

## RESEARCH PAPER

# Torcetrapib-induced blood pressure elevation is independent of CETP inhibition and is accompanied by increased circulating levels of aldosterone

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**Background and purpose:** Inhibition of cholesteryl ester transfer protein (CETP) with torcetrapib in humans increases plasma high density lipoprotein (HDL) cholesterol levels but is associated with increased blood pressure. In a phase 3 clinical study, evaluating the effects of torcetrapib in atherosclerosis, there was an excess of deaths and adverse cardiovascular events in patients taking torcetrapib. The studies reported herein sought to evaluate off-target effects of torcetrapib.

**Experimental approach:** Cardiovascular effects of the CETP inhibitors torcetrapib and anacetrapib were evaluated in animal models.

**Key results:** Torcetrapib evoked an acute increase in blood pressure in all species evaluated whereas no increase was observed with anacetrapib. The pressor effect of torcetrapib was not diminished in the presence of adrenoceptor, angiotensin II or endothelin receptor antagonists. Torcetrapib did not have a contractile effect on vascular smooth muscle suggesting its effects *in vivo* are via the release of a secondary mediator. Treatment with torcetrapib was associated with an increase in plasma levels of aldosterone and corticosterone and, *in vitro*, was shown to release aldosterone from adrenocortical cells. Increased adrenal steroid levels were not observed with anacetrapib. Inhibition of adrenal steroid synthesis did not inhibit the pressor response to torcetrapib whereas adrenalectomy prevented the ability of torcetrapib to increase blood pressure in rats.

**Conclusions and implications:** Torcetrapib evoked an acute increase in blood pressure and an acute increase in plasma adrenal steroids. The acute pressor response to torcetrapib was not mediated by adrenal steroids but was dependent on intact adrenal glands.

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**Keywords:** cholesteryl ester transfer protein inhibitor; aldosterone; preclinical animal models

**Abbreviations:** CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein

## Introduction

The use of statins for lowering low-density lipoprotein (LDL) in humans has unequivocally been demonstrated to reduce the incidence of cardiovascular disease and increase longevity (Scandinavian Simvastatin Survival Study Group, 1994; Sacks *et al.*, 1996; Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group, 1998; Sever *et al.*,

2003). However, it is equally evident that greater clinical benefit can be achieved given that, at best, statins reduce the risk of major coronary events by only 30% (LaRosa *et al.*, 1999; Maron *et al.*, 2000). An alternative therapeutic strategy being pursued by many pharmaceutical companies is to increase circulating levels of high-density lipoprotein (HDL). This strategy is predicated on the strong epidemiologic data that document an inverse correlation between HDL levels and coronary heart disease (Gordon *et al.*, 1989; Assmann and Schulte, 1992).

A novel pharmacological approach for elevation of HDL levels is the use of inhibitors of cholesteryl ester transfer protein (CETP). Inhibitors of CETP prevent the transfer of

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cholesteryl esters from HDL to LDL and very low-density lipoprotein and the reciprocal transfer of triglycerides from LDL and very low-density lipoprotein to HDL. Numerous preclinical and clinical studies confirm that CETP inhibitors effectively increase circulating HDL levels (Barter *et al.*, 2003; Brousseau *et al.*, 2004; Clark *et al.*, 2004; Davidson *et al.*, 2006). Furthermore, preclinical studies with CETP inhibitors demonstrated beneficial antiatherosclerotic effects in animal models (Sugano *et al.*, 1998; Okamoto *et al.*, 2000).

The only human clinical trial to evaluate the effects of a CETP inhibitor on cardiovascular events is the Investigation of Lipid Level Management to Understand Its Impact in Atherosclerotic Events (ILLUMINATE) trial. In this study, the effect of a CETP inhibitor, torcetrapib (Pfizer, New York, NY, USA), in combination with atorvastatin was compared to treatment with atorvastatin alone. Unfortunately, the study was terminated prematurely because of an unexpected increase in cardiovascular events and death with the combination treatment versus atorvastatin alone (Barter *et al.*, 2007). The reason(s) for the increase in mortality is not known; however, it is known that torcetrapib administration in humans is associated with an increase in blood pressure (Davidson *et al.*, 2006; Barter *et al.*, 2007; Nissen *et al.*, 2007). Furthermore, examination of serum electrolyte values from the ILLUMINATE study revealed a decrease in potassium and increases in sodium and bicarbonate at the end of 12 months in patients taking torcetrapib plus atorvastatin compared with those taking atorvastatin alone. These findings prompted a *post hoc* examination of aldosterone levels, which were shown to be higher in patients who had taken torcetrapib for 3 months.

The studies described herein evaluate the acute haemodynamic effects of torcetrapib in a variety of preclinical models and species and compare the effects of torcetrapib with another experimental CETP inhibitor, anacetrapib (MK-0859). Administration of torcetrapib was shown to acutely increase blood pressure in both rodent and non-rodent species. In addition, in rats, administration of torcetrapib was associated with the release of aldosterone and corticosterone *in vivo* and *in vitro* from primary adrenocortical cells. The other CETP inhibitor, anacetrapib, did not increase blood pressure under equivalent conditions and was not associated with adrenal steroid release either *in vivo* or *in vitro*.

## Methods

### Animals

All animals were maintained in AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care)-accredited facilities at Merck Research Laboratories (Rahway, NJ, USA or West Point, PA, USA), and all experimental procedures were approved by the Institutional Animal Care and Use Committee and were in conformance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

C57BL/6NTac mice (B6) were purchased from Taconic (Hudson, NY, USA); C57BL/6-Tg(CETP)1Pnu/J mice (B6-Tg(CETP)) were purchased from Jackson (Bar Harbor, ME, USA) and bred at Merck Research Laboratories. Male

Sprague–Dawley rats (300–400 g body weight) with surgically implanted femoral artery and vein catheters were obtained from Charles River Laboratories (Raleigh, NC, USA). Male or female mixed breed mongrel dogs (approximately 8–12 kg) were obtained from either Covance Research Products Inc. (Cumberland, VA, USA) or Marshall BioResources (North Rose, NY, USA). Male rhesus monkeys (approximately 4–6 years old, body weight 6.5–7.5 kg) were obtained from Covance Research Products Inc. (Alice, TX, USA).

### Haemodynamics

Heart rate and blood pressure were measured in mice, rats, dogs and rhesus monkeys under a variety of experimental conditions.

