

**PERIPHERAL ADMINISTRATION OF A MELANOCORTIN 4-  
RECEPTOR INVERSE AGONIST PREVENTS LOSS OF LEAN BODY  
MASS IN TUMOUR-BEARING MICE**

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## ABSTRACT

Cachexia affects many different chronically ill patient populations including those with cancer. It results in loss of body weight, particularly of lean body mass (LBM), and is estimated to be responsible for over 20% of all cancer-related deaths. Currently available drugs are ineffective and new therapies are urgently needed. Melanocortin 4-receptor (MC4-R) blockade has recently been shown to be effective in preventing cancer cachexia in rodent models. In the present study we have tested a MC4-R blocker, ML00253764, (Vos *et al.*, 2004) *in vitro* and *in vivo*. In membranes of HEK-293 cells expressing human MC4-R, ML00253764 displaced NDP-alpha-melanocyte stimulating hormone binding with an IC<sub>50</sub> of 0.32μM. At concentrations above 1μM, ML00253764 decreased cAMP accumulation (maximum reduction of -20%) indicative of inverse agonist activity. ML00253764 was administered bi-daily (15mg/kg s.c.) for 13 days to C57BL6 mice bearing subcutaneous Lewis lung carcinoma tumours. Food intake and body weight were measured and body composition was assessed using magnetic resonance relaxometry. ML00253764 stimulated light-phase food intake relative to vehicle-treated controls ( $p < 0.05$ ) although no effect was observed on 24-hour food intake. During the 21 days of the experiment, the LBM of tumour-vehicle treated mice decreased ( $p < 0.05$ ). In contrast, the tumour-bearing mice treated with ML00253764 maintained their LBM. These data support the view that MC4-R blockade may be a suitable approach for the treatment of cancer cachexia and that MC4-R inverse agonists may have potential as drug candidates.

## INTRODUCTION

Cachexia affects many chronically ill patient populations including those with cancer, renal failure, heart failure, AIDS and rheumatoid arthritis. It is a syndrome which results in weight loss, in particular the loss of lean body mass (LBM), and is often accompanied by anorexia. The severity of cachexia is considered an important determinant of mortality and it has been estimated that cachexia is responsible for more than 20% of all cancer-related deaths (Bruera, 1998). Although there are some drugs available (e.g. megestrol acetate) they are mostly ineffective and new drugs and therapies for the treatment of cachexia are urgently needed (Hofbauer et al., 2005).

Although the aetiologies of the various diseases associated with cachexia are clearly different, a common element in all forms of cachexia is the patients` defence response. Both pre-clinical and clinical evidence has demonstrated elevated levels of pro-inflammatory cytokines in different forms of cachexia. Cytokines are known to act in the central nervous system to alter the functioning of feeding circuitry and thereby to influence appetite and metabolic rate. One central feeding circuit known to be affected by cytokines is the leptin/melanocortin 4-receptor (MC4-R) pathway. Indeed, it has been demonstrated that cytokine-induced anorexia may be prevented by both pharmacological and genetic blockade of central MC4-R, indicating that cytokines in the brain lead to activation of MC4-R which results in a decrease in food intake (Huang et al., 1999; Lawrence and Rothwell, 2001; Marks et al., 2001). Furthermore, it has been postulated that chronic elevation of cytokines in various disease states results in cachexia, via prolonged over-activation of central MC4-R (Marks et al., 2001).

The central melanocortin system has been recognised as an important regulator of energy balance for several years (see Cone, 2005, for review). Activation of hypothalamic MC4-R by the pro-opiomelanocortin (POMC) derived endogenous agonist  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) decreases food intake and leads to an increase in energy expenditure, whereas blockade of MC4-R by the endogenous MC4-R inverse agonist agouti-related protein (AgRP) increases food intake, decreases energy expenditure and leads to weight gain. The physiological importance of balance within the central melanocortin system has been demonstrated in mouse models of MC4-R knock-out (Huszar et al., 1997) and AgRP over-expression (Ollmann et al., 1997) where hyperphagia and obesity are the most obvious aspects of the phenotypes observed. Furthermore, humans who have mutations in MC4-R exhibit profound hyperphagia and obesity as a consequence of reduced MC4-R signalling (Farooqi et al., 2003). However, the physiological importance of the MC4-R extends beyond the regulation of food intake and a further consequence of blockade of MC4-R in both rodents and humans is increased linear growth including LBM (Huszar et al., 1997; Farooqi et al., 2003). Overall, this suggests that selective MC4-R blockade could be suitable approach for the treatment of cachexia. Indeed, MC4-R KO mice have been shown to be protected from tumour-induced (Marks et al., 2003), lipopolysaccharide-induced (Marks et al., 2001) and uraemia-associated cachexia (Cheung et al., 2005). Also, central administration of AgRP to mice (Marks et al., 2001) and the MC3/4-R antagonist SHU-9119 to rats, has been shown to protect against cancer-induced anorexia (Wisse et al., 2001). Finally, in a recent study by Markison et al. (2005) a low molecular weight, selective, MC4-R antagonist was administered peripherally to mice and was shown to protect against tumour-induced anorexia and to increase LBM relative to vehicle-treated controls.

In the present study we have investigated the effect of chronic peripheral administration of a MC4-R ligand, ML00253764 (Vos et al. 2004), in a murine model of cancer cachexia. This

compound has previously been shown to increase body weight in CT-26 tumour-bearing BALB/c mice (Vos et al., 2004), and in the present study we have extended these findings by investigating the *in vitro* characteristics of this compound, and its *in vivo* effects on food intake and body composition in C57Bl6 mice bearing subcutaneous Lewis lung carcinoma tumours. Body composition was determined using a traditional chemical extraction method and the more recently established method magnetic resonance relaxometry (MRR) (Künnecke et al., 2004). We have found ML00253764 to be an inverse agonist at MC4-R *in vitro*. Inverse agonists act not only by antagonizing the effect of agonists but also by reducing constitutive activity of receptors. Consequently, they can reduce receptor activity in the absence of agonists and display increased efficacy in comparison to antagonists. When administered to mice, ML00253764 increased light-phase food intake and prevented tumour-induced loss of LBM. The data presented support the view that MC4-R blockade may be a suitable therapeutic approach for the treatment of cachexia, and suggest that MC4-R inverse agonists may have potential as drug candidates.

## METHODS

### *In Vitro Studies*

#### **Preparation of membranes containing melanocortin receptors**

HEK-293 cells expressing human melanocortin receptors were suspended in ice-cold buffer (50mM HEPES-NaOH pH 7.4, 250mM sucrose, complete protease inhibitors, in analytical H<sub>2</sub>O), and passed through a 25G syringe needle ten times at a flow rate of 40ml/min. Lysis of the cells was monitored by microscopy. Debris was removed from the lysate by a short centrifugation step (2500g, 10min, 4°C). The pellet was discarded and the supernatant was centrifuged again in a high-speed centrifuge (47'000g, 90min, 4°C). The resulting pellet was re-suspended in ice-cold buffer followed by 3 passages through a 25G syringe needle. Aliquots of the preparation were snap-frozen in dry ice and stored at -80°C until use.

