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# Matrix metalloproteinase inhibition affects adipose tissue mass in obese mice

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## SUMMARY

**1. Because the development of adipose tissue involves remodelling of the extracellular matrix (ECM), which requires matrix metalloproteinase (MMP) activity, we examined whether MMP inhibitors may have the potential to affect adipose tissue mass in obese mice.**

**2. Administration of the relatively gelatinase-specific MMP inhibitor tolylsam ((R)-3-methyl-2-[4-(3-p-tolyl-[1,2,4]oxadiazol-5-yl)-benzenesulphonylamino]-butyric acid; 100 mg/kg per day) for 7 weeks to obese wild-type mice on a high-fat diet resulted in significantly lower bodyweight ($P < 0.05$), lower subcutaneous (SC) and gonadal (GON) adipose tissue mass (both $P < 0.05$) and smaller adipocytes in both SC ($P < 0.005$) and GON ($P < 0.0005$) adipose tissues.**

**3. Magnetic resonance imaging confirmed a lower total body fat content in tolylsam-treated mice ($P < 0.0005$). In addition, tolylsam treatment of wild-type mice was associated with a marked enhancement in metabolic rate.**

**4. Electron microscopy analysis of tissue sections at the end of the 7 week feeding period revealed significantly higher collagen accumulation in the ECM of SC adipose tissues of tolylsam-treated mice ($P < 0.001$).**

**5. Thus, the relatively gelatinase-specific MMP inhibitor tolylsam has the potential to affect fat tissue growth in obese mice.**

**Key words: adipocyte, adipose tissue, gelatinase, matrix metalloproteinases, obesity.**

## INTRODUCTION

The development of adipose tissue is a complex process in which several growth factors, cytokines, hormones and proteinases contribute to adipogenesis, angiogenesis and proteolytic remodelling of the extracellular matrix (ECM).[1] The matrix metalloproteinase (MMP) system plays an important role in these processes, suggesting that modulation of the activity of specific MMPs may affect adipose tissue development.[2] Several lines of evidence indicate a functional role for the gelatinases (gelatinase A or MMP-2 and gelatinase B or MMP-9). Thus, *in vitro* studies with proteinase inhibitors and neutralizing antibodies have revealed that MMP-2 and MMP-9 play a key role in adipocyte differentiation.[3] Furthermore, MMP-2-deficient, but not MMP-9-deficient, mice kept on a high-fat diet (HFD) develop significantly less adipose tissue than their wild-type littermates.[4,5] Previous studies have shown the potential to impair adipose tissue development in young mice by using broad-spectrum MMP inhibitors, such as galardin,[6] Bay 12-9566[7] or Ro 28-2653.[8] We have shown that the more gelatinase-specific inhibitors tolylsam[4] and ABT-518[9] impair bodyweight gain and adipose tissue development in young wild-type mice kept on an HFD. However, it is not known whether such inhibitors have the potential to affect established obesity, which would be more relevant clinically.

In the present study, we investigated the potential of tolylsam ($IC_{50}$ 5 nmol/L for MMP-2, 49 nmol/L for MMP-9 and 34 nmol/L for MMP-12)[10] to affect adipose tissue mass and composition in mice with established obesity.

## METHODS

### Diet-induced obesity model

Five-week-old male wild-type mice (C57Bl/6 genetic background) were kept in microisolation cages on a 12 h light–dark cycle and fed *ad libitum* with an HFD (TD 88137; Harlan Teklad, Zeist, The Netherlands; 42% kcal as fat, 20.1 kJ/g) for 20 weeks. Then, five obese mice were given the HFD supplemented with 100 mg/kg per day tolylsam for an additional period of 7 weeks, with another five mice continuing on the HFD alone (control group). Tolylsam ((*R*)-3-methyl-2-[4-(3-p-tolyl-[1,2,4]oxadiazol-5-yl)-benzenesulphonylamino]-butyric acid), an MMP-2, MMP-9 and MMP-12 inhibitor,[10] was the kind gift of Shionogi (Osaka, Japan). The weight and food intake of the mice were measured at weekly intervals. Fat pad volumes were measured 1 week after the start (i.e. 26 weeks of age) and at the end (i.e. 32 weeks of age) of the experiments using non-invasive magnetic resonance imaging (MRI), performed as described previously.[11] After an overnight fast, mice were killed by intraperitoneal injection of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL, USA). Blood was collected via the retro-orbital sinus on trisodium citrate (final concentration 0.01 mol/L) and plasma was stored at −80°C. Intra-abdominal (gonadal; GON) and inguinal subcutaneous (SC) fat pads were removed and weighed, and

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portions were snap-frozen in liquid nitrogen for histological analysis, RNA or protein extraction.[4] Other organs, including the kidneys, lungs, spleen, pancreas, liver and heart, were also removed and weighed. Zymography of adipose tissue extracts on gelatin-containing gels was performed as described elsewhere.[4,12]

Spontaneous physical activity was evaluated by placing the mice in a separate cage equipped with a turning wheel linked to a computer to register full turning cycles. Data thus obtained are expressed as the number of cycles per night (i.e. over 12 h). Body temperature was measured using a rectal probe (TR-100; Fine Science Tools, Foster City, CA, USA). Data from three readings per mouse were averaged, with the mean ± SEM then calculated for the number of mice investigated in each group.

