Journal of Steroid Biochemistry & Molecular Biology 114 (2009) 167–173

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Influence of testosterone and a novel SARM on gene expression in whole blood of *Macaca fascicularis*☆

Irmgard Riedmaier*, Ales Tichopad, Martina Reiter, Michael W. Pfaffl, Heinrich H.D. Meyer

Physiology Weihenstephan, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany

ARTICLE INFO

Article history:
Received 5 November 2008
Received in revised form 26 January 2009
Accepted 28 January 2009

Keywords:
Testosterone
SARM
Biomarker
Gene expression
Real-time qRT-PCR

ABSTRACT

Anabolic hormones, including testosterone, have been suggested as a therapy for aging-related conditions, such as osteoporosis and sarcopenia. These therapies are sometimes associated with severe androgenic side effects. A promising alternative to testosterone replacement therapy are selective androgen receptor modulators (SARMs). SARMs have the potential to mimic the desirable central and peripheral androgenic anabolic effects of testosterone without having its side effects.

In this study we evaluated the effects of LGD2941, in comparison to testosterone, on mRNA expression of selected target genes in whole blood in an non-human model. The regulated genes can act as potential blood biomarker candidates in future studies with AR ligands.

Cynomolgus monkeys (*Macaca fascicularis*) were treated either with testosterone or LGD2941 for 90 days in order to compare their effects on mRNA expression in blood. Blood samples were taken before SARM application, on day 16 and on day 90 of treatment.

Gene expression of 37 candidate genes was measured using quantitative real-time RT-PCR (qRT-PCR) technology.

Our study shows that both testosterone and LGD2941 influence mRNA expression of 6 selected genes out of 37 in whole blood. The apoptosis regulators CD30L, Fas, TNFR1 and TNFR2 and the interleukins IL-12B and IL-15 showed significant changes in gene expression between control and the treatment groups and represent potential biomarkers for androgen receptor ligands in whole blood.

1. Introduction

Over the last decades the proportion of elderly people in the population has increased [1]. This is the reason why the incidence of age-related conditions like sarcopenia and osteoporosis is rising and becoming one of the major topics in health care. Sarcopenia is the loss of muscle mass during the aging process that may lead to frailty [2–5]. Sarcopenia is commonly associated with osteoporosis, which is the age-related loss of bone mineral density. The combination of sarcopenia and osteoporosis results in a high incidence of bone fractures relating to accidental falls, which is a significant cause of morbidity and mortality in the elderly population.

Both conditions are associated with a decrease in the endogen production of anabolic hormones, including testosterone [4]. Testosterone treatment has been proposed as a therapy for osteoporosis and frailty in both men and women [6,7]. However, the androgen therapies available today are associated with androgenic side effects, such as skin virilization in women and prostate hypertrophy in men [8–10].

A promising alternative for testosterone replacement therapy is the development of selective androgen receptor modulators (SARMs) [6]. SARMs are synthetic molecules that bind to the androgen receptor exhibiting tissue-selective effects. An “ideal” SARM is an orally active compound that provides an increase in muscle mass and strength and has an anabolic effect on bone density without inducing undesirable androgenic side effects [6]. LGD2941 is a novel non-steroidal, orally active SARM, which has shown potent anabolic activity on bone and muscle in rats and monkeys, but reduced effects on the prostate [7].

It is already known that androgens cause changes in the biochemical pathways of different organs and tissues. Specific enzymes, receptors and cytokines can be activated or suppressed on the cellular mRNA expression level. Using appropriate specific and sensitive quantification methods, like quantitative real-time RT-PCR, such mRNA expression changes are measurable.

The aim of this study was to evaluate the effects of LGD2941, in comparison to testosterone, on mRNA expression of selected target genes in whole blood samples. Whole blood is chosen because samples can easily been taken from the living organism. Furthermore there is evidence in the literature that androgens affect gene

☆ The poster version of this manuscript was presented at the Congress in Seefeld, Tirol 2008.

* Corresponding author. Fax: +49 8161 714204.
E-mail address: irmgard.riedmaier@wzw.tum.de (I. Riedmaier).

0960-0760/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2009.01.019

expression of the different blood cells. The regulated genes have the potential to act as blood biomarkers in future studies with AR ligands.

2. Materials and methods

2.1. Animal experiment

24 male cynomolgus monkeys (*Macaca fascicularis*) were separated to four groups of six animals each. All animals were 5–6 years old, skeletally mature and had an average body weight of 6± kg. The treatments were group 1 (control or oral vehicle group), group 2 (reference group, testosterone group) 3.0 mg/kg Testosteronenanthate as Testoviron®-depot-250 (Schering, Berlin, Germany), dosed biweekly by intramuscular injection, group 3 (intermediate concentration group, SARM1) 1 mg/kg SARM LGD2941 daily and group 4 (high concentration group, SARM10) 10 mg/kg SARM LGD2941 daily. The oral vehicle control and the SARM were dosed once daily for 90 days.

Whole blood samples were taken at three time points. Predose samples were taken after study start without prior treatment. Further samples were taken at day 16 and day 90 of treatment. Duplicate blood samples (2.5 mL each) were transferred into PAXgene blood RNA tubes (BD, Heidelberg, Germany) gently shaken, incubated at room temperature for two hours and stored at −20 °C.

