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Evacetrapib reduces preβ-1 HDL in patients with atherosclerotic cardiovascular disease or diabetes

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HIGHLIGHTS

- Cholesteryl ester transfer protein (CETP) inhibitor, evacetrapib, significantly reduces preb1-HDL, which is involved in reverse cholesterol transport, while significantly induces larger HDLs and HDL-cholesterol. This could be one of reasons that the inhibitor has no effect on the protection of cardiovascular disease.
- A simple and sensitive method for preb1-HDL measurement has been developed. The relationship between preb1-HDL and cardiovascular diseases should be re-evaluated, because the new method is focus on lipids and density, but not only on apoA-I.
- Increasing preb1-HDL should be the focus of future study, in terms of anti-atherogenesis.

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ABSTRACT

Background and aims: Cholesteryl ester transfer protein (CETP) inhibitor-mediated induction of HDL-cholesterol has no effect on the protection from cardiovascular disease (CVD). However, the mechanism is still unknown. Data on the effects of this class of drugs on subclasses of HDL are either limited or insufficient. In this study, we investigated the effect of evacetrapib, a CETP inhibitor, on subclasses of HDL in patients with atherosclerotic cardiovascular disease or diabetes.

Methods: Baseline and 3-month post-treatment samples from atorvastatin 40 mg plus evacetrapib 130 mg (n = 70) and atorvastatin 40 mg plus placebo (n = 30) arms were used for this purpose. Four subclasses of HDL (large HDL, medium HDL, small HDL, and preβ-1 HDL) were separated according to their size and quantified by densitometry using a recently developed native polyacrylamide gel electrophoresis (PAGE) system.

Results: Relative to placebo, while evacetrapib treatment dramatically increased large HDL and medium HDL subclasses, it significantly reduced small HDL (27%) as well as preβ-1 HDL (36%) particles. Evacetrapib treatment reduced total LDL, but also resulted in polydisperse LDL with LDL particles larger and smaller than the LDL subclasses of the placebo group.

Conclusion: Evacetrapib reduced preβ-1 HDL and small HDL in patients with ASCVD or diabetes on statin. Preβ-1 HDL and medium HDL are negatively interrelated. The results could give a clue to understand the effect of CETP inhibitors on cardiovascular outcomes.

1. Introduction

Epidemiological studies have shown high-density lipoprotein cholesterol (HDL-C) levels to be independently and inversely correlated with cardiovascular disease (CVD) [1]. This relationship is thought to be largely mediated by the ability of HDL to transport excess cholesterol from peripheral tissues back to the liver for excretion, a process known as reverse cholesterol transport [2]. Potent cholesteryl ester transfer

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protein (CETP) inhibitors, like torcetrapib, evacetrapib and anacetrapib, significantly increase HDL-C and decrease LDL-C levels, as monotherapy and in combination with statins [3–6]. However, except anacetrapib [7], these lipoprotein changes have not resulted in reduction of major cardiovascular events (MACE) [8–10]. It has been hypothesized that small increase in blood pressure and hsCRP, as well as qualitative changes in HDL subclasses observed with CETP inhibitors, could partly offset the beneficial effect of LDL-C reduction [11].

It is well known that HDL particles are very heterogeneous [12], and many HDL subclasses have been described depending on the analytical technique used for separation. One of these subclasses, preβ1-HDL, plays a critical role in reverse cholesterol transport but it is particularly challenging to quantify. Two-dimensional nondenaturing linear gel electrophoresis followed by apoA-I immunoblotting and image analysis were first utilized to identify preβ1-HDL, preβ2-HDL, and α-HDL [13]. Subsequently, a further separation of lipid-free apoA-I from preβ1-HDL using the same technique was described [14]. However, other investigators have not been able to distinguish lipid-free monomolecular apoA-I from preβ1-HDL using an in-house sandwich enzyme immunoassay [15,16]. A modified two-dimensional gel system has also been used to define 12 subfractions of HDL, including preβ1-HDL and preβ2-HDL [17], however, there was no clear evidence to show that the method can distinquish preβ1-HDL and lipid free apoA-I [17]. We have recently established a native polyacrylamide gel electrophoresis (PAGE) system with lipid pre-staining for quantification of preβ1-HDL and other HDL subclasses, as well as LDL, in human serum [18]. In the present study, we utilized this method to evaluate the effect of evacetrapib on HDL subclasses in patients with atherosclerotic cardiovascular disease (ASCVD) or diabetes, on background of atorvastatin 40 mg daily, enrolled in the ACCENTUATE trial [19].

