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Enhanced Evaluation of Selective Androgen Receptor Modulators *In Vivo*

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Abstract

Selective AR modulators (SARMs) are a class of drugs that control the activity of the androgen receptor (AR), which mediates the response to androgens, in a tissue-selective fashion. They are specifically designed to reduce the possible complications that result from the systemic inhibition or activation of AR in patients with diseases that involve androgen signaling. However, there are no ideal *in vivo* models for evaluating candidate SARMs. Therefore, we created a panel of androgen responsive genes in clinically-relevant AR expressing tissues including prostate, skin, bone, fat, muscle, brain, and kidney. We used select genes from this panel to compare transcriptional changes in response to the full agonist dihydrotestosterone (DHT) and the SARM bolandiol at 16h and 6wks. We identified several genes in each tissue whose expression at each of these time points correlates with the known tissue-specific effects of these compounds. For example, in the prostate we found four genes whose expression was much lower in animals treated with bolandiol compared to animals treated with DHT for 6wks, which correlated well with differences in prostate weight. We demonstrate that adding molecular measurements (androgen regulated gene expression) to the traditional physiological measurements (tissue weights, etc) makes the evaluation of potential SARMs more accurate, thorough, and perhaps more rapid by allowing measurement of selectivity after only 16 hours of drug treatment.

Keywords

selective androgen receptor modulator; androgen receptor; androgens; androgen replacement therapy

Introduction

The androgen receptor (AR) is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily. AR activation by androgens can have disparate effects at the cellular level, driving proliferation in some cells and terminal differentiation in others. These varied responses to androgen at the cellular level manifest as distinct physiological outcomes at the tissue level including maintenance of bone mineral density (BMD) and muscle mass and control of prostate growth (Keller, et al., 1996). Not surprisingly, changes in AR signaling are associated with various clinical diseases. Unfortunately, current treatments for these diseases often have adverse side-effects due to changes in AR activity in non-diseased tissue, impacting a patient's life drastically (Sprenkle and Fisch, 2007). Many mechanisms have been proposed to account for the tissue specific effects of androgens, including tissue specific expression of 5 α -reductase, an enzyme that converts serum

testosterone into the higher affinity AR ligand, dihydrotestosterone (DHT), and tissue specific cofactor expression. However, our understanding of the tissue-specific AR activity on a molecular level is far from complete.

New treatment options that target AR activity in specific tissues would provide clinical benefit for a number of different conditions. Selective AR modulators (SARMs) represent a promising new class of drugs with the potential to reduce the side-effects associated with treatments that target the AR (Negro-Vilar, 1999). These molecules can activate (or potentially inhibit) AR in some, but not all AR-expressing tissues. Perhaps the greatest challenge to the developments of SARMs is the slow and difficult process of accurately defining the tissue-selective effects *in vivo*. Currently, promising compounds are tested *in vivo* using some adaptation of the Hershberger assay, which, in its original form, uses changes in weight of several tissues to assess tissue-selective androgenic response (Hershberger, et al., 1953). Although useful as a screening tool, this method has some important limitations. The most often employed adaptation of the Hershberger assay uses the weight of several androgen sensitive tissues, including the ventral prostate and levator ani muscle, and changes in bone physiology, usually BMD (Kearbey, et al., 2007), to assess the tissue-selective androgenic activity of potential SARMs. While androgens are known to control the growth and maintenance of the prostate, skeletal muscle, and bone, the intensity and kinetics of androgen-related changes are very different in these tissues (Bagatell and Bremner, 1996). In addition, often the potential effects of SARMs are not examined in other important androgen-responsive tissues, which raises safety concerns. Furthermore, the assay requires exact dissection of very small tissues, which is difficult and can influence the accuracy of the results. Finally, while changes in prostate weight can be detected within 7–14 days, changes in other physiological parameters such as BMD and body composition often require several additional weeks of treatment, slowing preclinical development of SARMs.

