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MAPPING OF BIGUANIDE TRANSPORTERS IN HUMAN FAT CELLS AND THEIR IMPACT ON LIPOLYSIS

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Abstract

AIMS.—The anti-lipolytic actions of biguanides in fat cells may contribute to their antidiabetic effects. Biguanides use membrane transporters to act intracellularly. The transporters involved in mediating the antilipolytic effect in human fat cells are unknown and were presently examined.

MATERIALS AND METHODS.—Gene expression of biguanide transporters was mapped in human subcutaneous adipose tissue and in adipocytes before and after differentiation. Those expressed in mature fat cells were knocked down by RNA interference and the antilipolytic effect of metformin and two novel, highly potent biguanides, NT1014 and NT1044, was examined.

RESULTS.—Transporter affinity of biguanides in HEK293 cells overexpressing individual transporters showed that NT1014 and NT1044 displayed >10 times higher affinity compared with metformin. Studies in animals showed that NT1014 was >5 times more potent than metformin in lowering plasma glucose in mice. In human fat cells, the novel biguanides displayed higher AMPK activation and antilipolytic efficacy than metformin. Five transporters, OCT1 (*SLC22A1*), OCTN1 (*SLC22A4*), OCT3 (*SLC22A3*), PMAT (*SLC29A4*) and MATE1 (*SLC47A1*) were detectable in fat cells but only OCT3, PMAT and MATE1 increased during adipogenesis *in vitro* and were enriched in fat cells compared with other adipose cell types. Gene knockdown by RNA interference showed that MATE1 and PMAT reduction attenuated the anti-lipolytic effect of metformin but only PMAT knockdown decreased the effect of all three biguanides.

CONCLUSIONS.—While human fat cells primarily express three biguanide transporters, our data suggest that PMAT is the primary target for development of fat cell-specific antilipolytic biguanides with high sensitivity and potency. (250 words)

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Conflict of Interest

K.B. and J.L. are employed at NovaTarg Therapeutics Inc. None of the other authors have any conflict of interest to report.

INTRODUCTION

The biguanide metformin is the most commonly used pharmacological compound for treatment of type 2 diabetes [1]. Its blood glucose lowering effect is mainly ascribed to improved insulin-mediated suppression of glucose production in the liver [2], pleiotropic effects in the gastrointestinal tract [3] and to a lesser extent by increased glucose uptake in skeletal muscle [4]. However, metformin also suppresses lipolysis in adipose tissue leading to decreased output of fatty acids which may also improve insulin action [4]. The antilipolytic effect of metformin was first and directly demonstrated in human subcutaneous adipose tissue using microdialysis measures of glycerol (an end product of lipolysis) [5, 6]. This suggests that metformin, at relevant *in vivo* concentrations, can attenuate fat cell lipolysis in man. Several *in vitro* studies of isolated human or rodent adipocytes have subsequently confirmed that metformin inhibits catecholamine-stimulated lipolysis, with maximal effects reaching up to 30–40% inhibition [7–11]. These reports have also delineated the intracellular mechanisms of the antilipolytic effect, which is due to inhibition of several steps in cyclic AMP-mediated lipolysis activation. At least in human fat cells, the most important action seems to be stimulation of AMP-activated protein kinase (AMPK) which counteracts the pro-lipolytic effect of cyclic AMP [10].

Given the marked polarity of metformin, an important aspect in mediating its action is the cellular uptake via membrane transporters [12]. A large number of such transporters have been identified, including organic cation transporters-1, -2, and -3 (OCT1–3, encoded by *SLC22A1*, *SLC22A2* and *SLC22A3*, respectively), organic cation transporter novel type 1/ergothioneine transporter (OCTN1/ETT, encoded by *SLC22A4*), multidrug and toxin extrusion transporters-1 and -2 (MATE1 and -2, encoded by *SLC47A1* and *SLC47A2*, respectively) as well as plasma membrane monoamine transporter (PMAT, encoded by *SLC29A4*) [13]. While the role of these transporters for the antilipolytic effect of metformin is unknown, some OCTs have been investigated in human fat cells with regard to lipogenesis and AMPK activity [14]. This showed that OCT1, but not OCT2, was expressed in adipocytes and that cimetidine (a non-specific inhibitor of OCTs and MATEs) counteracted the effects of metformin.

