Electronic Supplementary Material (ESI) for Chemical Communications

Allosteric inhibitors of HMG-CoA reductase, the key enzyme involved in cholesterol biosynthesis

Adi Haber[1], Amona Abu-Younis Ali[1], Michael Aviram[2], and Zeev Gross[1,*]

Material and Methods

General procedures

Materials

Metallocorroles were prepared according to previously described procedures,[1, 2] and metalloporphyrins were purchased from Frontier Scintific. Fluvastatin, NADPH, HMG-CoA, $NADP^+$, CoA and HMGCR were all purchased from Sigma-Aldrich. The HMGCR was supplied as the catalytic domain fused to the GST protein.[3] DMEM and FCS were purchased from Biological Industries LTD.

Cell cultures

Murine J774.A1 macrophages were cultured in DMEM containing 5% FCS in a humidified incubator.

Cholesterol biosynthesis measurement

Cellular cholesterol biosynthesis was measured as previously described with minor adjustments.[4] Briefly, cells were incubated overnight with DMEM containing 2% BSA, followed by incubation for 3 hours at 37 °C in DMEM containing 2% BSA and 1mCi/ml of [^{3}H]acetate or [^{14}C]mevalonolactone (Amersham International, Bucks, UK). Cellular lipids were then extracted in hexane:isopropanol (3:2, v:v), and the upper phase separated on silica gel plates with hexane:ether:acetic acid (80:20:1.5, v:v:v). Unesterified cholesterol spots were visualized by iodine vapor (using appropriate standard), scraped into scintillation vials and counted in a β-counter. 0.1M NaOH were added to the remains of the cells, and cellular proteins were measured the following day by the Lowry method.[5]

Cholesterol mass measurement

Cellular lipids were then extracted in hexane:isopropanol (3:2, v:v) and analyzed for total cholesterol by commercially available kits (Roche Diagnostics).

Corrole chemiluminescence measurement

Corrole chemiluminescence was measured as previously described.[6] Briefly, prior to chemiluminescent detection, cells were thoroughly washed with PBS, suspended in distilled water and frozen until measurement. Emission at 430 nm was followed in a kinetic detection mode after the addition of 1 mM luminol and 10 mM H_2O_2 (pH = 13) to the ruptured cells. For qualitative analysis the plots were integrated from t = 0 to t = 12 min and intracellular concentration determined relative to a standard curve.

HPLC separation of HMGCR reaction components

HPLC separation was performed on a Waters system equipped with a 600 controller, 717plus autosampler, 2996 photodiode array detector and a Millennium software. Samples were separated on a reverse phase GraceSmart RP18 (5µm, 250mm x 4.6mm) column with the detector set at 260 nm. A gradient of 10 mM ammonium acetate buffer pH 5.8-6 containing 10mM tetrabutyl ammonium acetate (solvent A) and acetonitrile (solvent B) were used at a flow rate of 1.5 mL/min: 10%-30% B up to 15 min, 30%-85% B up to 30 min and back to 10% B at 38 min.

HMGCR mRNA expression

Total RNA was extracted with TRI-reagent (Molecular Research Center, Inc.). Final total RNA concentration was determined spectrophotometrically by measuring the absorbency at 260 nm. cDNA was generated from 1 µg of total RNA using M-MLV reverse transcriptase (RT) (Promega) and random primers (Promega). The RT reaction was carried out at 37 °C for 60 min followed by 5 min at 95 °C. Products of the RT reaction were subjected to PCR amplification: first step at 94°C for 1 min, second step at 95°C for 1 min, third step at 55°C for 1 min, fourth step performed at 72°C for 1.5 min (steps 2, 3 and 4 were cycled for 35 times), and the last step was performed at 72°C for 8 min. The amplified transcripts were separated on a 1% (w/vol.) agarose gel containing ethidium bromide and visualized with ultraviolet transillumination.

Primer for the HMG CoA reductase:

Forward: 5′-GGGACGGTGACACTTACCATCTGTATGATG-3′

Reverse: 5′-ATCATCTTGGAGAGATAAAACTGCCA-3′.

Primers for the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH):

Forward: 5′-CTGCCATTTGCAGTGGCAAAGTGG-3′

Reverse: 5′-TTGTCATGGATGACCTTGGCCAGG-3′.