The animal attendance and blood sampling were done by Covance Laboratories GmbH (Münster, Germany) and was conducted with permission from the local veterinary authorities and in accordance with accepted standards of Humane Animal Care.

2.2. RNA preparation and qRT-PCR

RNA from blood samples was extracted using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

To quantify the amount of total RNA extracted, optical density (OD) was measured with the Biophotometer (Eppendorf Biophotometer, Hamburg, Germany) for each sample. RNA purity was calculated with the $OD_{260/280}$ ratio.

RNA integrity and quality control was performed via capillary electrophoresis in the Bioanalyzer 2100 (Agilent Technology, Palo Alto, USA). Eukaryotic total RNA Nano Assay (Agilent Technology) was taken for sample analysis and the RNA Integrity Number (RIN) served as RNA quality parameter. Agilent Bioanalyzer 2100 calculated the RIN value based on a numbering system from 1 to 10 (1 being the most degraded profile, 10 being the most intact) for all samples. A RIN ≥ 6 should be achieved to assure good results in qRT-PCR [11,12].

Candidate genes were chosen by screening the respective literature for androgen and inflammation-related effects on blood cells. Their expression was investigated using listed primers (Table 1). All primers were designed using published human nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Primer design and optimization was done with primer design program of MWG Biotech (MWG, Ebersberg, Germany) and primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with regard to primer dimer formation, self-priming formation and a constant primer annealing temperature of 60 °C. Newly designed primers were ordered and synthesized at MWG Biotech (Ebersberg, Germany) or Invitrogen (Karlsruhe, Germany). Primer performance testing was done with six optional untreated samples and a no template control (NTC contains only RNAse free water) for each primer set.

Quantitative real-time RT-PCR was performed using SuperScript III Platinum SYBR Green One-Step qPCR Kit (Invitrogen, Carlsbad, USA) by a standard protocol, recommended by the manufacturer. With the kit the master mix was prepared as follows: for one sample it is 5 μL 2× SYBR Green Reaction Mix, 0.5 μL forward primer (10 pmol/μL), 0.5 μL reverse primer (10 pmol/μL) and 0.2 μL SYBR Green One-Step Enzyme Mix (Invitrogen, Carlsbad, USA). 6.2 μL of the master mix was filled in the special 100 μL tubes and 3.8 μL RNA (concentration 1 ng/μL respectively 10 ng/μL) was added. Tubes were closed, placed into the Rotor-Gene 3000 and Analysis Software v6.0 was started (Corbett Life Science, Sydney, Australia). The following one-step qRT-PCR temperature cycling program was used for all genes: Reverse transcription took place at 55 °C for 10 min. After 5 min of denaturation at 95 °C, 40 cycles of real-time PCR with 3-segment amplification were performed consisting of 15 s at 95 °C for denaturation, 30 s at primer dependent temperature for annealing and 20 s at 68 °C for polymerase elongation. The melting step was then performed with slow heating starting at 60 °C with a rate of 0.5 °C per second up to 95 °C with continuous measurement of fluorescence.

Take off points (Ct) and melting curves were acquired by using the "*Comparative quantitation*" and "*Melting curve*" program of the Rotor-Gene 3000 Analysis software v6.0. Only genes with melting curves showing a single peak and no primer dimers were taken for further data analysis. Samples that showed irregular melting peaks were excluded from the quantification procedure.

2.3. Selection of target genes

Candidate genes that might be biomarkers in blood were chosen by screening the respective literature for androgen and inflammation-related effects on blood cells. Androgens are known to down-regulate proliferation of lymphocytes [13,14]. Therefore the different pro- and anti-inflammatory interleukins (IL) IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12B, IL-13 and IL-15 and the growth factors tumor growth factor β (TGF-β), insulin growth factor 1 receptor (IGF-1R) were selected for analysis. It was already shown that testosterone influences the rate of apoptotic blood cells [15–17]. Therefore different apoptosis regulators were chosen for analysis: the TNF receptor superfamily member 6 (Fas), its ligand FasL, tumor necrosis factor receptor (TNFR) 1 and 2, their ligand tumor necrosis factor α (TNF-α), B-cell CLL/lymphoma 2 (BCL-2), BCL2-like 1 (BCL-XL), Caspase 3 (Casp 3), Caspase 8 (Casp 8), CD30 Ligand (CD30L), the inflammatory factor nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFκB) and its inhibitor IκB. To determine if the treatment also has an influence on the amount of the different white blood cells, the expression of the cell specific CD Antigens CD4 (T helper cells), CD8 (cytotoxic T cells), CD11b (granulocytes), CD14 (monocytes), CD20 (B-cells), CD25 (activated T cells) and CD69 were measured. Further leukocyte genes that were measured are androgen receptor (AR), tumor necrosis factor β (TNF-β) and CD27 Ligand (CD27L). As genes expressed in reticulocytes, haemoglobin alpha (α-globin), haemoglobin beta (β-globin) and their transcription factors and stabilization factors transcription factor CP2 (CP2), acid phosphatase 1 (αCP1) and upstream transcription factor 1 (USF-1) were chosen. As reference gene candidates β-Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured, whereas β-Actin and GAPDH were chosen as best reference genes by using GenEx Ver 4.3.3 Software (multiD Analyses AB, Gothenburg, Sweden).