For the assessment of cardiovascular function in conscious mice, animals were surgically implanted with catheters in a carotid artery and a jugular vein at least 24 h prior to study. On the day of study a Becton Dickinson (Franklin Lakes, NJ, USA) DTX pressure transducer was attached to the arterial catheter and subsequently to a Gould ACQ-7700 data acquisition system using Ponemah software. Heart rate was derived from the arterial pulse wave. Heart rate and arterial pressure were measured between 30 and 60 min to establish baseline values. Subsequently, test compound or vehicle (2% dimethyl sulphoxide:4% Cremophor EL:94% saline) was administered in a volume of 0.2 mL per mouse via a 30 min infusion. Cardiovascular data were recorded continuously and are reported as average values over 1 min intervals. Equivalent procedures were used for the measurement of haemodynamics in rats, with the exception that studies were performed in anaesthetized animals (sodium pentobarbital; 55 mg kg<sup>-1</sup>, i.p.). In some studies, rats were pithed according to the method of Gillespie *et al.* (1970). Test compound or vehicle (2% dimethyl sulphoxide:4% Cremophor EL:94% saline or 50% dimethylacetamide:50% polyethylene glycol 200) was administered in a volume of 1 mL per rat via an i.v. infusion of 20–30 min duration.

Haemodynamic studies were performed in dogs anaesthetized with sodium pentobarbital (35 mg kg<sup>-1</sup>, i.v. loading dose plus 6 mg kg<sup>-1</sup> h<sup>-1</sup> maintenance infusion). An endotracheal tube was inserted into the trachea and artificial respiration with ambient room air was employed utilizing a large animal ventilator (Harvard Apparatus, Boston, MA, USA). Body temperature was maintained using an automated heating surgical table and infrared lamp. Bilateral femoral vein catheters were implanted for the i.v. administration of anaesthetics and test compounds. Arterial blood pressure was captured via a catheter inserted into a femoral artery and connected to a disposable type pressure transducer. Heart rate was derived from the pulsatile blood pressure or from the ECG signal. A CA Recorder Systems (DISS LLC) data acquisition system was used to collect and analyse input signals.

Haemodynamic parameters were collected from conscious rhesus monkeys implanted with Konigsberg cardiovascular total implants. Data were transmitted via radiotelemetry and recorded by CA Recorder Systems (DISS LLC). Arterial blood pressure and heart rate were collected for  $\geq 24$  h prior to vehicle dosing,  $\geq 2$  h prior to oral dosing with test compound and  $\geq 24$  h postdosing. Data were collected as

15-min mean values and are reported as mean 1 h values  $\pm$  s.e. of the mean.

#### *Adrenalectomy*

Rats were anaesthetized with sodium pentobarbital ( $55 \text{ mg kg}^{-1}$ , i.p.). The back of the animal was shaved and a 1.5-cm dorsal midline incision made with its midpoint centered over the thirteenth rib. The underlying muscle wall on either side of the spinal column was dissected, and the adrenal glands were located and removed. Skin incisions were closed with stainless steel wound clips and acute haemodynamic measurements made as described above.

#### *Isolated vascular tissue experiments*

The descending thoracic aorta was removed from rats, cleaned of connective tissue and cut into 3 mm rings. The endothelium was left intact, and rings were subsequently mounted (using stainless steel triangle mounts) in 5 mL isolated organ chambers (Radnoti Glass, Monrovia, CA, USA) containing modified Krebs solution (in mM: 120 NaCl, 5.9 KCl, 2.5  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 1.2  $\text{KH}_2\text{PO}_4$ , 25  $\text{NaHCO}_3$ , 10 glucose) at  $37^\circ\text{C}$  and aerated with 95% $\text{O}_2$ :5% $\text{CO}_2$ . Mounted rings were connected to isometric force transducers (Radnoti Glass). Passive tension was set to an optimal resting tension of 0.5–1 g, and rings were allowed to equilibrate for 30 min. Isometric tension was monitored and recorded using IOX data acquisition software (EMKA Technologies Inc., Falls Church, VA, USA). Rings were tested for viability using 120 mM KCl, washed and allowed to re-equilibrate for an additional 30 min before performing concentration–response curves to test compounds.

#### *Perfused hindlimb preparation*

Rats were anaesthetized with urethane ( $800 \text{ mg kg}^{-1}$ , i.p.) and  $\alpha$ -chloralose ( $40 \text{ mg kg}^{-1}$ , i.p.). A midline incision (3 cm long) was made in the lower abdomen to expose the bifurcation of the abdominal aorta. The right iliac artery and vein were isolated and cannulated with modified PE-50 catheters. The arterial catheter was connected to a peristaltic pump (ML 172; ADInstruments, Newton, NH, USA), the speed of which was set to generate a perfusion pressure of 60–70 mm Hg. Perfusion pressure was monitored using a pressure transducer (DTX plus TNF-R; Becton Dickinson) connected to a side arm of the arterial catheter. Initially, the hindlimb was perfused with buffer alone (Krebs-Henseleit solution) to obtain a steady baseline. Subsequently, the hindlimb was sequentially perfused with torcetrapib (3–30  $\mu\text{M}$ ) followed by phenylephrine (5  $\mu\text{M}$ ), each for a duration of 5 min with a 2 min interval between treatments. The pressure signal was captured by a Ponemah physiological data acquisition system (P3 Plus; Data Science International, St Paul, MN, USA). Mean perfusion pressure was derived by the software automatically and recorded continuously throughout the study.

#### *Measurement of plasma adrenal steroids*

Adrenal steroid concentrations in plasma samples were determined using commercially available ELISA kits. The

aldosterone assay was obtained from Alpha Diagnostics (San Antonio, TX, USA) and the corticosterone assay from Cayman Chemical Company (Ann Arbor, MI, USA).

