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Effect of gelatinase inhibition on adipogenesis and adipose tissue development

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SUMMARY

1. The potential of the matrix metalloproteinase (MMP) inhibitor ABT-518 to affect pre-adipocyte differentiation *in vitro* and adipose tissue development *in vivo* was investigated using mouse models of adipogenesis and obesity.

2. Differentiation of 3T3-F442A pre-adipocytes into mature adipocytes was enhanced in a dose-dependent manner by the addition of ABT-518 (0–100 μmol/L). This was associated with increased expression of the adipogenic markers adipocyte fatty acid-binding protein 2 (AP2), peroxisome proliferator-activated receptor γ and adiponectin.

3. Feeding 5-week-old male wild-type mice with a high-fat diet, with or without ABT-518 (to achieve a dose of 100 mg/kg per day), for 16 weeks resulted in a significant reduction in bodyweight throughout the experimental period. Magnetic resonance spectroscopy revealed that the lipid : water ratio was significantly lower in ABT-518-treated mice. The total weight of isolated subcutaneous or gonadal fat depots did not differ significantly following ABT-518 treatment, but adipocyte and blood vessel size were significantly reduced in the gonadal fat.

4. Administration of ABT-518–2 (100 mg/kg per day for 10 weeks) to 5-week-old male wild-type mice with established obesity maintained on a high-fat diet had no effect on total bodyweight at the end of the experiment, but was associated with reduced blood vessel size in the fat depots.

5. Thus, the MMP inhibitor ABT-518 stimulates differentiation of 3T3-F442A pre-adipocytes *in vitro*. It mildly reduces bodyweight gain in a murine model of diet-induced obesity, but does not affect established obesity.

Key words: adipocyte, adipogenesis, adipose tissue, gelatinase, matrix metalloproteinase, matrix metalloproteinase inhibitor, obesity.

INTRODUCTION

The matrix metalloproteinase (MMP) family of zinc-dependent endopeptidases comprises over 20 members in humans and is counterbalanced by four tissue-specific inhibitors of MMPs (TIMP-1–4).[1] Several recent studies have suggested a functional role of the MMP system in obesity, by affecting adipogenesis, angiogenesis and/or extracellular maxtrix (ECM) remodelling.[2,3]

Thus, a deficiency of MMP-3 (stromelysin-1), MMP-11 (stromelysin-3) or MMP-19 in mice leads to enhanced development of adipose tissue when the mice are fed a high-fat diet.[4–6] In contrast, a deficiency of MMP-14 (membrane type 1 MMP) and MMP-2 (gelatinase A) leads to impaired adipose tissue development[7,8] and deficiency of MMP-9 (gelatinase B) or MMP-10 (stromelysin-2) has no apparent effect on adiposity.[9,10] Furthermore, TIMP-1-deficient mice on a high-fat diet gain less weight than their wild-type littermates and developed less adipose tissue.[11] In contrast, TIMP-2-deficient mice gained more weight than wild-type controls, both on standard chow and a high-fat diet.[12]

Previous studies have shown the potential to impair adipose tissue development in mice by using broad-spectrum MMP inhibitors.[3] Furthermore, the relatively gelatinase-specific inhibitor Tolylsam (Shionogi Research Laboratories, Osaka, Japan) has been shown to reduce bodyweight and adipose tissue mass in a nutritionally induced obesity model in young wild-type mice, as well as in leptin-deficient (*ob/ob*) mice.[8,13] A functional role of gelatinases was also suggested in the differentiation of pre-adipocytes into mature adipocytes.[14] Thus, the available evidence suggests a functional role for gelatinases (mainly MMP-2) in adiposity, although no real specific inhibitors were available at the time the studies were performed. In the present study, we wanted to show the effect of ABT-518, an MMP inhibitor with high selectivity and potency against gelatinases (IC_{50} 0.78 nmol/L for MMP-2 and 0.5 nmol/L for MMP-9), on adipogenesis and adipose tissue development in mouse models of obesity. Our aim was to determine whether the gelatinases promote the development of adipose tissue *in vivo*. Furthermore, we hypothesised that gelatinase inhibition would have the potential to induce regression of adipose tissue in mice with established obesity.

METHODS

Matrix metalloproteinase inhibitory activity of ABT-518

ABT-518 (*N*-[1(*S*)-[2,2-dimethyl-1,3-dioxolan-4(*S*)-yl]-2-[4-[4-(trifluoromethoxy) phenoxyl]phenylsulphonyl]ethyl]-*N*-hydroxyformamide)[15] was custom synthesized at Oxygen Healthcare (Cambridge, UK) and dissolved in dimethylsulphoxide (DMSO; final concentrations 0–5 nmol/L). Recombinant murine MMP-2 was obtained from Abcam (Cambridge, UK) and the MMP-2 fluorogenic substrate M-2350 (Mca-Lys-Pro-Leu-Gly-Leu-Dap (Dnp)-Ala-Arg-NH_2) was obtained from Bachem (Bubendorf, Switzerland).

The *in vitro* inhibitory potential of ABT-518 was confirmed by measuring the K_i value against murine MMP-2. Briefly, murine MMP-2 (final concentration 0.4 nmol/L) was pre-incubated with ABT-518 (final concentration

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0–5 nmol/L) for 5 min to construct a calibration curve. The M-2350 was then added to a final concentration of 10 μmol/L and initial rates of substrate hydrolysis were determined by monitoring the increase in fluorescence (λ_{ex} = 328 nm; λ_{em} = 393 nm). The K_i value, computed from non-linear regression analysis using the Morrison equation (Eqn 1) for tight binding inhibitors[16] was found to be approximately 70 pmol/L. All measurements were performed at 37°C in TNCB buffer (50 mmol/L Tris, 10 mmol/L $CaCl_2$, 150 mmol/L NaCl, 0.05% w/v Brij-35, pH 7.5).