2.4. Data analysis and statistics

Statistical description of the expression data as well as statistical tests were produced with SAS v. 9.1.3 for Windows. The raw data were the Ct values obtained from each qPCR sample. Each qRT-PCR sample was associated with a blood sample whereas for each experimental animal two blood samples were analysed. Since the

Table 1
List of primer pairs used for qRT-PCR analysis.

Group	Gene	Primer name	Primer sequence 5′ → 3′	Product length
Reference genes	Ubiquitin C	UBC_for	TGA AGA CTC TGA CTG GTA AGA CC	128 bp
		UBC_rev	CAT CCA GCA AAG ATC AGC CTC	
	Actin-β	ActB_for	AGT CCT GTG GCA TCC ACG AA	148 bp
		ActB_rev	GCA GTG ATC TCC TTC TGC ATC	
	GAPDH	GAPDH_for	GAA GGT GAA GGT CGG AGT CAA	233 bp
		GAPDH_rev	GCT CCT GGA AGA TGG TGA TG	
Interleukins	IL-1β	IL1beta_for	GGA CAG GAT ATG GAG CAA CAA G	121 bp
		IL1beta_rev	AAC ACG CAG GAC AGG TAC AG	
	IL-2	IL2_for2	GCA ACT CCT GTC TTG CAT TGC	165 bp
		IL2_rev2	CAT CCT GGT GAG TTT GGG ATT C	
	IL-4	IL4_for3	TTC CCC CTC TGT TCT TCC TG	121 bp
		IL4_rev3	GTT GTG TTC TTC TGC TCT GTG AG	
	IL-6	IL6_for5	AGG AGA CTT GCC TGG TGA AA	179 bp
		IL6_rev5	CAG GGG TGG TTA TTG CAT CT	
	IL-10	IL10_for2	AGC CTTCGTC TGA GAT GAT CCA G	190 bp
		IL10_rev2	CAT TCT TCA CCT GCT CCA CG	
	IL-12b (p40)	IL12B_for	AAG GAG GCG AGG TTC TAA GC	213 bp
		IL12B_rev	AAG AGC CTC TGC TGC TTT TGA C	
	IL-13	IL-13_for2	AAT GGC AGC ATG GTA TGG AGC	124 bp
		IL-13_rev2	AGA ATC CGC TCA GCA TCC TC	
	IL-15	IL-15_for2	TCC AGT GCT ACT TGT GTT TAC TTC	93 bp
		IL-15_rev2	TAG GAA GCC CTG CAC TGA AAC	
Apoptosis regulators	Fas	FasR_for4	TTC TGC CAT AAG CCC TGT CC	174 bp
		FasR_rev4	CCA CTT CTA AGC CAT GTC CTT C	
	Fas ligand	FasL_for2	GGC CTG TGT CTC CTT GTC AT	162 bp
		FasL_rev2	GTG GCC TAT TTG CTT CTC CAA AG	
	TNFR1	TNFR1_for	AGC TGC TCC AAA TGC CGA AAG	147 bp
		TNFR1_rev	CAG AGG CTG CAA TTG AAG CAC	
	TNFR2	TNFR2_for	TGA CCA GAC AGC TCA GAT GTG	99 bp
		TNFR2_rev	TCC TCA CAG GAG TCA CAC AC	
	bcl-2	bcl2_for2	GAG GAT TGT GGC CTT CTT TGA G	170 bp
		bcl2_rev2	ACA GTT CCA CAA AGG CAT CCC	
	TNF-α	TNFa_for	AGG GAC CTC TCT CTA ATC AGC	104 bp
		TNFa_rev	CTC AGC TTG AGG GTT TGC TAC	
	Caspase 3	Casp3_for	GAA TTG ATG CGT GAT GTT TC	198 bp
		Casp3_rev	GCA GGC CTG AAT AAT GAA AAG	
	Caspase 8	Casp8_for	TGG CAC TGA TGG ACA GGA G	230 bp
		Casp8_rev	GCA GAA AGT CAG CCT CAT CC	
	bcl-xl	bcl-xl_for	TAA ACT GGG GTC GCA TTG TG	145 bp
		bcl-xl_rev	TGG ATC CAA GGC TCT AGG TG	
	CD30L	CD30L_for	CAT TCC CAA CTC ACC TGA CAA C	281 bp
		CD30L_rev	GCT CCA ACT TCA GAT CGA CAG	
Growth factors	TGF-β	TGFb_for	TAC TAC GCC AAG GAG GTC AC	239 bp
		TGFb_rev	AGG TAT CGC CAG GAA TTG TTG C	
	IGF-1R	IGF1R_for	CAT TTC ACC TCC ACC ACC AC	151 bp
		IGF1R_rev	AGG CAT CCT GCC CAT CAT AC	
CD antigens	CD4	CD4_for3	CTA AGC TCC AGA TGG GCA AG	154 bp
		CD4_rev3	TGA GTG GCT CTC ATC ACC AC	
	CD8	CD8_for	GGA CTT CGC CTG TGA TAT CTA C	112 bp
		CD8_rev	AAA CAC GTC TTC GGT TCC TGT G	
	CD11b	CD11b_for	GAG AAC AAC ATG CCC AGA ACC	246 bp
		CD11b_rev	CGG TCC CAT ATG ACA GTC TG	
	CD14	CD14_for	AGA ACC TTG TGA GCT GGA CG	115 bp
		CD14_rev	ATG GAT CTC CAC CTC TAC TGC	
	CD20	CD20_for	CAA CTG TGA ACC AGC TAA TCC C	163 bp
		CD20_rev	CCA TTC ATT CTC AAC GAT GCC AG	
	CD25	CD25_for	ATC AGT GCG TCC AGG GAT AC	196 bp
		CD25_rev	ACG AGG CAG GAA GTC TCA C	
	CD69	CD69_for2	TTG GCT ACC AGA GGA AAT GCC	164 bp
		CD69_rev2	CAG TCC AAC CCA GTG TTC CT	
Transcription factors	NFκB	NFkB_for2	ATC ATC CAC CTT CAT TCT CAA CTT G	149 bp
		NFkB_rev2	ATC CTC CAC CAC ATC TTC CTG	
	IκB α	IkappaB_for	AAC AGG AGG TGA TCG ATA AGC TG	138 bp
		IkappaB_rev	CCT TGT AGA TAT CCG CCT GG	
Reticulocyte genes	α-globin	alpha-gl._for	AGA CCT ACT TCC CGC ACT TC	275 bp
		alpha-gl._rev	CAG AAG CCA GGA ACT TGT CC	
	β-globin	beta-gl._for	GTC CAC TCC TGA TGC TGT TAT G	240 bp
		beta-gl. rev	TGT CAC AGT GCA GCT ACA TC	
	αCP1	aCP1_for	CCA CCC ATG AAC TCA CCA TTC	160 bp
		aCP1_rev	GCA GAG CCA GTG ATA GTA ACC	
	USF1	USF1_for	AGA TTC AGG AAG GTG CAG TGG	121 bp
		USF1_rev	CCA TTC TCA GTT CGG AAG ACG	