#### **Measurement of MC4-R binding**

Binding of ligands to human (h) MC4-R, MC3-R, and MC5-R was measured using a homogenous, non-radioactive competition assay. In brief, suitable dilutions of test ligands were prepared in dimethyl sulfoxide (DMSO) and 1µl per well was transferred into a black 384 well plate with a non-binding surface (#3654, Corning Inc, NY). 29µl of 0.77nM Cy3B-NDP-αMSH fluorescent tracer (custom synthesis by Thermo Electron, Ulm, Germany) in assay buffer (50mM HEPES-NaOH, 2.5mM CaCl<sub>2</sub>, 0.1% BSA, complete protease inhibitors, in H<sub>2</sub>O) and 10µl MC-receptor membrane preparation containing 1.6 - 6µg membrane protein (equivalent to 5.2fmol receptor per well) were added. The assay was placed for 60min at 24°C, overnight at 4°C, and 3.5 hours at 24°C prior to reading fluorescence polarization (FP) in a FARCyte (Amersham Biosciences UK Ltd) or TECAN Ultra (Tecan, Männedorf, Switzerland) plate reader using an FP filter set with 535nm excitation / 590nm emission wavelengths, 40µs integration and 0µs lag times.

### **Measurement of the MC4-R-induced Functional Activity (cAMP production)**

cAMP levels were measured in a homogenous, non-radioactive cAMP agonist assay using membrane preparations instead of whole cells. Assay conditions (Allen *et al.* 2002) were as follows: suitable dilutions of test ligands were prepared in DMSO and 1 $\mu$ l per well was transferred into a black 384 well plate with a non-binding surface (Corning #3654). Per well, 1.8 $\mu$ g MC4-R membrane protein diluted in 9 $\mu$ l membrane buffer (55mM HEPES-NaOH pH7.4, 11mM MgCl<sub>2</sub>, 110mM NaCl, EDTA-free complete protease inhibitors, in analytical H<sub>2</sub>O) was added and the plate was placed on ice immediately. After a pre-incubation of 20min on ice, 10 $\mu$ l stimulation buffer (50mM HEPES-NaOH pH7.4, 10mM MgCl<sub>2</sub>, 100mM NaCl, 100 $\mu$ M ATP, 2 $\mu$ M GTP, 200 $\mu$ M isobutyl-methyl-xanthine, in H<sub>2</sub>O) containing 0.33 $\mu$ l anti-cAMP antibody (FPA202002KT assay kit, Perkin Elmer Life Sciences, Boston, MA) was added and the assay was transferred from ice to 37°C. After 45min the reaction was stopped by transferring the assay to ice and addition of 20 $\mu$ l of ice-cold detection buffer (FP1087c, provided with the FPA202002 kit) containing 3.3nM fluorescein-cAMP (Perkin Elmer FPA202002KT assay kit or BLG-F002, BioLog, Bremen, Germany). After two hours on ice, the assay was allowed to warm up to 24°C for 10-15min. Immediately afterwards, fluorescence polarization (FP) was read in a FARCyte (Amersham Biosciences UK Ltd) or TECAN Ultra (Tecan, Männedorf, Switzerland) plate reader using an FP filter set with 485nm excitation / 535nm emission wavelengths, 60 $\mu$ s integration and 0 $\mu$ s lag times.

### **Data processing of *In Vitro* Results**

FP raw data was obtained using the XFLUOR4 plug-in (Tecan, Männedorf, Switzerland) for Excel (Microsoft Zurich, Switzerland). The raw data was processed in custom Excel worksheets using the XLfit4 curve-fitting package (IDBS, Guildford, UK) for calculation of the result charts.

### ***In Vitro* Reagents**

Reagents were purchased from Fluka Chemie GmbH / Sigma-Aldrich Corporation, Buchs, Switzerland (sucrose, CaCl<sub>2</sub>, MgCl<sub>2</sub>, H<sub>2</sub>O, BSA, ATP, GTP); Acros Organics, Geel, Belgium (DMSO, IBMX); VWR International AG Life Science, Lucerne, Switzerland (HEPES); and Roche Diagnostics, Basel, Switzerland (complete protease inhibitor tablets).

### ***In Vivo* Studies**

#### **Animals**

Male C57BL6 mice (~20g) were obtained from RCC (Füllinsdorf, Switzerland) and were individually housed for at least 7 days before the start of experiments under conditions of controlled temperature (21-22°C) and a 12:12-h light-dark cycle (lights on from 06:00h to 18:00h). The mice were placed on a standard *ad libitum* chow diet (NAFAG 3432, 3.0 kcal/g, 61.6% of total calories from carbohydrate, 24.8% of total calories from protein and 13.6% of total calories from fat) and had free access to tap water. All experiments were performed in accordance with the Swiss regulations for animal experimentation.

#### **Tumour Cell Culture and Implantation**

Lewis lung carcinoma (LLC) cells were purchased from American Type Culture Collection (Molsheim, France) and were maintained as a primary culture as recommended by the supplier. Cells were harvested during the exponential growth phase and were suspended in phosphate buffered saline for injection. For the implantation of tumour cells, mice were momentarily anaesthetised with isoflurane (4% with medicinal O<sub>2</sub> as the carrier gas) and received a s.c. injection of 1x10<sup>6</sup> cells in 100µl volume in the upper flank. Sham control mice underwent the same procedure but received 100µl PBS.

### **Compound Administration**

ML00253764 (2-{2-[2-(5-Bromo-2-methoxyphenyl)-ethyl]-3-fluorophenyl}-4,5-dihydro-1H-imidazolium hydrochloride) was synthesized at Santhera Pharmaceuticals, Liestal, Switzerland. The compound was weighed out and dissolved in PEG200: saline (1:10) just prior to each application and was injected s.c. in a volume of 10ml/kg. The dose of 15mg/kg was selected based on the results of the study by Vos *et al.* (2004). Applications were b.i.d. during the early light-phase (2-3 hours following lights-on) and during the last hour before the onset of the dark-cycle from day 8 post implantation of the LLC cells until the end of the study (day 21).

### **Measurement of Light-phase Food Intake**

All measurements of food intake were conducted in the home cage. At the start of the study enough chow was placed into each food hopper for the duration of the study, and this was weighed and returned to the hopper twice daily, at the time of each injection, in order to determine light-phase and 24-hour food intake.

### **Assessment of Tumour Size**

Quantitative assessment of tumour size was carried out upon completion of the study by weighing the tumour which had been dissected from each animal.

### **Measurement of Body Composition**

Body composition was measured in conscious mice by magnetic resonance relaxometry (MRR) during the light-phase just prior to tumour inoculation (day 1) and 21 days post implantation of the tumour cells. The second measurement was taken post mortem in mice that had been killed by CO<sub>2</sub> asphyxiation and had had their subcutaneous tumours removed.

Sham mice underwent the same procedure, including opening of the skin. Care was taken to ensure that the same time ensued between death and the second MRR measurement for all animals.

#### **(a) MRR Method**

Nuclear magnetic resonance (NMR) relaxometry measurements were carried out on a Bruker Biospec 70/20 (7T, 300MHz) using a commercial whole-body  $^1\text{H}$  resonator (Bruker, Germany). Whole body transversal relaxation was used for body composition analysis of conscious mice. The method used has been described previously (Künnecke *et al.*, 2004). Few changes were necessary to transfer the method to the spectrometer used in the present study. At the beginning of each measurement series the magnetic field was homogenized by using the inbuilt Bruker autoshim procedure on the first mouse. If necessary the shim was improved manually until the half line width of the water proton line was below 250Hz. For each measurement sequence the probe was tuned and matched and the pulse power was individually adjusted using the inbuilt pulse power calibration routine. A Carr-Purcell-Meiboom-Gill (CPMG) multispinecho sequence was used. The initial  $90^\circ$  excitation was performed by a rectangular 150 $\mu\text{s}$  pulse. The following 256 refocusing  $180^\circ$  rectangular pulses had a duration of 300 $\mu\text{s}$  and were each separated by an echo time of 3ms. Two averages were taken for one sequence with a repetition time of 10s. Three sequences were averaged for each mouse.