All animal experiments were approved by the local ethics committee (KU Leuven; P06022) and were performed in accordance with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (1996; see http://www.nap.edu/catalog.php?record_id=12910, accessed December 2011), as well as the guidelines of the International Society on Thrombosis and Haemostasis.[13]

### Microscopy analysis

The size and density of adipocytes were determined on 10 $\mu$m paraffin sections of adipose tissue, stained with haematoxylin–eosin under standard conditions.[4] For each mouse, at least 10 areas in 12 sections were measured using a computerized image analyser; data are expressed as the mean ± SEM for the number of mice studied. Blood vessels were stained using biotinylated *Bandeiraea (Griffonia) simplicifolia* BSI lectin (Sigma-Aldrich, Bornem, Belgium),[14] followed by signal amplification with the Tyramide Signal Amplification Cyanine System (Perkin Elmer, Boston, MA, USA). The results were analysed using computer-assisted image analysis, with blood vessel density normalized against adipocyte number.[15]

Macrophage infiltration in adipose tissues was quantified after Mac3 staining and is expressed as a percentage of section area. Collagen was stained using Sirius red and was quantified as percentage stained area per total tissue section area. The quality of the collagen fibres was estimated by Sirius red polarization microscopy, which enables quantification of thick, tightly packed collagen fibres (orange–red) and thin, loosely assembled fibres (yellow–green).[16]

Alternatively, collagen content was monitored by transmission electron microscopy. Samples were fixed by immersion in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 90 min at room temperature and post-fixed with 1% osmium tetroxide. After fixation, samples were dehydrated in ethanol and embedded in epoxy resin. Ultrathin sections (70–110 nm) were cut using a diamond knife, picked up on Formvar-coated 100-mesh nickel grids and stained with uranyl acetate and lead citrate. Specimens were examined using a Hitachi (Tokyo, Japan; model H-7600) transmission electron microscope. Collagen-rich areas were measured using ImageJ software (NIH; see http://imagej.nih.gov/ij, accessed April 2011).[17]

### Analysis of mRNA expression

Adipose tissues were homogenized using lysing matrix tubes (Qbiogene, Carlsbad, CA, USA) in a Hybaid ribolyser (Thermo, Waltham, MA, USA). Total DNA-free RNA was extracted using an RNA Easy kit (Qiagen, Valencia, CA, USA) and RNA concentrations were determined with the RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR, USA). Samples were aliquoted and stored at −80°C.

Reverse transcription reactions were performed from 10 ng total RNA with thermostable reverse transcriptase (*rTth*) at 70°C for 15 min, followed by 2 min incubation at 95°C for denaturation of RNA–DNA heteroduplexes, using the GeneAmp Thermostable RNA PCR Kit (Applied Biosystems, Foster City, CA, USA) and target-specific antisense primers. Amplification was started with 15 s at 94°C, 20 s at 68°C and 10 s at 72°C (35 cycles) and terminated by 2 min at 72°C using a GeneAmp PCR System 9700 thermocycler (Perkin Elmer, Waltham, MA, USA).

Expression of mRNA was measured by real-time polymerase chain reaction (PCR) using TaqMan Gene Expression Assay products (product numbers Mm99999915_g1 (*GAPDH*), Mm00607939_s1 (*β-actin*), Mm00439506_m1 (*MMP-2*), Mm01240562_g1 (*MMP-9*), Mm00441818_m1 (tissue-specific inhibitor of metalloproteinase-1; *TIMP-1*), Mm00441825_m1 (*TIMP-2*), Mm00434759_m1 (*Leptin*); Applied Biosystems) on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The mRNA levels were normalized against those of endogenous *GAPDH* or *β-actin* mRNA and are expressed as arbitrary units (a.u.).

### Metabolic parameters

Blood glucose concentrations were measured using Glucocard strips (Menarini Diagnostics, Firenze, Italy). Plasma levels of triglycerides, total cholesterol, high-density lipoprotein–cholesterol (HDL-C) and low-density lipoprotein–cholesterol (LDL-C) were determined using standard laboratory assays. Insulin (Mercodia, Uppsala, Sweden) and leptin (R&D Systems, Oxon, UK) levels were determined using commercially available ELISAs. Faecal triglyceride levels were determined using a Triglycerides FS kit (DiaSys Diagnostic Systems, Holzheim, Germany).