#### *Adrenal steroid release assay*

The procedure for isolating rat adrenal cells was developed by modifying previously published methods (Fredlund *et al.*, 1975). Adrenal glands were removed from male rats, asphyxiated by  $\text{CO}_2$  and placed in ice-cold Dulbecco's phosphate-buffered saline. The glands were dissected free of all fat and connective tissue. Each gland was dissected in half, and the outer capsule separated. Each capsule was placed in 1.2 mL of room temperature medium (one part Krebs-Ringer bicarbonate (mM: 120 NaCl, 4.6 KCl, 0.5  $\text{MgCl}_2$ , 0.7  $\text{Na}_2\text{HPO}_4$ , 1.5  $\text{NaH}_2\text{PO}_4$ , 10 glucose) with two parts Medium-199 and two parts L-15 plus 5 mM HEPES) containing collagenase ( $2 \text{ mg mL}^{-1}$ ) and DNase ( $0.4 \text{ mg mL}^{-1}$ ). The capsules were teased apart in this solution and incubated with gentle rocking for 70 min at room temperature. Every 20 min, cells were gently dispersed by drawing the solution up and down into a silicon tubing of 10 inch length. The suspensions were washed twice in Dulbecco's phosphate buffered saline following centrifugation at 100 g for 10 min. Finally, the cells were re-suspended in 1.0 mL medium containing BSA ( $4 \text{ mg mL}^{-1}$ ) and soybean trypsin inhibitor ( $2 \text{ mg mL}^{-1}$ ). The final cell concentration was approximately 200 000 cells per mL. Cell suspensions (500  $\mu\text{L}$  per well) were placed in a 24-well polystyrene plate to which stimuli were added. The plate was incubated in a water bath at  $37^\circ\text{C}$  for 2 h. Cell suspensions were centrifuged at 1000 g for 10 min, and the media assayed for aldosterone and corticosterone as described above.

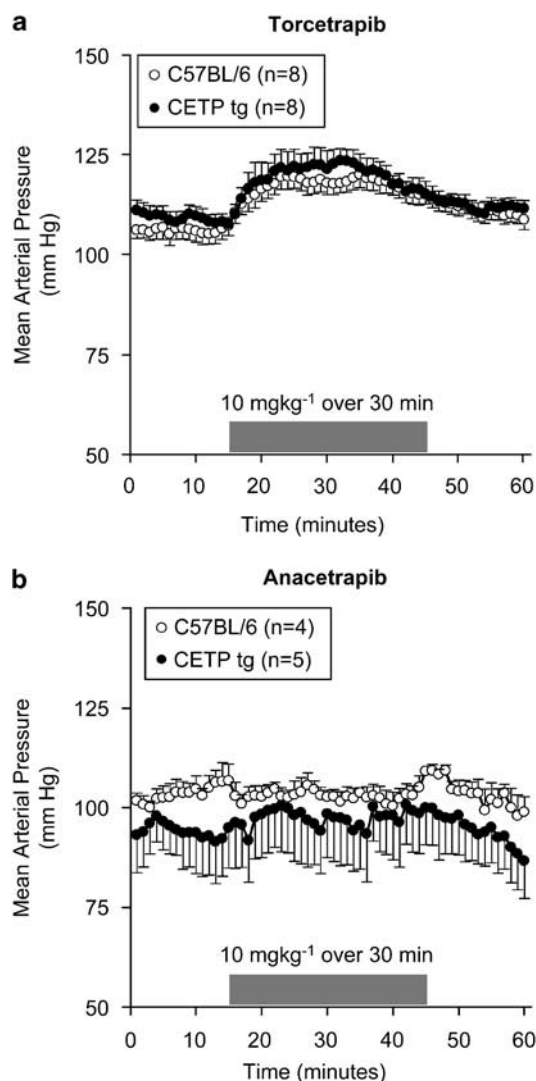
#### *Test compounds*

Torcetrapib, anacetrapib and E-3174 were synthesized at Merck Research Laboratories. The endothelin ( $\text{ET}_A/\text{ET}_B$ ) antagonist, compound A (Nishikibe *et al.*, 1999), was synthesized at Banyu Pharmaceuticals, Tsukuba, Japan. Trilostane was purchased from Chempacific (Baltimore, MD, USA).

## Results

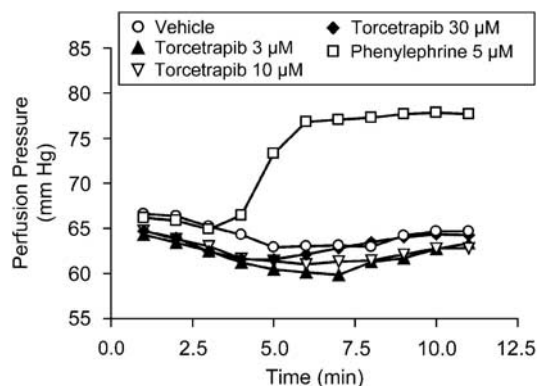
#### *Effects of CETP inhibitors on vascular haemodynamics*

The effects of torcetrapib and anacetrapib on blood pressure were evaluated in normal mice that do not contain CETP and in transgenic mice expressing the cynomolgus CETP gene (Figure 1). Administration of torcetrapib ( $10 \text{ mg kg}^{-1}$ ) by i.v. infusion of 30 min duration evoked a significant increase in mean arterial blood pressure of approximately 15 mm Hg. The increase in blood pressure was transient, returning to near baseline levels by the conclusion of the infusion. In both normal B6 mice and B6-Tg(CETP) mice, heart rate decreased transiently by approximately 20% (data not shown) following administration of torcetrapib, presumably a reflexogenic response to the increase in blood pressure. In contrast, administration of anacetrapib ( $10 \text{ mg kg}^{-1}$ ) or



**Figure 1** Torcetrapib increased blood pressure in normal and CETP transgenic mice. Mean arterial blood pressure was measured in conscious B6 mice and B6-Tg(CETP) transgenic mice. Torcetrapib ( $10 \text{ mg kg}^{-1}$ ) or anacetrapib ( $10 \text{ mg kg}^{-1}$ ) was administered as an i.v. infusion of 30 min duration. Blood pressure was monitored continuously and is shown as average values at 1 min intervals. Each point represents the mean, and vertical lines represent the s.e. Numbers of animals per group are shown in parentheses. CETP, cholesteryl ester transfer protein.

