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Design, synthesis and identification of a new class of triarylmethyl amine compounds as inhibitors of apolipoprotein E production

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ABSTRACT

We have identified a new class of triarylmethyl amine compounds that can inhibit apolipoprotein E (apoE) production. ApoE is a cholesterol- and lipid-carrier protein implicated in aging, atherosclerosis, Alzheimer's Disease (AD), and other neurological and lipid-related disorders. Attenuation of apoE production is generally considered to be of therapeutic value. A majority of the apoE in the brain is produced by astrocytes. Here, we describe the design, synthesis, and biological screening of a small library of compounds that led to the identification of four triarylmethyl amines as potent inhibitors of apoE production in CCF-STTG1 astrocytoma cells.

Apolipoprotein E (apoE) is a cholesterol- and lipid-carrier that has been implicated in aging, atherosclerosis and several neurological diseases including Alzheimer Disease (AD).[1] ApoE genotype is the biggest risk factor for AD and may account for 60–90% of the genetic variance (V_G) associated with AD.[2] There are three common isoforms (alleles) of apoE in humans—apoE2, apoE3, and apoE4—which contribute to the pleiotropic effects observed in human cognition and neurodegenerative diseases.[3] In contrast, there is only one common apoE allele in other primates.[4] Increase in levels of murine apoE in a mouse model of AD by Bexarotene, an FDA-approved cancer drug, has been considered to be therapeutic because the treatment attenuated Aβ plaque burden.[5] However, the human apoE4 is hypofunctional and is considered to be the 'bad' apoE, while the human apoE3 is the most common isoform and is considered to be the 'normal' apoE.[1,3,6] Moreover, high levels of plasma and brain apoE are risk-factors for AD independent of the apoE genotype.[7] Thus, there is a strong interest in the biomedical community to find therapies that can reduce apoE levels. These therapies are of value to all individuals, and especially to subjects that have apoE4 allele(s).

We have found a class of small molecules that inhibit apoE production in the human CCF-STTG1 astrocytoma cell line. The initial screening was performed on a small library of compounds (Chart 1; scaffolds **A**–**C**) which was followed up using traditional structure-activity-relationship (SAR) approaches on scaffold **A** (Chart 2). The three scaffolds, **A**–**C** were chosen to provide with simple, readily available and chemically modifiable molecular backbones that offer the versatility for further functionalization to enable future SAR studies should any of these series yields any hit. Herein, we report on the design, synthesis and biological study leading to the identification of the triarylmethyl amine compounds that showed the greatest reduction in the amount of apoE present in the conditioned medium from the CCF-STTG1 cells.

The synthesis of the triarylmethyl amine series started with the formation of a Grignard reagent from the corresponding aryl bromide, followed by the subsequent reaction with benzophenone to generate the tertiary alcohol (Scheme 1).[8] The alcohol was in turn converted into the chloride using acetyl chloride, which was substituted with an amine nucleophile to yield the final triarylmethyl amine compound.[9] Most of the molecules in the scaffold **A** series (**1**–**4** and **10**–**42**, Chart 2) were purified by standard procedures, viz., crystallization, column chromatography, and preparative thin layer chromatography (prep-TLC). However, the triarylmethyl amines with *para*-methoxyphenyl moieties (compounds **21**–**32** in Chart 3) proved to be less stable than the other two aromatic groups used for this scaffold during purification processes. We attribute this instability to the ease of triarylmethyl carbocation formation due to the presence of a strong electron-donating methoxy group

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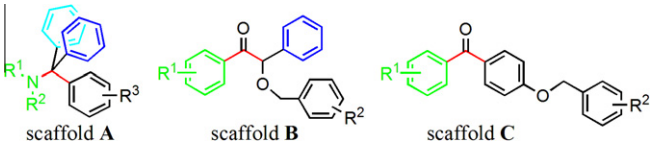


Chart 1. Scaffolds **A**–**C**, tested for their effects on apoE production.