The antilipolytic actions of metformin are observed *in vivo* at therapeutic doses [5, 6] but the *in vitro* effects are only observed using high millimolar concentrations of the drug. It could be argued that, at these levels, the effect of metformin is purely pharmacological. Recently, more potent biguanide analogues have been developed for the treatment of metabolic disorders and cancer. Thus, NT1014 and NT1044 (Figure 1A) have been evaluated in models for ovarian cancer and endometrial cancer [15]. These studies showed that both compounds suppressed cancer cell growth through their effects on the AMPK/mTor pathways resulting in G1 cell cycle arrest and apoptosis.

Herein, we compared the antilipolytic effect of metformin in human adipocytes with that of NT1014 and NT1044 and found that the two novel biguanides display significantly more potent antilipolytic effects compared with metformin. In order to better understand whether these differences could be explained by cellular uptake, we mapped all known biguanide transporters in human subcutaneous fat cells. Furthermore, we downregulated the adipocyte-

expressed transporters by RNAi and compared the changes in the antilipolytic effects of metformin, NT1014 and NT1044.

Methods

Subjects

Freshly isolated adipocytes were obtained from 14 subjects. Clinical characteristics are in Table 1. Five subjects were diagnosed with type 2 diabetes, all were life-style treated and none were on insulin or glitazone treatment. All remaining subjects were healthy. Subjects were consecutively recruited to our out-patient clinic and were not selected based on BMI, age or gender. Exclusion criteria were on-going treatment with metformin, glitazones and/or insulin and uncontrolled hypertension. Following the clinical examination, adipocytes were isolated from subcutaneous abdominal fat biopsies as described below. All samples were handled by the same research nurse and technician. The study was approved by the regional ethics board and informed written consent was obtained from all participants.

Adipocyte isolation from adipose tissue

Adipose tissue was rinsed repeatedly in saline and visual blood vessels and cell debris were removed. Tissue specimens (about 1 gram) were divided into portions, one of which was subjected to collagenase treatment to obtain isolated adipocytes as described [16]. The remaining part of the adipose samples (~300 mg) was immediately frozen in liquid nitrogen for subsequent RNA isolation as described below.

Lipolysis in mature adipocytes isolated from tissue biopsies

Diluted adipocyte suspensions (2% vol/vol) were incubated in duplicates for 2 h with air as the gas phase at 37°C in Krebs-Ringer phosphate buffer (pH 7.4) supplemented with glucose (8.6 mmol/l), ascorbic acid (0.1 mg/ml) and bovine serum albumin (BSA) (20 mg/ml). Cells were treated with 5 nmol/l of isoprenaline in the absence or presence of increasing concentrations of metformin (Sigma Aldrich, St. Louis, MO), NT1014 and NT1044 (the latter two from NovaTarg Therapeutics Inc, Research Triangle Park, NC). During lipolysis, intracellular triglycerides are broken down into non-esterified fatty acids and glycerol. After the incubation period, glycerol release was determined as an index of lipolysis. Results were expressed as amount of glycerol in relation to control cells incubated without biguanides. Glycerol measurements were performed using a well-established bioluminescence method [17].

Lipolysis in *in vitro* differentiated adipocytes

For assessments of non-esterified fatty acid release, human subcutaneous preadipocytes (ZenBio Inc., Research Triangle Park, NC) were cultured in 96-well plates and differentiated into adipocytes by the Zen Bio protocol (Instruction Manual ZBM0001.04, ZenBio, Inc.). The adipocytes were then incubated as described in lipolysis buffer assay (Instruction manual ZB LIP-2-NC, ZenBio, Inc.) in the absence or presence of increasing concentrations of metformin, NT1014 or NT1044. Lipolysis was stimulated by the addition of 0.5 µmol/l isoprenaline and allowed to proceed for 3 h when non-esterified fatty acids were measured as described (Instruction manual ZB LIP-2-NC, ZenBio, Inc.).

For assessment of lipolysis in siRNA-transfected human adipose-derived stem cells (hASCs), cells were isolated, grown, and differentiated as previously described [18]. In brief, cells were incubated for 3 h in DMEM/F12 medium supplemented with 20 mg/ml of BSA, isoprenaline (50 nmol/l) with or without metformin, NT1014 and NT1044. Glycerol in media was measured using Free Glycerol Reagent (Sigma Aldrich) and Amplex UltraRed® (Invitrogen). Amplex Ultra Red was diluted 100-fold in Free Glycerol Reagent, mixed with 20 µl of conditioned medium in a 96-well plate, incubated at room temperature for 15 min and fluorescence was measured (Ex/Em 530/590) using an Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland). Upon collection of medium, cells from the same wells were lysed in RIPA buffer and protein concentration was measured. Glycerol levels were then corrected for total protein content.