We hypothesized that measurement of androgen-regulated gene expression by Q-PCR could circumvent many of the problems with the traditional model of SARM evaluation. In previous work, we created a microarray profile of androgen responsive genes in relevant AR expressing tissues (Otto-Duessel, et al., 2012). In this study, we examined the expression of a subset of these genes (Supplementary Table 1) by Q-PCR in animals treated with the full agonist DHT or the SARM bolandiol. We specifically sought genes whose expression either after 16hrs or six weeks of treatment correlated with the tissue-specific physiological effects of DHT and bolandiol as these genes are most likely to be useful in assessing the tissue selectivity of androgens. Bolandiol is a synthetic androgen that is reported to have prostate sparing agonist activity within a particular dose range (Page, et al., 2008) and was therefore, an ideal androgen for use in this comparative selectivity study. Our data suggest that Q-PCR-based measurement of androgen regulated gene expression can be used as an additional measure of the tissue-selective effects of SARMs and may expedite the *in vivo* screening of SARMs by allowing selectivity measurements to be made after only 16h of treatment.

Materials and Method

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were performed under the IACUC approval of City of Hope. Animals were purchased from Jackson Laboratory (Bar Harbor, Maine). Forty-eight, eight-week-old male Sprague Dawley rats were divided into 8 groups (n=6) and treated for either 6 weeks or 16h as follows; intact, castrate + vehicle, castrate + DHT, and castrate + bolandiol (19-Nor-4-

androstene-3 β , 17 β -diol). Animals were castrated and implanted with silastic capsules containing drugs. Following treatment, animals were euthanized and organs harvested, weighed and immediately processed for quantitative RT-PCR of candidate genes. For the 6 week experiment micro CT scans and biochemical assays were performed.

Drug Treatment

DHT was administered s.c. by a 5cm Silastic implant (1.57mm $\times$ 2.41mm; Silclear tubing, Tuzki, BO). The DHT capsule was designed to have a release rate of 150 μ g/d (5cm), administering slightly supra-physiological levels of DHT (Liu, et al., 2009, Wang, et al., 2007). Bolandiol (Sternaloids RI) was delivered s.c. in a 4 cm long Silastic capsule (Nantermet, et al., 2005). A 4cm capsule has shown to increase BMD but not increase prostate growth in castrated rats (Page, Marck, Tolliver and Matsumoto, 2008). All capsules were implanted s.c. at the day of castration and the treatment period lasted six weeks. For the 16 h experiment animals received one single i.p. injection of DHT (10mg/kg) or bolandiol (8mg/kg).

Reverse transcription and quantitative polymerase chain reaction (RT-PCR)

Dissected tissue was placed in RNA Later (Qiagen, CA) before being mechanically homogenized less than an hour later. Following homogenization, RNA was isolated with an RNeasy kit (Qiagen, CA). RNA then was reverse transcribed (Promega, WI) and transcripts identified in Supplementary Table 1 were amplified (Qiagen Taq and reagents) on a 7300 Real Time PCR System (Applied Biosystems, CA), using SYBR green (Invitrogen, CA) as the detecting dye and Rox (Invitrogen, CA) as the reference dye. Differences between experimental (x) and control (y) samples were normalized to RPL19 transcript levels (androgen unresponsive) and determined with the following calculation:

$$(2^{-(Ct_{x_{\text{gene1}}} - Ct_{y_{\text{gene1}}})}) / (2^{-(Ct_{x_{\text{RPL19}}} - Ct_{y_{\text{RPL19}}})}).$$

Micro computed tomography (Micro-CT)

Micro-CT analyses were performed at the Keck School of Medicine, USC. Rat femurs were scanned with a Scanco microCT50 system (Scanco Medical AG, Bassersdorf, Switzerland). All femur bones were placed in a 19mm sample holder and scanned transversely. The samples were scanned with an energy setting of 70kVp, 200microAmperes, 300ms integration time and 20micron voxels. Whole femurs were scanned and consisted of approximately 1500–2200 slices depending on the size of the sample. The bone mineral density (BMD) of the central shaft was determined using 600–700 slices and the ROI drawn contained only cortical bone excluding any trabecular bone present in the slices towards the ends. The BMD of the distal knee joint was determined using 150–200 slices. After preprocessing the images with a Gaussian filter (sigma=0.8, support=1), the images were thresholded and analyzed using the μ CT Evaluation Program (Scanco Medical AG, Bassersdorf, Switzerland) with the Bone Midshaft Evaluation and Bone Trabecular Morphometry scripts for central shaft and distal knee BMD analyses, respectively.