HMGCR protein expression and phosphorylation

HMGCR protein expression and phosphorylation and protein expression of the house keeping gene β-actin were detected by western blot analysis using cell lysates containing 20 µg protein on a 10% SDS-PAGE. The membrane was blocked with 2% BSA in TBST for 1.5 h, followed by treatment with a primary antibody (in TBST containing 1 % BSA) for 2 h and a secondary antibody (in TBST containing 1 % BSA) for 1 h, all in at room temperature. The membrane was then developed using an EZ-ECL kit. The antibodies used were as follows:

protein	primary antibody	secondary antibody
HMGCR	Goat anti HMGCR (1:300) Santa cruz	Rabbit anti goat (1:5000) Jackson Laboratories
Phospho-HMGCR	Rabbit anti Phospho-HMGCR (1:1000) Upstate	Goat anti rabbit (1:5000) Jackson Laboratories
β-actin	Mouse anti β-actin (1:5000)	Rabbit anti goat (1:10000)

	Sigma	Jackson Laboratories

Statistical analysis

Each separate experiment was performed in triplicate, and each individual experiment was replicated 3 times (n=3). Statistical analyses were performed using Student's t test for comparing differences between the 2 groups.

In vivo experiments

Mice treatment. The study protocol was approved by the Committee for the supervision of animal experiments of the Technion Israel Institute of Technology (approval number IL-046-04-2008) and was conducted in accordance with the guiding principles in the care and use of laboratory animals. At an age of 30 weeks, 10 male E^0 mice were divided randomly to 2 groups of 5 mice each. Treated mice received 10mg/kg of **1-Fe** within their drinking water. After 12 weeks the mice were sacrificed and heart with attached aorta and mouse peritoneal macrophages (MPM) were collected from all mice.

Mouse peritoneal macrophages (MPM). MPM were harvested 4 days after intra-peritoneal injection of 3 mL thioglycolate (40 g/L). The cells were washed with PBS, diluted to+ 10^6 cells/mL in DMEM supplemented with fetal calf serum, plated in 6-well plates and incubated at 5% CO_2 and 37 ºC. The next day, cholesterol biosynthesis and cholesterol content were measured as described.

Histopathology development of aortic atherosclerosis lesions. Heart and entire aorta were rapidly dissected out from each mouse and immersion-fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.01% calcium chloride, pH 7.4, at room temperature. The first 4 mm of the aortic arch was stained with osmium tetroxide, which colors all the lipid components a dark brown-black color thus enabling delineation of the lesion with greater accuracy. The blocks were embedded in epon resin and thin transverse sections were cut to allow greater resolution of the lesion details. The area covered by the lesion was determined by image analysis [7].

In vitro experiments

Dose dependent effect of 1-Fe on cholesterol biosynthesis in macrophages

J774.A1 macrophages ($2x10^6$ cells/well) were incubated for 24 h with or without 5, 20 or 50 μM of **1-Fe**. After washing the cells, cholesterol biosynthesis was determined. Intracellular corrole concentration was determined in parallel following the same treatment.

The effect of 1-Fe on HMGCR mRNA expression, protein expression and protein phosphorylation

J774.A1 macrophages ($2x10^6$ cells/well) were incubated for 24 h with or without 20 µM of **1-Fe**. After washing the cells HMGCR mRNA expression, HMGCR protein expression or HMGCR protein phosphorylation were determined.

The effects of potential inhibitors on HMG-CoA consumption by purified HMGCR

NADPH (400 µM) and HMG-CoA (200 µM) were mixed in activity buffer (100 mM sodium phosphate buffer containing 1 mM EDTA, 10 mM DTT, 2% DMSO, and 1 mM magnesium sulphate, pH 6.8). Fluvastatin (FS), **1-Fe**, **1-Ga**, **1-Mn** or FeTPPS were added to the substrate mixture at various concentrations, followed by addition of HMGCR (0.5 µM, that equals 380 µg/mL). Reactions were then incubated at 37 ºC for 15 min, followed by injection of 80 µl to the HPLC by the method described above. The yield of each reaction was calculated from the amount of CoA formed in the reaction after subtracting the baseline amount of CoA present as an impurity in the commercial HMG-CoA determined by injecting the substrate mixture alone. The inhibition was calculated from the yield of each reaction relative to the yield of the enzymatic reaction without the addition of inhibitor.