AMPK activity assay

Human subcutaneous preadipocytes (ZenBio Inc.) and hASC were cultured and differentiated to adipocytes as described for the lipolysis assay. AMPK activation was measured following a 2 h incubation with the indicated concentration of biguanide or vehicle. Instructions were followed for the ELISA kit#7959 from Cell Signaling Technologies (Danvers, MA, USA) that determines p-AMPK on Thr-172 in the cell extract. Total AMPK α was measured in parallel using ELISA kit ##7961C (Cell Signaling) and pAMPK α values were normalized to the total AMPK α values. Data were analyzed as outlined by the manufacturer.

Measurements of oxygen consumption rate

Oxygen consumption rate (OCR) was measured in HEK293 cells expressing the indicated human biguanide transporter using the MitoXpress Xtra reagent as described for dual read time-resolved fluorescence for adherent cells (MitoXpress manual, Luxcell Biosciences, Cork, Ireland). In these studies dual real-time fluorescence measurements were made every 2 min over a 2 h period. Lifetime slopes were calculated using lifetime measurement over time. The IC₅₀ values were determined by plotting lifetime slope values versus compound concentration. Given that a fundamental action of biguanides on mitochondria is the inhibition of mitochondrial complex 1 and/or mitochondrial glycerophosphate dehydrogenase [19, 20], effects on OCR can be considered a “functional” measure of the affinity of a biguanide for a specific transporter. The effects of the biguanides on OCR were not secondary to cell toxicity. This was demonstrated in separate experiments of HEK293 cells over-expressing OCT1 or OCT2 where only incubations with high concentrations of metformin or phenformin (corresponding to 20–1000 times higher than the IC₅₀ for the OCR-effect) for up to 24 hrs had effects on cell proliferation. For metformin, even these high concentrations had small effects on cell proliferation (15–30% reduction).

Gene knockdown experiments and viability assays

Studies were performed in hASCs cultured as described above. Cells were transfected using a Neon electroporator (Invitrogen, Carlsbad, CA) at day 4 of differentiation according to the manufacturer's protocol. In brief, 1 million cells were mixed with 40 nmol/l ON-TARGETplus SMARTpool small interfering RNAs (siRNAs) targeting either *SLC22A3*, *SLC29A4* or *SLC47A1* or a non-targeting siRNA pool (Dharmacon, Lafayette, CO) and

electroporated using a 100 µl NEON electroporation tip. Electroporation conditions were 1600 Volts, 20 ms width, 1 pulse. Subsequently, cells were plated in antibiotic-free medium at a density of 60.000 cells/well in 48-well plates. Medium was replaced 24 h post-transfection and subsequently every second day. The cells were cultured until day 12 of differentiation upon which lipolysis experiments were performed and protein/RNA/medium was collected. Apart from assessing cell viability by cell morphology, protein concentrations (in total cell lysates) or gene expression (e.g. of *18S*rRNA), determination of lactate dehydrogenase activity (a measure of cell membrane integrity) was also performed in the conditioned media using Cytotoxicity Detection Kit (Roche). This showed no significant effect of our treatments (data not shown).

RT-qPCR and gene expression analyses

WAT specimens from the clinical samples were disrupted mechanically and RNA isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The hASCs were collected at day 12 after the induction of *in vitro* differentiation for isolation of RNA. For time course samples, hASCs were collected immediately after induction of adipogenic differentiation and at day 1, 2, 4, 8 and 13. Total RNA from the hASCs was extracted using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Concentration and purity of RNA were measured using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, US). Reverse transcription was performed using the iScript cDNA synthesis kit (Qiagen) and random hexamer primers (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed using commercial TaqMan probes (Thermo Fisher Scientific). Gene expression was normalized to the internal reference *18S*rRNA. Relative expression was calculated using the 2^{-C_t} method [21]. For *SLC22A1* amplification, two different TaqMan kits were used (#Hs00427552_m1 and #Hs00427550_m1), both yielding similar Ct values (>35) in adipose tissue and *in vitro* differentiated hASCs. Ct values between 35–40 correspond to an expression of <1 transcript per cell and are not considered physiologically relevant [22]. For expression studies of biguanide transporters in human preadipocytes and adipocytes (Zenbio Inc.), total RNA was isolated and cDNA was generated using 2 µg of RNA (ABI High Capacity cDNA reverse transcription kit #4368814, Waltham, MA). Quantitative PCR for each cDNA was conducted with Taqman probes using iTaq Universal Probe Supermix (BioRad, Hercules, CA). Microarray data of different resident cells in human subcutaneous adipose tissue were generated as described [23].