Biochemical Assays

Serum testosterone, cholesterol, and triglycerides were quantified using 96-well fluorometric assays (Cayman Chemical Company, MI) followed by reading wells with a plate reader (Tecan, Infinite M1000, Switzerland). All samples were run in duplicate.

Statistical Analysis

All results are presented as means $\pm$ standard deviations. One-way analysis of variance was applied to determine statistical differences among groups using post-hoc Dunnett's test. $P < 0.05$ was considered significant.

Results

Identification of genes with the potential to predict the physiological response to SARMs at six weeks

DHT treatment is known to prevent castration-induced changes in nearly all tissues studied. Bolandiol has been shown to prevent castration-induced changes in anabolic tissues like the bone and muscle, but not in reproductive tissues like the prostate and seminal vesicles (SV) (Attardi, et al., 2010). Thus, these two androgens are ideal controls for correlating tissue-specific gene expression changes with tissue-specific physiological changes. As expected, castration significantly reduced the weight of the prostate ($p=0.01$), levator ani (LA) muscle ($p<0.01$) and seminal vesicles ($p<0.01$) (Figure 1). DHT treatment maintained the weight of all three tissues compared to castrated animals and actually stimulated the growth of these tissues compared to intact controls. Bolandiol treatment was not able to maintain prostate and SV weight but did maintain LA muscle weight, though only 79% as effectively as DHT. Significant reduction in body weight compared to intact controls was observed in the castration group ($p=0.01$) and the castrate + DHT group ($p=0.03$). The origin of the weight difference is not clear, but may be related to body composition, lean muscle mass, and fat mass. Body weight was factored in for the weight assessments of the other organs.

The *ex vivo* micro-CT measurements taken after a six week treatment period allowed us to observe structural bone changes. Cortical BMD, both tissue density and apparent density, was significantly lower in castrated group ($p<0.01$) compared to the control group (Table 1). BMD was also significantly lower in the castrated group compared to the animals that received DHT or bolandiol (data not shown). The same trend was also true for trabecular BMD although the difference was not significant (data not shown). There were significant differences in microstructural parameters of cortical bone, including bone volume/tissue volume (BV/TV), cortical number (CN), and cortical spacing (C.S) between the castrated group and the other groups, indicating a dramatic bone loss. Cortical thickness (CT) was the only parameter not different among the groups. Bolandiol treatment was about half as effective in maintaining cortical bone density when compared to DHT treatment (Table 1). However, bolandiol treatment worked equally well as DHT in maintaining BV/TV, CN, CS, and CT. Several studies have used serum levels of cholesterol and/or triglycerides as a measure of the androgenic potency of compounds in fat (Goss, et al., 2007, Isidori, et al., 2005). Thus, we analyzed these parameters in our experiment. The results of the serum analyses are depicted in Table 2. Serum testosterone levels were significantly decreased in all three groups ($p=0.01$) when compared to control group. It was impossible to determine if administration of DHT or bolandiol further decreased the serum testosterone concentration compared to castration alone as the testosterone values were at or below the limit of detection of our assay. In castrated animals, serum cholesterol levels were significantly elevated ($p=0.01$). In contrast, DHT as well as bolandiol treatment effectively prevented an increase in cholesterol levels. Triglyceride levels were decreased in the castrated group, whereas increased levels were observed in bolandiol and DHT treated animals. However, the changes in triglyceride levels were not statistically different among the groups.

To correlate these physiological changes with changes in expression of androgen regulated genes, we isolated RNA from the prostate, muscle, bone, fat, kidney, brain and skin. Using a list of androgen regulated genes identified by our previous microarray studies (Otto-Duessel, He and Jones, 2012), we screened these tissues for genes whose expression (as measured by QPCR) correlated with the known physiological effects of DHT and bolandiol. We chose genes from the microarray data based on the strength of the DHT regulation on the arrays, paying special attention to those genes with a known role in the physiology of the tissue being studied and those genes previously reported to be androgen-regulated. We first identified genes whose expression significantly differed or trended toward significance