Table 1 (*Continued*)

Group	Gene	Primer name	Primer sequence 5′ → 3′	Product length
	CP2	CP2_for3	TCT TCG TTT ACC ATG CCA TCT ATC	178 bp
		CP2_rev3	CAT GCT TCT TCC TGA AAG TTC TG	
Other genes	Androgen receptor	AR_for	CCA CTT CCT CCA AGG ACA ATT AC	126 bp
		AR_rev	TGG ACT CAG ATG CTC CAA CG	
	TNFβ	TNFb_for	TGC TCA CCT CAT TGG AGA CC	149 bp
		TNFb_rev	AGT AGA CGA AGT AGA TGC CAC TG	
	CD27L	CD27L_for	ACA GGA CCT CAG CAG GAC	272 bp
		CD27L_rev	GAG GCA ATG GTA CAA CCT TGG	

amplification efficiency was not known, the assumption of identical amplification efficiency 100% was made, allowing more simple quantification model.

The Ct values of each gene were averaged by arithmetic mean for each animal. The obtained mean Ct values were then translated to normalized expression quantities using two reference genes in a form of normalization index. The normalization index was calculated as an arithmetic mean of the Ct values of the two reference genes:

$$\text{reference index} = \text{mean}(\text{CtACTB}, \text{CtGAPDH}) \quad (1)$$

Then, the expression of every target gene was calculated relatively to the expression of the housekeeping gene as:

$$\text{normalized expression} = \frac{2^{\text{reference index}}}{2^{\text{Ct target gene}}}, \quad (2)$$

where the 2 represents the 100% amplification efficiency. The normalized expressions of the timepoints 16 and 90 days were then divided with the normalized expressions of the baseline (predose), generating the expression ratio R as:

$$R_{\text{timepoint/baseline}} = \frac{\text{normalized expression}_{\text{timepoint}}}{\text{normalized expression}_{\text{baseline}}} \quad (3)$$

The expression ratio R was then analysed statistically. The Box–Whisker plot was constructed to facilitate visual screening of regulated genes (Figs. 1–3).

The objective of the statistical analysis was to disclose genes with significant regulation between control group and any of the treated groups. It was not intended to perform all treatment-to-treatment tests for all genes in order to avoid statistical type I error (false positive difference). Hence, ANOVA model was calculated on the $\log_2$ transformed R values employing the SAS procedure GLM with contrast sentence defining the control group as the contrast group for all treatment groups, thus adjusting the overall test confidence level to the number of relevant comparisons only. Further adjustment of the overall confidence level with respect to number of investigated genes was not performed. Hence, this study is to be considered as purely explorative whereas significant findings here indicate candidate biomarkers. Tests generating significant ($p < 0.05$) results were reviewed based on descriptive parameters of the compared groups and visually by means of the Box–Whisker plots to disclose possible outliers. As comparable trends were observed between the three treatment groups, no further test were produced.

To disclose multivariate response to the treatment, the method of principal component analysis (PCA) was employed using GenEx v. 4.3.3 (multiD Analyses AB, Göteborg, Sweden). PCA involves a mathematical procedure that transforms a number of variables (here normalized expression values) into a smaller number of uncorrelated variables called principal components. By this the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot, here two dimensions. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. Normalized expression values of all responding genes were taken as the initial variables and reduced to two principal components only, facilitating thus resolution of treatment clusters in the scatter plot (Fig. 4). Similarly, also each gene was analyzed by PCA taking its response in each sample as the initial variable and plotted in two dimensional scatter plot. This facilitated resolution of co-regulated genes (Fig. 5).