Analysis of protein expression

Human ASCs were transfected with siRNA as described above and plated in 6-well plates at a density of 500.000 cells. At day 12 of differentiation, cells were lysed in RIPA buffer as described [24]. Total liver protein was obtained from Novus Biologicals (CO, USA). 20–25 µg of total protein was separated by SDS-PAGE and Western blot was performed according to standard procedures. The membranes were blocked in 3 % ECL Advance™ Blocking Agent (GE Healthcare, Buckinghamshire, UK). Primary antibodies were OCT1-rabbit IgG (ab181022, Abcam, Cambridge, UK) used at a concentration of 1:2500, OCT1-rabbit IgG (LS-C354446, LifeSpan Biosciences, Inc., WA, USA) (1:500), OCT3-goat IgG (42–915, ProSci Inc., CA, USA) (1:500), MATE1-rabbit IgG (#14550, Cell Signaling Technologies,

Danvers, MA, USA) (1:1000), PMAT-rabbit IgG (HPA052829, Atlas AB, Bromma, Sweden) (1:200); GAPDH-rabbit IgG (1:2000) was used as a loading reference (Cell Signaling). Secondary rabbit-IgG/goat-IgG antibodies conjugated to horseradish peroxidase were used (Sigma-Aldrich). Proteins were detected by chemiluminescence using ECL™ Select Western Blotting Detection Kit (GE Healthcare) in Chemidoc XRS system (Bio-Rad, Hercules, CA) and quantified by Quantity One software (Bio-Rad).

Glucose Tolerance Tests in animals

Oral glucose tolerance tests (OGTT) studies in mice were conducted to compare NT1014 with metformin. The work was carried out by Charles River Laboratories and were approved by the regional board of ethics. 24 male C57Bl/6 mice were studied at ~9 weeks of age. Animals were sourced from Charles River (Wilmington, MA) and group housed in larger cages at 8/cage. Standard rodent chow (Purina Lab Diet 5001) and drinking water were provided ad libitum prior to study day. Vehicle was compared with metformin orally dosed at 250 mg/kg and with NT1014 orally dosed at 50 mg/kg. Compounds were formulated in 0.5% methylcellulose the morning of the dose and were administered to the appropriate animals by oral gavage. The dose volume for each animal (10 mL/kg) was based on the most recent body weight measurement taken just prior to dosing. Tests were conducted following a 5 hr fast (food removed ~0800 hrs). In brief, at ~1300 hrs, a baseline blood glucose was checked via handheld glucometers on all mice. Mice were randomized into treatment groups based on the 5 hr fasted blood glucose. At ~1330 hrs all mice were gavaged with their respective compound dose and 30 minutes later (~1400 hrs) were gavaged with glucose at 3 g/kg (10 mL/kg) at a pace of 1 minute per mouse. Blood glucose was checked via glucometer at the following times relative to glucose dose: baseline (same time as 5hr fasted bleed ~1300 hrs, prior to compound dose), 15, 30, 60, 90, and 120 min.

Statistics

Values are given as mean±S.D. in text and mean±S.E.M in figures. Standard statistical tests were used including t-test, repeated measures and one-way ANOVA and Fisher's post-hoc tests as indicated in figure legends. GraphPadPrism v7.02 from GraphPad Software Inc. was used.

Results

Antilipolytic effects of metformin and biguanide analogues

In vitro differentiated human adipocytes were incubated with the β -adrenergic agonist isoprenaline in the presence of increasing concentrations of metformin, NT1014 and NT1044 (Fig. 1B). For all three agents there was a concentration-dependent effect resulting in >80% attenuation of NEFA release. The drug concentration causing half-maximum inhibition (IC₅₀) for NT1014 and NT1044 were approximately ten times lower than that for metformin where NT1044 provided the most potent inhibition of lipolysis with IC₅₀ values of 0.05 mmol/l, 0.23 mmol/l and 2.0 mmol/l for NT1044, NT1014 and metformin, respectively. Maximal inhibitory effects of NT1014 and NT1044 were observed at 1 mmol/l versus 10 mmol/l for metformin. Similar differences in potency were observed in the same cell model system with regard to AMPK activation (Fig. 1C). Based on the findings in