between castrate and castrate + DHT groups. We then determined how bolandiol regulated each gene in relation to DHT (Table 3). For the prostate, we found several genes whose expression was more strongly regulated by DHT than bolandiol, just as DHT more strongly stimulated the growth of this tissue. For instance, DHT increased the expression of the *Sqle* gene 5.8x compared to the castrate group while bolandiol only increased it 1.2x, or 3% as much as DHT. This is very similar to the changes observed in prostate weight, as bolandiol was only 5% as effective as DHT. A similar trend was observed for the genes *Sox4*, *Zfp361l* and *Ayl2*, although the correlations to physiological changes were less strong, as bolandiol regulated the expression of each 20–30% as much as DHT. In the muscle, DHT caused a 2.9x higher expression of *Amd1* when compared to castration. Bolandiol treatment elevated *Amd1* expression 2.5x compared to castration, which corresponds to 74% of the DHT response. This was similar to the effects on LA muscle weight, where muscles from bolandiol-treated rats weighed 79% of DHT treated ones. In the bone, DHT elevated *Pou2af1* gene expression 1.8x compared to castration whereas bolandiol increased its expression by 1.5x or 60% as much as DHT. This parallels changes in BMD where bolandiol was 46% as effective as DHT in maintaining cortical tissue density. In the fat, DHT elevated *Eno3* expression levels by 4x compared to castration, while bolandiol increased its expression 1.5x, or 16% as much as DHT. A similar response was observed for *Mt1a* where bolandiol was 23% as effective as DHT. These results did not correlate well with effects on serum lipids where bolandiol had the same impact as DHT. However, it is possible that serum cholesterol and triglycerides are not good correlates for androgen-response in solid fat pads and that the expression of androgen-regulated genes in fat pads may more accurately reflect the activity of compounds in this tissue. It is also possible that fat pad weight would provide a better physiological correlate in this tissue.

There are no reports of the androgenic effects of bolandiol in the brain, skin, or kidney. While we had no physiological correlates, we found that bolandiol had an effect similar to DHT in regulating androgen-responsive genes in these tissues (Table 3). In the kidney, *Hrg* was actually stimulated to a greater extent by bolandiol than DHT. This was also true of *Igf1* in the brain and *Wnt5a* and *Ptgis* in the skin.

Identification of genes with the potential to predict the physiological response to SARMS after 16hr treatment

The ability to more rapidly determine the tissue-selectivity of a SARM would be advantageous for drug discovery efforts. Having identified several genes whose expression correlated with physiological changes induced by six weeks of treatment with DHT or bolandiol, we also identified genes whose expression following 16h of treatment correlated with physiological changes after 6 weeks of treatment (Table 4). We screened the same pool of genes as used in the six week treatment experiment (Supplementary Table 1). Interestingly, we found that a different subset of genes demonstrated the best correlation between 16hrs of treatment and later physiological changes. In the prostate we found that changes in expression of *Sox4* after only 16h of treatment predicted the tissue-selective response of bolandiol, similar to changes in expression of *Sox4* following six weeks of treatment. *Ayl2* had a similar regulation pattern after 16h and six weeks of treatment, but it was a better predictor of bolandiol effect at six weeks (28% of DHT) than at 16h (54% of DHT). We also found that *Odc1* was a good predictor of bolandiol effect at 16h, as bolandiol stimulated expression of this gene 13% as much as DHT.

In the muscle, we identified nine genes which were stimulated by bolandiol between 44 and 100% as effectively as DHT. Five of these genes (*Amd1*, *Acta1*, *Myot*, *Pappss2*, and *Arg2*) were ~50% as effective as DHT while the remaining four (*Igf1*, *Pvalb*, *Mafox*, and *Murf1*) were >98% as effective as DHT, representing an interesting dichotomy that, when averaged,

approximately predicts the physiological difference in response to DHT and bolandioli in the LA muscle (Figure 1). In the bone, DHT increased the expression of *Ccnd1* 7.2x compared to the castrate group, while bolandioli only increased it 2.4x, or 23% as much as DHT. As bolandioli maintains BMD ~50% as effectively as DHT, *Ccnd1* is a relatively good predictor of this physiological difference, though it certainly doesn't predict other aspects of bone physiology (BV/TV%, etc) where bolandioli is as effective as DHT. In the fat we found that *Mt1a* and *Ccnb1* were more strongly stimulated by bolandioli than DHT, mirroring the effect of bolandioli in serum lipid assays. However, these results differed from the trend in gene expression observed after six weeks of treatment, making the interpretation of gene expression data from fat difficult without a better physiological correlate. A similar situation was observed in the kidney where we identified three genes (*Aytl2*, *Enac*, *Hrg*) which were stimulated to a much lesser extent by bolandioli than DHT. One of these genes, *Hrg*, was repressed more strongly by bolandioli treatment than by DHT treatment at the six week mark, but only 14% as much as DHT at the 16hr mark. Again, it is difficult to assess which regulation pattern is accurate without a relevant physiological endpoint.