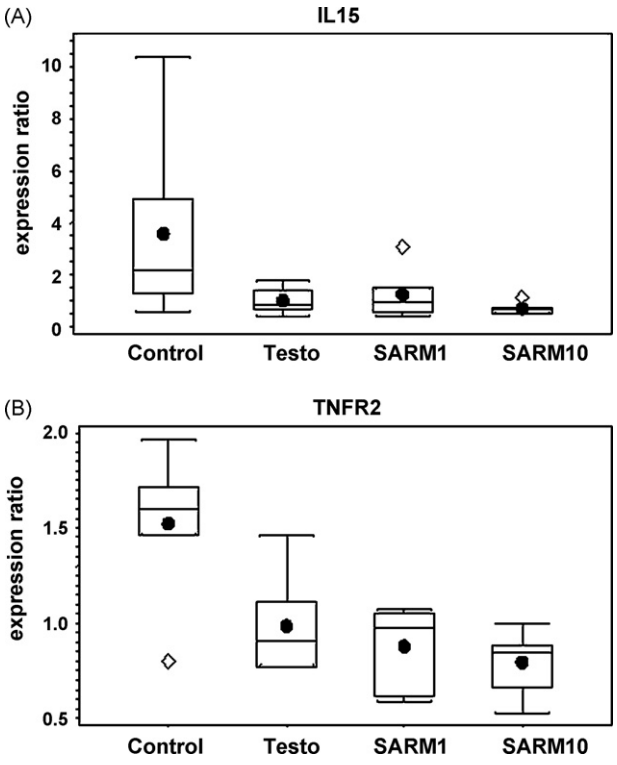

Fig. 1. Significant regulation for IL-15 (A) and TNFR2 (B) between control and treated samples after 16 days of treatment. Box plots show the median, mean (spot) and standard deviations.

3. Results

3.1. RNA quality

The mean (±std.dev.) RIN value of the blood samples were 7.5 (±4.8) at predose, 8.5 (±5.0) on day 16 and 7.7 (±4.2) at day 90 indicating a well intact RNA.

3.2. Primer testing and gel electrophoresis

Primer pairs of 40 genes were successfully used in qRT-PCR analysis to get single peaks and uniform melting curves, as well as a specific single band in high resolution agarose gel electrophoresis.

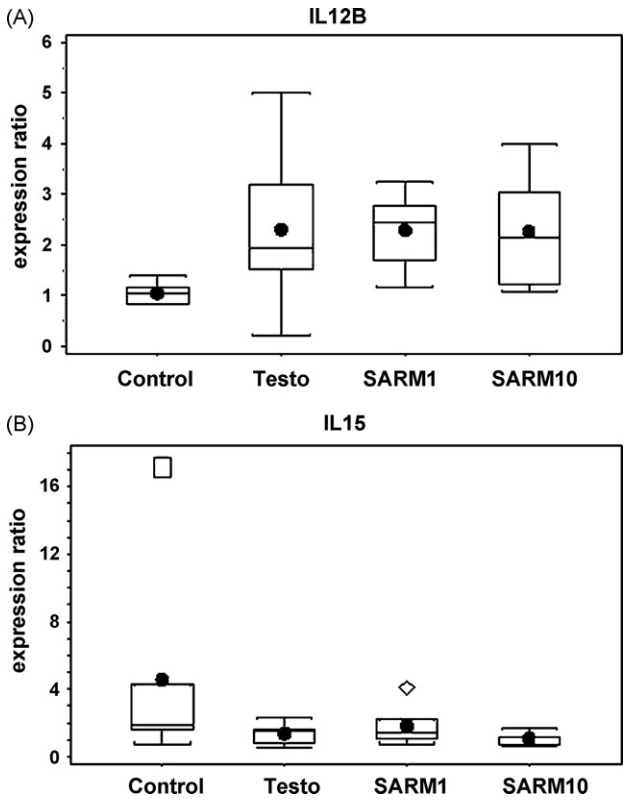

Fig. 2. Significant regulation for the proinflammatory interleukins IL-12B (A) and IL-15 (B), between control and treated samples after 90 days of treatment. Box plots show the median, mean (spot) and standard deviations.

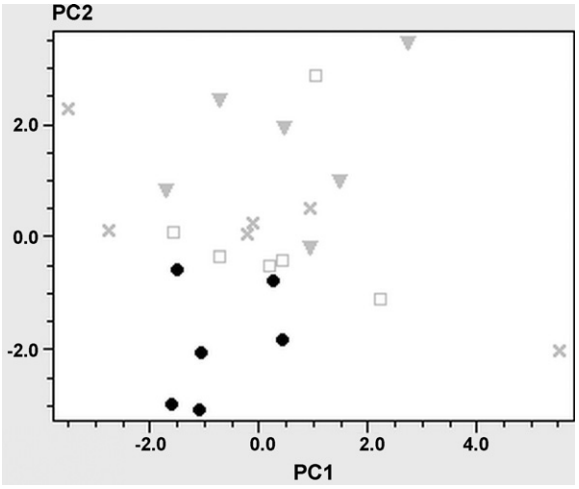

Fig. 4. Principal components analysis (PCA) for the six regulated genes IL-12B, IL-15, CD30L, Fas, TNFR1 and TNFR2 in the control group (black dots) the testosterone treated group (grey cross) the low dosed SARM group (grey squares) and the high dosed SARM group (grey triangle).