vehicle (data not shown) under the same dosing conditions was without effect on either blood pressure or heart rate. The lack of effect of anacetrapib on blood pressure cannot be explained on the basis of an insufficient dose of anacetrapib for the following reasons. In the study shown in Figure 1, the two compounds achieved comparable systemic exposure: in the wild-type mice, terminal compound levels were  $23 \pm 4 \mu\text{M}$  of torcetrapib and  $25 \pm 3 \mu\text{M}$  of anacetrapib. In the transgenic mice, the relevant values were  $12 \pm 7 \mu\text{M}$  of torcetrapib and  $9 \pm 3 \mu\text{M}$  of anacetrapib. Furthermore, the two compounds have similar intrinsic potencies against CETP. In an *in vitro* fluorogenic assay of CETP activity (Eveland *et al.*, 2007), torcetrapib had an  $\text{IC}_{50}$  of  $13 \pm 3 \text{ nM}$  ( $n=9$ ) and anacetrapib had an  $\text{IC}_{50}$  of  $17 \pm 5 \text{ nM}$  ( $n=10$ ). The two compounds also have similar potencies *in vivo*. In a study using the same



**Figure 2** Torcetrapib did not increase perfusion pressure in the isolated perfused rat hindlimb. The effects of torcetrapib or vehicle on perfusion pressure were evaluated in urethane ( $800 \text{ mg kg}^{-1}$ , i.p.)- and  $\alpha$ -chloralose ( $40 \text{ mg kg}^{-1}$ , i.p.)-anaesthetized rats. The femoral artery was cannulated and connected to a perfusion pump at a rate that produced a resting perfusion pressure of  $\sim 65 \text{ mm Hg}$ . Subsequently, animals were infused sequentially with torcetrapib ( $3\text{--}30 \mu\text{M}$ ) followed by phenylephrine ( $5 \mu\text{M}$ ). Each data point is the mean of determinations made in two animals.

strain of B6-Tg(CETP) mice used in Figure 1, 2 weeks of dosing of each compound (10 mice per group) at approximately  $50 \text{ mg kg}^{-1}$  (p.o.) yielded substantial increases in HDL compared to control animals:  $214 \pm 54\%$  for torcetrapib and  $318 \pm 51\%$  for anacetrapib. In this 2-week study, terminal compound levels were also comparable:  $4.6 \pm 3.2 \mu\text{M}$  for torcetrapib and  $9.4 \pm 3.1 \mu\text{M}$  for anacetrapib.

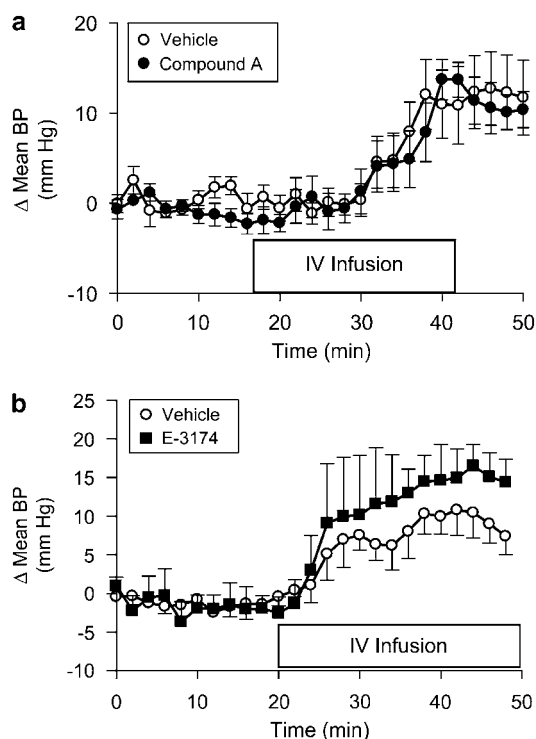
The effects of torcetrapib on blood pressure and heart rate were also evaluated in rats, dogs and rhesus monkeys, whereas anacetrapib was evaluated only in dogs and rhesus monkeys (Supplementary Figures 1–3). Torcetrapib increased mean arterial pressure in anaesthetized rats ( $5 \text{ mg kg}^{-1}$ , i.v.), anaesthetized dogs ( $1\text{--}10 \text{ mg kg}^{-1}$ , i.v.) and in conscious rhesus monkeys ( $500 \text{ mg kg}^{-1}$ , p.o.). Interestingly, torcetrapib had no effect on heart rate in either rats or rhesus monkeys but did produce increases in heart rate of approximately 35% in anaesthetized dogs. Anacetrapib was without effect on haemodynamic parameters in either dogs ( $1\text{--}10 \text{ mg kg}^{-1}$ , i.v.) or rhesus monkeys ( $500 \text{ mg kg}^{-1}$ , p.o.).

#### *Torcetrapib did not directly contract vascular smooth muscle*

A series of studies was performed to investigate the mechanism by which torcetrapib increases blood pressure. The ability of torcetrapib to directly contract vascular smooth muscle was evaluated using both isolated vascular smooth muscle preparations as well as a rat perfused hindlimb preparation. Torcetrapib ( $10^{-9}\text{--}10^{-5} \text{ M}$ ) did not exhibit any contractile activity when added to isolated rat aortic rings or isolated rat mesenteric artery rings, whereas methoxamine ( $10^{-6} \text{ M}$ ) evoked a robust contractile response of either aortic or mesenteric artery rings (data not shown). Furthermore, torcetrapib neither enhanced the contractile response to  $\alpha$ -adrenoceptor agonists or angiotensin II nor reduced the relaxant effect of ACh (data not shown). In a perfused rat hindlimb preparation (Figure 2), torcetrapib ( $3\text{--}30 \mu\text{M}$ ) had no effect on perfusion pressure, whereas phenylephrine evoked a marked increase in perfusion pressure.