Results in skin and brain were more consistent between 16h and six weeks of treatment. In the brain, we found that *Cdkn1a* was repressed more strongly by bolandioli than DHT, similar to the regulation pattern of *Igf1* at the six week mark. In the skin, *Scd3* was regulated more strongly by bolandioli than DHT at both 16h and six week time points. Interestingly, bolandioli and DHT induced *Scd3* expression at 16h but repressed it at six weeks. This appeared to be a common phenomenon in the skin as we observed a similar trend for several genes (Supplementary Table 2). *Ccnb1* was also regulated nearly as strongly by bolandioli as DHT after 16h treatment (92%), though we lacked data from the six week experiment to determine if its regulation was also correlated at that time point. Regardless of the direction of regulation, the genes we identified should be predictive of tissue selectivity, assuming bolandioli is equally effective as DHT in the skin.

Discussion

In this study, we used changes in the expression of androgen-regulated genes to enhance the standard assay for assessing the *in vivo* tissue-selectivity of androgens. The inclusion of molecular markers like gene expression in addition to physiological markers should improve the accuracy of *in vivo* selectivity measurements and may allow for more rapid assessment of lead compounds. The genes used for this study represent a subset of those identified in our previous study (Otto-Duessel, et al., 2012) (Otto-Duessel, He and Jones, 2012). It is important to note that we did not assess every gene identified in the previous study and not every androgen-responsive gene we did assess demonstrated an ability to predict tissue-specific responses (Supplementary Table 1). For instance, in the prostate the regulation of *Fut4* and *Cox6a2* was similar between bolandioli and DHT, both at 16h and at six weeks. The expression of some genes, while androgen responsive, did not directly correlate with physiological changes. We specifically sought genes that correlated well with our chosen physiological endpoints. It is possible that the expression of some genes is more sensitive to bolandioli treatment than physiological changes. Bolandioli at higher doses loses its tissue-selectivity and will maintain prostate weight following castration (Attardi, Page, Hild, Coss and Matsumoto, 2010). We expect changes in gene expression to also be dose-dependent, but these changes might occur on a slightly different dose range than physiological effects. Additional studies to determine the dose-dependence of gene expression is warranted.

Our study was modeled on a standard experiment in which the effects of a SARM (bolandioli in our study) are compared to that of a full androgen, DHT, after several weeks of treatment in Sprague-Dawley rats. We confirmed that DHT acts as an agonist in the muscle, bone, and reproductive tissues, while bolandioli acts as an agonist only in the muscle and some aspects

of bone physiology. Thus, bolandiol and DHT make excellent standards with which to compare changes in physiology to changes in gene expression.

In the prostate, we found that *Sqle* best correlated to the physiological difference between DHT and bolandiol after 6 weeks of treatment, as bolandiol was only 3% as effective as DHT in stimulating this gene, compared to 5% as effective at maintaining prostate wet weight. Importantly, *Sqle* is a known androgen regulated gene and is the rate limiting enzyme in sterol biosynthesis, which is essential for prostate function (Chugh, et al., 2003, Schirra, et al., 2007). *Sox4* and *Zfp3611* may also be useful in predicting selective response in the prostate, though they were each closer to 30% in bolandiol effective rate. When we looked at gene regulation in the prostate after only 16h of treatment, *Sox4* was the best predictor of selectivity, as bolandiol was only 11% as effective as DHT at stimulating *Sox4* expression at this time point. *Zfp3611* and *Sqle* were less predictive of selectivity at 16h than they were at 6 weeks of treatment, but experiments in larger cohorts may increase the ability of these genes to predict selectivity. In the 16h analysis, the *Odc1* gene became a good predictor of selectivity, as bolandiol stimulated the expression of this gene only 13% as much as DHT. *Odc1* is a documented androgen regulated gene and is the rate limiting enzyme involved in the synthesis of polyamines, which are thought to regulate cell proliferation and differentiation and thereby influence prostate size (Simoneau, et al., 2008).