In addition, MMP-2 and MMP-9 levels were determined using commercially available murine ELISAs (R&D Systems). Plasminogen activator inhibitor-1 (PAI-1) levels were measured using a specific ELISA, as described previously.[18]

To monitor metabolic rate, a second group of WT mice were kept on an HFD supplemented with ($n$ = 7) or without ($n$ = 8) 100 mg/kg per day tolylsam for 15 weeks. Mice were placed in an open-circuit indirect calorimeter equipped with a double-beam infrared $CO_2$ analyser to measure $CO_2$ production and a paragenetic $O_2$ analyser to measure $O_2$ consumption, as described in previously.[19] Heat production (kJ/h) was calculated as 16.18 $O_2$ (L/h) + 5.02 $CO_2$ (L/h) and is expressed per kg bodyweight.[20]

### Statistical analysis

Data are expressed as the mean ± SEM. Differences between groups were analysed using the non-parametric Mann–Whitney $U$-test, compatible with small sample size ($n$ = 5–8). Comparison of progress curves and the effect of treatment were evaluated by two-way repeated-measures ANOVA. All analyses were performed using Prism 4 (GraphPad Software, San Diego, CA, USA) and two-sided $P < 0.05$ was considered significant. Bonferroni correction was applied for multiple testing.

## RESULTS

### Effect of tolylsam on established adipose tissue

After 1 week on the HFD, MRI scanning failed to reveal any significant differences between control and tolylsam-treated mice in terms of total body fat (16.0 ± 0.7 *vs* 15.3 ± 0.5 $cm^3$, respectively), SC fat (10.1 ± 0.5 *vs* 9.8 ± 0.2 $cm^3$, respectively) and GON fat (5.9 ± 0.2 *vs* 5.5 ± 0.3 $cm^3$, respectively). However, as a result of the MRI procedure, mice in both groups lost weight (Fig. 1a; Table 1). Between Weeks 2 and 7 of the experiment, control mice gained weight (from 44.3 ± 0.9 to 49.3 ± 0.9 g; $P < 0.0001$), whereas the tolylsam-treated mice did not (from 42.9 ± 1.3 to 41.8 ± 1.9 g; $P = 0.29$), resulting in a significantly higher bodyweight of mice in the control group at the end of the experiment.

Food intake was comparable in the tolylsam-treated and control groups (3.9 ± 0.1 *vs* 3.7 ± 0.2 g/day, respectively; $P = 0.4$) and tolylsam treatment had no effect on physical activity (4566 ± 1344 *vs* 4704 ± 985 turns/12 h in the control and tolylsam-treated groups, respectively; $P = 0.9$) or body temperature (35.9 ± 0.2°C *vs* 35.8 ± 0.2°C in the control and tolylsam-treated groups, respectively; $P = 0.5$). The mass of isolated SC and GON fat depots was significantly lower in the tolylsam-treated group, whereas the spleen and kidneys appeared enlarged (Table 1). Following inhibitor (tolylsam) treatment, the proportion of SC fat to total bodyweight decreased compared with that in the control group (3.4 ± 0.2% *vs* 5.1 ± 0.1%, respectively; $P = 0.008$); the corresponding percentage contribution of GON fat was 4.1 ± 0.2% and 4.7 ± 0.1%, respectively ($P = 0.016$). Magnetic resonance imaging confirmed significant reductions in the volumes of total body fat (11.7 ± 0.8 *vs* 17.9 ± 0.7 $cm^3$; $P < 0.0005$), SC fat depots (7.6 ± 0.5 *vs* 12.0 ± 0.4 $cm^3$; $P < 0.0005$) and GON fat depots (4.1 ± 0.3 *vs* 6.0 ± 0.3 $cm^3$; $P < 0.005$) in the tolylsam-treated mice compared with the control group (Fig. 1b). Furthermore, total body volume was significantly lower in tolylsam-treated mice compared with control (33.9 ± 1.3 *vs* 41.3 ± 0.6 $cm^3$, respectively; $P < 0.005$).