*Torcetrapib did not increase blood pressure via a central mechanism, via sympathetic nervous system activation or via activation of angiotensin type I or endothelin receptors*

The possible central effects of torcetrapib, for elevation of blood pressure in rats, were evaluated by performing studies in control animals or in pithed rats. The acute administration of torcetrapib ( $5 \text{ mg kg}^{-1}$ , i.v.), infused over a 30-min time period, produced an equivalent increase in blood pressure in control and pithed rats (Supplementary Figure 4), indicating that torcetrapib was not increasing blood pressure via a central mechanism. In dogs, pretreatment with the  $\alpha_1$ -adrenoceptor antagonist (prazosin;  $1 \text{ mg kg}^{-1}$ ) or the  $\beta_1$ -adrenoceptor antagonist (atenolol;  $3 \text{ mg kg}^{-1}$ ) did not attenuate the pressor response to torcetrapib (Supplementary Figures 5a and 6a). Atenolol partly reduced the torcetrapib-evoked increase in heart rate (Supplementary Figure 6b), whereas hexamethonium ( $3 \text{ mg kg}^{-1}$ ) completely suppressed the increase in heart rate (Supplementary Figure 7b) but with only a minor reduction in the pressor response (Supplemen-

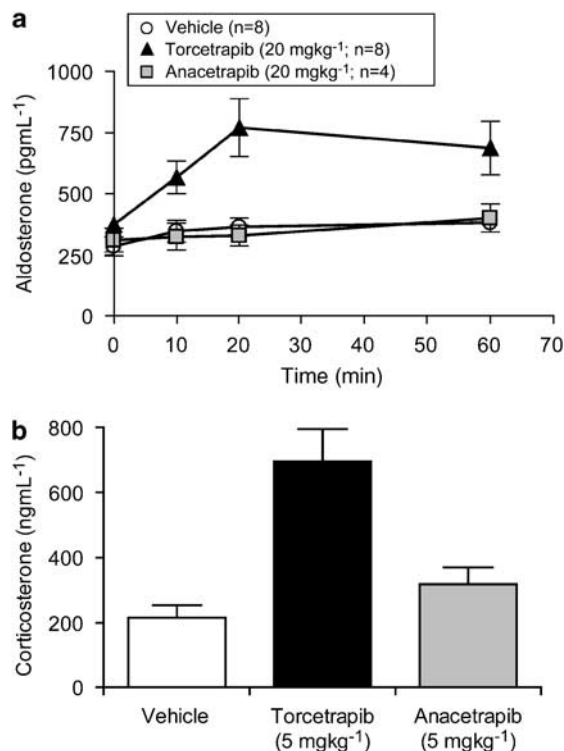


**Figure 3** Effects of endothelin or angiotensin II receptor antagonists. Mean arterial blood pressure was measured in pentobarbital ( $55 \text{ mg kg}^{-1}$ , i.p.)-anaesthetized rats. (a) Separate groups of animals were given either the  $\text{ET}_A/\text{ET}_B$  antagonist, compound A ( $3 \text{ mg kg}^{-1}$ , i.v.) or vehicle (saline) 60 min prior to the i.v. infusion of torcetrapib ( $10 \text{ mg kg}^{-1}$ ) in a volume of 1 mL per rat, over 30 min. Data are expressed as the change in pressure from the baseline period 20–40 min prior to test compound infusion. Each point represents the mean, and vertical lines represent the s.e. for groups of five rats each. (b) Mean arterial blood pressure was measured in pentobarbital ( $55 \text{ mg kg}^{-1}$ , i.p.)-anaesthetized rats. Separate groups of animals were given either the  $\text{AT}_1$  receptor antagonist, E-3174 ( $1 \text{ mg kg}^{-1}$ , i.v.) or vehicle (saline) 60 min prior to the i.v. infusion of torcetrapib ( $10 \text{ mg kg}^{-1}$ ) in a volume of 1 mL per rat over 30 min. Data are expressed as the change in blood pressure from the baseline period 20–40 min ( $\Delta$  mean BP) prior to giving test compounds. Each point represents the mean, and vertical lines represent the s.e. for groups of four rats each.

tary Figure 7a). A similar lack of effect of prazosin or phentolamine, to reduce the pressor response to torcetrapib, was observed in rats (data not shown). The pressor response to torcetrapib ( $10 \text{ mg kg}^{-1}$ , i.v.) was not reduced in rats pretreated with either a mixed  $\text{ET}_{A/B}$  receptor antagonist (compound A;  $3 \text{ mg kg}^{-1}$ , i.v.; Figure 3a) or an angiotensin II receptor blocker (E-3174;  $1 \text{ mg kg}^{-1}$ , i.v.; Figure 3b).

#### *Evaluation of adrenal steroid levels in rats following administration of CETP inhibitors*

To explore other potential mediators that could account for the hypertensive effects observed with torcetrapib, plasma levels of aldosterone prior to and after i.v. infusion of either torcetrapib or anacetrapib to conscious rats were measured. Administration of torcetrapib ( $20 \text{ mg kg}^{-1}$ ) by i.v. infusion for 30 min evoked a significant ( $P < 0.01$ ) increase in plasma aldosterone (Figure 4a) from baseline values to those measured 20 min post initiation of compound infusion and remained elevated for 30 min after completion of the infusion. In contrast to torcetrapib, neither vehicle nor anacetrapib evoked significant changes in plasma aldosterone levels. Subsequent studies demonstrated that the

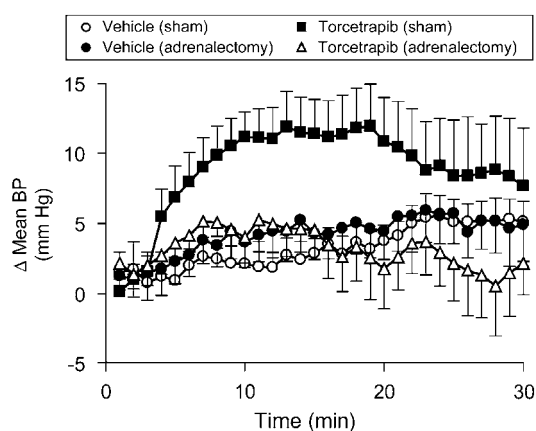


**Figure 4** Measurement of adrenal steroids. (a) Plasma levels of aldosterone were measured in plasma from conscious rats prior to and at the indicated times following the i.v. infusion of compounds at  $20 \text{ mg kg}^{-1}$  or vehicle (50% dimethylacetamide:50% polyethylene glycol 200) for 20 min. Each data point is the mean and vertical lines represent the s.e. for the numbers of animals indicated in parentheses. (b) Plasma levels of corticosterone were measured in plasma from conscious rats 20 min following the i.v. infusion of compounds at  $5 \text{ mg kg}^{-1}$  or vehicle (50% dimethylacetamide:50% polyethylene glycol 200) for 20 min. Each data point is the mean and vertical lines represent the s.e. for the numbers of animals indicated in parentheses.

effects of torcetrapib were not selective for aldosterone release as plasma corticosterone levels were also increased in animals that received torcetrapib (Figure 4b) but not following administration of anacetrapib.