In the muscle, we found that several genes known to play a role in muscle physiology were good predictors of the tissue-selective response to androgens. *Amd1* promotes muscle growth via androgen regulation (Lee and MacLean, 2011). Regulation of *Amd1* was similar independent of the length of treatment period, although changes in transcription levels of *Amd1* after six weeks of treatment better matched changes in weight. *Acta1* encodes skeletal muscle alpha actin, which is a main component of the skeletal muscle and is thought to mediate, in part, androgen anabolic function in the muscle (Hong, et al., 2008). *Myot* encodes myotilin, a Z-band protein that contributes to structural and functional integrity of striated muscle (Salmikangas, et al., 1999). Both genes are important for maintaining normal muscle health, making them ideal markers for predicting the response to androgens in the muscle. Both of these genes were good predictors of muscle response at 16h. Similarly, *Mafbx* and *Murfl* play an important role in muscle loss and castration induces expression of these genes (Jones, et al., 2010, Pires-Oliveira, et al., 2010). Both DHT and bolandiol decrease expression of these genes at 16h, suggesting that they too are relevant predictors of physiological response to androgens in the muscle.

In the bone, bolandiol equaled DHT in the ability to maintain many physiological parameters, but was not as capable of maintaining BMD following castration. Higher doses of bolandiol are reported to fully maintain BMD but also maintain prostate weight to a greater degree (Attardi, Page, Hild, Coss and Matsumoto, 2010). Thus, we chose to use a dose of bolandiol that would allow us to maximize differences in reproductive versus other tissues, but this complicated our comparative analysis in bone. Changes in *Pou2af1* gene regulation roughly paralleled changes in BMD induced by bolandiol and DHT after 6 weeks of treatment. *Pou2af1* is a B-cell-specific transcriptional co-activator and we likely detected this gene in our bone samples because many B-cells are present in the bone marrow (Nielsen, et al., 1996). However, it is not clear if and how it relates to the parameters of bone physiology we measured. *Ccnd1* encodes Cyclin D1, which not only plays an important role in the cell cycle, but it has also been reported that reduced levels of cyclin D1 are associated with oestoblast growth arrest, which could have a direct effect on bone physiology (Datta, et al., 2005). *Ccnd1* was the gene that best correlated with differences in BMD maintenance at 16h, though it was not perfect and did not hold true at six weeks. Our model would benefit from the identification of additional genes that can predict the physiological response to androgen, especially those that can discriminate among distinct physiological processes. A

recent study identified two genes that correlated with bone physiology changes in response to androgens (Schmidt, et al., 2010). In this study, it was reported that *Col2a1* was induced and *Scd1* was repressed in the bone by androgens. In our study, the regulation of those genes followed the same trend, but neither was significantly regulated by DHT. However, it is possible that studies with larger cohorts of animals would show a significant regulation of these genes by DHT in our studies and we could add them to our predictive model of androgen action in bone.

Both DHT and bolandiol maintained serum lipids at levels equivalent to intact animals following castration. The two genes that best reflect this physiological response were *Ccnb1* and *Mt1a*. However, they were similar to the physiological response only at the 16h mark, not the six week mark, where we found no genes that suggested bolandiol was as effective as DHT. Although the link between total body fat and serum lipids has been made, it might be that the changes in serum lipids do not accurately reflect changes in fat pads and that using the weight of the fat pad or determining changes in fat body mass by dual x-ray absorptiometry (Kearbey, Gao, Narayanan, Fisher, Wu, Miller and Dalton, 2007) might be more appropriate endpoints.

