dihydroartemisinin was δ 4.76 ppm ($J_{9\text{-}10}$=9.2 Hz), while the chemical shift of H-1 of compound **19b** was δ 5.17 ppm ($J_{1\text{-}2}$=2.0Hz), H-10 on dihydroartemisinin of 1**9b** was δ 5.11 ppm ($J_{9\text{-}10}$=3.2 Hz). The chemical shift and the characteristics coupling constants of these protons were consistent with the literature.[18-19] The configuration of glucoside bonds of **19c**-**19f** were also determined by NMR spectroscopic method.[18] The configuration of **19c**, **19d** and **19f** were assigned to 1α-xyloside, 1β-arabinoside and 1α-simaltoside respectively. Although, it is difficult to determine the configuration of glucoside bond of rhamnoside (**19e**) by simply comparing the coupling constant, the conformation analysis of rhamnoside (**Figure 7)** indicated the chemical shift of H-1 of 1α-rhamnoside should shift towards the lower field due to the induction effect of axial 3-OH. Hence, the chemical shift of H-1 and H-10 of **19e** justified the 1β, 10α configuration (**Table 1**).

Table 1. Selected NMR data of dihydroartemisinin glycosides

	4a	**4b**	**19a**	**19b**	**19c**	**19d**	**19e**	**19f**
$\delta_{H\text{-}1}$ (J_{Hz})	5.22 (3.7)	5.25 (3.9)	5.05 (1.3)	5.19 (1.6)	5.16 (3.6)	5.23 (3.1)	5.14 (1.6)	5.22 (3.8)
$\delta_{H\text{-}10}$ (J_{Hz})	5.09 (3.2)	5.09 (3.3)	4.78 (9.4)	5.13 (3.4)	5.01 (3.3)	5.02 (3.3)	4.82 (9.4)	5.16 (3.7)
configuration	1α,10β	1α, 10β	1β,10α	1α, 10β	1α, 10β	1β, 10β	1β,10α	1α, 10β

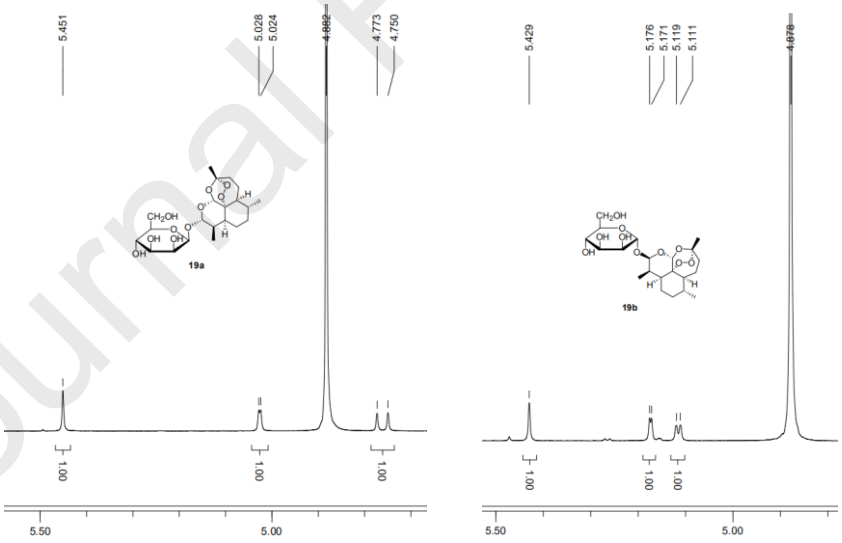
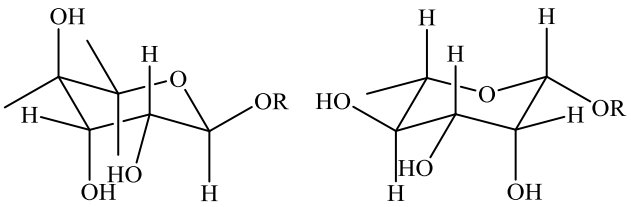


Figure 6. The characteristic ^{1}H-NMR of **19a** and **19b**



1α-arhamnoside 1β- rhamnoside

Figure 7. Conformation analysis of rhamnoside

Table 2. Dihydroartemisinin glycosides inhibiting T cells proliferation *in vitro*

Compounds	$CD4^+$ T Cell IC_{50} (μM)	$CD8^+$ T Cell IC_{50} (μM)
4a	17.85	19.41
4b	10.41	8.63
19a	3.49	2.65
19b	>32	>32
19c	10.85	9.52
19d	14.07	12.95
19e	3.83	3.36
19f	9.09	4.00
DHA	4.03	5.53
SM934	1.05	1.36

The dihydroartemisinin glycosides synthesized were assessed for their proliferation inhibitory activities [13] on human T cells and the results were depicted in **Table 2**.