Figure 1B, the effects of 10 mmol/l metformin and 1 mmol/l biguanide analogues were determined in freshly isolated abdominal subcutaneous fat cells obtained from 14 donors (Table 1) where isoprenaline-induced lipolysis was determined as glycerol release (Fig. 1D). While the attenuating effect on lipolysis of all three biguanides was less pronounced than that observed in *in vitro* differentiated adipocytes, cells incubated with NT1044 or NT1014 displayed significantly stronger inhibition of glycerol release (~40%) compared with cells treated with metformin (~20%). In cells from these subjects, there was a trend ($p=0.088$) for a negative association ($r=-0.47$) between the degree of metformin-induced inhibition of lipolysis and circulating non-esterified fatty acid levels (S-NEFA) (graph not shown). This suggests that subjects with elevated S-NEFA concentrations may display a more pronounced effect of metformin. In contrast, the antilipolytic effects of NT1014 and NT1044 showed no relationship with NEFA levels (graphs not shown). Five of the subjects were diagnosed with type 2 diabetes, however, the effects of the three biguanides was similar in subjects with or without diabetes (graphs not shown).

Affinity of biguanides to cellular transporters

The relative affinity of specific biguanide transporters for cellular uptake was compared in HEK293 cells overexpressing individual transporters (Table 2). These cells express negligible endogenous levels of each transporter. Effects were determined by assessing the inhibitory effects of metformin, NT1014 and NT1044 on oxygen consumption rate (OCR), providing a “functional” measure of biguanide affinity for each transporter, and were expressed as IC_{50} . All three biguanides reduced OCR in a concentration-responsive manner (graphs not shown). Under these experimental conditions, the “affinity” for metformin and both biguanide analogues were highest for OCT1–3 and PMAT and lowest for OCTN1 and MATE1. The potency of NT1014 and NT1044 versus metformin was again underscored by the approximately ten times lower IC_{50} observed for all transporters (except OCTN1 for NT1044). Importantly, the IC_{50} values observed in this artificial cell system cannot be extrapolated to treatments in cells expressing endogenous transporter levels.

In vivo effects of biguanides

The efficacy of the novel biguanides was tested in an animal model. To this end, mice underwent glucose tolerance tests following administration of vehicle, metformin (250 mg/kg) or NT1014 (50 mg/kg). In concordance with the *in vitro* data in adipocytes, a similar reduction in individual glucose values (Fig. S1A) and area under the curve (Fig. S1B) was observed between the two biguanides despite administering NT1014 at one fifth of the dose of metformin. This supports the notion that the novel biguanides are effective in improving metabolic control *in vivo* at lower doses than metformin. It was outside the scope of the present work to determine the contribution of biguanide effects in different tissues.

Gene and protein expression of biguanide transporters in human adipocytes

In order to identify transporters of importance for mediating the antilipolytic effects in human adipocytes, the gene expression of all seven hitherto described biguanide transporters was determined by qPCR in human adipose-derived stem cells during adipocyte differentiation *in vitro*. *SLC22A2* (OCT2) and *SLC47A2* (MATE2) were not detectable while *SLC22A1* (OCT1) was expressed at very low levels ($Ct\text{-values}>35$) at all time points.

The latter was not considered physiologically relevant. For the remaining four genes there was a marked difference in expression levels (Fig. 2). Thus, *SLC22A4* (OCTN1) displayed a peak at day 1 but was subsequently down-regulated (Fig. 2A), while *SLC22A3* (OCT3), *SLC29A4* (PMAT) and *SLC47A1* (MATE1) were significantly upregulated during adipogenesis (Fig 2B-D). Congruent gene expression results were observed in comparisons of human preadipocytes before and after differentiation (Fig. S2) and in the publically available Human Protein Atlas (www.proteinatlas.org, data not shown). We also analyzed microarray data from different resident cells of white adipose tissue (Fig. 3A-E). This confirmed that *SLC22A3* and *SLC29A4* were virtually selective for mature fat cells and that *SLC47A1* was clearly enriched in the adipocyte fraction (Fig. 3C-E). In concordance with the qPCR results, *SLC22A1* was expressed at very low levels (array signal<30) with no clear cellular enrichment (Fig. 3A) while *SLC22A4* expression was comparatively low in the adipocyte fraction (Fig. 3B). Gene expression was compared with the levels of the corresponding proteins in *in vitro* differentiated adipocytes by Western blot analysis (Fig. S3A-D). This showed that OCT3, PMAT and MATE1 were clearly detectable (Fig. S3A-C). Despite the low expression of *SLC22A1*, based on the data described above [14], we chose to analyze OCT1 protein as well and compared that to the levels in liver, an organ that is well established to express OCT1. Using one commercially available antibody, a band corresponding to the size of OCT1 (61 kD) was observed in liver while a stronger band with a slightly larger size was observed in differentiated and non-differentiated adipocytes in both membrane and cytosol fractions (Fig. S3D). We therefore opted to test a second OCT1-directed antibody. This showed that while there was a clear band in liver, there was no corresponding band in *in vitro* differentiated adipocytes (Fig. S3D). Taken together, this suggests that OCT1 protein is not expressed to any significant degree in human adipocytes and further studies were therefore not pursued on this transporter.