$$v_i = v_o\left[1 - \frac{([E] + [I] + K_i) - \sqrt{([E] + [I] + K_i)^2 - 4[E][I]}}{2[E]}\right] \quad (1)$$

where [*E*] and [*I*] are the concentrations of enzyme and ABT-518, respectively. The presence of free (unbound) ABT-518 in plasma or adipose tissue extracts of ABT-518-treated mice was assayed as follows. Mice were treated for three consecutive days with 100 mg/kg per day ABT-518 mixed with their food. Plasma samples were collected at four different times during the day (0930, 1200, 1600 and 2000 h). Extracts were prepared from adipose tissues collected from mice treated with ABT-518 (100 mg/kg per day) for 4 weeks or from control mice (see below). A 500 μL aliquot of the samples was dialyzed against 750 μL TCNB+ (50 mmol/L Tris, 50 mmol/L CaCl2, 150 mmol/L NaCl, 0.05% w/v Brij-35, pH 7.5) using rapid equilibrium dialysis. The presence of ABT-518 in the dialysate was then detected by its ability to inhibit MMP-2 (final concentration 0.6 nmol/L) in the activity assay described above using different sample dilutions. On average, the concentration of free circulating ABT-518 in the plasma was in the range 50–170 nmol/L (25–85 ng/mL) range. In adipose tissue extracts, concentrations of active ABT-518 ranged between 150 and 180 nmol/L for SC and between 80 and 180 nmol/L for GON fat.

Cell culture

Murine 3T3-F442A preadipocytes were grown in basal medium (Dulbecco's modified Eagle's medium (DMEM); Invitrogen, Paisley, UK) supplemented with 10% bovine calf serum (BCS; iron supplemented; Hyclone, Logan, UT, USA) and 1% PenStrep (Invitrogen). Cells were passaged when preconfluent. To induce differentiation, cells were seeded at a density of 10×10^3 cells/cm^2 and grown to confluency in basal medium in an atmosphere of 95% humified air–5% CO2 at 37°C. The culture medium was replaced with fresh medium every 2 days, until Day 12. Experimental Day 0 was defined as the day when the cells reached confluence. After 2 days, cells were induced to differentiate for 48 h with induction medium (DMEM supplemented with 10% BCS, 17 nmol/L insulin, 2 nmol/L tri-iodothyronine (T_3), 100 nmol/L dexamethasone (DEX) and 100 μmol/L methylisobutylxanthine (IBMX)). On experimental Day 4, cultures were switched to differentiation medium (DMEM with 10% BCS, 17 nmol/L insulin and 2 nmol/L T_3). Alternatively, post-confluent cells were incubated in medium containing different concentrations of the synthetic MMP inhibitor ABT-518 (0–100 μmol/L), diluted in dimethylsulphoxide (DMSO), or containing DMSO only (final concentration 1/1000). Every 2 days during the differentiation procedure, samples were collected from independently prepared vials of cells and used for mRNA expression analysis.

On experimental Day 12, the extent of differentiation was assessed by quantification of Oil Red O uptake by lipid-containing cells. Cells were washed with phosphate-buffered saline (PBS), fixed in 1.5% glutaraldehyde in PBS for 5 min, stained for 3 h with a 0.2% Oil Red O solution (Sigma-Aldrich, Bornem, Belgium), washed and kept in tissue culture water. The stained fat droplets in the cells were visualized by light microscopy and photographed. For spectrophotometric quantification of lipid accumulation, the Oil Red O dye was extracted with isopropanol in a 96-well plate and the absorbance of the solution was read at 510 nm on an EL808 plate reader using KC4 DATA ANALYSIS software (Bio-tek Instruments, Winooski, VT, USA). Cellular viability was assessed by a Trypan blue dye exclusion assay. Briefly, on experimental Day 12, cells were trypsinized and stained with 0.4% Trypan blue. The number of blue cells was counted and is expressed as a percentage of total cells.

Analysis of mRNA expression

DNA-free total RNA was extracted from 3T3-F442A cells using the RNeasy kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total RNA samples were stored at −80°C. Complementary DNA was prepared from total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The polymerase chain reaction (PCR) was performed from 10 ng/μL total RNA at 25°C for 10 min, followed by amplification at 48°C for 1 h and finally 5 min at 95°C. Quantitative real-time PCR was performed in the ABI 7500 Fast Sequence detector using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Expression of the different adipocyte factors was determined using the primers and 6-carboxy-fluorescein (FAM)-labelled probes or gene assays (Applied Biosystems) listed in Table 1.

Transcript levels were determined in duplicate and normalized against the house-keeping gene β-actin. Data were obtained as cycle threshold (Ct) values

Table 1 Primers and probes used for mRNA expression analysis

Gene	Primer (5′–3′)	Probe	Applied Biosystems code
AP2	Forward: ccttcaaactgggcgtgg Reverse: cgttttctctttattgtggtcgact	atgctcttcaccttcctgtcgtctgcg	
PPARγ	Forward: ctgtcggtttcagaagtgcct Reverse: atctccgccaacagcttctc	cccaaacctgatggcattgtgagaca	
Pref-1	Forward: aaccatggcagtgcatctg Reverse: agcattcgtactggcctttc	aaatagacgttcgggcttgcacctc	
Adiponectin			Mm00456425_m1
MMP-2			Mm00439498_m1
MMP-9			Mm00442991_m1
MMP-14			Mm01318969_g1
TIMP-1			Mm00441818_m1
TIMP-2			Mm00441825_m1
VEGF-A			Mm00437304_m1
β-Actin			Mm00607939_s1

AP2, adipocyte fatty acid-binding protein 2; PPARγ, peroxisome proliferator-activated receptor γ; MMP, matrix metalloproteinase; TIMP, Tissue-specific inhibitor of metalloproteinase.

and are expressed as the copy number of target mRNA relative to 10^5 copies of β-actin.