#### Pressor responses to torcetrapib are absent in adrenalectomized rats

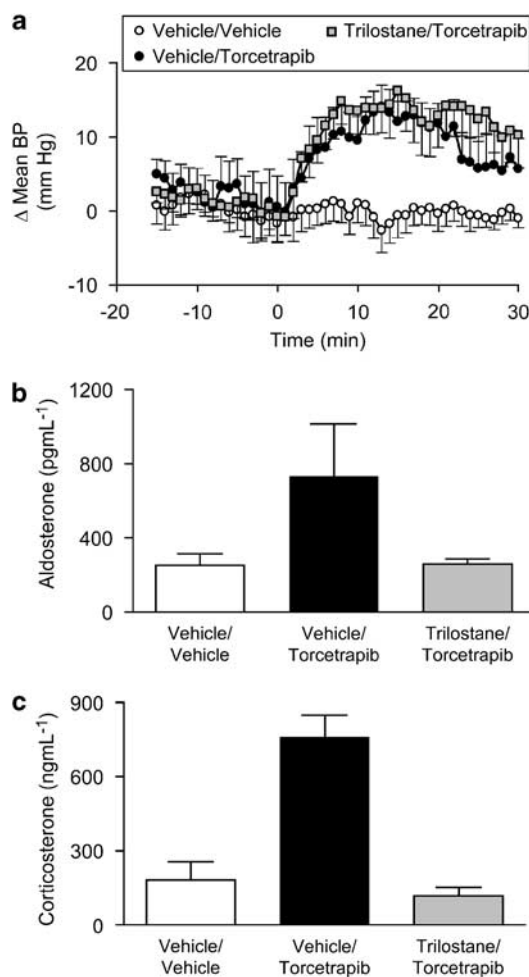
Having observed an acute increase in plasma aldosterone and corticosterone in response to administration of torcetrapib, we sought to explore further the relationship between adrenal steroids and the acute hypertensive response to torcetrapib. In acutely adrenalectomized, anaesthetized rats, torcetrapib ( $5 \text{ mg kg}^{-1}$ , i.v. infused over 30 min) did not increase blood pressure, whereas administration of torcetrapib to sham-operated animals evoked a robust pressor response (Figure 5). Pressor responses to acute angiotensin II challenge ( $50 \text{ ng kg}^{-1}$ , i.v.) were equivalent in both adrenalectomized and sham-operated animals (data not shown). At the conclusion of torcetrapib infusion, plasma aldosterone levels were  $488 \pm 101 \text{ pg mL}^{-1}$  in sham-operated animals and  $126 \pm 17 \text{ pg mL}^{-1}$  (mean  $\pm$  s.e.mean,  $n = 7$ ) in acutely adrenalectomized animals. The relationship between the acute pressor effect of torcetrapib and the release of aldosterone and corticosterone was explored by administration of the  $3\beta$ -hydroxysteroid dehydrogenase inhibitor, trilostane. Torcetrapib ( $5 \text{ mg kg}^{-1}$ , i.v.) evoked equivalent increases in blood pressure (Figure 6a) in animals administered trilostane or control animals administered vehicle (50% DMA:50% PEG200). However, trilostane completely inhibited the torcetrapib-induced increase in plasma aldosterone (Figure 6b) and plasma corticosterone (Figure 6c). Furthermore, administration of exogenous aldosterone ( $1$  and  $10 \mu\text{g kg}^{-1}$ ) to rats did not increase blood pressure, whereas administration of torcetrapib ( $5 \text{ mg kg}^{-1}$ ) to these same animals evoked a robust increase in blood pressure of 10–15 mm Hg (Figure 7).



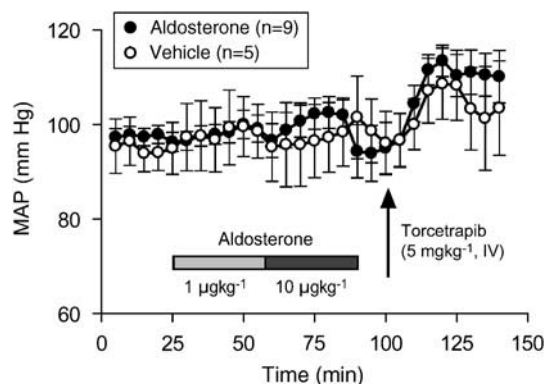
**Figure 5** Adrenalectomy prevented the pressor response to torcetrapib. The effects of torcetrapib infusion ( $5 \text{ mg kg}^{-1} \times 20 \text{ min}$ ) or vehicle (2% DMSO:4% Cremophor EL:94% saline) on mean arterial pressure were evaluated in pentobarbital ( $55 \text{ mg kg}^{-1}$ , i.p.)-anaesthetized rats following acute adrenalectomy or in animals that were sham-operated. Data are expressed as the change in blood pressure ( $\Delta$  mean BP) from the baseline period 20 min prior to giving test compounds. Each point represents the mean and vertical lines represent the s.e. for groups of six to nine animals. DMSO, dimethyl sulphoxide.

#### Effects on isolated primary adrenocortical cells

The mechanism of torcetrapib-evoked aldosterone release *in vivo* is not known. Possibilities include a direct secretagogue action of torcetrapib or an indirect effect via known stimuli such as angiotensin II, adrenocorticotrophic hormone or potassium. To determine if torcetrapib has a direct effect on the adrenal gland, primary adrenocortical cells were isolated from rat adrenal glands and exposed *in vitro* to either torcetrapib (Figure 8a) or anacetrapib (Figure 8b). Angiotensin II ( $0.1 \mu\text{M}$ ) served as a positive control and produced a robust increase in aldosterone release into the medium. Torcetrapib also stimulated aldosterone release, whereas



**Figure 6**  $3\beta$ -hydroxysteroid dehydrogenase inhibition does not block the pressor effect of torcetrapib. (a) The effects of torcetrapib infusion ( $5 \text{ mg kg}^{-1} \times 30 \text{ min}$ ) or vehicle (2% DMSO:4% Cremophor EL:94% saline) on mean arterial pressure were evaluated in conscious rats 60 min after the administration of trilostane ( $10 \text{ mg kg}^{-1}$ , i.v.) or an equivalent volume ( $1 \text{ mL kg}^{-1}$ ) of vehicle (50% dimethylacetamide:50% polyethylene glycol 200). Data are expressed as the change in blood pressure ( $\Delta$  mean BP) from the baseline period 15–30 min prior to giving test compounds. Each point represents the mean and vertical lines represent the s.e. for groups of eight animals. (b) Plasma levels of aldosterone were measured in a subgroup ( $n = 4$ ) of the animals in (a). Plasma samples were prepared from blood obtained at the conclusion of the infusion of torcetrapib or vehicle. (c) Plasma levels of corticosterone were measured in a subgroup ( $n = 4$ ) of the animals in (a). Plasma samples were prepared from blood obtained at the conclusion of the infusion of torcetrapib or vehicle. DMSO, dimethyl sulphoxide.