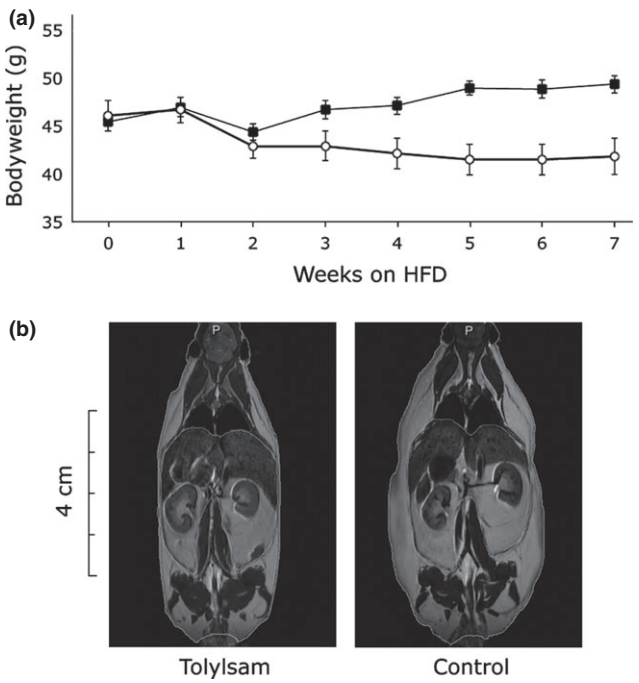


**Fig. 1** (a) Effects of tolylsam treatment on bodyweight of obese C57Bl/6 mice kept on a high-fat diet (HFD) for 7 weeks. (○), tolylsam-treated mice; (■), control group. Data are the mean ± SEM ($n = 5$ mice in each group). Two-way repeated-measures ANOVA confirmed significantly lower bodyweight ($P < 0.0001$) in the tolylsam-treated group. (b) Representative magnetic resonance imaging analysis of control and tolylsam-treated mice at the end of the experiments. Subcutaneous and abdominal fat are red and blue, respectively; the yellow and orange lines delineate total body area and the abdominal cavity, respectively.

**Table 1** Effects of tolylsam on adipose tissue and organ weights of obese mice kept on a high-fat diet for 7 weeks

| | Control | Tolylsam |
|---|---|---|
| Bodyweight (g) | | |
| Start of experiment | 45.4 ± 1.0 | 46.0 ± 1.6 |
| At 2 weeks | 44.3 ± 0.9 | 42.9 ± 1.3 |
| End of experiment | 49.3 ± 0.9 | 41.8 ± 1.9* |
| SC fat (g) | 2.4 ± 0.1 | 1.4 ± 0.2* |
| GON fat (g) | 2.2 ± 0.1 | 1.6 ± 0.1* |
| Lungs (mg) | 196 ± 15 | 227 ± 16 |
| Spleen (mg) | 117 ± 3 | 168 ± 12* |
| Heart (mg) | 157 ± 3 | 167 ± 7 |
| Pancreas (mg) | 180 ± 21 | 205 ± 13 |
| Liver (g) | 3.7 ± 0.2 | 3.5 ± 0.2 |
| Kidneys (mg) | 386 ± 9 | 444 ± 21* |

Data are the mean ± SEM ($n = 5$ mice in each group).

*$P < 0.05$ compared with control (non-parametric Mann–Whitney *U*-test).

SC, subcutaneous; GON, gonadal.

Quantitative analysis of isolated SC and GON fat depots revealed significant adipocyte hypotrophy, associated with higher adipocyte density, following tolylsam treatment (Table 2). There were no significant differences in blood vessel size or blood vessel density normalized for adipocyte density.

Sirius red staining revealed that tolylsam treatment preserved collagen compared with collagen content in the control group. Specifically, the total collagen content in SC adipose tissues from tolylsam-treated and control mice was 7.7 ± 0.7% and 4.4 ± 0.5%, respectively ($P = 0.005$); the corresponding values for GON adipose tissues were 3.4 ± 0.4% and 2.3 ± 0.3% ($P = 0.07$). Structural disorganization of collagen, monitored as the ratio of thick : thin fibres, was less prominent in tolylsam-treated SC adipose tissue (0.42 ± 0.07 *vs* 0.22 ± 0.02; $P = 0.05$) and GON adipose tissue (0.43 ± 0.05 *vs* 0.26 ± 0.06; $P = 0.06$) compared with control. In addition, quantitative analysis of electron micrographs (Fig. 2) confirmed significantly more collagen-rich areas in SC adipose tissues of tolylsam-treated compared with control mice (0.32 ± 0.04 *vs* 0.006 ± 0.001 $\mu m^2$, respectively; $P < 0.0001$), but not in GON adipose tissue (0.17 ± 0.03 *vs* 0.14 ± 0.03 $\mu m^2$, respectively; $P = 0.6$). Furthermore, there were no significant differences between the tolylsam-treated and control groups in terms of macrophage accumulation in SC adipose tissues (2.86 ± 1.05% *vs* 0.99 ± 0.41%, respectively; $P = 0.22$) or GON adipose tissues (1.35 ± 0.59% *vs* 0.76 ± 0.27%, respectively; $P = 0.42$).