2. Materials and methods

2.1. Study design

Patient disposition and characteristics, as well as baseline and post-treatment laboratory value for the ACCENTUATE trial (ClinicalTrials.gov NCT02227784), have previously been reported [19]. Samples were available for 30 patients on atorvastatin 40 mg daily and 70 patients on atorvastatin 40 mg plus evacetrapib 130 mg daily, at baseline and after 90 days of treatment. Eli Lilly and Company provided Xian-Cheng Jiang's laboratory at SUNY Downstate Medical Center with the samples and some information from the ACCENTUATE study for the analyses under a Material Transfer Agreement between Lilly and SUNY Downstate Medical Center in 2017. Submission of this manuscript for publication has followed the terms of that Agreement.

The measurement in the study was in a blind fashion.

2.2. Study procedure

We followed an established system [18] for the measurement of subclasses of HDL. Briefly, 1) Solution A: Acrylamide (9.75 g; Fluca) and 0.25 g N,N'-methylenebisacrylamide (Fluca) in deionized water to 100 ml final volume. 2) Solution B: Tris (18.30 g) to 24 ml of 1 N HCl and diluted to 100 ml final volume with deionized water. 3) Solution C: Acrylamide (19.60 g) and 0.40 g N,N'-methylenebisacrylamide in deionized water to 100 ml final volume. 4) Solution D: Tris (6.06 g) and 1.17 g EDTA-Na_2 in deionized water to 100 ml final volume. 5) Staining solution: Sudan Black-B (0.125 g; Sigma) to 25 ml isopropanol:ethylene glycol (4:1, V/V), mixed well, and incubated in a water bath at 37 °C overnight. 6) Running buffer: Tris (6.0 g) and 28.8 g glycine in deionized water to 1000 ml final volume, and then diluted 10-fold with deionized water before use.

The gradient gels in 6 × 100 mm glass tubes were prepared as indicated in previously [18]. Each tube gel comprised three layers differing in polyacrylamide concentration (7.0%, 3.6%, and 3.0%) and height (45 mm, 40 mm, and 7 mm, respectively). Each gel tube was inserted vertically into an electrophoretic cell (DYY-III 27B Model, Liuyi Inc., Beijing). The upper and lower chambers of the electrophoretic cell contained ~400 ml and ~600 ml running buffer, respectively. Serum (100 μl) was mixed with 10 μl of staining solution, and 50 μl was loaded on the top of the tube gel; electrophoresis was carried out for 3.0 h at 100 V. Gel image was acquired with a customized scanner (Model BeneScan-1000 from BENEFI and MICROTEK, Inc. China). The lipid-staining area under the curve (AUC) was calculated automatically by the densitometer.

2.3. Statistical analysis

Continuous variables were reported as means ± standard deviation (SD) and were compared by paired Student's t-test between before treatment and after treatment. Δparameter (ΔPreβ1-HDL, Δlarge HDL, Δmedium HDL, Δsmall HDL and ΔLDL) was the difference between the parameter value after treatment and that before treatment. For univariate analyses, Pearson's correlation was used to determine the correlation of ΔPreβ1-HDL with other lipid parameters. p values < 0.05 were considered statistically significant. All analyses were done using SPSS version 16 analytical software (SPSS Inc., Chicago).

3. Results

As shown in Fig. 1A, our tube native PAGE system can separate HDL (HDL total) into four fractions, preβ1-HDL, large HDL (HDL1), medium HDL (HDL2), and small HDL (HDL3). The percentage of each HDL subclass in total HDL is preβ1-HDL (about 5%), HDL1 (about 10%), HDL2 (about 50%), and HDL3 (about 35%) (Fig. 1B). HDL1, HDL2, and HDL3 were confirmed by their densities, as previously described [18]. Preβ1-HDL was also confirmed by the treatment with 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), an LCAT inhibitor. Serum was incubated in the absence or presence of 2 mM DTNB, preβ1-HDL further conversion could be blocked after DTNB treatment (Fig. 1C).