Proton relaxations were obtained by picking the maximal echo amplitude of each echo signal of the CPMG signal. Body composition was calculated by processing the time domain of proton relaxation by an inverse Laplace transformation as provided by CONTIN software (Provencher, 1982). The area of the two separated peaks was assumed to be proportional to the number of protons of tissue water and of fat. Conversion to absolute weight (grams) was

performed using agarose phantoms (Künnecke et al., 2004) whose transversal relaxation times ( $T_2$ ) were adjusted to ~31ms and ~200ms for LBM and fat respectively.

### **(b) Chemical Analysis of Body Composition**

Following the second MRR measurement body composition was assessed by chemical extraction as described by Markewicz et al. (1993). The abdominal cavity of each mouse was opened, the bladder was emptied and the gastrointestinal (GI) tract was removed. The GI tract content was pushed out of the tract which was then returned to the carcass of each mouse. Care was taken not to lose blood during this procedure. The carcasses were then weighed to establish the “wet weight” of each animal. Mice were placed into pre-weighed metal containers, covered with aluminium foil and placed into an oven at 65°C for one week. After one week, three daily weight measurements were taken and when they gave consistent values, the carcasses were considered to be completely dry. The final dry measurement taken was considered the “dry weight” of the mice, and the “water mass” of each animal was calculated by subtracting “wet weight” from the “dry weight”. Mouse carcasses were then wrapped in fat-free paper and exposed to chloroform extraction of fat using standard Soxhlet’s apparatus. Following fat extraction, carcasses were placed in a hood overnight to allow for the complete evaporation of chloroform, and were then re-weighed to establish “fat free dry mass”. Subtraction of the “fat free dry mass” from the “dry mass” gave a measure of “fat mass” for each mouse and the sum of the “fat free dry mass” and the calculated “water mass” was considered to be a measure of “lean body mass”.

### **Statistical Methods**

Light-phase food intake in mice was analysed using the unpaired two-tailed Student’s t-test. Analysis of body composition data was done using two-way repeated measures ANOVA with time and treatment as the measured variables. Post-hoc analysis was carried out using the

Student-Newman-Keuls method. Statistical tests were performed using SigmaStat and Excel software. A statistical comparison of the MRR and chemical extraction methods was conducted according to the method of Bland and Altman (1986).

## RESULTS

### *In Vitro* Pharmacology

In a non-radioactive assay using HEK-293 cells which expressed hMC4-R, ML00253764 displaced NDP- $\alpha$ -MSH binding with a mean IC<sub>50</sub> of 0.32 $\mu$ M. With hMC3-R and hMC5-R, IC<sub>50</sub> values of 0.81 $\mu$ M and 2.12 $\mu$ M were obtained indicating approximately 2.5-fold selectivity for MC4-R over MC3-R and approximately 7-fold over MC5-R (Figure 2a). In the cAMP functional activity assay on membranes expressing human MC4-R, ML00253764 decreased cAMP accumulation at concentrations above 1 $\mu$ M with a maximum decrease of -20%. AgRP(83-132) decreased cAMP accumulation in this assay at concentrations above 1nM with a maximum decrease of -40% (Figure 2b). On membrane preparations from cells expressing human MC3- or MC5-R, ML00253764 did not show any induction of cAMP activity at concentrations up to 100 $\mu$ M.

### Food Intake

Mice were treated with ML00253764 from day 8 post implantation of the tumour cells until the end of the study. Cumulative 13-day light-phase food intake was significantly increased by ML00253764 treatment in both sham and tumour-bearing mice ( $p < 0.05$ , Figure 3a). There was, however, no effect of ML00253764 on cumulative 13-day 24-hour food intake either in the sham or the tumour treated groups ( $p = 0.80$  and  $0.08$  respectively, Figure 3b).

### Body Weight

Both sham-vehicle and sham-ML00253764 treatment groups tended to gain body weight during the 21 days of the study (21.79 $\pm$ 0.25g to 22.34 $\pm$ 0.34g ( $p = 0.18$ ) and 21.85 $\pm$ 0.15g to 22.35 $\pm$ 0.30g ( $p = 0.23$ ) for sham-vehicle and sham-ML00253764-treated mice respectively).

In the tumour groups, the body weight of the vehicle-treated mice tended to decrease ( $21.56 \pm 0.20\text{g}$  to  $20.85 \pm 0.37\text{g}$ ) and the body weight of the tumour-ML00253764-treated mice decreased significantly during the 21 day period ( $21.80 \pm 0.27$  to  $20.94 \pm 0.35\text{g}$ ;  $p < 0.05$ ). The extent of this decrease did not differ between tumour-vehicle and tumour-ML00253764 treatment groups ( $p = 0.82$ ).

### **Body Composition as Assessed by MRR**

#### *Fat Mass*

Sham control mice treated with vehicle and ML00253764 gained fat mass (FM) during the course of the study ( $p < 0.001$ , Figure 4a) ( $+28.4 \pm 6.9\%$  and  $+27.5 \pm 6.9\%$  in sham-vehicle and sham-ML00253764 treated mice respectively). The extent of this increase was not different between the vehicle and ML00253764-treated groups ( $p = 0.55$ ). Tumour-vehicle treated mice also gained FM during the course of the experiment ( $+21.5 \pm 8.7\%$ ;  $p < 0.01$ ) an effect which was significantly different ( $p < 0.001$ ) to that observed in the tumour-ML00253764 treated group whose FM did not change significantly ( $-3.3 \pm 5.0\%$ ,  $p = 0.72$ ; Figure 4b).

#### *Lean Body Mass*

In sham control mice treated with vehicle or ML00253764, LBM did not change significantly during the experimental period (Figure 4c). The percentage change in LBM was  $-2.6 \pm 1.6\%$  and  $-0.6 \pm 2.0\%$  for sham-vehicle and sham-ML00253764-treated mice respectively ( $p=0.18$ ). In contrast, LBM decreased in tumour-vehicle treated mice ( $-3.4 \pm 1.3\%$ ,  $p < 0.05$ ), a finding which differed significantly ( $p < 0.01$ ) to that observed in the tumour-ML00253764 group whose LBM did not change significantly during the course of the study ( $+0.8 \pm 1.1\%$ ,  $p = 0.65$ , Figure 4d).

### **Body Composition as Assessed by Chemical Extraction and Comparison with MRR**

Figure 5 shows the differences between the values obtained by MRR and chemical extraction for (a) LBM and (b) FM. For LBM measurements the mean difference between the two methods was -1.99g with a 95% confidence interval of -2.43 to -1.55g. The limits of agreement for lean body mass were -4.59 to +0.61g. The mean difference between the fat measurements was 0.34g with 95% a confidence interval of 0.28 to 0.40g. The limits of agreement were -0.08 to +0.76g. According to the method and Bland and Altman (1986) the two methods provided comparable results.

### **Tumour Size**

At the end of the study, tumour size did not differ between the tumour-vehicle and the tumour-ML00253764 treatment groups. Mean dissected tumour weights were  $1.65 \pm 0.12$ g and  $1.68 \pm 0.19$ g for vehicle and ML00253764 treated groups respectively ( $p = 0.90$ ).