Obesity models

For the diet-induced obesity (DIO) model, 5-week-old male mice (C57Bl/6 genetic background) were housed in individual microisolation cages on a 12 h light–dark cycle. All mice had *ad libitum* access to drinking water and a high-fat diet (HFD; TD 88137; Harlan Teklad, Zeist, the Netherlands; 42% kcal as fat, 20.1 kJ/g) for 16 weeks, with (n = 10) or without (n = 10) the addition of 100 mg/kg per day ABT-518. Bodyweight and food intake were measured between 0800 and 1000 h at weekly intervals. Body temperature was measured at 0800 h (three times at 2 weekly intervals) using a rectal probe (TR-100; Fine Science Tools, Foster City, CA, USA). Physical activity was measured 1 week before the end of the experiment over three consecutive days after a 1 day acclimatization period. To this end, mice were placed in individual cages equipped with a turning wheel linked to a computer to record full turns at night (1900–0700 h). Data were averaged per mouse and are given as the mean±SEM for the number of animals studied. Mice were anaesthetized after overnight fasting by intraperitoneal injection (between 0800 and 1000 h) of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL, USA). Blood was collected immediately via the retro-orbital sinus on trisodium citrate (final concentration 0.01 mol/L) and plasma was stored at −80°C. Gonadal (GON) and inguinal subcutaneous (SC) adipose tissue was removed and weighed. Paraffin sections (10 μm) were prepared for histology. Triglycerides in the faeces (collected at in the morning) and in liver extracts, prepared from equivalent amounts of tissues at the end of the experiments, were measured using the Triglycerides FS kit (DiaSys Diagnostic Systems, Holzheim, Germany). Apoptosis was monitored by staining adipose tissue sections with rabbit anti-active mouse caspase 3 (R&D Systems, Minneapolis, MN, USA). The size and density of adipocytes were determined by staining with haematoxylin–eosin under standard conditions. Blood vessels were stained using the biotinylated Bandeiraea (Griffonia) Simplicifolia BSI lectin (Sigma-Aldrich), followed by signal amplification with the Tyramide Signal Amplification Cyanine System (Perkin Elmer, Boston, MA, USA).[17] Blood vessel density was normalized against adipocyte number. Analysis was performed using a Zeiss Axioplan 2 microscope with the AXIOVISION REL. 4.6 software (Carl Zeiss, Oberkochen, Germany). Other organs, including kidneys, lungs, spleen, pancreas, liver and heart, were also removed and weighed.

Blood glucose concentrations were measured using Glucocard Strips (Menarini Diagnostics, Firenze, Italy). Levels of triglycerides, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein–cholesterol (LDL-C) and liver enzymes, namely alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), in the plasma were determined using standard laboratory assays. Insulin (Mercodia, Uppsala, Sweden) and leptin (R&D Systems, Oxon, UK) levels were determined using commercially available ELISA.

For the diet-established obesity (DEO) model, ABT-518 (100 mg/kg per day in HFD) was administered to obese mice (previously kept on the HFD for 20 weeks) over a period of 10 weeks to determine whether regression of adipose tissue mass could be achieved. Mice were killed and analysed as described above.

All animal experiments were approved by the local Ethics Committee of Katholieke Universiteit Leuven (Project P0622, KULeuven, Leuven, Belgium) and performed in accordance with the EU Directive 86/609 EEC and the guidelines of the International Society of Thrombosis and Haemostasis.[18]

Magnetic resonance experiments

Magnetic resonance (MR) studies were performed using a Bruker Biospec 9.4 Tesla small animal MR scanner (Bruker Biospin, Ettlingen, Germany) equipped with an actively shielded gradient set of 600 mT/m using a 0.2 cm quadrature resonator (Bruker Biospin). Mice were anaesthetized using isofluorane. Temperature and respiration were monitored and maintained at 37°C and > 60 /min, respectively. After acquisition of a localization MR image consisting of orthogonal slices to confirm central positioning of the animals in the magnet and resonator, a single pulse [^{1}H]-MR spectrum was acquired (repetition time 15 s, one average, receiver gain identical for all animals). The total protocol took < 2 min per animal. The MR spectra were processed using TOPSPIN software (Bruker Biospin). Relative lipid : water ratios were determined after phase and baseline correction using the water resonance at 4.7 p.p.m. and the lipid CH_2 resonance at 1.3 p.p.m. Absolute integral values were also compared.

Statistical analysis

Data are expressed as the mean±SEM. Differences between two groups were analysed with the non-parametric Mann–Whitney U-test, compatible with small sample sizes (n = 10). Comparison of progress curves and evaluation of the effect of time or treatment were performed by two-way repeated-measures ANOVA. Correlation analysis was performed using the non-parametric Spearman rank correlation test. Analyses were performed using PRISM 4 (GraphPad Software, San Diego, CA, USA). Two-sided $P < 0.05$ was considered significant; for ANOVA analysis, Bonferroni correction was applied for the vertical pairwise contrasts.[19]

RESULTS

Effect of ABT-518 on *in vitro* adipocyte differentiation

A dose–response curve of the effect of ABT-518 (0–100 μmol/L) on the differentiation of 3T3-F442A pre-adipocytes, revealed enhanced differentiation with a maximum at 25 μmol/L ABT-518, as monitored by Oil Red O staining: at 10, 25 and 50 μmol/L ABT-518, absorbance at 510 nm increased by 1.5 ± 0.1-, 1.9 ± 0.2- and 1.3 ± 0.1-fold compared with control, respectively (n = 3; all $P < 0.05$, non-parametric Mann–Whitney U-test). The addition of equivalent DMSO concentrations had no effect on 3T3-F442A pre-adipocyte differentiation. In contrast, the addition of 100 μmol/L ABT-518 resulted in 3.3 ± 0.8-fold reduction (P = 0.002) in Oil Red O staining. Trypan blue staining revealed that this was due to enhanced apoptosis at 100 μmol/L ABT-518 (66% dead cells vs 15% in the control and DMSO vehicle groups). At lower concentrations, ABT-518 was not cytotoxic for 3T3-F442A cells.