Fig. 2A shows typical results from three patients treated with atorvastatin plus evacetrapib and three treated with atorvastatin plus placebo. Evacetrapib treatment reduced preβ1-HDL, HDL3, and LDL, and dramatically increased HDL2 and HDL1, with no changes observed in the patients on placebo. In Fig. 2A, results from 2 patients with CETP homozygous deficiency (the G→A mutation in the first position of intron 14 of the *CETP* gene) [20] are shown for comparison. Like patients treated with evacetrapib, these 2 patients had no detectable preβ1-HDL, reduced HDL3, and major increase in larger HDL.

We next sought to quantify the changes in lipoproteins. Evacetrapib treatment dramatically increased total HDL by 2.7 folds, HDL1 by 3.9 folds, and HDL2 by 4.6 folds, and reduced preβ1-HDL by 36% and HDL3 by 27% (Table 1and Fig. 3). Moreover, evacetrapib treatment reduced LDL by 36% (Table 1), with appearance of larger and smaller LDL particles (polydisperse LDL) as compared to placebo treated patients.

Univariate correlations of Δpreβ1-HDL (after treatment – before treatment) with other lipoprotein subclasses are shown in Table 2. Δpreβ1-HDL positively correlated with ΔHDL3 and ΔLDL, and negatively correlated with ΔHDL2 only in the evacetrapib treated group.

4. Discussion

The novel finding of the present study is the reduction of preβ-1 HDL particles with evacetrapib treatment in patients with ASCVD or diabetes on atorvastatin 40 mg per day, when a recently developed native PAGE system was used to quantify this particular HDL species. Moreover, we found that preβ-1 HDL and medium HDL are negatively interrelated. As previously shown by other authors, here we also observe a dramatic increase of large HDL [21–24]. Finally, our native PAGE system clearly shows that evacetrapib treatment results in