3.3. qRT-PCR results and data analysis

The calculation of the expression ratios (formula (1)) produced non-normally distributed data with frequent extreme values. Some of the extreme values can be outliers and were indicated in the Box–Whisker plot as squares outside the beyond inter quartile range (box). Nonetheless, no exclusion of extreme values/outliers was performed.

Significant down-regulation of gene expression of the treatment groups compared to the control group could be identified for IL-15 ($p = 0.0093$) and TNFR2 ($p < 0.0001$) after 16 days (Fig. 1)

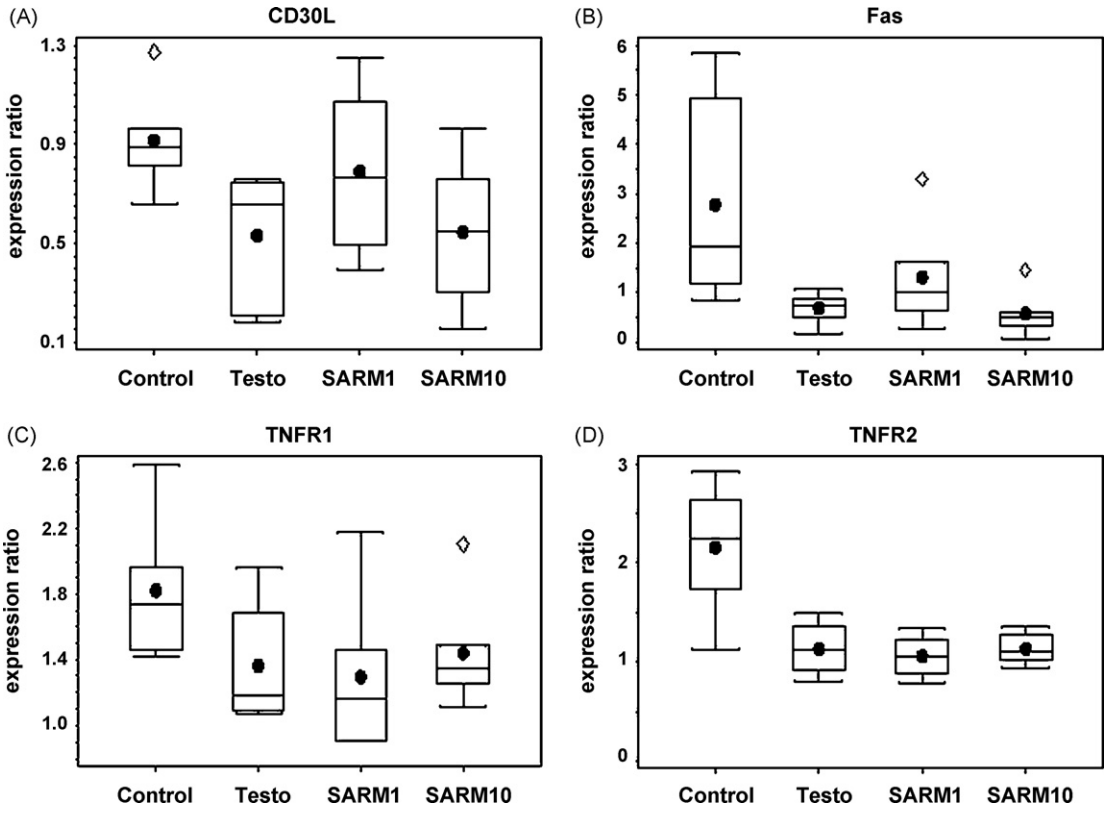

Fig. 3. Significant regulation for the apoptosis regulators CD30L (A), Fas (B), TNFR1 (C) and TNFR2 (D) between control and treated samples after 90 days of treatment. Box plots show the median, mean (spot) and standard deviations.

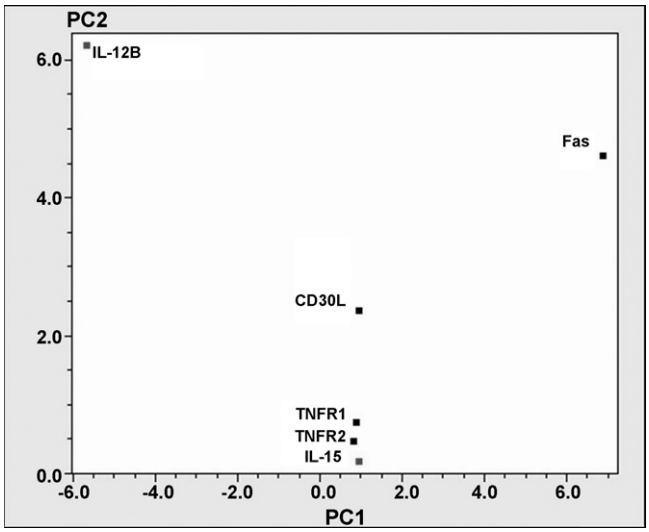

Fig. 5. Principle components analysis (PCA) for the regulated genes in all four groups. Grey spots show the interleukins and black spots show the apoptosis regulators.

and for IL-15 ($p = 0.0498$), CD30L ($p = 0.0435$), Fas ($p = 0.0032$), TNFR1 ($p = 0.0308$) and TNFR2 ($p < 0.0001$) after 90 days of treatment. Significant up-regulation of gene expression of the treatment groups compared to the control group could be observed for IL-12B ($p = 0.0240$) after 90 days of treatment (Figs. 2 and 3).