Down regulation of biguanide transporters in human adipocytes

The role in mediating the antilipolytic effects of biguanides was tested by knocking down the corresponding transporter genes by siRNA in human adipocytes followed by incubations with isoprenaline with or without metformin, NT1014 or NT1044 (Fig. 4). RNAi efficiently reduced the levels of *SLC22A3*, *SLC29A4* and *SLC47A1* by >85 % (Fig S3E). This was reflected by significantly attenuated levels of MATE1 and PMAT protein (>65%) although knockdown of OCT3 was less pronounced (~30%) (Fig. S3A-C, E). Downregulation of *SLC22A3* (OCT3) did not affect the antilipolytic effects of any of the biguanides (Fig. 4A, biguanide concentrations same as in Fig. 1D). In contrast, siRNAs against *SLC29A4* (PMAT) resulted in an attenuated effect by all three biguanides (Fig. 4B) while downregulation of *SLC47A1* (MATE1) only influenced the effects of metformin (Fig. 4C). PMAT knockdown abrogated AMPK-activation by NT1014/-1044 confirming that this transporter is essential for mediating the effects of the novel biguanides (Fig. S4). Altogether, this suggests that PMAT mediates uptake of all three biguanides while MATE1 is relevant for metformin uptake, at least at the supra-pharmacological concentrations used herein.

Discussion

Several cationic transporters mediating metformin uptake have been described in recent years [13]. However, their inter-individual importance, particularly in adipose tissue, is still unclear. Given that the antilipolytic effects of biguanides may contribute to their antidiabetic actions, we mapped cell membrane transporters of functional relevance in human adipocytes. Our data demonstrate that five genes are detectable in WAT, three of which are expressed at physiologically relevant levels and upregulated during adipocyte differentiation. Among the latter, two (OCT3 and PMAT) are specific for adipocytes while MATE1 is enriched in the same cell fraction. A caveat when assessing the effects of metformin in experimental models is that suprapharmacological doses are often used. In the present study we therefore assessed the effects of NT1014 and NT1044, two biguanide analogues displaying significantly higher affinity to all cell membrane transporters as well as higher potency and efficacy on fat cell AMPK activation and anti-lipolysis compared with metformin. We performed a set of functional assays in order to determine the transporters primarily mediating biguanide uptake in human adipocytes. As selective inhibitors of cationic transporters are not available, we studied the effects of the biguanides following gene knockdown by siRNA. Our findings confirm that the compounds display differential sensitivity to specific transporters and that PMAT appears to be of particular relevance in mediating uptake and thereby the anti-lipolytic effects of both metformin and novel biguanides in human adipocytes.

Despite more than 6 decades of clinical use, the exact mechanism of action of biguanides remains elusive. Although it has been contested by some groups [25], AMPK activation is still suggested by many independent investigators to mediate most of the effects induced by metformin including antilipolysis [10]. In line with this notion we could confirm that metformin as well as NT1014 and NT1044 increased AMPK activation in human adipocytes *in vitro*. The novel biguanides have been synthesized to improve their ability to elicit positive changes in metabolism in both metabolic disorders and cancer. The guiding principle for the design of these agents is to increase transmembrane transport into metabolic target organs like liver, skeletal muscle and adipose tissue when compared to metformin while not increasing their excretion by the kidney. The increases in transporter activity for NT1014 and NT1044 over that of metformin stem from the aromatic heterocycle attached to the biguanide group via an acyl linker which was found during the testing of over 260 novel biguanides synthesized by NovaTarg Therapeutics. That this approach is valid is demonstrated by the fact that metformin induced a small increase in AMPK activation at a high concentration (10 mmol/l), while NT1014 and NT1044 displayed significantly larger effects at lower concentrations (0.1 mmol/l). Overall, these results support the notion that NT1014 and NT1044 produce a greater response than metformin in the AMPK signaling pathway through their increased intracellular uptake. That this translates into more potent effects is supported by our *in vivo* studies demonstrating that NT1014 exerted similar glucose-lowering effects as metformin at one fifth of the dose.