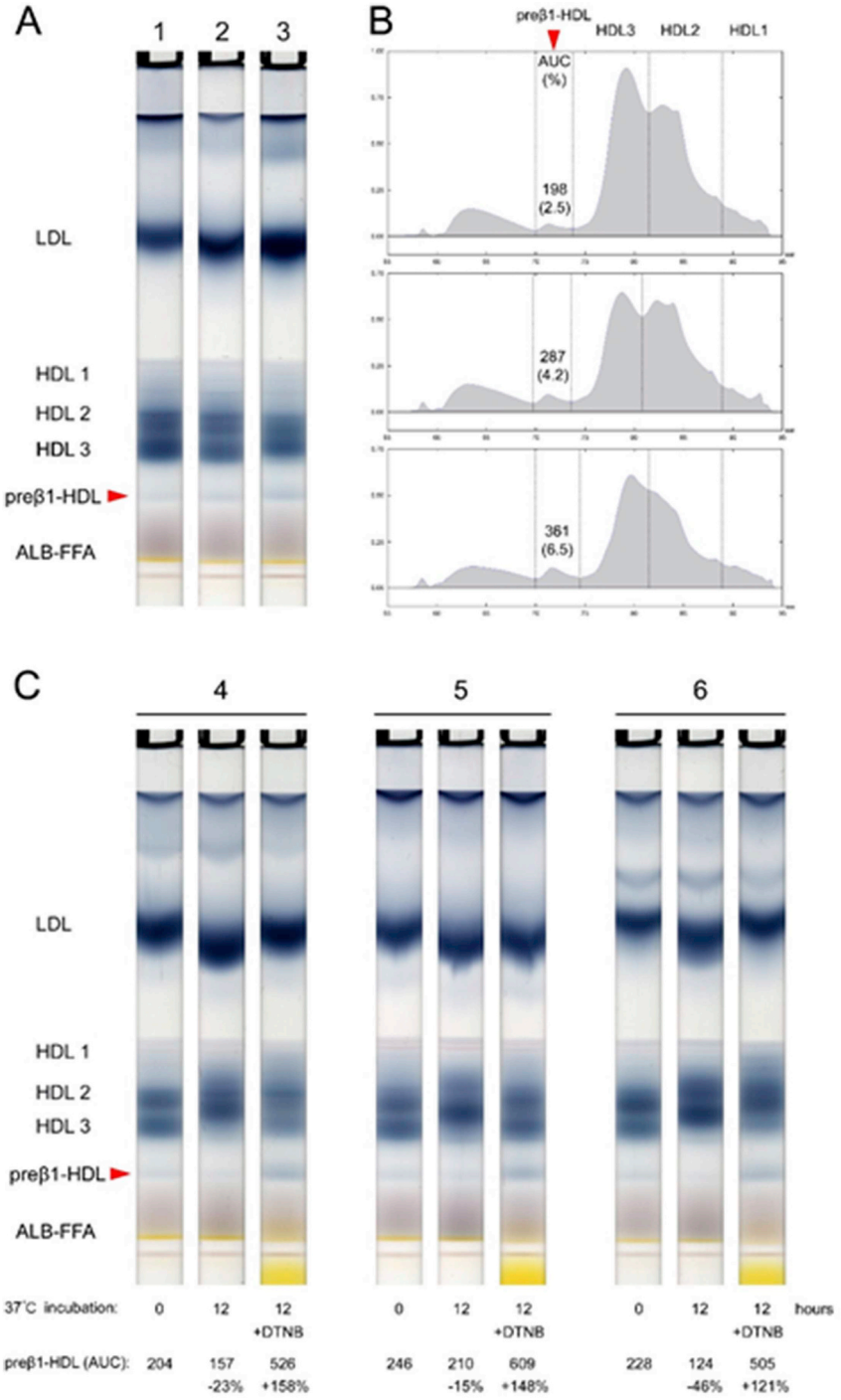

Fig. 1. Native PAGE system to separate HDL subclasses.
Human serum pre-stained with Sudan Black. (A) Three representative samples: LDL and HDL1, HDL2, HDL3, and preβ1-HDL bands are indicated. (B) Corresponding densitometric scans. (C) Human serum treated with or without DTNB (2.0 mM), at 37 °C for 12 h, then pre-stained with Sudan Black. Gel image was acquired by a customized scanner. The lipid-staining area under the curve (AUC) was calculated automatically by the densitometer.

qualitative changes in the distribution of LDL species with prevalence of very large and very small particles, thus recapitulating the polydisperse LDL phenotype in CETP-deficient subjects [25].

Previous studies have shown that preβ1-HDL particles increased after treatment with CETP inhibitors, such as evacetrapib and TA-8995, in subjects with mild dyslipidemia on atorvastatin 20 mg/d or rosuvastatin 10 mg/d, respectively [23,24]. These studies used non-denaturing two-dimensional gel electrophoresis (2DGE) [24] and/or immunofixation [23] to quantify preβ1-HDL particles. Here, we report a reduction of preβ-1 HDL particles with evacetrapib in a different population (ASCVD or diabetes) on higher atorvastatin dose (40 mg/d), using a different method to measure this HDL class.

Our native PAGE system and the traditional 2DGE clearly have a different ability to detect and quantify preβ1-HDL, with the latter appearing less suitable for this measurement. Traditional 2DGE had been used to show that serum of patients with Tangier disease contains preβ1-HDL, but not mature HDL [26]. However, this technique could have a limitation in distinguishing preβ1-HDL from free apoA-I [17]. In contrast, our native PAGE system does not detect any preβ1-HDL in serum of Tangier patients, in support of the concept that the absence of