**Figure 7** Exogenous aldosterone did not increase blood pressure. Mean arterial blood pressure was measured in pentobarbital (55 mg kg<sup>-1</sup>, i.p.)-anaesthetized rats that were administered aldosterone as sequential 30 min infusions at 1 and 10 µg kg<sup>-1</sup> or equivalent volumes of vehicle (saline). Fifteen minutes later, both groups of animals were administered torcetrapib (5 mg kg<sup>-1</sup>) as an i.v. infusion of 30 min duration. Each point represents the mean and vertical lines represent the s.e. for the numbers of animals indicated in parentheses.

anacetrapib was without effect at concentrations up to 10 µM. Attempts to measure corticosterone levels by enzyme immunoassay in isolated adrenal cells were not successful using torcetrapib, anacetrapib or angiotensin II as agonists.

## Discussion

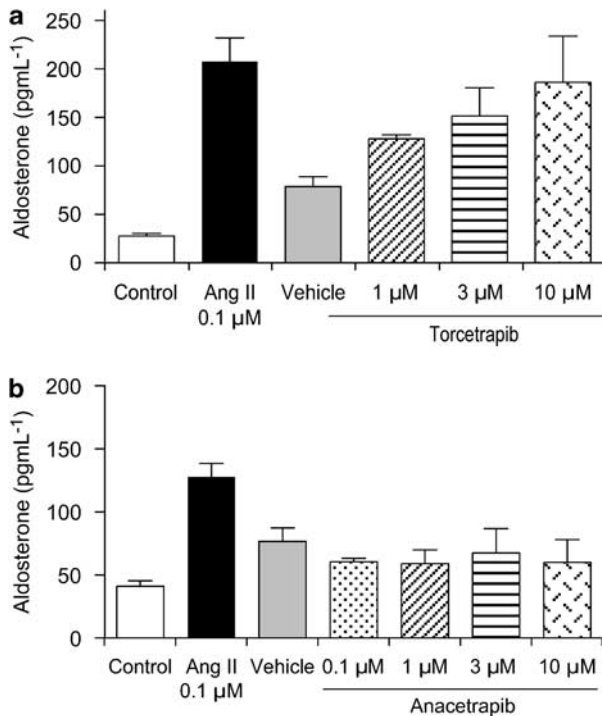
Reduction of circulating LDL levels through the use of statins is a mainstay of the pharmacological management of atherosclerosis. However, despite their unequivocal efficacy, there is still considerable room for additional cardiovascular risk reduction in patients. Raising plasma HDL levels is an attractive objective to complement LDL-lowering drugs, particularly in light of the strong epidemiological relationship between increased HDL levels and reduced cardiovascular risk. There exist many potential strategies by which levels of HDL can be increased. Recently, in phase I and II studies, CETP inhibitors produced robust dose-dependent elevation of HDL levels (Grooth *et al.*, 2002; Clark *et al.*, 2004). However, early clinical studies with the CETP inhibitor torcetrapib, either alone or in combination with atorvastatin, demonstrated mild increases in systolic and diastolic blood pressure in humans (Davidson *et al.*, 2006; McKenney *et al.*, 2006). More recently, a phase III study of torcetrapib in combination with atorvastatin was terminated prematurely after approximately 18 months when the incidence of serious cardiovascular events, including death, was greater in the torcetrapib-atorvastatin group compared with subjects receiving atorvastatin alone (Barter *et al.*, 2007). In this phase III study, there was also reported an increase in blood pressure and hypertensive adverse events in patients treated with torcetrapib. An evaluation of serum electrolytes revealed a decrease in serum potassium and increases in serum sodium and bicarbonate. The biochemical changes, along with the increase in blood pressure, suggested a possible mineralocorticoid excess that was further substantiated by determining increases in serum aldosterone. In the present study, we report an acute increase in blood

pressure following torcetrapib administration in a variety of preclinical animal species. Furthermore, we showed that torcetrapib administration caused an acute increase in plasma aldosterone and corticosterone levels in rats and that torcetrapib released aldosterone via a direct action on rat adrenocortical cells.

The blood pressure response to torcetrapib is independent of inhibition of CETP. This conclusion is derived from two lines of evidence. The first is from data obtained with normal mice that do not express CETP and a line of transgenic mice expressing simian CETP. Torcetrapib increases HDL levels in the CETP transgenic mice but not in normal mice. However, torcetrapib increased blood pressure to an equivalent extent in both normal and CETP transgenic mice. The second line of evidence is that anacetrapib raises HDL to an equivalent extent as torcetrapib in CETP transgenic mice and at similar exposure levels as torcetrapib but, in contrast to torcetrapib, anacetrapib has no effect on blood pressure in either strain of mouse.

Our observation that anacetrapib does not increase plasma adrenal steroids, at exposures that increase plasma HDL levels, strongly suggests that the mechanism of torcetrapib-induced adrenal steroid elevation is also not via a mechanism dependent on CETP inhibition. This was further substantiated by demonstrating that torcetrapib can directly release aldosterone from cultured primary rat adrenocortical cells *in vitro* in the absence of serum. Furthermore, anacetrapib had no effect on aldosterone release in this *in vitro* assay. The biochemical pathway(s) by which torcetrapib evokes aldosterone release are under investigation. Although the first biochemical step in the synthesis of aldosterone is the conversion of cholesterol to pregnenolone, the true rate-limiting step of hormone-stimulated steroidogenesis is the delivery of the substrate, cholesterol, to the inner mitochondrial membrane (Stocco and Clark, 1996). Whether torcetrapib is able to influence this transfer is not known but this would be a pathway worthy of further investigation.