During differentiation in the presence of 25 μmol/L ABT-518, expression of adiponectin, adipocyte fatty acid-binding protein 2 (AP2) and peroxisome proliferator-activated receptor (PPAR) γ increased with time, whereas that of pre-adipocyte factor 1 (Pref-1) decreased. At the end of differentiation (Day 12), cells treated with 25 μmol/L ABT-518 were exhibiting significantly greater expression of AP2, PPARγ and adiponectin ($P < 0.05$, non-parametric Mann–Whitney U-test), whereas the expression of Pref-1 was comparable to that in control cells (Fig. 1). Analysis by two-way repeated-measures ANOVA of the evolution with time revealed a significant effect of 25 μmol/L ABT-518 on the expression of Pref-1 (P = 0.02), PPARγ ($P < 0.0001$), adiponectin (P = 0.0001) and AP2 (P = 0.0002). After Bonferroni correction (for seven comparisons), the effect of treatment remained significant for the expression of PPARγ ($P < 0.001$), adiponectin ($P < 0.001$) and AP2 ($P < 0.002$). Monitoring of the time-course of the expression of MMPs and TIMPs revealed that expression of TIMP-1, TIMP-2, MMP-2 and MMP-14 at the end of the experiment was downregulated by 25 μmol/L ABT-518, whereas that of vascular endothelial growth factor (VEGF-A) was upregulated (Fig. 2). Analysis by two-way repeated-measures ANOVA of the evolution with time revealed a significant effect of 25 μmol/L ABT-518 on the expression of MMP-2 ($P < 0.0001$), MMP-9 (P = 0.02), MMP-14 ($P < 0.0001$), TIMP-1 (P = 0.005), TIMP-2

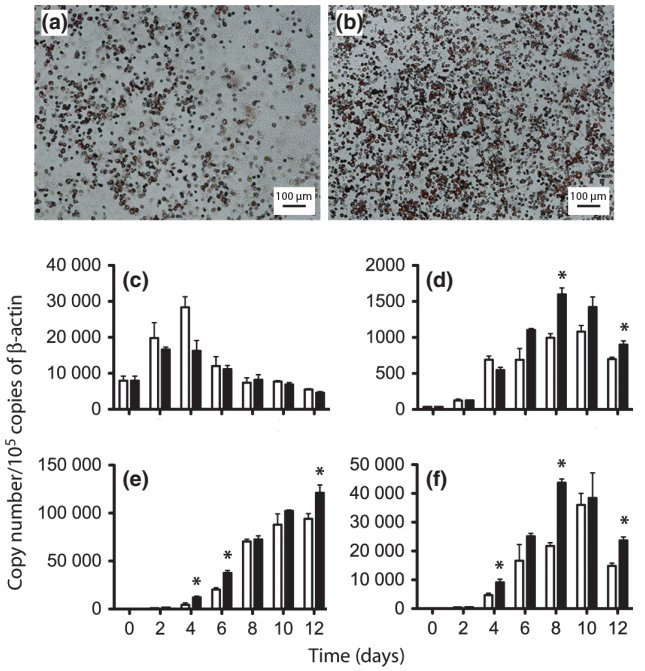

Fig. 1 Effect of ABT-518 on the extent of *in vitro* differentiation of 3T3-F442A pre-adipocytes into mature adipocytes. (a, b) Oil Red O staining at Day 12 of differentiation without (a) or with (b) the addition of 25 µmol/L ABT-518. (c–f) Time-course of the expression of preadipocyte factor 1 (Pref-1; c), peroxisome proliferator-activated receptor (PPAR) γ (d), adiponectin (e) and adipocyte fatty acid-binding protein 2 (AP2; f) during differentiation in the presence of 25 µmol/L ABT-518 (■) or dimethylsulphoxide vehicle (DMSO: □). Data are the mean±SEM of three experiments. *$P < 0.05$ compared with DMSO (non-parametric Mann–Whitney *U*-test).

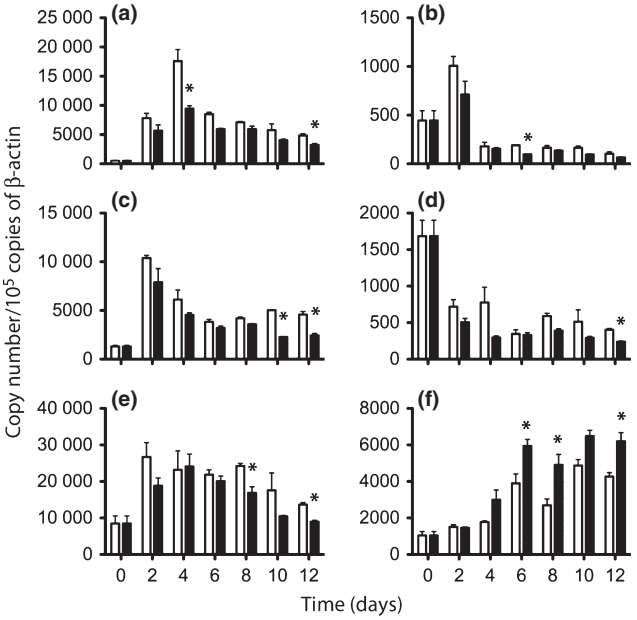

Fig. 2 Time-course of the expression of (a) matrix metalloproteinase (MMP)-2, (b) MMP-9, (c) MMP-14, (d) tissue-specific inhibitors of metalloproteinases (TIMP)-1, (e) TIMP-2 and (f) vascular endothelial growth factor (VEGF-A) during differentiation of 3T3-F442A pre-adipocytes in the presence of 25 µmol/L ABT-518 (■) or dimethylsulphoxide vehicle (DMSO: □). Data are the mean±SEM of three experiments. *$P < 0.05$ compared with DMSO (non-parametric Mann–Whitney *U*-test).

($P = 0.007$) and VEGF-A ($P < 0.0001$). After Bonferroni correction (seven comparisons), the effect of treatment remained significant for MMP-2 ($P < 0.001$), MMP-14 ($P < 0.001$), TIMP-1 ($P < 0.05$), MMP-2 ($P < 0.05$) and VEGF-A ($P < 0.001$).

Effect of ABT-518 on adipose tissue development in the DIO model

A pilot study of the administration of 10 mg/kg per day ABT-518 to 5-week-old wild-type mice for 15 weeks ($n = 10$) did not reveal significant differences compared with controls ($n = 9$). For example, total bodyweight in ABT-518-treated mice increased from 24.2 ± 0.4 to 44.3 ± 1.0 g, compared with an increase from 24.1 ± 0.4 to 44.8 ± 1.1 g in the control group. Food intake was not affected by ABT-518 either (3.17 ± 0.03 vs 3.06 ± 0.03 g/day in the control group). Furthermore, ABT-518-treated and control mice had comparable weights of both the SC (1650 ± 45 vs 1630 ± 54 mg, respectively) and GON (2270 ± 81 vs 2300 ± 52 mg, respectively) fat depots. The weight of the other organs was also very similar between the two groups (data not shown).