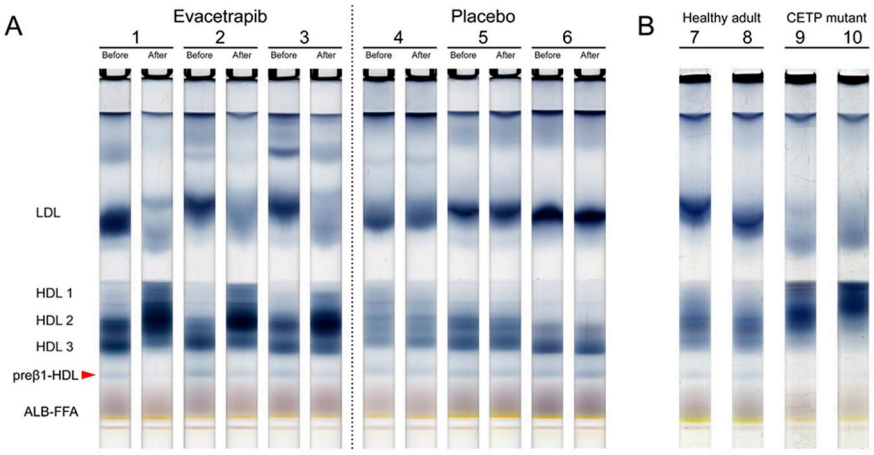

Fig. 2. Representative samples showing the effect of evacetrapib on LDL and HDL subclasses.
(A) Three representative samples of patients on evacetrapib and three on placebo. (B) Two representative samples of healthy subjects and two of patients with CETP homozygous deficiency (the G→A mutation in the first position of intron 14 of the *CETP* gene).

Table 1
Effect of evacetrapib on human serum lipoproteins.

	Variable	Before	After	*p* value
Placebo	Total HDL	6642 ± 2375	6815 ± 2830	0.40
	Preβ-1 HDL	370 ± 90	384 ± 104	0.39
	HDL3	2805 ± 922	2888 ± 1025	0.46
	HDL2	2942 ± 1176	3089 ± 1462	0.42
	HDL1	425 ± 186	455 ± 239	0.24
	LDL	6163 ± 2101	6913 ± 2699	0.07
Evacetrapib	Total HDL	5953 ± 2202	15818 ± 8216	< 0.0001
	Preβ-1 HDL	394 ± 101	254 ± 97	< 0.0001
	HDL3	2517 ± 817	1832 ± 635	< 0.0001
	HDL2	2631 ± 1126	12175 ± 6034	< 0.0001
	HDL1	411 ± 158	1589 ± 1451	< 0.0001
	LDL	6003 ± 1910	3866 ± 939	< 0.0001

After electrophoresis, each tube was subject to densitometry (604 nm; Model CS-9301, Shimadzu, Inc. Japan) and peak area for each lipoprotein was obtained and then compared. Value (Arbitrary Unit) for variables are given as means ± SD and evaluated using paired *t*-test. HDL1, large HDL; HDL2, medium HDL; HDL3, small HDL.

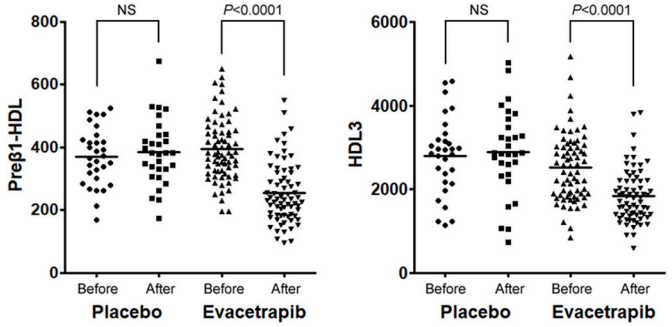

Fig. 3. Evacetrapib treatment significantly decreased preβ-1 HDL and HDL3. Dot plotting for all the samples. HDL3, small HDL.

ABCA1 in these patients results in lack of free apoA-I lipidation and subsequent preβ1-HDL formation [18]. Like Tangier patients, evacetrapib-treated patients in the present study had very low levels of preβ1-HDL. It is well known that "preβ1-HDL-like" lipid-poor apoA-I or nascent HDL is formed when lipid-free apoA-1 retrieves phospholipids and cholesterol from cells in a process mediated by ABCA1 [27,28]. Thus, the levels of preβ1-HDL should be an indicator of the capacity for nascent HDL formation and initiation of reverse cholesterol transport.

Another key finding of this study is that even though evacetrapib treatment increases the number of large HDL, it actually reduces small dense HDL particles. A decrease of small HDL particles has been observed by NMR with dalcetrapib at the highest dose (900 mg/d) [22]. Evacetrapib and TA-8995 also decreased small α-3 and preα-3 HDL as measured by 2DGE, in both monotherapy and combination therapy [23,24]. Small, dense, and protein-rich HDL are currently believed to possess potent biological and atheroprotective activities [29,30]. For instance, HDL3 particles are enriched with specific sphingolipids, such as sphingomyelin and sphingosine-1-phosphate [29], which are involved in nitric oxide generation and protection against oxidative stress-associated endothelium dysfunction [31]. Very recent studies suggested that age acts as a confounding factor associated with both low HDL3 cholesterol and increased carotid atherosclerosis [32,33]. Moreover, HDL3 particles displayed greater efficacy in removing cellular cholesterol as compared with other HDL subpopulations [29].