The effects of 1-Fe and 1-Ga on NADPH consumption by purified HMGCR

NADPH (400 µM) and HMGCR (0.1 µM, that equals 76 µg/mL) were mixed in activity buffer (100 mM sodium phosphate buffer containing 1 mM EDTA, 10 mM DTT, 2% DMSO, and 1 mM magnesium sulphate, pH 6.8). **1-Fe** or **1-Ga** were added to the mixture at concentration of 0, 1, 1.5 or 2 µM, and the reactions were initiated by the addition of HMG-CoA (25, 50, 100, 200, or 400 µM). Reaction kinetics was followed on a SpectraMax M2 microplate reader (Molecular Devices) for 10 min at 37 ºC by measuring the disappearance of the 340 nm absorbance characteristic of NADPH. The slope of the linear part of each curve was used for determining V_0, and Lineweaver-Burk plots allowed for identification of the inhibition mode.

Figures S1-S4

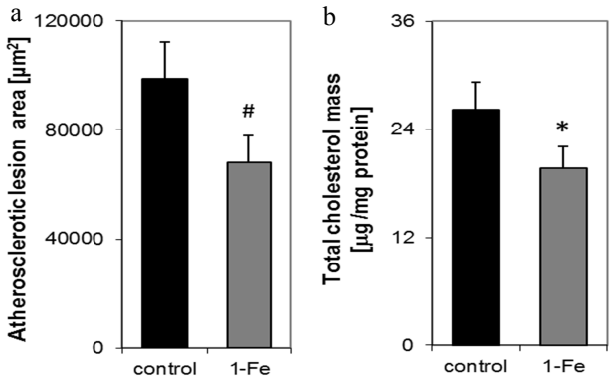

Fig. S1 The effect of oral administration of **1-Fe** on aged mice. Thirty weeks old mice were treated for 12 weeks with or without 10 mg/kg/day **1-Fe** within their drinking water. Variables determined at the end of treatment were (a) aortic arch lesion area, and (b) MPM total cholesterol mass. Results are mean (n=5) ± SD. $^{*}p < 0.01$ relative to control. $^{\#}p < 0.05$ relative to control.

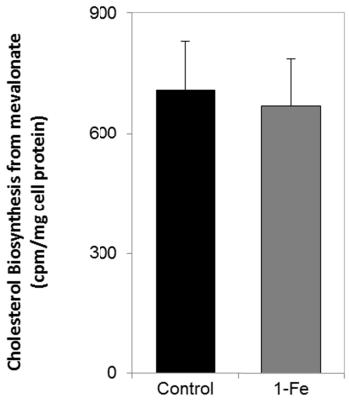

Fig. S2 The effect of **1-Fe** on macrophage cholesterol biosynthesis from mevalonate. J774.A1 macrophages were incubated for 24 h with or without 20 μM of **1-Fe**, washed to remove extracellular-remaining inhibitor, and cholesterol biosynthesis from mevalonate was determined.