To further establish a potential effect of ABT-518 on *in vivo* adipose tissue, the dose was increased to 100 mg/kg per day. Mice treated with 100 mg/kg per day ABT-518 gained somewhat less weight compared with the control group (20.6 ± 1.1 vs 23.5 ± 0.7 g, respectively; $n = 10$ in each group; $P = 0.08$, non-parametric Mann–Whitney *U*-test; Table 2). Analysis by two-way repeated-measures

Table 2 Effect of ABT-518 (100 mg/kg per day) on adipose tissue and organ weights of young mice kept on a high-fat diet (HFD) for 16 weeks (diet-induced obesity model) or obese mice kept on an HFD for 10 weeks (diet-established obesity model)

	DIO model		DEO model	
	Control	ABT-518	Control	ABT-518
Bodyweight (g)				
At start	21.0 ± 0.5	21 ± 1	44 ± 1	44 ± 1
At end	45 ± 1	41 ± 2	51 ± 1	50 ± 2
SC fat (g)	1.6 ± 0.1	1.6 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
GON fat (g)	2.4 ± 0.1	2.1 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
Lungs (mg)	190 ± 14	200 ± 11	220 ± 14	190 ± 10
Spleen (mg)	120 ± 10	110 ± 3	170 ± 20	120 ± 4
Heart (mg)	150 ± 6	150 ± 4	170 ± 9	170 ± 4
Pancreas (mg)	330 ± 16	330 ± 10	400 ± 24	380 ± 11
Liver (g)	4.1 ± 0.3	3.3 ± 0.2*	5.2 ± 0.2	4.8 ± 0.3
Kidneys (mg)	440 ± 13	460 ± 11	530 ± 18	500 ± 17

Data are the mean±SEM of 10 experiments in each group. *$P < 0.05$ compared with controls (non-parametric Mann–Whitney *U*-test).

DIO, diet-induced obesity (5-week-old mice kept on an HFD for 16 weeks); DEO, diet-established obesity (5-week-old mice first fed the HFD for 20 weeks to establish obesity and then maintained for a further 10 weeks on the HFD); SC, inguinal subcutaneous adipose tissue; GON, gonadal adipose tissue.

ANOVA of the evolution of bodyweight with time (Fig. 3a) revealed a significant effect of 100 mg/kg per day ABT-518 ($P < 0.0001$ vs control). This analysis further confirmed a significant effect of time on the difference in bodyweight ($P < 0.0001$), but no interaction

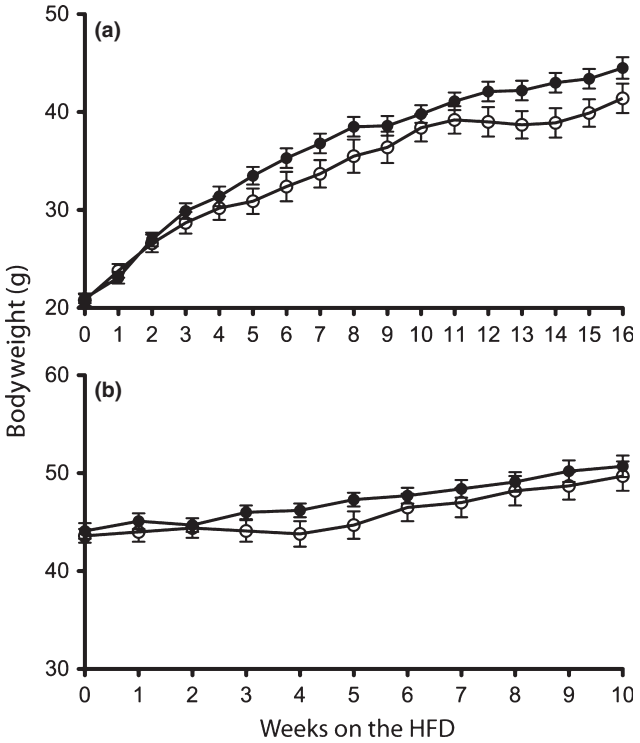

Fig. 3 Effect of ABT-518 (100 mg/kg per day) on the bodyweight of (a) young mice kept on a high-fat diet (HFD) for 16 weeks (diet-induced obesity model) or (b) obese mice maintained on the HFD for a further 10 weeks (diet-established obesity model). (○), ABT-518-treated mice; (●), placebo-treated controls. Data are the mean±SEM of 10 experiments in each group.

between time and dose ($P = 0.80$). After Bonferroni correction (for 17 comparisons), the difference remained significant ($P < 0.002$). Magnetic resonance spectroscopy after 13 weeks confirmed a significantly lower lipid : water ratio following inhibitor treatment compared with the control group (58 ± 6% vs 73 ± 4%, respectively; $P < 0.0001$, non-parametric Mann–Whitney *U*-test). Although absolute quantification was not intended, arbitrary units for the integration of the MR spectra indicate only marginal variability for the water signals ($2.00 \pm 0.12 \times 10^8$ vs $1.96 \pm 0.12 \times 10^8$ for control and inhibitor treatment, respectively) but tentatively lower values for the lipid signal intensities in treated animals ($1.47 \pm 0.11 \times 10^8$ vs $1.15 \pm 0.13 \times 10^8$ for control and inhibitor treatment, respectively; $P < 0.0001$). Lipid : water ratios were correlated with leptin levels in ABT-518-treated mice ($r = 0.68$; $P = 0.03$), but not in the control group.

