The effect of VBP6, a steroid-sparing agent, on growth and bone of glucocorticoid-treated *mdx* mice
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Background

Duchenne muscular dystrophy (DMD) affects 1 in 4000 live male births and is a severe and ultimately fatal, X-linked recessive disease[1]. Short stature and osteoporosis are common findings and may become key concerns for patients as life expectancy improves [2]. Progression of DMD can be slowed with the administration of glucocorticoids (GC) but these are associated with further growth retardation and skeletal fragility[3]. Finding an alternative compound to traditional GC that has equivalent anti-inflammatory effects, but a reduced skeletal side-effect profile could have huge clinical impact in DMD. Traditional GC have an 11-beta-hydroxy or an 11-keto group whereas a new class of compounds have been designed with a key conversion from the hydroxyl group to a double bond between carbons 9 and 11, leading to a Δ9,11 C ring as the backbone. This modification still enables the compound to effectively inhibit nuclear factor kappa light chain enhancer of activated B cells (NF-κB) activity and thus have an anti-inflammatory role, but it appears to do so without inducing glucocorticoid receptor element (GRE)-mediated transcription and thus avoiding many of the classical GC side-effects [4–6]. Some rudimentary bone and growth data have previously been reported in a study comparing one of these Δ9,11 steroids (VPB15/vamorolone) to prednisolone in x-linked muscular dystrophy (*mdx)* mice[7]. Although these data are encouraging, further studies are essential to gain a full understanding of its effects (or lack of) in the skeleton[6]. This present study uses VBP6, a metabolic precursor for VBP15[6] because VBP15 is presently unavailable for use in pre-clinical work as it is used in a clinical trial[8]. This study was designed to compare the effects of prednisolone compared to VBP6 on muscle function, bone growth and skeletal structure in the *mdx* mouse model. This approach will provide crucial additional insight to support the clinical trial outcomes, where changes in bone growth and structure are very difficult to accurately quantify, given the short time frames involved.

Methods

Four-week-old *mdx* mice were used as a model of DMD. They were treated with vehicle, prednisolone or VBP6 (both at 20mg/kg/day via cherry syrup gavage) for 4 weeks and compared to similarly treated wild-type (WT) mice to study growth and skeletal structure. Bodyweight (BW), crown-to-rump and tail length measurements were taken twice weekly from first day of intervention until cull. Forelimb grip strength testing was performed prior to cull, using a grip strength meter with a specialised mouse grid. Serum creatine kinase (CK) activity was measured by a CK assay kit (Pointe Scientific, Stroud, UK). Serum P1NP and αCTX concentrations were measured by ELISA according to manufacturer's instructions. Micro-computed tomography imaging (μCT) was performed to quantify trabecular architecture, cortical bone geometry, tissue mineral density (TMD) and tibial length. Three-point bending analysis of the cortical region of the left tibiae was completed after μCT analysis, using a Lloyd LRX5 materials testing machine. Static histomorphometry was performed on paraffin-embedded, decalcified sections of proximal tibiae. Tartrate-resistant acid phosphatase (TRAP) activity with fast-red staining was used to identify osteoclasts (TRAP+ve) as multinucleated cells lying on the bone surface. Slides were stained with Goldners trichrome using standard protocols to identify osteoblasts.

Results

Mean % weight gain of the WT mice treated with 20mg/kg/day of prednisolone was significantly lower during the study period compared to their vehicle controls (12.9% in pred-treated mice v 59.2% gain in vehicle control mice, $p<0.001$. The same trend was observed in the *mdx* mice but did not reach statistical significance. The WT mice administered VBP6 gained significantly more weight (%) than their respective vehicle controls (mean 94.0% gain v 59.2% ($p<0.01$). There was no difference in the % weight gain of the *mdx* mice given VBP6 compared to their vehicle controls. Total growth plate (GP) and proliferative zone (PZ) heights were lower in the pred-treated WT mice than those given syrup alone.

Four weeks of VBP6 did not change the total height of the GP or its individual zones in WT mice. There were no significant changes to GP height or height of its individual maturational zones in *mdx* mice given either pred or VBP6 compared to their respective vehicle controls. Tibial length at 8 weeks-of-age was less (0.57 mm difference in WT ($p<0.05$) and 0.88 mm in *mdx* ($p<0.05$) mice) in all pred-treated mice. Tibial length was unaffected by daily VBP6 in either WT or *mdx* mice. Neither absolute nor BW-normalised grip strength values differed by treatment group in WT. However, normalised grip strength was higher in pred-treated *mdx* mice compared to their vehicle controls. The administration of 4 weeks of VBP6 did not significantly change grip strength compared to their vehicle controls in either WT or *mdx*. As expected, greater levels of inflammation and muscle cell regeneration were noted in *mdx* compared to WT. VBP6 reduced cumulative muscle damage in tibialis anterior of *mdx*. As expected, CK values were higher in the untreated *mdx* compared to WT. There was no difference in serum CK values after 4 weeks of either pred or VBP6, compared to their respective vehicle controls.