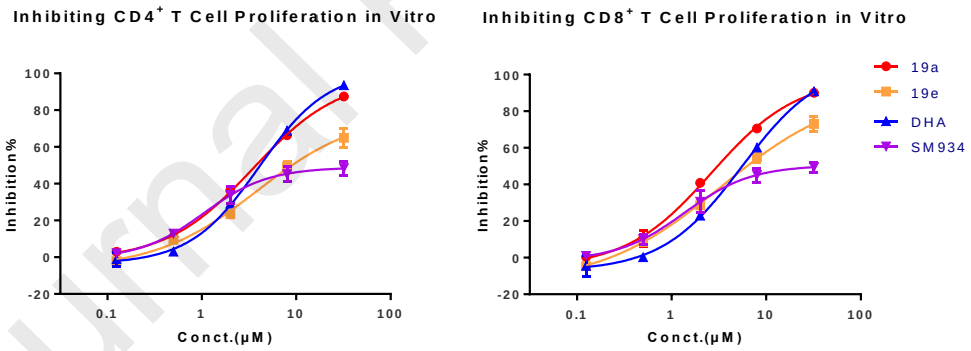


Figure 8. Anti-proliferation activity of **19a** and **19e**

*dose response curves fitted by 4 parameters non-linear regression(GraphPad Prism). Error bar expressed as mean±SD (n=3).

As shown in **Table 2,** both **19a** and **19e** demonstrated enhanced *in vitro* activity in suppressing T cell proliferation in a dose dependent manner (**Figure 8**). The IC_{50} of **19a** and **19e** inhibiting $CD4^+$ T cells proliferation was 3.49、3.83 μM, and 2.65、3.36 μM inhibiting $CD8^+$ T cells respectively. (**Table 2**).

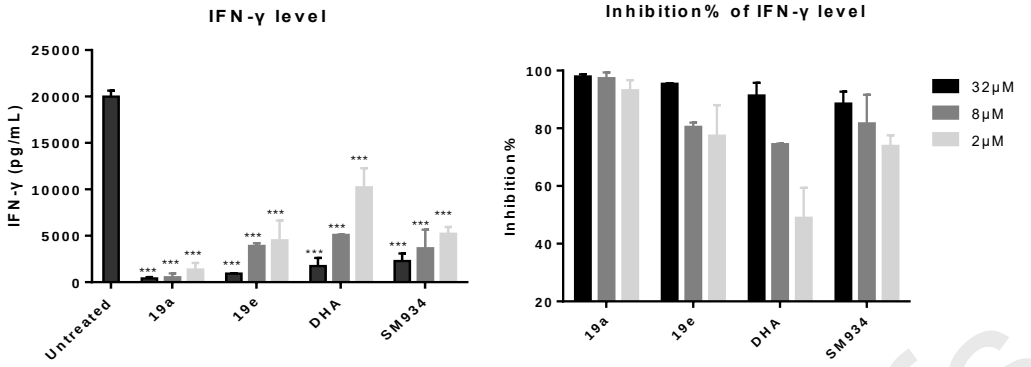


Figure 9. **19a** and **19e** impaired IFN-γ level.
Error bar represent mean±SD(n=3). *** P < 0.0001

Immune cell dysfunction and imbalance of pro- and anti- inflammatory processes are key features of autoimmune diseases. Interferon gamma (IFN-γ) is a pivotal pro-inflammatory cytokine involved in immune response and inflammation. Artemisinin analogs have been reported to ameliorate IFN-γ levels and alter Th1 cell response in RA, SLE and multiple sclerosis murine models. [20-23] Thus, it is of interest to explore whether **19a** and **19e** demonstrate similar activity in T cell activation process. Human primary T cells were isolated from PBMC and co-incubated with above compounds for 120 hrs under the stimulation of CD3/CD28 T cell activator. IFN-γ levels in the cell culture media was tested through ELISA. As depicted in **Table 3**, **19a** and **19e** decreased IFN-γ recovery in the cell media dose-dependently, concurring with the inhibition effect of T cell proliferation. Among compounds tested, **19a** demonstrated the highest potentency and efficacy in inhibiting T cell proliferation and reducing IFN-γ levels. (**Figure 9**)

In addition, there are two interesting observations: 1). **19a** and **19b** are diastereomers, however, **19a** was more potent than **19b** in decreasing cell media IFN-γ. 2). Both **19a** and **19e** have 10α-dihydroartemisinin moiety with different glycoside configurations and are more potent compared to other derivatives. Therefore, we hypothesizes that the configuration of dihydroartemisinin plays an important role in immunosuppressive activity, and dihydroartemisinin glycosides with 10α -configuration exhibit an improved activity.