Food intake throughout the experimental period was not affected by the high dose of ABT-518 compared with control (3.17 ± 0.03 vs 3.06 ± 0.03 g/day, respectively). Furthermore, physical activity (4900 ± 980 vs 5300 ± 1250 turns/12 h for ABT-518-treated vs control mice, respectively) and body temperature (36.0 ± 0.2 vs 35.8 ± 0.1°C for ABT-518-treated vs control mice, respectively) were comparable between the two groups. Similarly, faecal triglyceride content did not differ significantly between ABT-518-treated and control mice (10 ± 1 vs 9.9 ± 1.1 mg/g, respectively). However, triglyceride levels in liver extracts were significantly lower in ABT-518-treated mice than in the control group (19 ± 3 vs 31 ± 1 mg/g tissue, respectively; $P = 0.002$, non-parametric Mann–Whitney *U*-test). A weak positive correlation was found between liver triglycerides and liver weight ($r = 0.42$; $P = 0.07$) and

Table 3 Effect of ABT-518 (100 mg/kg per day) on adipocyte and blood vessel size and density in adipose tissue of young mice kept on a high-fat diet (HFD) for 16 weeks (diet-induced obesity model) or obese mice kept on an HFD for 10 weeks (diet-established obesity model)

	DIO model		DEO model	
	Controls	ABT-518	Controls	ABT-518
Adipocyte size (μm^2)				
SC fat	4100 ± 110	4500 ± 160	4400 ± 180	4500 ± 160
GON fat	7500 ± 160	6600 ± 330*	6300 ± 260	6000 ± 300
Adipocyte density ($\times 10^{-6}/\mu m^2$)				
SC fat	250 ± 7	230 ± 9.0	240 ± 9	230 ± 8
GON fat	130 ± 3	160 ± 10*	160 ± 8	170 ± 9
Blood vessel size (μm^2)				
SC fat	42 ± 2	48 ± 2	46 ± 2	39 ± 2*
GON fat	52 ± 2	46 ± 4*	58 ± 3	48 ± 3*
Blood vessel density ($\times 10^{-6}/\mu m^2$)				
SC fat	370 ± 7	340 ± 8	340 ± 17	310 ± 12
GON fat	250 ± 9	210 ± 6	260 ± 10	260 ± 15
Normalized blood vessel density				
SC fat	1.48 ± 0.05	1.46 ± 0.04	1.46 ± 0.09	1.34 ± 0.06
GON fat	1.85 ± 0.06	1.34 ± 0.06**	1.75 ± 0.08	1.51 ± 0.07*
Apoptotic cell density ($\times 10^{-6}/\mu m^2$)				
SC fat	3.5 ± 0.5	4.9 ± 0.6	8.0 ± 1.5	7.5 ± 1.3
GON fat	3.6 ± 0.4	3.8 ± 0.7	7.1 ± 1.2	11 ± 1*

Data are the mean±SEM of 10 experiments in each group. *$P < 0.05$, **$P < 0.0005$ compared with controls (non-parametric Mann–Whitney *U*-test).

DIO, diet-induced obesity (5-week-old mice kept on an HFD for 16 weeks); DEO, diet-established obesity (5-week-old mice first fed the HFD for 20 weeks to establish obesity and then maintained for a further 10 weeks on the HFD); SC, inguinal subcutaneous adipose tissue; GON, gonadal adipose tissue.

total liver triglyceride content remained lower in ABT-518-treated compared with control mice (68 ± 13 vs 125 ± 5 mg, respectively; $P = 0.001$, non-parametric Mann–Whitney *U*-test).

There were no significant differences in SC and GON fat mass between the two groups and the weight of the other organs (except liver weight) was not affected by ABT-518 treatment (Table 2).

Histological analysis of adipose tissue sections revealed significantly smaller adipocyte size and higher density in GON but not in SC fat (Table 3). In addition, blood vessel size and density normalized against adipocyte density were significantly lower in ABT-518-treated GON adipose tissues (Fig. 4). Apoptosis in SC or GON adipose tissues was not significantly affected by ABT518 treatment (Table 3).

Analysis of metabolic parameters revealed significantly lower total cholesterol, HDL and LDL-C levels following inhibitor treatment, as well as decreased AST and ALT levels (Table 4). Both insulin and leptin plasma levels were lower in treated mice and leptin levels were positively correlated with bodyweight ($P = 0.002$; $r = 0.60$).

Effect of ABT-518 on adipose tissue in the DEO model

Total bodyweight gain of obese mice kept on the HFD for a further 10 weeks was not affected by ABT-518 treatment, resulting in comparable total bodyweight in the ABT-518-treated and control groups at the end of the experiment (Table 2; Fig. 3b). However, analysis by two-way repeated-measures ANOVA of the evolution of bodyweight

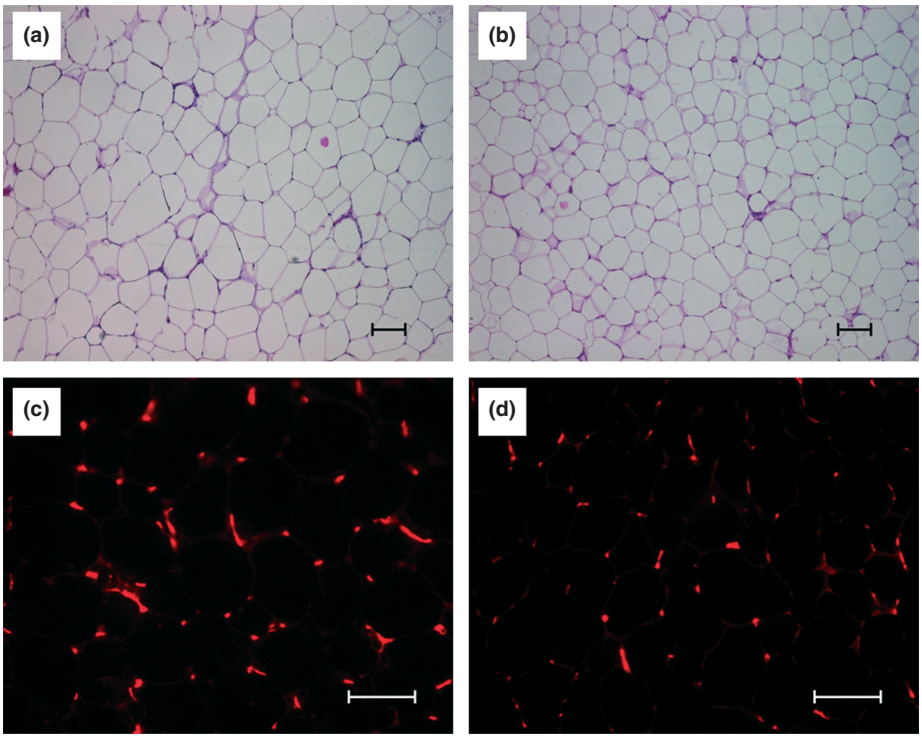

Fig. 4 (a, b) Haematoxylin–eosin and (c, d) lectin staining of gonadal (GON) adipose tissue sections from control (a, c) or ABT-518-treated (b, d) mice kept on a HFD for 16 weeks (diet-induced obesity model). Blood vessels are coloured red (c, d). Bars, 100 μm.