Our analyses of gene expression showed that OCT3, PMAT and MATE1 were expressed at clearly detectable levels and increased during adipocyte differentiation *in vitro*. Together with their cellular expression in different cell fractions of WAT, this suggested that all three

may play a metabolically relevant role in fat cells. This hypothesis was directly tested by gene knockdowns in human adipocytes achieved using siRNA protocols. These were effective at both the mRNA and protein level for PMAT and MATE1. In contrast, the effects on OCT3 expression were smaller, suggesting that efficient OCT3 knockdown in human adipocytes may require other approaches. Thus, we cannot establish the importance of OCT3 in mediating the antilipolytic effects of biguanides in our present cell system.

Interestingly, previous studies have suggested that OCT1 is involved in mediating AMPK activation and anti-inflammatory effects of metformin in human adipocytes [14]. In that study, the authors showed that *SLC22A1* was expressed in human adipose tissue and *in vitro* differentiated adipocytes (commercially obtained from Zenbio) using qPCR. At present, we cannot explain why we observed very low levels of *SLC22A1* expression in our arrays, findings which were validated using two different TaqMan probes and were congruent with tissue expression data from the Human Protein Atlas. Furthermore, while a band of approximately the size of OCT1 was detectable in human adipocytes using one antibody, another antibody detected a band of the correct size in liver but not in *in vitro* differentiated adipocytes. It is therefore likely that the band detected by some commercial antibodies in adipocytes may represent another protein. At the moment, this remains a speculation as we have not sequenced the bands. Importantly, our findings do not question the relevance of OCT1 in mediating biguanide effects in non-adipose tissues. Thus, biguanides display a high sensitivity to OCT1 in HEK293 cells overexpressing this transporter (Table 2). Moreover, *Slc22a1* deletion or genetic variants in the *SLC22A1* gene result in abrogated or reduced effects of metformin in mice and humans, respectively [26]. However, none of these studies have specifically looked at OCT1 and its link to the antilipolytic effects in fat cells.

The antilipolytic action of metformin has so far been shown in only a limited number of studies. While we demonstrate this effect and identify cellular transporters in adipocytes from a large number of individuals (of both genders and with a broad BMI range), the size and composition of the cohort does not allow us to establish whether the actions of biguanides are influenced by factors such as age, gender, body fat mass/region or insulin resistance/type 2 diabetes. In preliminary analyses of published gene microarray data from subcutaneous adipose tissue in two independent cohorts of non-obese and obese women [27, 28], we have not identified any obvious relationship between gene expression of different biguanide transporters and obesity or weight loss (data not shown). Admittedly, as these data were obtained in whole tissue samples, this does not exclude that differences in expression may be present in fat cells. Given the pleiotropic effects of biguanides in different tissues (liver, muscle, gut), another caveat with the present work is that we cannot establish the contribution of lipolysis-inhibition to improvements in glucose homeostasis induced by biguanides. Based on the broad affinity of biguanides to several cationic transporters expressed in different metabolic tissues, such studies would require the development of knockout animals where the expression of multiple transporters is concomitantly deleted in a tissue-specific manner. The non-specific effects of such an approach would make any interpretation very difficult. Admittedly, our experimental data are solely based on cell models of either freshly isolated human fat cells or *in vitro* differentiated human adipocytes. However, as recently discussed [29], both systems are well-established in studies of lipolysis and show good correlations with measures of lipolysis *in vivo*. In addition, anti-lipolytic

effects of metformin in adipose tissue have been demonstrated *in vivo* in at least two independent studies [5, 6].

Finally, while it was outside the scope of the present study, it would be of interest to compare the effects of metformin, NT1014 and NT1044 in different cell types *in vitro* as well as in different tissues *in vivo*. According to the Human Protein Atlas, liver/gallbladder primarily expresses OCT1, OCTN1, OCT3 and MATE1 while muscle primarily expresses OCT1, OCT3 and MATE1. It should be noted that neither tissue expresses PMAT to any significant extent; in fact, PMAT appears to be quite specific for adipose tissue.