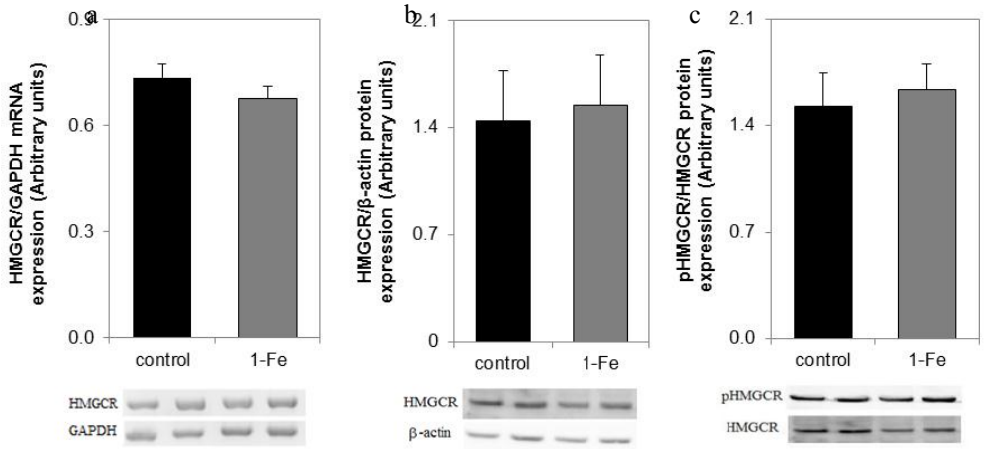

Fig. S3 The effect of **1-Fe** on HMGCR expression and phosphorylation. J774.A1 macrophages were incubated for 24 h with or without 20 μM of **1-Fe**, washed to remove extracellular-remaining corrole, and then HMGCR mRNA expression (a), HMGCR protein expression (b) or HMGCR protein phosphorylation (c) were determined.

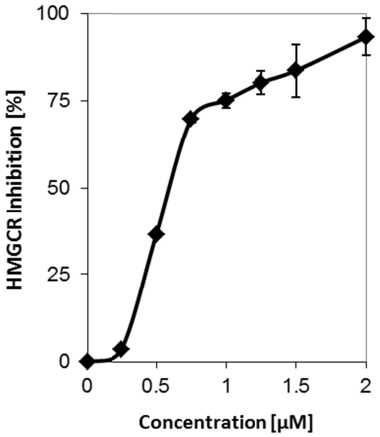

Fig. S4 The dose-dependent effect of **1-Fe** on the isolated HMGCR-catalyzed reaction.

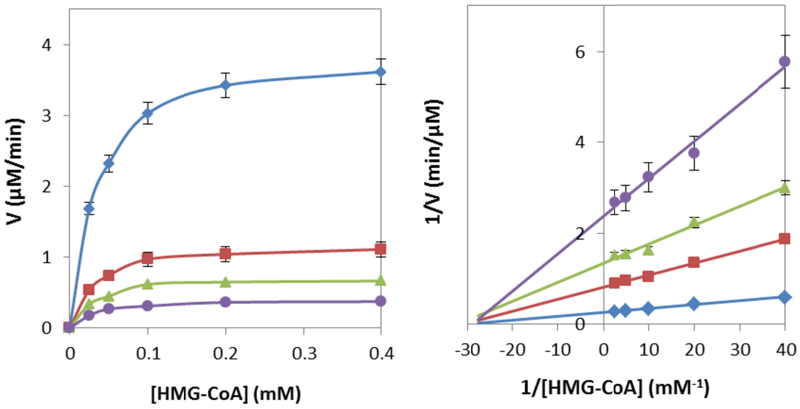

Fig. S5 Michaelis-Menten (a) and Lineweaver-Burk (b) plots for the kinetics of the isolated HMGCR-catalyzed reaction in the presence of **1-Ga**. NADPH (400 µM) and HMGCR (0.1 µM, 76 µg/mL) were mixed with **1-Ga** at: 0 µM (blue diamonds), 1 µM (red squares), 1.5 µM (green triangles), or 2 µM (purple circles). Reactions were initiated by the addition of HMG-CoA (25, 50, 100, 200, or 400 µM), and kinetics followed at 340 nm for 10 min at 37 ºC. Results are mean (n=4) ± SD.

References

1. A. Mahammed and Z. Gross, *Angew. Chem. Int. Ed.*, 2006, **45**, 6544.
2. I. Saltsman, A. Mahammed, I. Goldberg, E. Tkachenko, M. Botoshansky and Z. Gross, *J. Am. Chem. Soc.*, 2002, **124**, 7411.
3. J. P. Perchellet, E. M. Perchellet, K. R. Crow, K. R. Buszek, N. Brown, S. Ellappan, G. Gao, D. Luo, M. Minatoya and G. H. Lushington, *Int. J. Mol. Med.*, 2009, **24**, 633.
4. B. Fuhrman, A. Elis and M. Aviram, *Biochem. Biophys. Res. Comm.*, 1997, **233**, 658.
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.
6. A. Haber, M. Aviram and Z. Gross, *Inorg. Chem.*, 2012, **51**, 28.
7. R. Coleman, T. Hayek, S. Keidar and M. Aviram, *Acta histochemica*, 2006, **108**, 415.