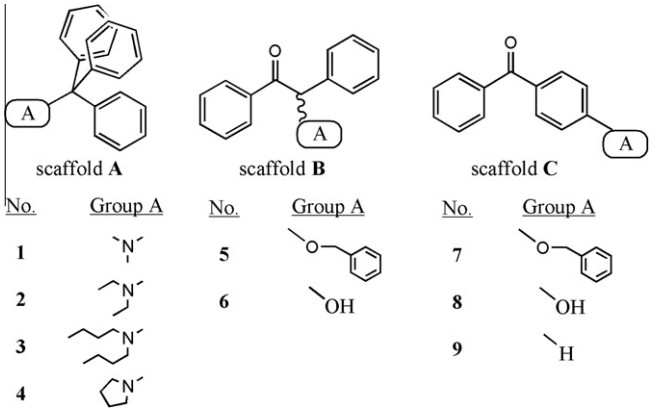
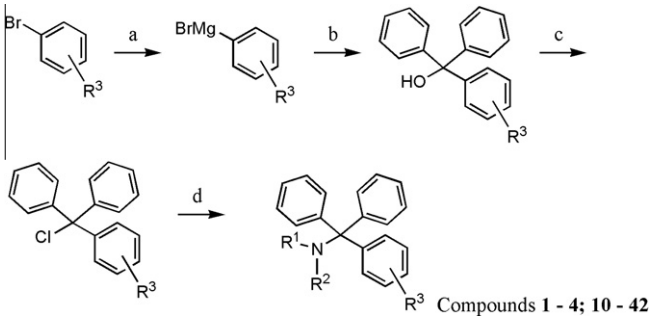


Chart 2. Triarylmethyl amine- (**1**–**4**), benzoin- (**5** and **6**) and benzophenone-based (**7**–**9**) compounds.



Scheme 1. Reagents and conditions: (a) Mg, anhyd. ether, reflux, 2 h; (b) benzophenone, reflux, 24 h; (c) acetyl chloride, benzene, reflux, 40 min; (d) R^1R^2NH, triethylamine, methylene chloride, rt, 15 h. Overall yield for steps a-d: 39–85%.[6]

on one aryl group. Successful purification of these final compounds was effected by pre-soaking the silica gel with eluent (ethyl acetate:hexane mixture) containing small amounts of triethylamine while performing flash columns, preparatory TLCs and radial chromatography.

The human CCF-STTG1 astrocytoma cells (ATCC, CRL-1718) were maintained in RPMI medium (Mediatech) supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1% penicillin/streptomycin, and 10% bovine growth serum (HyClone) at 37 °C with 5% CO_2. All compounds were dissolved in DMSO at a stock concentration of 10 mM. For ELISA assays, 1.6×10^4 cells / 96-well were grown for 1 day in RPMI, followed by a 24 h equilibration in serum-free Opti-MEM (Life Technologies). Treatments were carried out in quadruplicate with 100 µL of fresh Opti-MEM containing 10 µM of compound or vehicle (0.1% DMSO) for 24 h. Conditioned medium from these samples were then diluted twofold with incubation buffer (PBS + 0.05% Tween-20 + 0.1% BSA) and analyzed with a human apoE HRP ELISA kit (Mabtech) according to the manufacturer's protocol. A standard curve was used to derive sample apoE concentrations. The cells in the 96-well plates were used to determine the total cell number using CyQUANT reagent (Life Technologies). Sample apoE concentrations were adjusted for total cell number and then normalized to DMSO treated wells. Results for each set of compounds tested in parallel ($n = 3$) were analyzed by a one-tailed t-test.

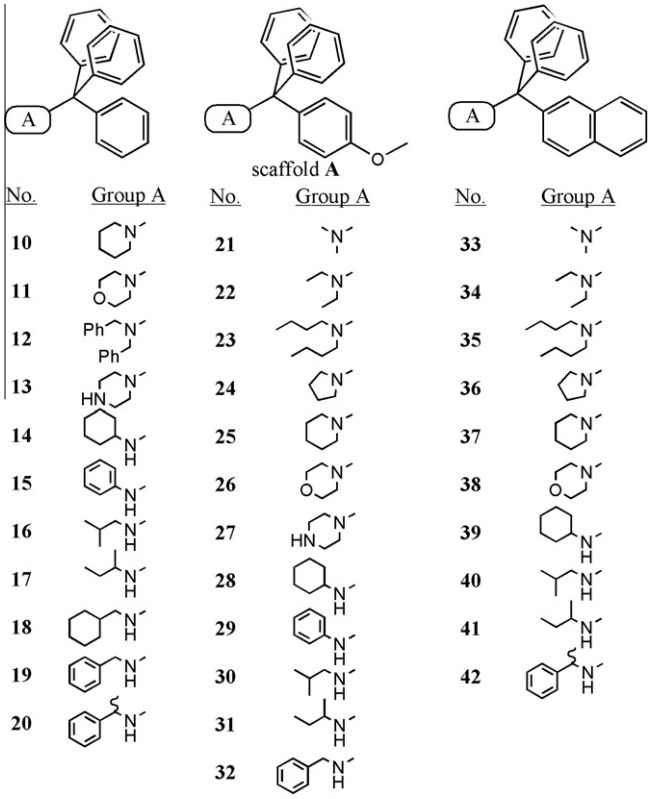


Chart 3. Triarylmethyl amine compounds with 3 aryl variants and 15 amines.