**Table 2** Effects of tolylsam on adipocyte and blood vessel size and density in adipose tissue of obese mice kept on a high-fat diet for 7 weeks

| | Control | Tolylsam |
|---|---|---|
| Adipocyte size ($\mu m^2$) | | |
| SC fat | 5160 ± 161 | 3710 ± 248** |
| GON fat | 6530 ± 186 | 4910 ± 94*** |
| Adipocyte density ($\times$ €$10^{-6}$ /$\mu m^2$) | | |
| SC fat | 199 ± 7 | 283 ± 20* |
| GON fat | 155 ± 5 | 205 ± 4*** |
| Blood vessel size ($\mu m^2$) | | |
| SC fat | 51 ± 2 | 54 ± 5 |
| GON fat | 50 ± 4 | 52 ± 4 |
| Blood vessel density ($\times$ €$10^{-6}$ /$\mu m^2$) | | |
| SC fat | 242 ± 19 | 334 ± 27* |
| GON fat | 175 ± 9 | 237 ± 13** |
| Normalized† blood vessel density | | |
| SC fat | 1.2 ± 0.1 | 1.2 ± 0.1 |
| GON fat | 1.1 ± 0.1 | 1.2 ± 0.1 |

Data are the mean ± SEM ($n$ = 5 mice in each group).
*$P < 0.05$,
**$P < 0.005$,
***$P < 0.0005$ compared with control (non-parametric Mann–Whitney $U$-test).
†Blood vessel density was normalized against adipocyte number.
SC, subcutaneous; GON, gonadal.

### Effect of tolylsam on MMP expression

Gelatin zymography of SC or GON adipose tissue extracts in the presence of EDTA (10 mmol/L) or tolylsam (200 $\mu$mol/L) confirmed the potential of tolylsam to inhibit adipose tissue-related MMP-2 and MMP-9 (Fig. 3). Gene expression analysis revealed upregulation of *MMP-2* mRNA in SC ($P$ = 0.016), but not in GON ($P$ = 0.56), adipose tissues treated with tolylsam. In contrast, tolylsam treatment significantly upregulated *MMP-9* mRNA expression in both SC ($P$ = 0.046) and GON ($P$ = 0.013; Fig. 4a, b). However, tolylsam treatment had no effect on *TIMP-1* or *TIMP-2* mRNA expression in SC or GON adipose tissues (data not shown). In agreement with these data, tolylsam treatment enhanced MMP-2 antigen levels in SC adipose tissue extracts ($P$ = 0.004), but not in GON adipose tissue extracts ($P$ = 0.56), whereas MMP-9 antigen levels were enhanced in both SC ($P$ = 0.002) and GON ($P$ = 0.076) adipose tissue extracts following tolylsam treatment (Fig. 4c,d). In contrast, tolylsam treatment had no effect, compared with the control, group on plasma antigen levels of either MMP-2 (105 ± 3 *vs* 114 ± 4 ng/mL, respectively; $P$ = 0.12) or MMP-9 (67 ± 8 *vs* 54 ± 5 ng/mL, respectively; $P$ = 0.21).

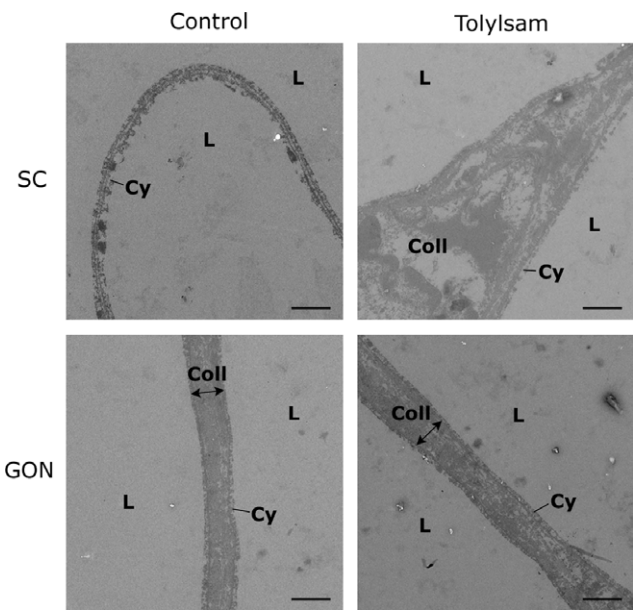


**Fig. 2** Electron microscopy analysis of subcutaneous (SC) and gonadal (GON) adipose tissue in obese mice kept on a high-fat diet with or without tolylsam treatment. Representative micrographs show adipocytes containing a large lipid droplet (L) and a thin rim of cytoplasm (Cy). In tolylsam-treated tissues, collagen (Coll) has accumulated in the intracellular space between adipocytes. Bars, 2 $\mu$m.