In tissues for which we did not have physiological measurements, we found that bolandiol and DHT regulated most genes examined to the same extent, suggesting that bolandiol is as effective as DHT in the brain, skin, and kidney. It would, however, be beneficial to include physiological endpoints to confirm the similarity between bolandiol and DHT in these tissues, and we plan to assess potential physiological correlates in future studies. Androgens control both sexual desire and sexual function, the prior being controlled by androgen action in the brain and the later by androgen action in reproductive tissues (Shah, et al., 2004). Behavioral studies that monitor mounting/intromission versus ejaculation can be used to monitor difference in androgen action in the brain versus reproductive tissues (Miner, et al., 2007). Based on our studies, we would predict bolandiol would maintain sexual desire in castrate rats but not sexual performance. Likewise, androgens have been shown to influence kidney weight (Broulik, et al., 1975), and we would predict that bolandiol would be just as effective as DHT at maintaining kidney weight in castrated rats. Finally, sebaceous gland number and sebum production have been used to measure androgen action in skin (Schmidt, et al., 2009) and may prove useful in our studies. A previous study identified two genes, *Scd3* and *Sqle*, as surrogate markers of androgen action in skin (Schmidt, Kimmel, Bai, Scafanas, Rutledge, Vogel, McElwee-Witmer, Chen, Nantermet, Kasparcova, Leu, Zhang, Duggan, Gentile, Hodor, Pennypacker, Masarachia, Opas, Adamski, Cusick, Wang, Mitchell, Kim, Prueksaritanont, Perkins, Meissner, Hartman, Freedman, Harada and Ray, 2010). In our study, *Scd3* was significantly regulated by DHT at 16h and bolandiol was an even more effective inducer of transcription. However, we found that this gene was not significantly regulated at six weeks of treatment. Regulation of *Sqle* in our study was similar between bolandiol and DHT at both 16h and six weeks, but at neither time point was *Sqle* significantly regulated. Experiments in larger cohorts of animals may increase the significance of the regulation of these genes and we could add them to our predictive model of androgen action in the skin. Again, we expect that bolandiol would be equal to DHT in maintaining physiological parameters in brain, skin, and kidney in castrated rats. However, if we find that bolandiol is not as effective at maintaining physiological parameters in these tissues as DHT, we can screen our lists of androgen-regulated genes for those that better correlate with the physiological observations.

In conclusion, we enhanced the *in vivo* model for SARM evaluation, using androgen-regulated gene transcription. By adding measurements of gene expression, we made the model more accurate and thorough than the usual implementation of the Hershberger assay. We also identified genes that can predict the long-term physiological effects of androgens

after only 16h of treatment. We propose that these genes can be used as a first pass screen to expedite the testing of large numbers of potential SARMS *in vivo*. Though our study was based on cohorts of only six animals, we had a thorough process for identifying and selecting genes in each tissue to use for the analysis. Where possible, we chose genes that were known to be androgen regulated and known to be involved in the physiology of the tissue in which they were being studied. In this way, the genes we used to assess tissue-selective activity are more likely to accurately reflect downstream androgen-induced physiological changes. Studies with additional SARMS with well-documented tissue-specific effects, especially those representing distinct structural classes, would help to validate the genes we have chosen to assess in our model and might generate a structure-independent SARM response profile. Furthermore, studies with larger cohorts of animals will likely increase the statistical significance of our findings and may allow us to include additional genes in the predictive model for each tissue. Such studies are underway. We are using this model to test several SARMS we have discovered in our lab. We also plan to extend this model to include female tissues, such as the ovary, uterus, and mammary glands. Such experiments will increase the breadth of the selectivity model and will improve the preclinical evaluation of SARMS for use in women as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparison of body and organ weights following 6wks of treatment

While there was no significant difference between the body weight of intact and bolandiol treated animals, DHT treatment and castration significantly reduced body weight (Fig. 1A). As expected, castration reduced the weight of the levator ani significantly. Bolandiol maintained muscle weight, while DHT increased muscle mass significantly (Fig. 1 B.) There was a significant difference in prostate weight following treatment. In bolandiol and castrated animals prostate weights were decreased significantly, in contrast a significant increase in prostate weight was observed in DHT treated animals (Fig. 1C). Bolandiol and castration negatively affected seminal vesicle weight causing a significant reduction. DHT maintained seminal vesicle weight (Fig. 1D). Values are given as mean $\pm$ standard deviation. * $p < 0.05$ by analysis of variance.

Table 1

Cortical bone parameters.

	Control	DHT + ORX	SARM + ORX	ORX	SARM relative to DHT response (%)
BV/TV	25.18 ± 2.33	26.45 ± 3.03	26.17 ± 2.34	19.87 ± 3.47*	96
CT (mm)	0.6 ± 0.05	0.59 ± 0.04	0.56 ± 0.07	0.57 ± 0.03	na
CN/mm	0.42 ± 0.04	0.45 ± 0.03	0.44 ± 0.03	0.35 ± 0.05*	90
CS (mm)	1.8 ± 0.23	1.65 ± 0.18	1.66 ± 0.14	2.34 ± 0.43*	100
App D (mg/ml)	1090.2 ± 36.9	1092.1 ± 20	1061.5 ± 23.3	1035.1 ± 10.7*	46
Tissue D (mg/ml)	1160.4 ± 32.7	1147.4 ± 34.4	1129.4 ± 20	1106.2 ± 10.4*	43

Apparent Density (App D), bone volume/tissue volume (BV/TV), cortical number (CN), cortical spacing (CS), orchiectomy (ORX).