Several mechanisms were proposed for immunosuppressive activities of artemisinin derivatives.[24] In addition, Zuo J. P. et al demonstrated that artemether impaired T cells activation and proliferation, decreased IFN-γ and IL-2 secretion through ERK1/2 inhibition.[25] Wang H. et al observed similar results on artesunate. They also proved that artesunate increases the percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg). [26] SM934 (NCT03951259) was found to ameliorate the symptoms of RA, reduced percentage of Th1 and Th17 populations in collagen-induced arthritis mice model.[27,28] However, it is still unknow how the stereochemistry of dihydroartemisinin glycosides influences their immunosuppressive activities. Since **19a** has superior activity of inhibiting IFN-γ secretion and T cell proliferation than SM934 and DHA, we believe **19a** worth for further *in vivo* studies on autoimmune diseases animal models.

In summary, we synthesized eight novel dihydroartemisinin-O-glycosides, of which **19a** and

19e showed foremost inhibitory effect towards T cell proliferation and IFN-γ media recovery. Hence, **19a** and **19e** may have the potential to exhibit similar therapeutic effect *in vivo*.

Acknowledgments

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Declaration of interests

☒ 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.

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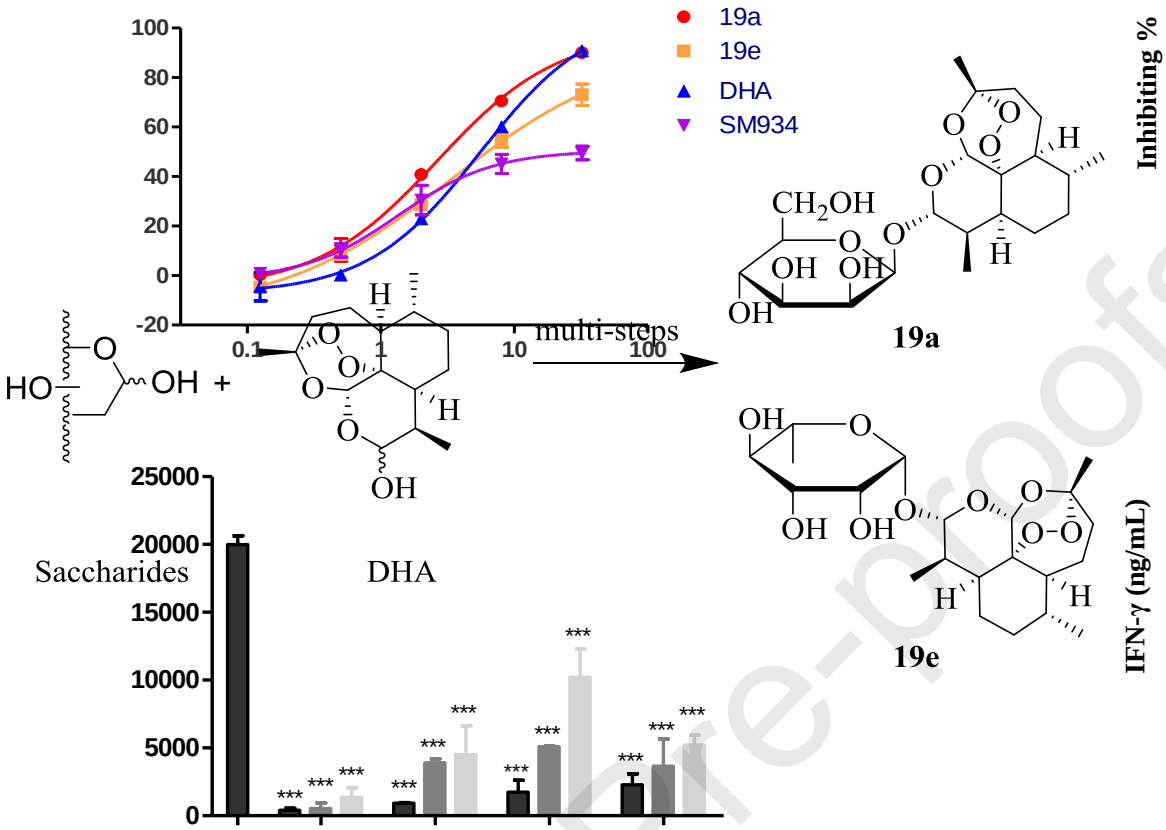

Inhibiting CD8+ T Cell Proli

Concentr

IFN-γ l

Untreated

19a

19e