### Effect of tolylsam on metabolic parameters

Analysis of plasma metabolic parameters at the end of the experiments did not reveal significant differences between the tolylsam-treated and control mice for glucose, insulin or HDL-C levels, whereas triglyceride, total cholesterol and LDL-C levels were significantly reduced by tolylsam (Table 3). Faecal triglyceride levels were similar in the tolylsam-treated and control mice (6.1 ± 1.7 *vs* 5.0 ± 2.3 mg/g faeces, respectively; $P$ = 0.7).

Plasma leptin levels were significantly lower in tolylsam-treated compared with control mice (Table 3) and were positively correlated with bodyweight ($r$ = 0.79; $P$ = 0.009) and the weight of SC fat ($r$ = 0.73; $P$ = 0.02), as well as GON fat ($r$ = 0.85; $P$ = 0.003), for both groups combined. Furthermore, although there was a tendency for reductions in *leptin* mRNA expression in tolylsam-treated compared with control adipose tissues for SC fat (7232 ± 848 *vs* 11 376 ± 1793 a.u., respectively; $P$ = 0.08) and GON fat (6518 ± 1026 *vs* 8196 ± 1491 a.u., respectively; $P$ = 0.38), the differences failed to reach statistical significance.

The pattern of heat production, as monitored by calorimetry, revealed that tolylsam-treated mice (bodyweight 37.1 ± 0.7 g) produce markedly more heat per kg bodyweight that did the control mice (bodyweight 47.7 ± 0.8 g), especially at night during the active period (Fig. 5). Comparison of the curves using repeated-measures ANOVA confirmed significantly higher heat production with tolylsam treatment ($P$ = 0.008) and revealed a significant difference between measurements during the day and

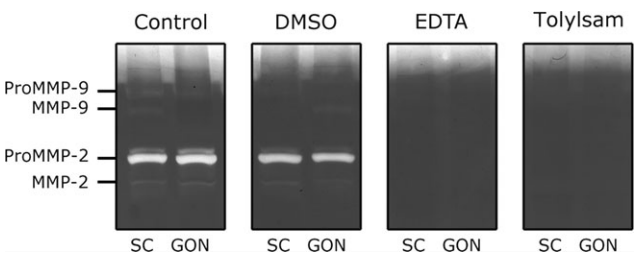


**Fig. 3** Gelatin zymography of extracts of subcutaneous (SC) and gonadal (GON) adipose tissues of obese mice in the presence of phosphate-buffered saline (control), 0.1% dimethylsulphoxide (DMSO), 10 mmol/L EDTA or 200 $\mu$mol/L tolylsam in the developing buffer. ProMMP, promatrix metalloproteinase; MMP, matrix metalloproteinase.

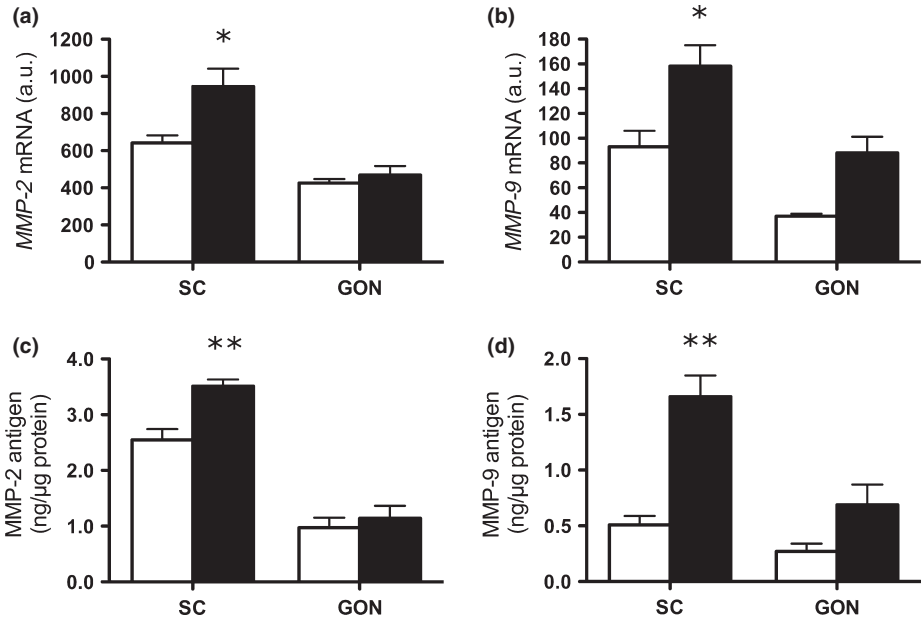


**Fig. 4** (a, c) Matrix metalloproteinase (MMP)-2 and (b,d) MMP-9 mRNA (a,b) and protein (c,d) levels in subcutaneous (SC) and gonadal (GON) adipose tissue extracts from control (□) and tolylsam-treated (■) mice. Data are the mean ± SEM ($n$ = 5 mice in each group). *$P$ < 0.05, **$P$ < 0.005 compared with controls.

at night ($P$ < 0.0001). After Bonferroni correction for multiple sampling, heat production with tolylsam treatment was found to be higher at night compared with the control group, but not during the day (Fig. 5).