We have demonstrated that administration of torcetrapib to rats evokes an acute increase in blood pressure accompanied by a rise in plasma adrenal steroids. We believe it is unlikely that the acute increase in blood pressure is mediated via aldosterone or corticosterone for the following reasons. First, as shown in Figure 7, acute administration of aldosterone (1 or 10 µg kg<sup>-1</sup>, i.v.) in rats had no effect on blood pressure under conditions where there was an acute blood pressure response to torcetrapib. Second, administration of the mineralocorticoid receptor antagonist eplerenone (20 mg kg<sup>-1</sup>, p.o.) did not reduce the pressor effect of torcetrapib (data not shown). Finally, administration of the 3β-hydroxysteroid dehydrogenase inhibitor, trilostane, completely prevented the torcetrapib-induced increase in plasma aldosterone and corticosterone but did not reduce the torcetrapib-induced increase in acute blood pressure (Figure 6). It remains to be determined whether chronic administration of torcetrapib to rats would have a sustained effect on increasing blood pressure or adrenal steroid levels. It is conceivable, that chronic elevation of aldosterone or corticosterone in response to torcetrapib administration would provide additional pathways for increasing blood pressure via either genomic or non-genomic pathways (Ullian, 1999; Schiffrin, 2006).



**Figure 8** Torcetrapib released aldosterone from isolated rat adrenal cells. Aldosterone release was measured from primary adrenocortical cells isolated from rat adrenal glands. Cells (~200 000 per sample) were incubated with angiotensin II (0.1 µM), torcetrapib (1–30 µM), anacetrapib (0.1–10 µM) or appropriate vehicle for 2 h at 37 °C. Following centrifugation, supernatants were assayed for aldosterone by an enzyme-linked immunoassay. Each bar represents the mean and vertical lines represent the s.e. for four samples.

Several lines of evidence indicate a relationship between elevated plasma aldosterone concentration, hypertension and increased cardiovascular events (Rocha and Funder, 2002; Vasan *et al.*, 2004; Connell and Davies, 2005). The relevance of the acute blood pressure elevation and increase in plasma aldosterone in response to torcetrapib reported herein to the blood pressure elevation and adverse cardiovascular events described in the ILLUMINATE trial is uncertain. Aldosterone is a major regulator of sodium and water homeostasis, thus contributing to the maintenance of normal blood volume and blood pressure. Increased aldosterone levels promote increased sodium reabsorption with subsequent facilitated potassium and hydrogen ion exchange, volume expansion and elevated blood pressure. These classical effects of aldosterone are mediated through binding of aldosterone to the well-studied mineralocorticoid receptor (NR3C2) in epithelial cells, primarily in the distal nephron of the kidney. More recently, it has become appreciated that there are non-classical aspects of aldosterone biology including synthesis at sites other than the adrenal cortex; the presence of mineralocorticoid receptors in non-epithelial tissues including heart, brain and blood vessels; and actions of aldosterone via non-genomic mechanisms (Rocha and Funder, 2002; Connell and Davies, 2005).

The strongest evidence that aldosterone is a contributor to adverse cardiovascular events comes from clinical trials with mineralocorticoid receptor antagonists. Two key studies, Randomized Aldactone Evaluation Study (Pitt *et al.*, 1999)

and Eplerenone Post-acute myocardial infarction Heart failure Efficacy and Survival study (Pitt *et al.*, 2003) demonstrated benefits of mineralocorticoid receptor blockade in patients with heart failure. Interestingly, in both studies, benefits were observed shortly after randomization, suggesting that aldosterone receptor blockade could be acting to limit cardiac electrical disturbances.

Collectively, a considerable body of evidence is available to support the concept that elevated plasma aldosterone concentration is associated with adverse cardiovascular effects and that reducing either the levels of aldosterone or the effects of aldosterone is beneficial. Whether the increased incidence of death and adverse cardiovascular events reported in the ILLUMINATE trial were attributable to the reported increase in plasma aldosterone is unknown. The studies reported herein have shown an acute increase in plasma aldosterone in rodents given torcetrapib. We are yet to determine whether aldosterone levels remain elevated with chronic administration of torcetrapib, and we do not know whether this phenomenon is observed across a broad range of species.

The mechanism for the acute blood pressure effect of torcetrapib remains to be determined. Torcetrapib did not have a direct action on vascular tone, as it did not contract isolated arterial vascular segments *in vitro* and did not enhance responses to other vasoactive agents. Torcetrapib also did not affect the vascular endothelial nitric oxide system as demonstrated by a lack of effect on ACh-induced relaxation in isolated vascular preparations. Possible effects of torcetrapib on smaller resistance vessels seem unlikely, given that torcetrapib did not increase pressure in an isolated perfused hindlimb preparation. Possible effects on cardiac output were evaluated using high-frequency ultrasound at the level of the ascending aorta. These studies showed no remarkable change in cardiac output of anaesthetized rats at doses where blood pressure is increased (data not shown). Collectively, these data indicate that the mechanism does not involve a direct effect on the cardiovascular system and is most likely via a hormonal or endogenous mediator system. Pharmacological studies have excluded activation of angiotensin AT<sub>1</sub> receptors, endothelin receptors or α<sub>1</sub>/α<sub>2</sub>-adrenoceptors. Possible central effects of torcetrapib were excluded because equivalent pressor effects of torcetrapib are present in both normal and pithed anaesthetized rats in which all central pathways are eliminated.

Administration of torcetrapib in a variety of animal models evoked an acute increase in blood pressure and in rats it was shown to increase plasma aldosterone and corticosterone levels. Whether either or both of these properties contributed to the adverse cardiovascular events reported in the ILLUMINATE trial is unclear. It is also unclear whether these properties of torcetrapib conspired against determining significant beneficial effects of torcetrapib on HDL and atherosclerotic plaque volume. In atherosclerosis-prone mice, mineralocorticoid receptor blockade with eplerenone not only reduced blood pressure but also significantly reduced atherosclerotic lesion area by 35% (Keidar *et al.*, 2003). Thus, it is possible that elevated aldosterone levels contribute to the development of atherosclerotic plaque. The question as to whether HDL elevation



through inhibition of CETP will be beneficial for the treatment of atherosclerosis remains open and should be evaluated with a CETP inhibitor that does not carry inherent cardiovascular liabilities.

## Conflict of interest

All authors of this manuscript are currently or previously (HV) were employees of Merck and Co., Whitehouse Station, NJ, USA.

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