In summary, the present work suggests that antilipolytic effects of two novel biguanides can be obtained with pharmacologically relevant concentrations and that development of potent analogues with high affinity to the cationic transporter PMAT could be useful as novel antidiabetic agents targeting lipolysis in adipocytes. Comparisons of biguanides with different transporter affinities could help decipher the tissue-specific relevance of biguanide-induced effects *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effects of biguanides in human fat cells.

A. Structure of NT1014 and NT1044, respectively. **B.** Concentration-dependent antilipolytic effects of indicated biguanides in *in vitro* differentiated adipocytes from Zenbio expressed as release of non-esterified fatty acids (NEFA). Data based on three independent experiments. **C.** Concentration-dependent effects on AMPK activation in *in vitro* differentiated adipocytes from Zenbio. Data based on three independent experiments. Results were first analyzed by ANOVA to confirm differences between treatments, asterisks denote significant differences in post-hoc tests (paired t-test) compared with control cells. **D.** Antilipolytic effects of biguanides in mature fat cells isolated from 14 individuals. Overall p-value using ANOVA is shown, b indicates statistically different from control ($p < 0.05$) and c indicates statistically different from metformin incubated cells ($p < 0.05$) in post-hoc tests (paired t-test). *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

Figure 2. Gene expression of cationic transporters during adipocyte differentiation. Human adipose-derived stem cells were differentiated into adipocytes and RNA was collected before and at the indicated days post-induction. Quantitative RT-PCR was performed using TaqMan probes against **A.** *SLC22A4* (OCTN1), **B.** *SLC22A3* (OCT3), **C.** *SLC29A4* (PMAT) and **D.** *SLC47A1* (MATE1). Data are based on cells isolated from three independent experiments.

Figure 3. Gene expression of cationic transporters in resident adipose tissue cells.

Gene microarray data from adipocyte progenitors, adipose tissue macrophages (ATM), T-cells, CD4+ T-cells, CD8+ T-cells, M1 ATM, M2 ATM and mature adipocytes were retrieved for the indicated genes. Data are based on pooled analyses of cells isolated from 4 independent donors.

Figure 4. Effects of RNAi-mediated knockdown of cationic transporters.

Human adipose-derived stem cells were transfected with non-targeting siRNA oligonucleotides (siNegC) or indicated siRNAs targeting specific transporters **A.** *SLC22A3* (OCT3), **B.** *SLC29A4* (PMAT) and **C.** *SLC47A1* (MATE1). Cells were incubated with isoprenaline (ISO) and the antilipolytic effects of metformin (10 mmol/l), NT1014 (1 mmol/l) and NT1044 (1 mmol/l) were determined. Asterisks indicate significant differences compared with siNegC transfected cells under the same condition using paired t-test. Results are based on four individual experiments with each condition performed at least in triplicates. **= $p < 0.01$, ***= $p < 0.001$.

Table 1.

Clinical characteristics of the 14 individuals where effects of metformin and novel biguanides were determined in mature fat cells.

Parameter	Nr of mean±S.D
Gender, female/male (nr)	13/1
Age, years	52.7±12
Body weight, kg	74.9±9.3
BMI, kg/m ²	28.3±2.8
Obese/overweight/lean (nr)	4/7/3
Waist-hip-ratio	0.91±0.05
T2DM, yes/no (nr)	5/9
Systolic blood pressure, mm Hg	131±20
HbA1c, mmol/mol	39±6
Total cholesterol, mmol/l	4.3±0.8
HDL-cholesterol, mmol/l	1.47±0.3
Triglycerides, mmol/l	0.73 ±0.18
Non-esterified fatty acids, mmol/l	0.57±0.17
Log HOMA IR	0.15±0.17
Fat cell volume, pl	520±167

Table 2.

Oxygen Consumption Rate (OCR) inhibition (IC₅₀) by metformin, NT1014 and NT1044. All experiments performed in HEK293 cells overexpressing each individual transporter. N.D.=not determined.

	OCR Inhibition, IC ₅₀ (micromol/l)					
Compounds	OCT1	OCT2	OCT3	OCTN1	PMAT	MATE1
Metformin	240	241	330	4003	288	5993
NT1014	11	14	49	340	20	365
NT1044	10	12	12	>5000	11	ND