## DISCUSSION

In the present study we have tested the effect of chronic peripheral application of a MC4-R inverse agonist on food intake and body composition in a murine model of tumour-induced cachexia. We report that chronic application of ML00253764 stimulates light-phase food intake and prevents the loss of LBM induced by subcutaneous LLC tumour burden.

In our *in vitro* membrane assay we have observed a comparable binding affinity of ML00253764 for MC4-R to that reported by Vos et al. (2004). ML00253764 was also found to decrease cAMP induction, indicative of inverse agonism, although this effect was found to be only 50% of that observed for AgRP(83-132). Constitutive activity of MC4-R has been reported *in vitro* (Nijenhuis et al., 2001) and its physiological importance has recently been confirmed in humans (Srinivasan et al., 2004). Because of the inverse agonist activity of ML00253764, the positive effects on LBM thus described could be the result of blockade of endogenous agonist-induced MC4-R activity as well as a reduction in intrinsic MC4-R activity.

We report that ML00253764 displays only 7- and 2.5-fold differences in affinity for MC4-R over MC5-R and MC3-R respectively. Like MC4-R, MC3-R are abundant in rat brain hypothalamus and although they seem not to be directly involved in food intake (Kask et al., 2000), they are thought to play a role in energy partitioning (Butler et al., 2000). It has also been proposed that MC3-R may function as inhibitory auto-receptors on POMC neurons of the arcuate nucleus of the hypothalamus (Bagnol et al., 1999). Inhibitory auto-receptors are located presynaptically and function to negatively modify neurotransmitter release. Activation of such inhibitory auto-receptors would lead to a decrease in MC4-R activity whereas blockade would lead to increased MC4-R activity. Consequently, blockade of MC3-

R would be expected to lead to enhanced symptoms of cachexia and this has been demonstrated in animal models (Marks et al., 2003). Because of the lack of selectivity of ML00253764 for MC4-R, the involvement of MC3-R in the *in vivo* results thus described cannot be excluded, although in view of the positive results thus described, we speculate that the involvement of MC3-R is likely to have been minimal. Of the other melanocortin receptor subtypes with possible involvement in the results described, the low binding affinity at MC5-R ( $IC_{50}$  of  $2\mu M$ ), and at MC1-R ( $K_i$   $65\mu M$ , T.J. Vos, personal communication) make involvement of these receptors unlikely.

In our *in vivo* studies, although ML00253764 stimulated light-phase food intake it did not affect 24-h food intake. This effect was observed in sham control mice as well as in tumour-bearing mice and is likely to be due to pharmacokinetic properties of ML00253764. Vos et al. (2004) have studied the brain concentration versus time profile of ML00253764 following subcutaneous administration and report that at 30mg/kg, ML00253764 achieved brain concentrations in excess of its functional MC4-R  $IC_{50}$  for 6 hours. Presumably this time profile would be reduced at the dose used in the present study (15mg/kg) and is a likely explanation for the lack of cumulative effect on 24-hour food intake and body weight that we observed.

For the determination of body composition, MRR was used in addition to a traditional chemical extraction method and our analysis using the statistical method of Bland and Altman (1986) confirmed the similarity of the results obtained using these two methods, with the limits of agreement being within acceptable boundaries. The MRR method has recently been adapted to provide quantitative body composition analysis in rodents (Künnecke et al., 2004) and provides numerous advantages over other methods of body composition analysis including increased precision and speed (Taicher et al., 2003). Furthermore, MRR may be

performed in conscious mice with no need for anaesthesia. Anaesthesia disrupts diurnal rhythms and results in decreased food intake which may lead to changes in body composition; therefore, the lack of requirement for anaesthesia may be considered a great advantage. Additionally, using MRR, sequential measurements of the same animal are possible, although in the present study, due to the complicating presence of a growing tumour, only two measurements were conducted at the start and end of the study with the latter being conducted in mice post mortem with the tumours removed. In other animal models of cachexia where tumour growth is not a complicating factor, it could be of interest to take several MRR measurements of body composition per mouse during the course of the experiment. Such assessment could provide important information regarding the progression of the disease and the optimal time to begin drug treatment. It is noteworthy that MRR does not measure bone mass or the contents of the gastrointestinal tract and therefore the sum of LBM and FM as measured by MRR would not be expected to equal total body weight.

MRR analysis indicated that ML00253764 administration did not affect body composition in sham control mice - both sham control mice treated with vehicle and with ML00253764 gained fat during the 3 week experimental period with no change in LBM, and no differences between the experimental groups. In contrast, differences in body composition were observed between the tumour-bearing groups. The tumour-vehicle mice lost LBM (indicative of a cachexic state), while displaying an increase in FM. The tumour-ML00253764 treatment group, however, were protected from this loss of LBM (indicating protection from cachexia) with no change in their FM. Since cumulative energy intake was the same in the two tumour-bearing groups, this suggests that metabolic changes were induced in the tumour-bearing mice as a consequence of ML00253764 treatment which may have affected the manner in which energy was used and stored. Consequently, in the tumour-ML00253764 treated mice, LBM was maintained rather than lost and FM maintained rather than increased. The data also

suggest that treatment of cachexia using MC4-R blockade need not increase overall energy consumption in order to be effective.

The difference between the effect of ML00253764 on body composition in the sham and the tumour-bearing mice suggests that, despite the absence of anorexia, an imbalance existed in the tumour-bearing mice, which was partially reversed by ML00253764. Such an imbalance might have been brought about by increased levels of cytokines and other inflammatory mediators which are likely to be present in this model (Marks and Cone, 2003; Argiles et al., 2005).

It has been postulated that cachexia due to chronic over-activation of MC4-R is common to many forms of cachexia (Marks et al., 2001). Therefore, in terms of pharmacotherapy, the MC4-R may be a suitable target because it constitutes a final common pathway in the aetiology of this disorder. Preclinical evidence supports this hypothesis as MC4-R blockade has recently been shown to prevent cachexia occurring from a number of different causes. Cytokine (Lawrence and Rothwell 2001; Huang et al., 1999; Marks et al., 2001), uraemia (Cheung et al., 2005) and various forms of tumour-induced cachexia, in rats and mice (Wisse et al., 2001; Marks et al., 2001; Markison et al., 2005) have been shown to be prevented by both pharmacological and genetic blockade of MC4-R.

In summary, we report that the loss of LBM that occurs in tumour-induced cachexia in mice may be prevented by chronic dosing of an MC3/4-R inverse agonist. This finding supports the view that MC4-R blockade may be a suitable approach for the treatment of cachexia.

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## FIGURE LEGENDS

### Figure 1

Chemical structure of ML00253764 (Vos et al., 2004).

### Figure 2

Binding and functional activity data for ML00253764. (a) Concentration-dependent displacement of NDP- $\alpha$ -MSH binding by ML00253764 in HEK-293 cells expressing human MC3-R, MC4-R and MC5-R. (b) Functional activity of MC4-R as assessed by cAMP production in a cell membrane preparation. Data are expressed as mean  $\pm$  SEM from triplicate observations.

### Figure 3

Cumulative light-phase and 24-hour food intake in sham control and tumour-bearing mice. Mice were injected s.c. b.i.d. with 15mg/kg ML00253764 during the early light-phase and just before the onset of the dark-phase, from day 8 post implantation of the tumour cells until the termination of the study (day 21). Cumulative light-phase food intake (a) and cumulative 24-hour food intake (b) for the 13 day treatment period are shown. Data are presented as mean + SEM and statistical analysis was carried out using the unpaired Student's t-test; \*  $p < 0.05$ .