## DISCUSSION

Obesity is a chronic disorder for which no real effective therapeutic treatment is available. Excess weight increases the risk of multiple conditions, including cardiovascular and cerebrovascular disease, hypertension, type 2 diabetes, certain types of cancer, gallstones and osteoarthritis. Obesity is frequently associated with metabolic abnormalities, such as impaired glucose tolerance, hyperinsulinaemia, dyslipidaemia with elevated triglyceride levels and decreased HDL-C concentrations (metabolic syndrome). In addition, obesity negatively affects physical functioning, vitality and general quality of life. Yet, at present there are few drugs that are effective against excess fat and those that are seem to be associated with unpleasant side-effects.[21]

The development and growth of adipose tissue involves adipogenesis, angiogenesis and ECM degradation.[1] Based on the rationale that MMPs play a functional role in these processes, we have evaluated the effect of the relatively gelatinase-specific inhibitor tolylsam on fat mass in mice with established obesity. The antigelatinolytic activity of tolylsam was confirmed by blocking MMP-2 and MMP-9 activity in adipose tissue extracts. Tolylsam was used previously at a dose of 50 mg/kg per day in a cerebral aneurysm model in the rat.[10] However, because the serum concentration of

**Table 3** Effects of tolylsam on plasma metabolic parameters in obese mice kept on a high-fat diet for 7 weeks

| | Control | Tolylsam |
|---|---|---|
| Glucose (mg/dL) | 134 ± 27 | 121 ± 28 |
| Insulin (ng/mL) | 1.8 ± 0.2 | 2.2 ± 0.4 |
| Leptin (ng/mL) | 22 ± 2 | 8.0 ± 1.8** |
| Triglycerides (mg/dL) | 96 ± 12 | 42 ± 4* |
| TC (mg/dL) | 372 ± 12 | 194 ± 13*** |
| HDL-C (mg/dL) | 181 ± 25 | 134 ± 11 |
| LDL-C (mg/dL) | 172 ± 22 | 52 ± 5** |
| PAI-1 (ng/mL) | 16 ± 2 | 13 ± 1 |

Data are the mean ± SEM ($n$ = 5 mice in each group).
*$P$ < 0.05,
**$P$ < 0.005,
***$P$ < 0.0005 compared with control (non-parametric Mann–Whitney $U$-test).

TC, total cholesterol; HDL-C, high-density lipoprotein–cholesterol; LDL-C, low-density lipoprotein–cholesterol; PAI-1, plasminogen activator inhibitor-1.

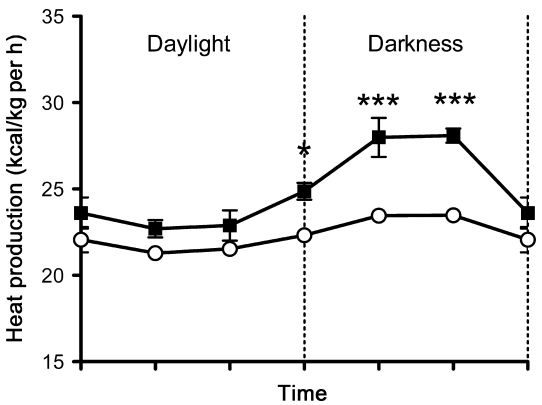


**Fig. 5** Heat production by C57Bl/6 mice kept on a high-fat diet, with (■) or without (○) tolylsam treatment, at night (1900–0700 hours) or during the day (0700–1900 hours). Data are the mean ± SEM of three consecutive measurements periods. *$P$ < 0.05, ***$P$ < 0.001 compared with control (two-way repeated-measures ANOVA with Bonferroni correction).

tolylsam achieved following oral gavage is several-fold lower in mice than in rats, we used a dose of 100 mg/kg per day in the present study. This dose has been shown previously to reduce bodyweight and adipose tissue development in a nutritionally induced obesity model in young wild-type mice.[4]

Overall, tolylsam treatment of obese mice was well tolerated in the present study and resulted in lower body and fat pad weights after 7 weeks feeding of an HFD. In SC and GON adipose tissues, we observed marked adipocyte hypotrophy and higher adipocyte density with tolylsam treatment. A significant positive correlation was found between bodyweight and SC fat mass ($r = 0.89$; $P = 0.001$) or GON fat mass ($r = 0.85$; $P = 0.003$) for both groups combined. The significantly lower circulating plasma levels of leptin following tolylsam treatment did not affect food intake. Our previous finding that tolylsam also impaired adipose tissue development in leptin deficient *ob*/*ob* mice confirms that tolylsam acts independently of leptin.[22] Thus, the feeding efficiency (weight gain per kJ energy intake) was lower in the tolylsam-treated mice. Given the fact that food intake was comparable in both groups, tolylsam does not seem to act by increasing satiety. Physical activity and body temperature were comparable in both groups, but tolylsam treatment enhanced the metabolic rate, as monitored by calorimetry. Administration of tolylsam to obese mice impaired adipose tissue growth, without affecting the pre-existing vasculature. Indeed, blood vessel density was higher in tolylsam-treated fat tissues, but this difference disappeared after normalization against adipocyte density.