Table 4 Effect of ABT-518 (100 mg/kg per day) on plasma metabolic parameters and liver enzymes of young mice kept on a high-fat diet (HFD) for 16 weeks (diet-induced obesity model) or obese mice kept on an HFD for 10 weeks (diet-established obesity model)

	DIO model		DEO model	
	Controls	ABT-518	Controls	ABT-518
Glucose (mg/dL)	180 ± 14	150 ± 14	180 ± 32	200 ± 31
Insulin (ng/mL)	2.1 ± 0.4	1.2 ± 0.3*	1.6 ± 0.4	1.7 ± 0.4
Leptin (ng/mL)	40 ± 2	33 ± 2*	42 ± 4	41 ± 3
Triglycerides (mg/dL)	100 ± 14	140 ± 23	110 ± 11	170 ± 31
Total cholesterol (mg/dL)	420 ± 28	280 ± 11***	500 ± 26	410 ± 18*
HDL-C (mg/dL)	260 ± 13	170 ± 8***	270 ± 11	220 ± 14*
LDL-C (mg/dL)	130 ± 17	84 ± 7*	200 ± 18	150 ± 12
ALP (U/L)	520 ± 60	610 ± 35	670 ± 31	650 ± 41
AST (U/L)	280 ± 35	190 ± 20*	570 ± 42	380 ± 47**
ALT (U/L)	340 ± 52	130 ± 17***	610 ± 50	300 ± 38***

Data are the mean±SEM of 10 experiments in each group. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ compared with controls (non-parametric Mann–Whitney *U*-test).

DIO, diet-induced obesity (5-week-old mice kept on an HFD for 16 weeks); DEO, diet-established obesity (5-week-old mice first fed the HFD for 20 weeks to establish obesity and then maintained for a further 10 weeks on the HFD); HDL-C, high-density lipoprotein–cholesterol; LDL-C, low-density lipoprotein–cholesterol; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

with time revealed a significant lowering effect of ABT-518 on bodyweight ($P = 0.004$ vs control) and a significant effect of time on this difference ($P < 0.0001$), but no interaction between the two parameters. After Bonferroni correction (for 11 comparisons), the effect of time ($P < 0.002$) and treatment ($P = 0.046$) remained significant. No differences were observed between the two groups for SC or GON fat mass, or for the weight of other organs. Administration of ABT-518 did not affect food intake (3.38 ± 0.04 vs 3.21 ± 0.02 g/day in ABT-518-treated and control mice, respectively), physical activity (4300 ± 870 vs 4800 ± 960 turns/12 h in ABT-518-treated and control mice, respectively) or body temperature (35.9 ± 0.2 vs 35.9 ± 0.1°C in ABT-518-treated and control mice, respectively).

Histological analysis did not reveal significant effects of ABT-518 treatment on adipocyte size or density in SC or GON adipose tissues (Table 3). However, blood vessel size was significantly reduced in both SC and GON adipose tissues in ABT-518-treated mice. Apoptosis in SC fat was not affected by ABT-518 treatment, whereas it was enhanced in GON fat ($P = 0.002$; Table 3).

Analysis of metabolic parameters revealed lower cholesterol levels and reduced AST and ALT levels following inhibitor treatment (Table 4). Insulin and leptin levels were not affected by ABT-518 treatment of obese mice and leptin levels were positively correlated with bodyweight ($r = 0.50$; $P = 0.04$). Liver triglyceride levels did not differ significantly between ABT-518-treated and control mice (22 ± 2 vs 24 ± 1 mg/g tissue, respectively; $P = 0.27$, non-parametric Mann–Whitney *U*-test) and were not correlated with liver weight in either group ($r = 0.18$; $P = 0.4$). Thus, total liver triglyceride content was comparable for ABT-518-treated and control mice (107 ± 13 vs 126 ± 7 mg, respectively; $P = 0.4$).

DISCUSSION

Several previous studies have suggested a functional role for the gelatinases (gelatinase A, or MMP-2, and gelatinase B, or MMP-9) in the

development of obesity. Both MMP-2 and MMP-9 are secreted by adipose tissue and play a role in the clustering of adipocytes during differentiation.[14] Obese patients have enhanced plasma/serum levels of MMP-2 and MMP-9 compared with lean controls.[20] Experimental murine models of DIO indicate that MMP-2 deficiency is associated with markedly impaired adipose tissue development,[8] whereas MMP-9 deficiency has no significant effect.[9] Furthermore, the relatively gelatinase-specific inhibitor Tolylsam (IC_{50} 5 nmol/L for MMP-2, 49 nmol/L for MMP-9 and 34 nmol/L for MMP-12) was shown to reduce adipose tissue development, associated with adipocyte hypotrophy, in both young wild-type mice and leptin-deficient (*ob/ob*) mice kept on an HFD.[8,13]

To further substantiate a specific role of gelatinases in adiposity, in the present study we evaluated the effect of ABT-518 on adipocyte differentiation *in vitro* and adipose tissue development *in vivo*. ABT-518 is a potent gelatinase inhibitor (IC_{50} 0.78 nmol/L for MMP-2 and 0.5 nmol/L for MMP-9) that also efficiently inhibits MMP-3 (IC_{50} 12 nmol/L), MMP-8 (IC_{50} 5 nmol/L) and MMP-13 (IC_{50} 3 nmol/L). Thus, ABT-518 may be a more potent but less specific gelatinase inhibitor than Tolylsam. ABT-518 is a biaryl ether retrohydroxamate that inhibits MMP activity by binding reversibly to the zinc atom in the active site. It is a highly potent, orally available inhibitor[21,22] that has been shown to have cytostatic activity in preclinical experiments in mice following its administration twice daily at a dose of 10–30 mg/kg.[15] In the present study, we mixed ABT-518 homogeneously in the HFD to obtain a dose of 100 mg/kg per day.