The triarylmethyl amine molecules designated as Scaffold **A** inhibited apoE secretion with the greatest potency.

We have used apoE ELISA to monitor apoE production after a 24 h-treatment with a 10 µM concentration of each of the compounds in Scaffolds **A**–**C** (Fig. 1). Figure 1 depicts the generally

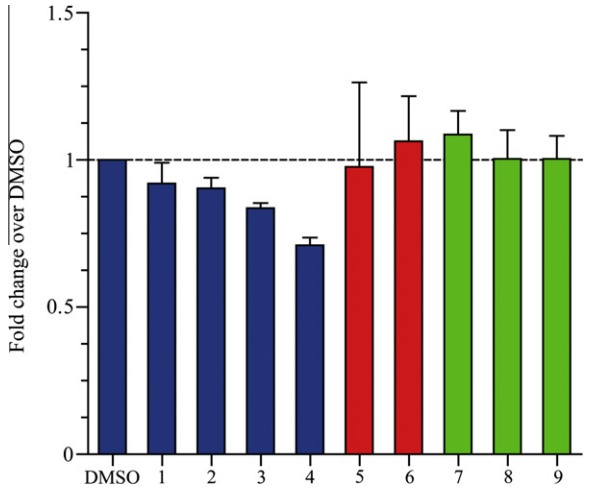


Figure 1. Effect of compounds, **1**–**9** from scaffolds **A**–**C** on apoE production in human CCF-STTG1 astrocytoma cells. The cells were treated with 10 µM compound or 0.1% DMSO (vehicle) in serum-free Opti-MEM for 24-h and the medium supernatant was used to quantify apoE production using a human apoE ELISA. Values are means of three independent experiments ± SEM and are normalized to the vehicle control.

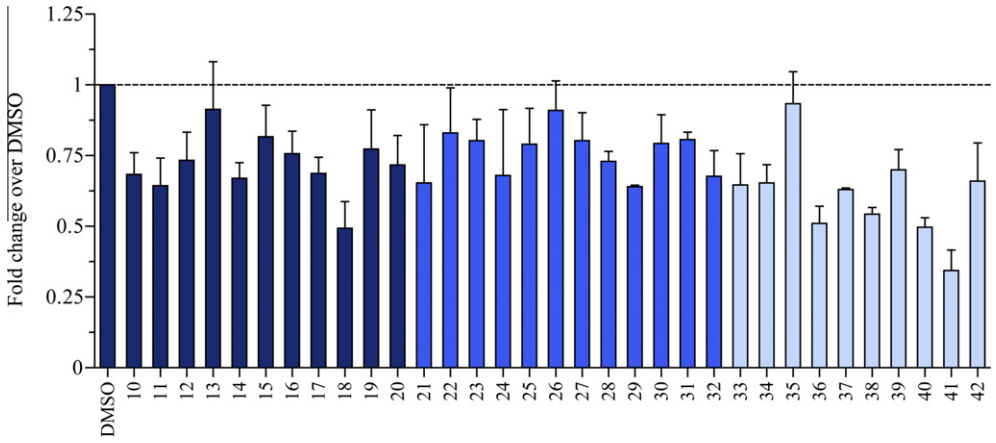


Figure 2. Effect of 33 compounds, **10**–**42** from scaffold **A** on apoE production in human CCF-STTG1 astrocytoma cells.