### Figure 4

Body composition analysis in sham control and tumour-bearing mice at the start (day 1) and end (day 21) of the study. Sham control and mice bearing s.c. Lewis lung carcinoma tumours were treated with vehicle or ML00253764 (15mg/kg, b.i.d. s.c. for 13 days). Fat content (a and b) and lean body mass (LBM) (c and d) were measured using magnetic resonance

relaxometry (MRR) in sham control and tumour-bearing mice with and without ML00253764 treatment. Data are presented as mean+SEM. Statistical analysis was carried out using two-way repeated measures ANOVA with Student-Newman-Keuls post-hoc analysis; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

### **Figure 5**

A statistical comparison of the two methods of body composition analysis using the method of Bland and Altman (1986) for (a) lean body mass (LBM) and (b) fat mass. Large dashed lines represent the mean differences between the methods and small dashed lines, the mean  $\pm$  2SD.

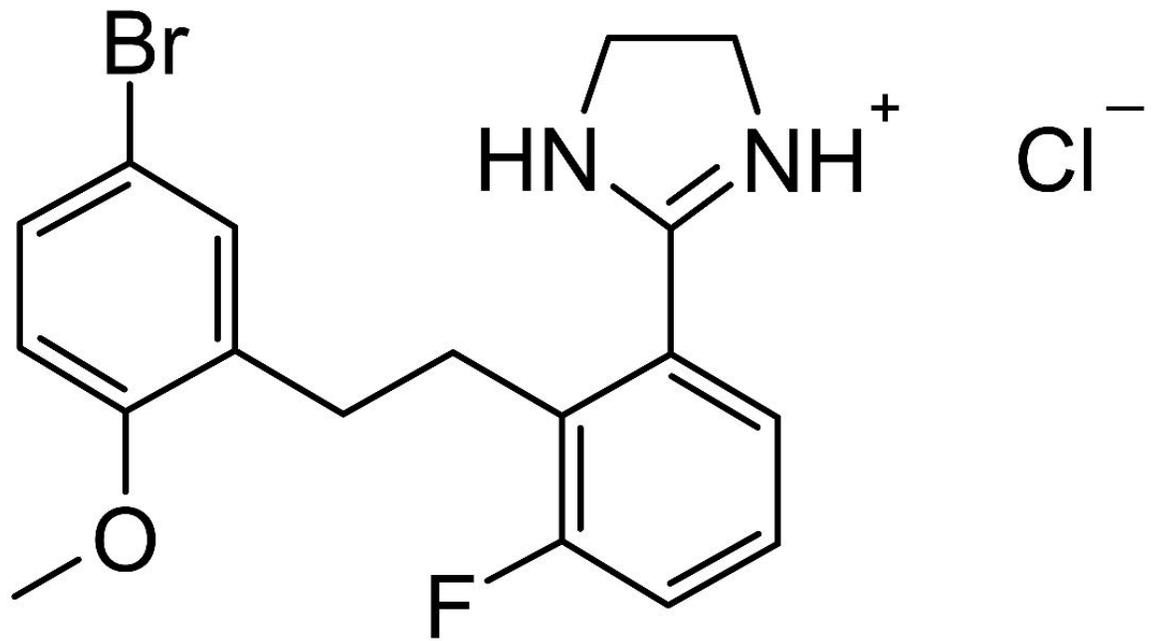
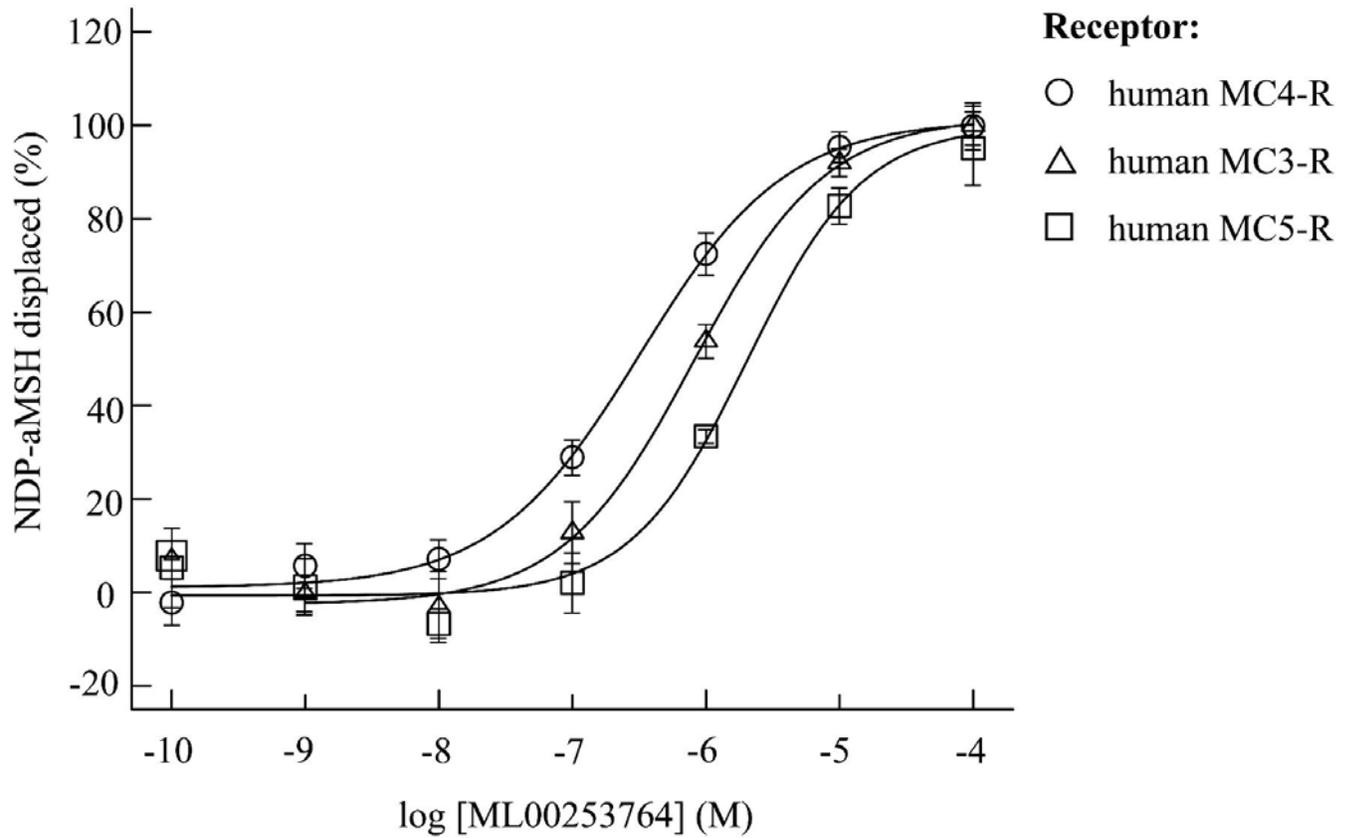


Figure 1

(a)



(b)