The reverse cholesterol transport is composed of two processes: 1) HDL-mediated process [34], and 2) LDL-receptor-mediated process [34]. CETP inhibitor blocks CE transfer from HDL to LDL and blocks the second process. Thus, accumulation of large HDL particles could be an indicator for an impairment of reverse cholesterol transport. The high inter-relationship between preβ1-HDL and large HDL could reflect a situation that CETP inhibition-mediated accumulation of large HDL can block the formation of preβ1-HDL and small HDL or promote their degradation.

Preβ1-HDL and small dense HDL particles play an important role in cholesterol efflux, the first step of reverse cholesterol transport [35–37],

Table 2
Univariate correlates of Δpreβ-1 HDL among total cohort, placebo group and evacetrapib group: Pearson's correlation was used to determine correlation of Δpreβ-1 HDL with other lipid parameters.

Variable	Total cohort (n = 100)		Placebo (n = 30)		Evacetrapib (n = 70)	
	r	*p*	r	*p*	r	*p*
ΔHDL3	0.474	< 0.0001	0.156	0.411	0.367	0.002
ΔHDL2	−0.618	< 0.0001	−0.050	0.793	−0.378	0.001
ΔHDL1	−0.307	< 0.0001	0.096	0.614	−0.084	0.489
ΔLDL	0.518	< 0.0001	−0.001	0.995	0.376	0.001

HDL1, large HDL; HDL2, medium HDL; HDL3, small HDL.

and in preventing oxidative stress-associated endothelium dysfunction [31]. We should promote the formation of preβ1-HDL and small dense HDL, from triglyceride-rich lipoproteins converting process [38,39] or cholesterol efflux process [40].

We used the same samples as the ACCENTUATE trial, which indicated an increase in cholesterol efflux capacity after treatment with CETP inhibitor [19]. We believe that CETP inhibitor-mediated increase of cholesterol efflux capacity is due to a dramatic induction of total HDLs and is not related to reverse cholesterol transport promotion. A similar study was conducted by Daniel J. Radar's group at the University of Pennsylvania. They reported that both phospholipid transfer protein (PLTP) transgenic and PLTP knockout mice have significantly reduced plasma HDL levels and both mice also have significantly reduced cholesterol efflux capacity. However, both PLTP overexpression and deficiency have no effect on macrophage reverse cholesterol transport [41]. Thus, plasma cholesterol efflux capacity measured by incubation of macrophages or cells overexpressing ABCA1 or ABCG1 in human or mouse plasma does not reflect the real situation of reverse cholesterol transport. The measured cholesterol efflux capacity should be greatly influenced by HDL in the plasma used in the experiment.

There is a limitation of the current study. We have only demonstrated a reduction in preβ1-HDL and small HDL but not a reduction in HDL functionality. We speculated that reduction of preβ1-HDL could be a reason why CETP inhibitor-mediated HDL-cholesterol raise has no effect on CVD prevention. Further work should be done, according to previous reports [42,43].

In summary, CETP inhibitor, evacetrapib, reduces preβ-1 HDL and small dense HDL in patients with ASCVD or diabetes on statin. Preβ-1 HDL and large HDL are negatively interrelated. Other CETP inhibitors could have similar effects. These results could give a clue to understand the effect of CETP inhibitors on cardiovascular outcomes. Moreover, we have to be aware of the limitation of the methods previously used for preβ-1 HDL quantification and, in fact, we should re-evaluate these measurements. We believe preβ-1 HDL is an anti-atherogenic [18,44] instead of pro-atherogenic [45–47] lipoprotein.

Trial registration

ClinicalTrials.gov NCT02227784

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

Y.C. and J.D. designed the study and performed most of experiments, and they contributed equally to this work; X.Z. and X.C. did some experiments and statistical analysis; L.W., H.C., and J.G. involved in designing the study and edited the paper; X.C.J. designed the study and wrote the manuscript.

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