consistent inhibitory effects with varying strengths from compounds **1** through **9** (Chart 2). The three scaffolds **A**–**C**, although fundamentally different, share some common features, viz., varying number of phenyl rings on minimalist scaffolds. The noteworthy difference between the three structures is the lack of an amine group in scaffolds **B** and **C**. The first four compounds have triarylmethyl amine scaffolds with all three phenyl groups. Four easily available amines, viz., dimethylamine, diethylamine, dibutylamine and pyrrolidine were used in compounds **1**–**4**, respectively. *O*-benzylbenzoin (**5**) was easily available from its precursor benzoin (**6**) through benzylation, and both the compounds (**5** and **6**) were selected for screening.[10,11] Compound **7**, a benzyloxy benzophenone, and two fragments, hydroxyl benzophenone (**8**) and benzophenone (**9**), were also screened in the biological assay.[12] Quantification of apoE in the conditioned medium of cells treated with these nine compounds identified the triarylmethyl amines (scaffold **A**) as the best apoE inhibitors over the benzoins (scaffold **B**) and benzophenones (scaffold **C**) (Fig. 1). Lack of apoE inhibitory activity observed in scaffolds **B** and **C** could be attributed to scaffold structure itself or, absent design elements may be crucial for the desired activity, viz., the amine moiety and the number of aryl groups. Triarylmethyl amine **4** containing a pyrrolidine group with a hydrogen-bond acceptor tertiary amine moiety exhibited maximum apoE inhibition in this early study.

The promising results from the pilot apoE screen (Fig. 1) prompted us to undertake further SAR studies to design, synthesize and screen more triarylmethyl amines (Chart 3). Attention was paid to include hydrogen bonding donor and acceptor amines in the final compounds, vary the size of the third aromatic ring and also include acyclic and cyclic amines of different sizes and shapes. Thus, each compound in this particular series has two phenyl rings, a third aryl ring, and a unique amine. *Para*-methoxyphenyl and naphthyl aromatic groups were selected for the new set of molecules in order to test the effect of varying size without altering polarity in this area of the molecule. Altogether, fifteen different primary and secondary amines provided with added diversity for the molecules. The amines included aliphatic acyclic amines such as dimethyl amine, diethyl amine, and dibutyl amine. Aliphatic, cyclic amines were also included in the study with the selection of pyrrolidine, piperidine and morpholine. Cyclohexyl amine, aniline, isobutyl amine, and *sec*-butyl amine represent the secondary amines. A racemic version of α-methylbenzyl amine moiety was the only chiral example of amine in this study which falls under the general secondary amine category. The initial nature of the selected amine dictated the hydrogen bonding capability of the final compounds. Some of these compounds, including **1** (Chart 2), **12**, **25** and **38** (Chart 3), have hydrogen-bond acceptor regions as tertiary amines, whereas others are secondary amines (**20**, **27**, **40** and **41** in Chart 3) in their final form. Although 45 molecules (15 amines with 3 aryl variations) were attempted for synthesis and subsequent biological screening, not every candidate was screened due to occasional challenges in isolation, purification and in certain cases with final acceptable purities (e.g., compounds comprising a naphthyl aryl group and one of the following amines: cyclohexylmethyl amine, dibenzylamine, and piperazine).

The effects of the 33 triarylmethyl amines on apoE production are shown in Figure 2. The expansion of the series yielded much greater apoE inhibitory activity than the initial screen. Both the new aryl groups fared better than the original phenyl series in terms of apoE inhibition, with the naphthalene moiety resulting in more pronounced inhibition of apoE production. Among all the triarylmethyl amine compounds comprising the naphthyl group (Group C, Chart 3), compounds **36**, **38**, **40** and **41** inhibited apoE production by 46%, 49%, 50% and 66%, respectively. Compound **18** from the phenyl series was the only compound that exhibited respectable apoE inhibition (51%). The corresponding amine functionalities in these four compounds are pyrrolidine (**36**), morpholine (**38**), isobutyl amine (**40**), *sec*-butyl amine (**41**), and cyclohexylmethyl amine (**18**). The presence of hydrogen bond donor vs acceptor groups at the amine functionality, or the acyclic vs cyclic nature of the amine did not cause any noticeable change in the inhibitory activity. Six of them (**33**–**38**) have hydrogen bond donor groups and the rest (**39**–**42**) have hydrogen bond acceptor groups and there is an equal mix of acyclic and cyclic structures around the amine group. The overall size of most of the amine groups might have played a role in determining the inhibition efficacy since all the four amines in compounds, **36**, **38**, **40**, and **41** comprise approximately 5–6 atoms. The cyclohexylmethyl amine in compound **18** is slightly larger than these four amines and the plans are in order to synthesize and screen the naphthyl version of the triarylmethyl amine molecule with cyclohexylmethyl amine.