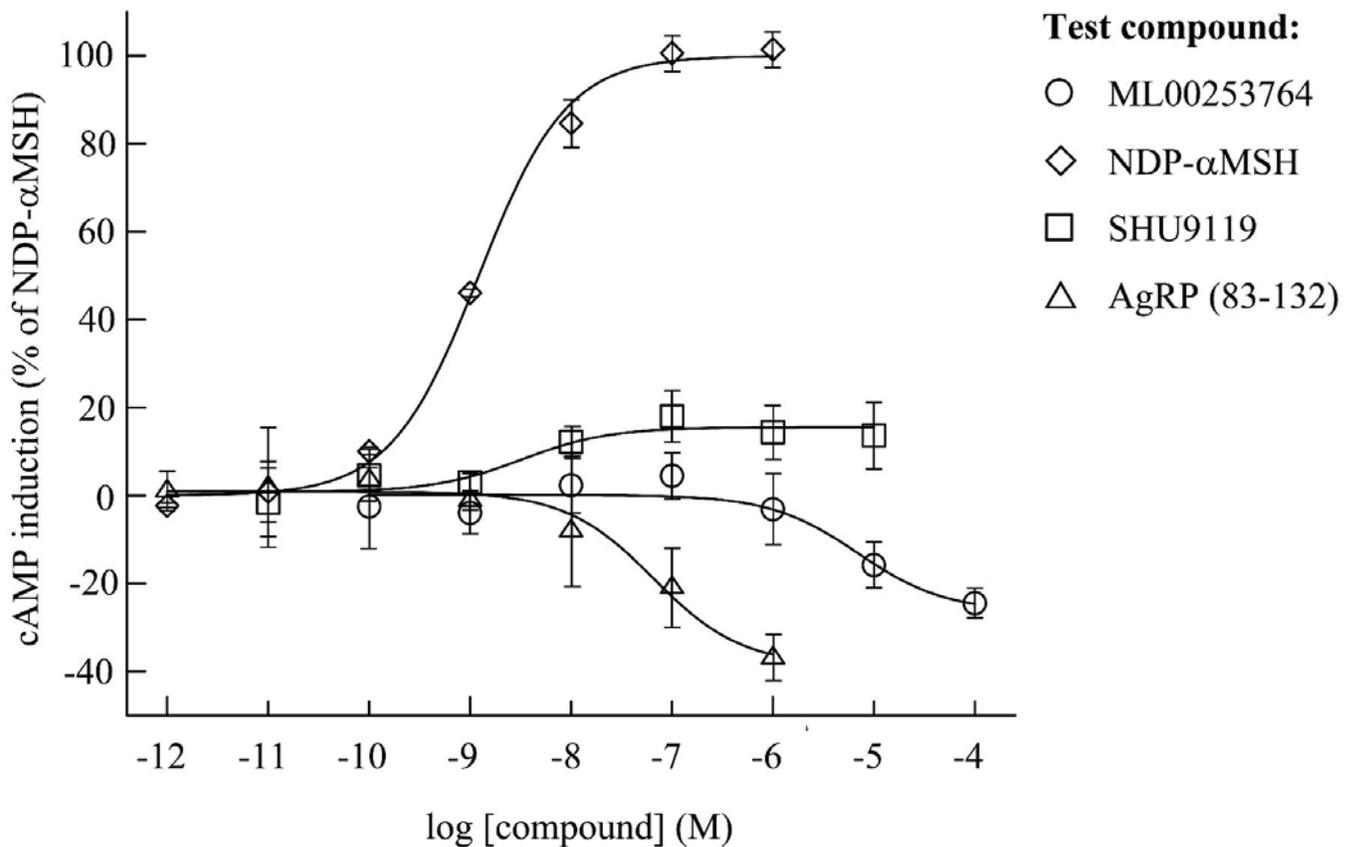


Figure 2

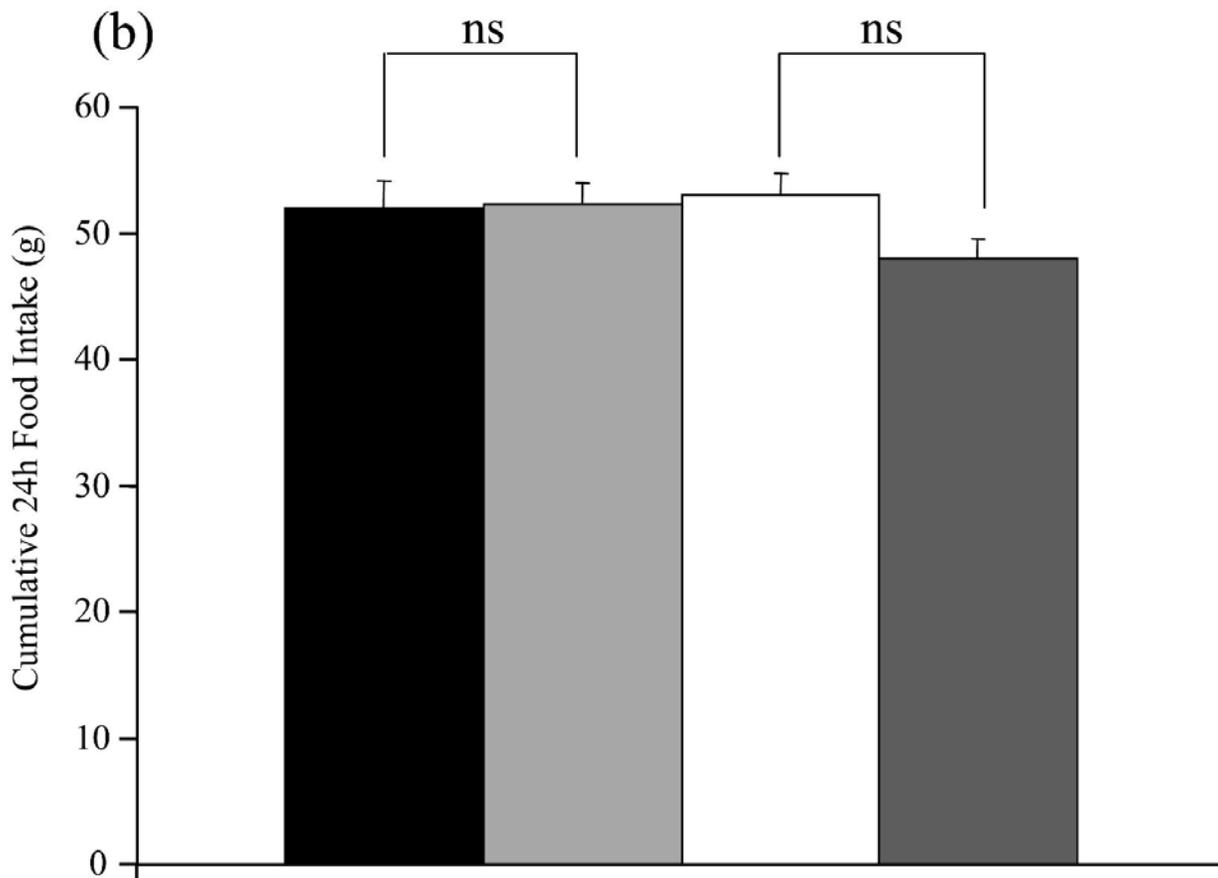
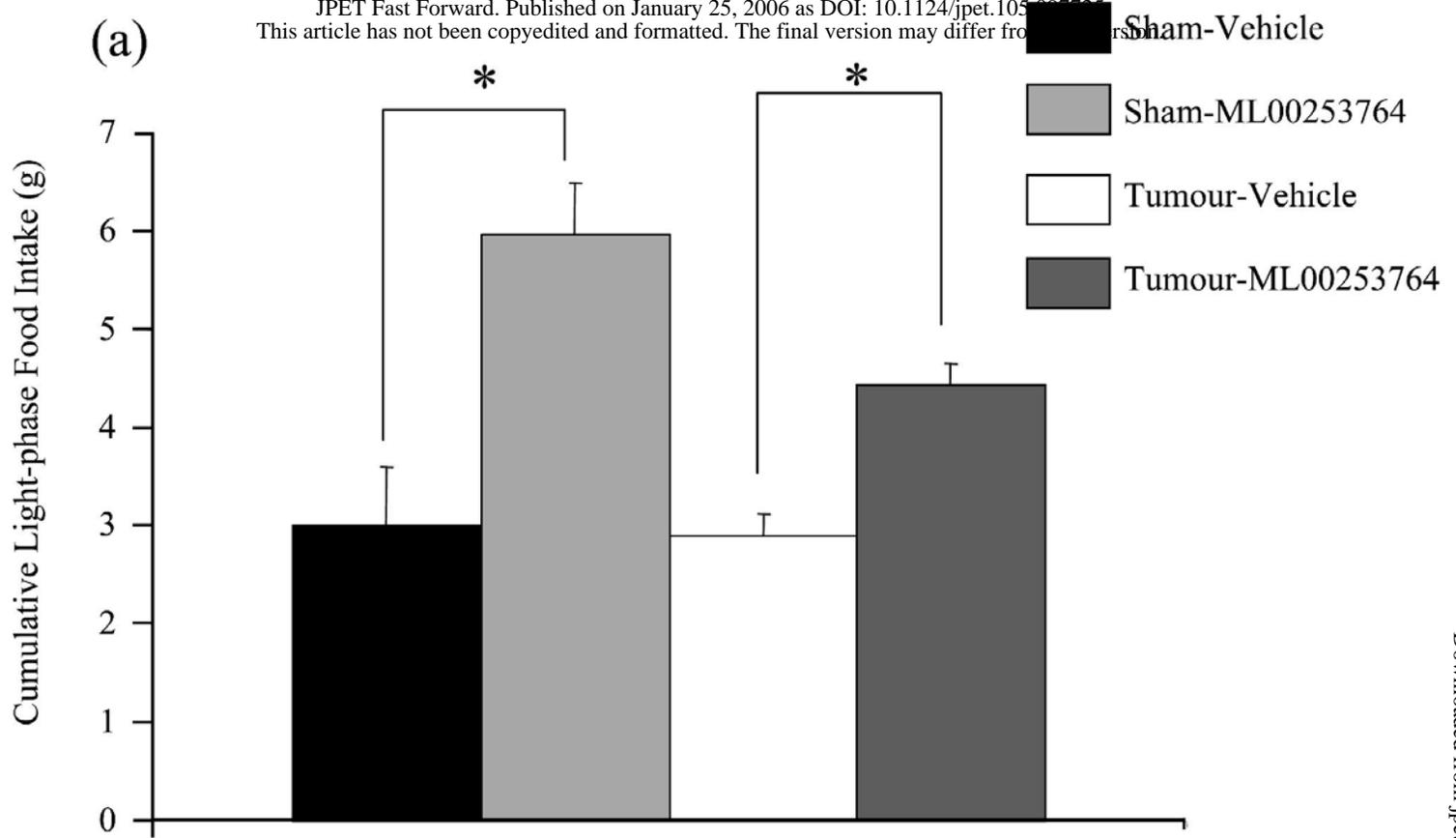