Overall, in the DIO model, treatment with ABT-518 resulted in a somewhat lower bodyweight after 16 weeks of HFD feeding. The observed difference in SC and GON fat mass does not account for the total difference in bodyweight, which may be due to the fact that not all fat pads are recovered and accounted for. Whole-body MR spectroscopy revealed a significantly lower fat : water ratio following inhibitor treatment, mainly as a result of reduced lipid content. Alternatively, the weight of other body parts may be affected; for example, liver weight was lower in ABT-518-treated mice. In the present study, levels of the liver enzymes AST and ALT were reduced after ABT-518 treatment, suggesting reduced liver toxicity of the HFD. We found that ABT-518 treatment reduced triglyceride levels in the liver, but we found no effect on the triglyceride content of faeces or plasma levels, whereas cholesterol levels were reduced in both models. It is unclear to what extent reduced gelatinase levels contribute to reduced hepatic triglyceride levels. Studies with broad-spectrum MMP inhibitors in diabetic rats did not show a significant effect on hepatic triglyceride or cholesterol levels.[23]

In the GON adipose tissues, we observed mild adipocyte hypotrophy and higher adipocyte density. Blood vessel size and density, normalized against adipocyte density, was lower in GON adipose tissues of ABT-518-treated mice, indicating a mild anti-angiogenic effect. It has been reported previously that MMP-9, which is inhibited by ABT-518, releases matrix-bound VEGF and thereby promotes angiogenesis.[24] Moreover, given the wide variety of bioactive molecules that are affected by MMPs, both positively and negatively, it is possible that other pro- or anti-angiogenic pathways are disturbed by ABT-518 and influence blood vessel formation. Because at high concentrations of ABT-518 *in vitro* we observed cytotoxicity for (pre) adipocytes, we confirmed that the inhibitor did not cause excess apoptosis in fat tissues at the steady state concentration achieved *in vivo*, except for an increase in the GON fat of obese mice.

In contrast with GON fat, blood vessels and adipocytes in SC fat were not affected by ABT-518 treatment. This underlines previous observations that, although fat depots throughout the body share structural characteristics, there are anatomical site-specific differences in adipocyte biology.[25] Indeed, GON (or visceral) fat has a lower basal lipolytic rate and the uptake of fatty acids is lower in GON compared with SC fat. Fat localized intra-abdominally also has higher PPARγ mRNA expression and adiponectin levels, but mRNA levels and secretion of leptin are lower.[25]

Administration of ABT-518 to mice with established obesity (DEO model) was associated with reduced blood vessel size in the fat depots, but did not result in a regression of adipose tissue mass. Indeed, although the progress curves of bodyweight versus time were different, as analysed by two-way repeated-measures ANOVA, the bodyweights at the start and end of the study were not different. In contrast with the DIO study, liver weight and total triglyceride content were not affected in the DEO model. This may be due to the fact that these mice were already obese at the start of the study.

Overall, administration of ABT-518 to wild-type mice did not reproduce the effects on adipose tissue development to the same extent as Tolylsam.[8] This may be due to the different inhibition profiles of both inhibitors. Despite the fact that both compounds are potent gelatinase inhibitors, ABT-518 also strongly inhibits MMP-3, which was shown previously to have an inhibitory role on adipose growth.[4] Enhanced suppression of MMP-3 activity may compensate for the inhibition of MMP-2. The inhibitory effect of Tolylsam on MMP-12 is unlikely to contribute to the observed differences because MMP-12-deficient mice do not show impaired development of adipose tissue (M Van Hul & HR Lijnen, unpubl. data, 2011).

In vitro differentiation of 3T3-F442A pre-adipocytes into mature adipocytes was enhanced in the presence of ABT-518, as monitored by Oil Red O staining and expression of adipogenic markers. However, *in vivo*, the development of SC and GON adipose tissue mass was not markedly affected by ABT-518 treatment, suggesting that in a three-dimensional matrix (in contrast to two-dimensional cell cultures) adipocyte differentiation is not affected (adipocyte density is comparable). Furthermore, *in vivo* angiogenesis and ECM degradation also contribute to adipose tissue formation. This implies that the mechanisms behind the effects of MMP on adipose tissue development should be evaluated in the complex *in vivo* context rather than only *in vitro*. Indeed, microarray data indicate that the cellular programmes associated with adipocyte differentiation are considerably more complex than previously appreciated.[25] Gene expression changes associated with adipocyte development *in vivo* and *in vitro*, although largely overlapping, are in some respects quite different.[26]

Moreover, conflicting data exist on the effect of MMP inhibition using this *in vitro* differentiation model. Treatment of 3T3-F442A pre-adipocytes with 10 μmol/L of the synthetic MMP inhibitors CT1746 or batimastat (BB-94) reduced adipose conversion *in vitro*.[14,27] Similar observations have been reported in 3T3-L1 pre-adipocytes with 10 μmol/L batimastat or 25 μmol/L ilomastat (galardin).[28,29] In contrast, Alexander *et al.*[30] reported that 10 μmol/L ilomastat promotes differentiation, rather than inhibiting it. Thus, it may be that in addition to the concentration of inhibitor, the time window wherein it is added to the cells and the concentration and origin of the serum used to supplement the medium may be crucial. Therefore, it may be necessary to use three-dimensional matrix systems as a more relevant model of differentiation *in vitro*.[31]

It should be kept in mind that none of the MMP inhibitors studied *in vitro* or *in vivo* is absolutely specific for a given MMP. The full inhibitory spectrum against all known MMPs is not available. Furthermore, because many MMPs play a functional role in important physiological processes, it remains to be determined whether inhibition of a specific MMP with the aim of affecting adipose tissue can be achieved without causing serious side-effects. However, given the high degree of redundance in MMP specificity, the function of a given MMP in a specific process may be compensated for by other MMPs.

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