The SAR studies have led to the identification of four compounds in the triarylmethyl amines series as preliminary hits for inhibiting the production of apoE protein. Results have pointed at the usefulness of the bicyclic naphthalene ring. Additionally, five specific amine functionalities, viz., pyrrolidine, morpholine, isobutylamine, *sec*-butylamine, and cyclohexylmethyl amine proved to be essential. These results also provide additional insights into structural features necessary for development of more potent and drug-like scaffolds and compounds to inhibit apoE production.

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8. Experimental. Triarylmethyl amine **38**: 4-(Naphthalen-2-yl-diphenyl-methyl)-morpholine: Scratched magnesium turnings (1.056 g, 43.4 mmol) were placed in a 250 mL round-bottomed, 3-neck flask at room temperature. A small portion of the solution of 2-bromonaphthalene (11.84 g, 47.7 mmol) in anhydrous ether (40 mL) was added through an addition funnel and the solution was heated to gentle reflux maintaining the temperature at 35 °C. The rest of the solution was added in small fractions over a period of 1 h, and the resulting reaction mixture was refluxed for an additional 1 h. Disappearance of all magnesium indicated the completion of the reaction. The solution containing benzophenone (7.04 g, 38.6 mmol) in anhydrous ether (20 mL) was added through an addition funnel over 2–4 min. The resulting reaction was gently refluxed for 24 h. The cooled mixture was slowly and carefully quenched (Note: exotherm!) by adding aq HCl (6M) to the reaction mixture until the pH was acidic to Litmus paper. The mixture was diluted with ether (30 mL) and the aqueous layer was removed. The ether layer was washed with water (30 mL), followed by brine (30 mL) and dried with anhydrous Na_2SO_4. After filtration, the solvent was removed under reduced pressure to provide a colorless solid (triarylmethyl alcohol). The crude triarylmethyl alcohol derivative (6.30 g, 20.3 mmol) was refluxed with acetyl chloride (2.2 mL, 30.45 mmol) in benzene (10 mL) for 5 min. An additional amount of acetyl chloride (2.5 mL) was added over the course of 10 min via an addition funnel, and the resulting solution was allowed to reflux for another 30 min. The solution was quickly cooled by holding the flask under a stream of running tap water while vigorously shaking the mixture. The mixture was concentrated under reduced pressure to give the triarylmethyl chloride derivative. The crude triarylmethyl chloride derivative and morpholine (5.0 mL, 57.9 mmol) in anhydrous CH_2Cl_2 (25 mL) were stirred at 25 °C for 18 h. The organic layer was washed with water (30 mL) followed by saturated $NaHCO_3$ solution (30 mL) and brine (30 mL), dried with anhydrous Na_2SO_4. The solution was filtered and the solvent was evaporated under reduced pressure to yield crude triarylmethyl amine, **38** as a dirty white solid. Flash chromatography using 230–400 mesh silica gel was performed to purify compound **38**. Elution of the column with 30% ethyl acetate in hexanes, followed by concentration of the appropriate fractions and subsequent evaporation of solvents yielded 6.88 g (47% overall) of triarylmethyl amine **38** as an off-white amorphous solid. ^{1}H NMR (400 MHz/$CDCl_3$) δ 2.88–1.81 (m, 4H), 4.10–3.66 (m, 4H), 7.21–7.15 (m, 2H), 7.35–7.23 (m, 4H), 7.86–7.36 (m, 10H) 7.44–7.41 (m, 2H), 7.61–7.46 (m, 4H) 7.66 (s, 1H), 7.72 (d, J = 8.4 Hz, 1H) 7.79–7.74 (m, 2H), 7.81 (d, J = 7.3 Hz, 1H), 7.96 (s, 1H); ^{13}C NMR (100 MHz/$CDCl_3$) δ 141.6 (C), 132.9, 131.8, 130.2, 129.2, 128.8, 128.1, 127.8, 127.7, 127.2, 126.7, 126.3, 125.9, 125.2, 77.6, 67.7, 48.7; IR ν (cm^{-1}): 3690, 2955, 2842, 1488, 1446, 1262, 1112, 1033, 998, 908, 877, 817, 743, 703, 571, 479; Mp: 113–114 °C; HRMS: (M^++Na^+) calcd for $C_{27}H_{25}NO$, 402.1828, found, 402.1818.
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