Figure 3

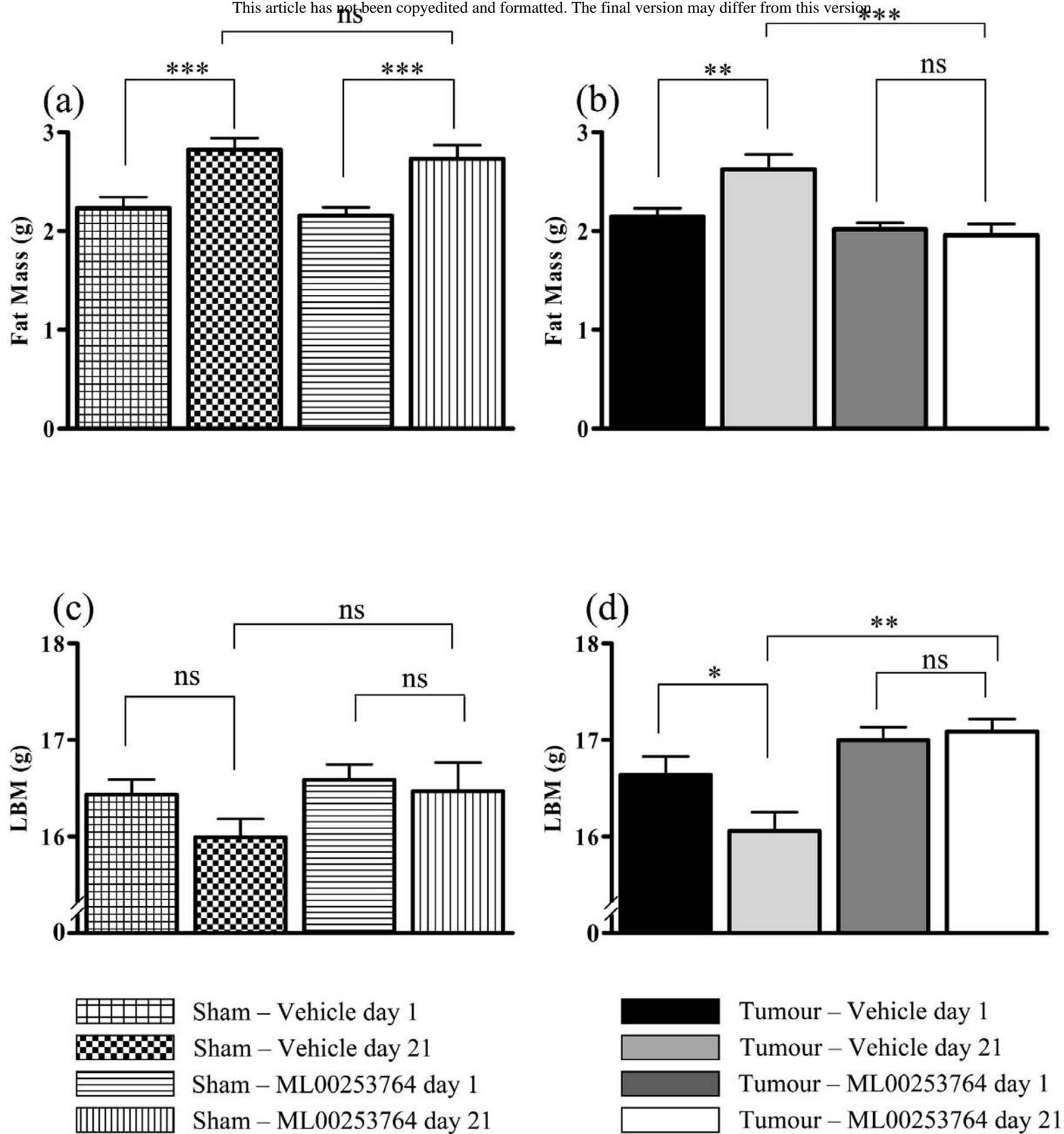
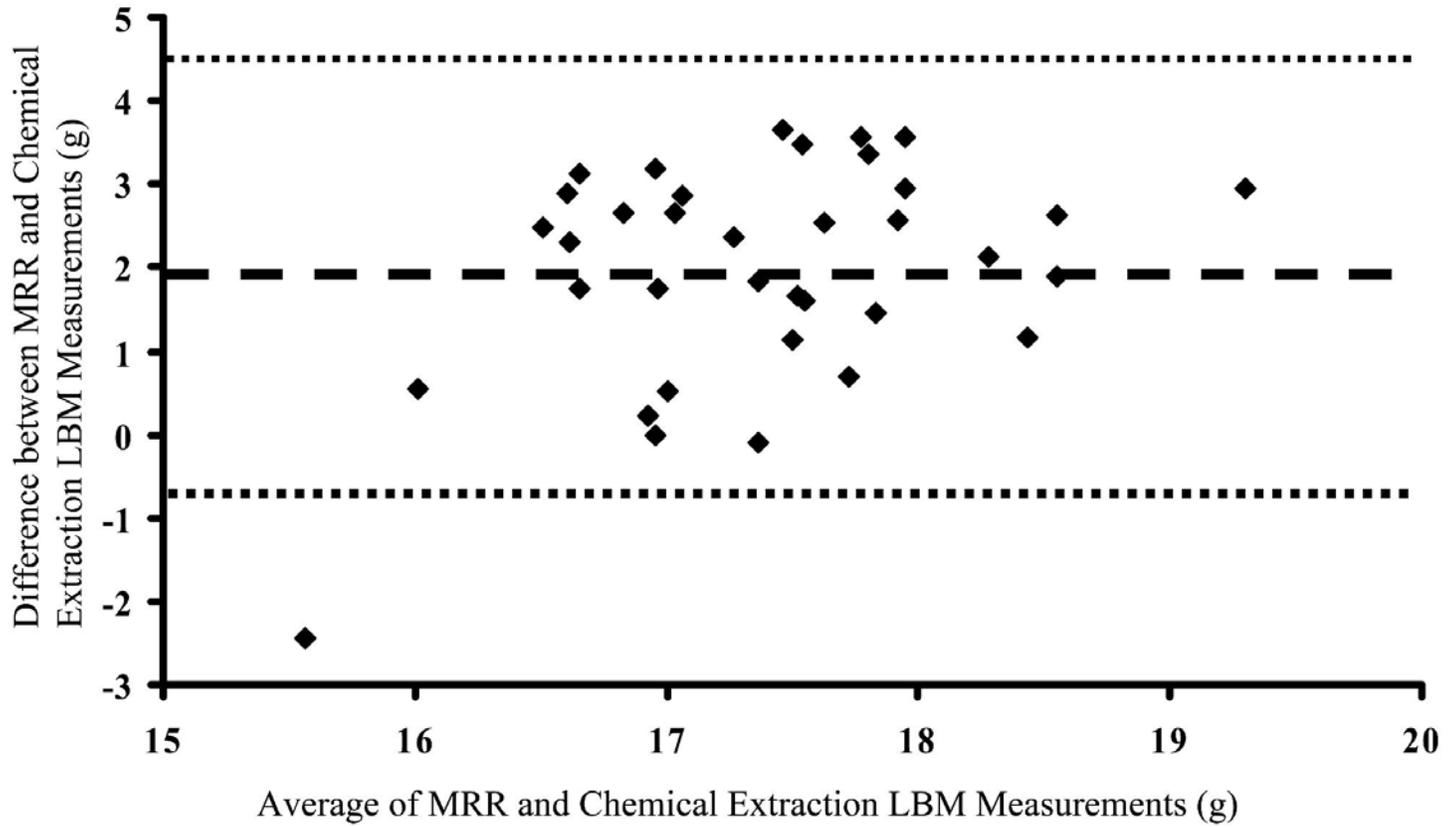