In the control group high variability could be observed compared to the treatment groups as indicated by the Box–Whisker plot. This reflects the natural variability of the non-induced expressing in each studied subject.

Principal components analysis (PCA) is a technique used to reduce multidimensional data sets to lower dimensions for analysis. Fig. 4 was obtained by plotting all samples of the four treatment groups by their two principal components obtained from the six responder genes. Black dots represent samples of the control group, grey crosses show the testosterone group, grey squares represent the SARM1 group and the grey triangles display the SARM10 group. A distinct control group can be seen, showing that there was a multi-transcriptional response to the treatment by any of the three drugs. In addition, the SARM1 neighbors to the control group, creating thus a transition to the Testosteron group and the SARM10 group. In Fig. 5 the six responder genes are clustered. Black dots show apoptosis regulators and grey spots display the interleukins. A distinct cluster of TNF receptors can be resolved.

4. Discussion

In this study changes of gene expression in blood cells caused by treatment with LGD2941 or testosterone were evaluated in order to compare the effects of both treatments on gene expression in blood cells. Further aims were the description of physiological effects and the identification of potential biomarkers for the treatment with AR ligands.

The main physiological effect that could be observed in this study is the down-regulation of various apoptotic marker genes in all three treatment groups. This is shown by the significant regulation ($p < 0.05$) of the apoptosis receptors Fas, TNFR1, TNFR2 and the apoptosis ligand CD30L. All regulated apoptosis factors belong either to the TNF Family (CD30L) or to the TNF-Receptor Family (TNFR1, TNFR2, Fas) [18]. It is already known that the death receptor Fas plays a dominant role in the programmed cell death of lymphocytes [18]. When B- and T-cells are activated they get sensitized to Fas mediated apoptosis. On resting peripheral lymphocytes Fas expression is low or even absent. Activation of B- and T-cells results in up-regulation of Fas mRNA [18–23]. Down-regulation of Fas after 90 days of treatment can be a hint to a down-regulating effect on the immune response. The death receptors TNFR1 and TNFR2 activate apoptosis via binding of TNF-α or TNF-β. Binding of the ligand to TNFR1 or TNFR2 can stimulate apoptosis and activate NFκB, whereas in most cases TNFR1 is responsible for these signals [18]. Ligand binding to TNFR2 leads to proliferation of thymocytes [24]. While TNFR2 expression is already regulated after 16 days of treatment, regulation of TNFR1 is only regulated after 90 days of treatment. A reason for this phenomenon could be that the mRNA expression of TNFR2 is inducible whereas expression of TNFR1 is not [24]. CD30L, a member of the TNF ligand superfamily is known to induce apoptosis by binding to its receptor CD30 and is expressed on activated T-cells [25,26]. Down-regulation of CD30L could also be observed after 90 days of treatment.

The down-regulation of these apoptosis regulators suggest that the immune response is suppressed by the treatment with testosterone and the SARM. This observation is consistent with the fact that testosterone has a suppressive effect on the immune system [27–29].

The gene expression of IL-12B – a subunit of IL12 – is up-regulated after 16 days of treatment. The main producers of IL-12 are monocytes, dendritic cells and activated macrophages. It promotes IFN-γ production by CD4 positive T-cells and stimulates proliferation and cytotoxic activity of T-cells and natural killer cells [30]. Gene expression of IL-15 is down-regulated after 16 and 90 days of treatment. It is produced by epithelial cells, fibroblasts, activated monocytes and dendritic cells. It acts as a T-cell activating factor but is not expressed by T-cells themselves [31]. Another important function of IL-15 is the up-regulation of natural killer cell survival and it promotes the production of IFN-γ, GM-CSF and TNF by natural killer cells [32–34].

Regarding the Box–Whisker plots it can be observed that the statistical variance in the control group is higher than in the treatment groups. The reason for this could be the natural variability of the non-induced expression in each studied subject. Suppression of gene expression by an external stimulus like treatment with testosterone or the SARM reduces natural variability of gene expression.

The PCA shows that both drugs show equivalent response and that the treatments differ from the control.

The second aim of this study was to find potential biomarkers for the use of the SARM. If the physiological effects of testosterone and the SARM are compared it became obvious that the SARM is active similar to natural androgens. The regulated genes found in this study can act as first biomarker candidates for the development of a screening pattern in whole blood. To confirm these biomarker candidate genes more studies will be helpful. In primary cell cultures or in further *in vivo* experiments it could be determined if the suggested parameters are independent of age, sex and immune status.

Acknowledgement

We thank TAP Pharmaceuticals Inc., Lake Forest, USA for supporting this study.