Paradoxically, gelatinase A (*MMP-2*) and gelatinase B (*MMP-9*) mRNA expression in adipose tissues was enhanced following inhibitor treatment. Thus, it cannot be excluded that gelatinase inhibition in adipose tissues is partially offset by increased expression. Nevertheless, we have confirmed the potential of tolylsam to inhibit adipose tissue-derived MMP-2 and MMP-9.

The administration of tolylsam was associated with preservation of ECM collagen in this model. The higher collagen levels in tolylsam-treated adipose tissues at the end of the experiment are compatible with reduced MMP activity. It is conceivable that the higher content of structurally intact collagen in tolylsam-treated fat hampers the hypertrophic growth of adipocytes, which allows continued weight gain in the HFD control group. A similar mechanism has been proposed previously to explain the effect of a broad-spectrum MMP inhibitor on adipose tissue development in a murine model of nutritionally induced obesity.[6]

In the present study, tolylsam treatment significantly reduced triglyceride levels in wild-type obese mice. In addition, cholesterol levels were also lower, possibly related to the fact that tolylsam significantly reduced SC and GON fat mass.

Measurement of heat production revealed enhanced energy expenditure in tolylsam-treated mice. Because the energy balance is the net result of energy intake and expenditure, and intake (= food consumption) is not affected by tolylsam, the enhanced energy expenditure may contribute to the lower weight gain and reduced fat mass in tolylsam-treated mice. Thus, a large proportion of the energy absorbed is dissipated as heat and not deposited as body fat. The mechanisms underlying this effect of tolylsam remain to be investigated.

In humans, MMP-9 plasma levels are enhanced in obese subjects,[23–25] whereas conflicting data have been reported for MMP-2, with some studies reporting no differences in circulating MMP-2 levels in obese subjects[26] and others observing a decrease in MMP-2 levels with obesity.[27] Although targeting MMP-2 may lead to a reduction in fat accumulation in mice, inhibiting MMP-2 as a potential therapeutic strategy to treat obesity in humans should be approached with considerable caution. Indeed, MMP-2 deficiency may be associated with serious side-effects. Although MMP-2-deficient mice are overtly normal, inactivating MMP-2 mutations in humans are associated with severe osteolysis and arthritis. For example, in a consanguineous family with two affected siblings displaying a nodulosis–arthropathy–osteolysis phenotype (NAO; Online Mendelian Inheritance in Man (OMIM) 605156), two family specific homoallelic nonsense mutations were identified in the *MMP-2* gene: R101H and Y244X.[28–30] Zankl *et al.*[31] reported a 21-year-old woman who suffered from a severe form of osteolysis that resembled the symptoms of Winchester syndrome (OMIM 277950) caused by a homozygous missense mutation (E404K) in the active site of MMP-2. Similarly, Rouzier *et al.*[32] described two sisters with a Winchester-like phenotype; a homozygous deletion in exon 8 of the *MMP-2* gene was identified in both patients. More recently, two affected brothers were reported with a novel mutation ($658 + 2T > C$).[33]

How MMP-2 inactivation causes the features of Torg syndrome, NAO and Winchester syndrome is unclear and it may seem contradictory that loss of proteinase activity promotes an osteolytic phenotype. However, MMP-2 has many other substrates that may affect osteoblast maturation or osteoclast activity, such as transforming growth factor-$\beta$1, which is involved in both processes.[34]

However, it should be kept in mind that complications caused by total MMP-2 deficiency from birth on may not necessarily occur following pharmacological inhibition at a later stage, when essential developmental processes have already occurred.

In conclusion, our data demonstrate the feasibility of impairing fat mass growth in obese mice by the oral administration of a relatively gelatinase-specific MMP inhibitor. This appears due, at least in part, to an effect on adipocyte growth and may be related to the preservation of collagen in the ECM. In addition, tolylsam administration was associated with enhanced metabolic rate. At present, it cannot be excluded that the *in vivo* effects of tolylsam are mediated by inhibition of other MMPs, in addition to MMP-2, MMP-9 and MMP-12, because its full inhibitory spectrum has not been determined.

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