Figure 4

(a)



(b)

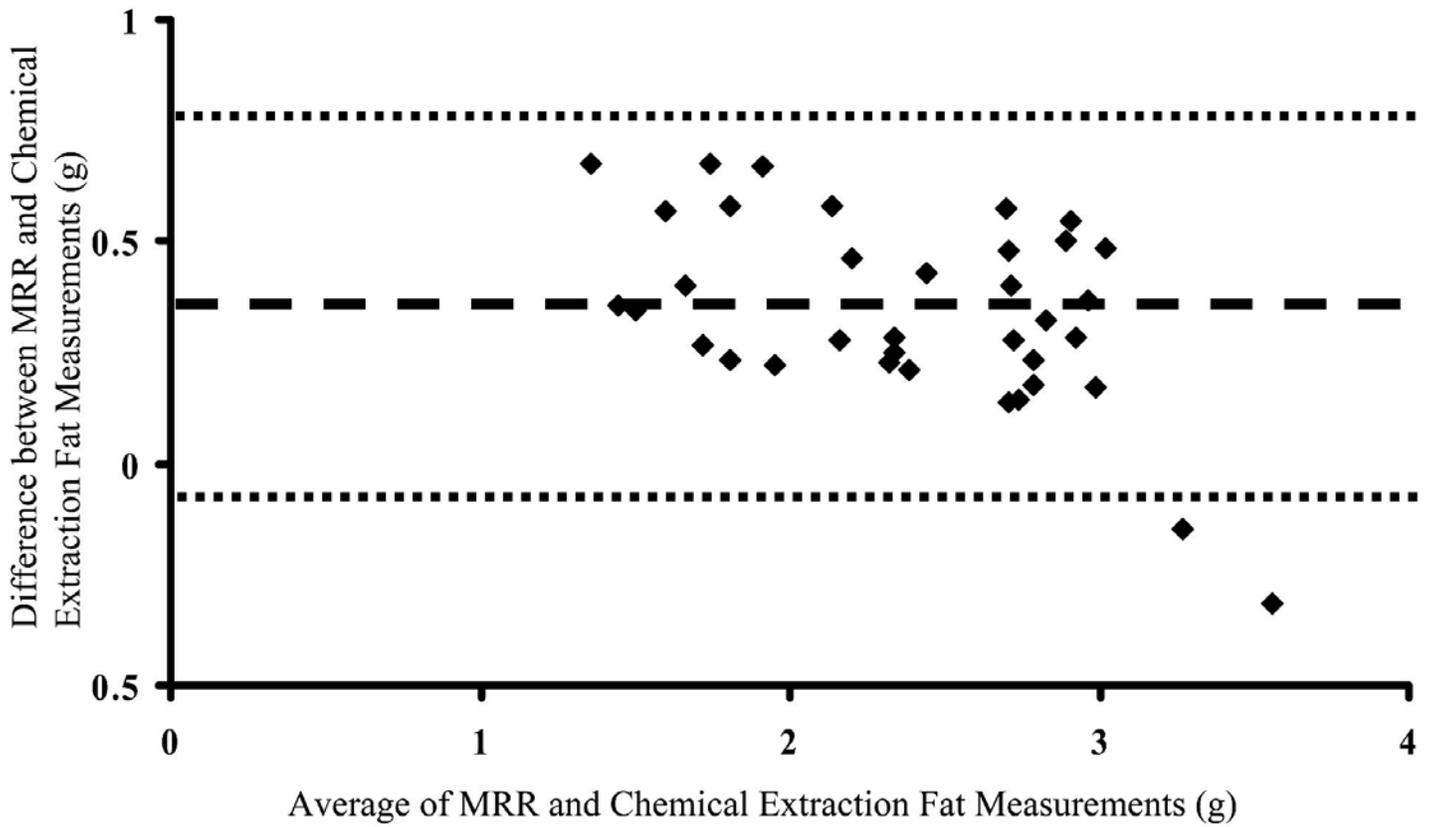


Figure 5