When analysing bone, no differences in trabecular bone parameters were noted in either WT or *mdx* that received VBP6 compared to their vehicle controls. Cortical bone area and volume were lower in both the pred-treated WT and pred-treated *mdx*. In keeping with this, mean cortical bone fraction was also less, at 57% in pred-treated WT v 63% in WT controls ($p<0.001$) and 59% in pred-treated *mdx* v 66% in *mdx* controls ($p<0.001$). There were no differences in all cortical bone parameters measured in either the WT or *mdx* that were administered VBP6 compared to their vehicle controls. There were no differences in cortical TMD by group, except in WT where the TMD was actually greater after 4 weeks of VBP6. There were no differences in biomechanical properties between either WT or *mdx* given pred or VBP6 regimens and their respective controls. P1NP values were lower in *mdx* than in WT (367.6 pg/ml in WT v 58.3 pg/ml in *mdx*, $p<0.001$). Both P1NP and αCTX were lower in the pred-treated WT ($p<0.05$). Four weeks of VBP6 treatment caused a reduction in P1NP levels, but there was no associated change in αCTX. These findings were not replicated in *mdx*. Chondrocyte proliferation rates were similar after pred or VBP6 in both WT and *mdx* as was osteoblast and osteoclast number/bone surface.

Conclusions

Growth retardation was evident in mice given pred at 20mg/kg/day. A significant reduction (46.3%) in the amount of weight gained during the intervention period was seen in the WT and the same trend was observed in the *mdx*. By contrast, VBP6 was protective against the GC-induced growth retardation as no reductions were seen in the anthropometric or histological parameters of growth. The absence of growth retardation is in keeping with results from other pre-clinical studies of VBP15 and further supports the concept that VBP6 and VBP15 are able to dissociate the GC-associated side-effects from their efficacy [6, 7, 9]. VBP6 at a dose of 20mg/kg/day was able to reduce cumulative muscle damage in the tibialis anterior muscle of *mdx* mice, without causing detectable skeletal side-effects.

In this study, the administration of 4 weeks of pred at 20mg/kg/day to both WT and *mdx* consistently demonstrated a reduction in cortical bone volume, tissue volume and cortical bone fraction, which was not observed in the mice given VBP6. In addition, a change in bone modelling/remodelling was suggested in the WT mice by a reduction in both bone resorption (CTX) and formation (P1NP) markers.

Data from this study confirm that VBP6 was able to reduce cumulative muscle damage in *mdx*, without causing detectable growth and skeletal side-effects. An analogue of VBP6 (VBP15 or vamorolone) has currently been investigated in a phase 2 clinical trial, and in keeping with the data reported here, early results have suggested an improvement in bone safety with vamorolone compared to traditional GC, which could offer a promising alternative to the current GC regimens that are used in DMD [8].

Benefit to applicant, department and endocrinology

This grant afforded me the opportunity to develop new laboratory skills, refine my micro-CT skills, and develop a mutually beneficial working relationship with Reveragen (supplier of VBP6 compound). This

work formed a substantial part of my PhD, and I was subsequently awarded the William Dick medal for the best PhD at Roslin Institute. I have since presented this work internationally, and also been invited to be a member of the DMD UK bone and endocrine working group and the Optimize DMD international consortium, set up to optimise management of endocrine complications in DMD. Working relationships formed as a consequence of membership of these groups has since led to several collaborative grant proposals and award of funding for related ongoing projects. These will hugely enhance my career prospects as an academic endocrinologist.

This pre-clinical work, alongside out additional analysis carried out by Reveragen, formed part of the large body of evidence which was presented alongside the results of the clinical studies of vamorolone (VBP15) that showed that the vamorolone improves muscle function outcomes compared with placebo in DMD and is associated with a better side-effect profile[10]. Since this work was carried out, Vamorolone has now been approved by the MHRA for use in DMD. It will now be vital that long term endocrine outcomes are studied in patients who start vamorolone, to determine whether the pre-clinical data translate to an improved side-effect profile in young people with DMD. If vamorolone does reduce the number and intensity of side-effects, compared to standard GC treatment this could have a significant impact on quality of life in young people with DMD. The results from this work could also be extended to other childhood diseases where inflammation affects growth, or where GC are required, including for example in the treatment of inflammatory bowel diseases and juvenile arthritis.

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