* p<0.05 compared with control group; data are presented as mean ± STD (n=6).

Table 2

Serum hormone and lipid levels.

	Control	DHT + ORX	SARM + ORX	ORX
Serum Testosterone (ng/ml)	2.2 ± 0.9	< 0.2*	< 0.2*	< 0.2*
Serum Cholesterol (mM)	1.4±0.54	1.4±0.4	1.2±0.95	2.7±0.52*
Serum Triglyceride (mg/dl)	115.9±34.18	131.1±58.44	119.1±58.0	95.9±32.12

* p< 0.05 compared with control group; data are presented as mean ± STD (n=5);

orchiectomy (ORX).

Table 3

Androgen-regulated gene expression after 6 weeks of treatment.

		Chronic Treatment				
Tissue	Gene	Ctrl:ORX (fold change)	SARM:ORX (fold change)	DHT:ORX (fold change)	SARM Relative to DHT response (%)	DHT vs ORX p values
Prostate	Sox4	-1.9	1.94	5.64	20	0.02
	Zfp36l1	13.00	-18.33	-60.04	29	<0.01
	Sqle	-8.11	1.16	5.84	3	0.45
	Ayrl2	2.87	-1.93	-4.32	28	<0.01
Muscle	Amd1	-2.46	2.47	2.89	74	0.39
Bone	Pou2af1	-1.47	-1.47	-1.79	60	0.33
	Ccnd1	-2.8	0.96	3.43	0	<0.01
Fat	Eno3	-1.02	1.49	3.99	16	0.25
	Mt1a	-3.82	3.05	10.06	23	<0.01
Kidney	Hrg	3.77	-9.68	-2.79	100	0.09
Forebrain	Igf1a	-1.91	11.80	12.76	92	0.25
Skin	Scd3	-1.15	1.13	1.49	27	0.28
	Wnt5	-1.38	2.84	2.62	100	0.27
	Ptgis	3.11	-2.56	-2.18	100	0.23

orchietomy (ORX).

Table 4

Androgen-regulated gene expression after 16 hours of treatment.

		16h Treatment					
Tissue	Gene	Ctrl:ORX (fold change)	SARM:ORX (fold change)	DHT:ORX (fold change)	SARM Relative to DHT response (%)	DHT vs ORX p values	
Prostate	Sox4	-3.07	1.36	4.27	11	0.28	
	Odc1	-5.11	1.15	2.15	13	0.021	
	Ayl2	3.44	-1.3	-1.55	54	0.08	
Muscle	Amd1	-2.01	9.72	20.67	44	0.16	
	Acta1	-2.91	2.16	3.43	48	0.07	
	Myot	-1.22	2.42	4.08	46	<0.01	
	Mafbx	21.91	-3.32	-1.34	100	0.22	
	Murf1	2.69	-1.37	-1.36	100	0.28	
	Pappb2	-1.15	2.49	3.67	53	<0.01	
	Igf1a	-3.7	2.24	3.06	60	0.03	
	Pvalb	-3.07	2.49	2.51	98	0.15	
Bone	Arg2	-3.6	16.14	15.38	100	0.06	
	Cend1	-2.85	2.41	7.2	23	<0.01	
Fat	Mt1a	1.55	-7.73	-5.86	100	0.02	
	Ccnb1	-1.54	2.37	1.97	100	<0.01	
Kidney	Ayl2	-3.19	1.31	2.76	18	0.14	
	Enac	4.19	-1.08	-4.05	3	0.06	
	Hrg	1.94	-1.31	-3.26	14	0.2	
Forebrain	Cdkn1a	61.86	-42.47	-22.04	100	0.18	
Skin	Scd3	-1.48	4.57	3.52	100	0.01	
	Ccnb1	-1.47	4.29	4.59	92	0.01	

orchietomy (ORX)