References

[1] M.C. Walsh, G.R. Hunter, M.B. Livingstone, Sarcopenia in premenopausal and postmenopausal women with osteopenia, osteoporosis and normal bone mineral density, Osteoporos. Int. 17 (1) (2006) 61–67.
[2] J.E. Morley, R.N. Baumgartner, R. Roubenoff, J. Mayer, K.S. Nair, Sarcopenia, J. Lab. Clin. Med. 137 (4) (2001) 231–243.
[3] J.E. Morley, Anorexia, sarcopenia, and aging, Nutrition 17 (7–8) (2001) 660–663.
[4] J.E. Morley, M.J. Kim, M.T. Haren, Frailty and hormones, Rev. Endocr. Metab. Disord. 6 (2) (2005) 101–108.
[5] T.J. Marcell, Sarcopenia: causes, consequences, and preventions, J. Gerontol. A Biol. Sci. Med. Sci. 58 (10) (2003) M911–M916.

[6] A. Negro-Vilar, Selective androgen receptor modulators (SARMs): a novel approach to androgen therapy for the new millennium, J. Clin. Endocrinol. Metab. 84 (10) (1999) 3459–3462.
[7] E. Martinborough, Y. Shen, A. Oeveren, Y.O. Long, T.L. Lau, K.B. Marschke, W.Y. Chang, F.J. Lopez, E.G. Vajda, P.J. Rix, O.H. Viveros, A. Negro-Vilar, L. Zhi, Substituted 6-(1-pyrrolidine)quinolin-2(1H)-ones as novel selective androgen receptor modulators, J. Med. Chem. 50 (21) (2007) 5049–5052.
[8] M.R. Deschenes, Effects of aging on muscle fibre type and size, Sports Med. 34 (12) (2004) 809–824.
[9] T.J. Doherty, Invited review: aging and sarcopenia, J. Appl. Physiol. 95 (4) (2003) 1717–1727.
[10] F.J. Lopez, New approaches to the treatment of osteoporosis, Curr. Opin. Chem. Biol. 4 (4) (2000) 383–393.
[11] S. Fleige, M.W. Pfaffl, RNA integrity and the effect on the real-time qRT-PCR performance, Mol. Aspects Med. 27 (2–3) (2006) 126–139.
[12] A. Schroeder, O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow, T. Ragg, The RIN: an RNA integrity number for assigning integrity values to RNA measurements, BMC Mol. Biol. 7 (2006) 3–17.
[13] D. Lehmann, K. Siebold, L.R. Emmons, H. Muller, Androgens inhibit proliferation of human peripheral blood lymphocytes in vitro, Clin. Immunol. Immunopathol. 46 (1) (1988) 122–128.
[14] J.D. Jacobson, M.A. Ansari, Immunomodulatory actions of gonadal steroids may be mediated by gonadotropin-releasing hormone, Endocrinology 145 (1) (2004) 330–336.
[15] S.A. Huber, J. Kupperman, M.K. Newell, Estradiol prevents and testosterone promotes Fas-dependent apoptosis in CD4+ Th2 cells by altering Bcl 2 expression, Lupus 8 (5) (1999) 384–387.
[16] R.W. McMurray, S. Suwannaroj, K. Ndebele, J.K. Jenkins, Differential effects of sex steroids on T and B cells: modulation of cell cycle phase distribution, apoptosis and bcl-2 protein levels, Pathobiology 69 (1) (2001) 44–58.
[17] M. Cutolo, S. Capellino, P. Montagna, P. Ghiorzo, A. Sulli, B. Villaggio, Sex hormone modulation of cell growth and apoptosis of the human monocytic/macrophage cell line, Arthritis Res. Ther. 7 (5) (2005) R1124–R1132.
[18] S. Nagata, Apoptosis by death factor, Cell 88 (3) (1997) 355–365.
[19] T.L. Rothstein, J.K.M. Wang, D.J. Panka, L.C. Foote, Z.H. Wang, B. Stanger, H. Cui, S.T. Ju, A. Marshakrothstein, Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B-cells, Nature 374 (6518) (1995) 163–165.
[20] P.H. Krammer, CD95's deadly mission in the immune system, Nature 407 (6805) (2000) 789–795.
[21] C. Klas, K.M. Debatin, R.R. Jonker, P.H. Krammer, Activation interferes with the Apo-1 pathway in mature human T-cells, Int. Immunol. 5 (6) (1993) 625–630.
[22] F. Leithauser, J. Dhein, G. Mechtersheimer, K. Koretz, S. Bruderlein, C. Henne, A. Schmidt, K.M. Debatin, P.H. Krammer, P. Moller, Constitutive and induced expression of Apo-1, a new member of the nerve growth-factor tumor-necrosis-factor receptor superfamily, in normal and neoplastic-cells, Lab. Invest. 69 (4) (1993) 415–429.
[23] H. Chan, D.P. Bartos, L.B. Owen-Schaub, Activation-dependent transcriptional regulation of the human fas promoter requires NF-kappa B p50–p65 recruitment, Mol. Cell. Biol. 19 (3) (1999) 2098–2108.
[24] P. Vandenabeele, W. Declercq, R. Beyaert, W. Fiers, Two tumour necrosis factor receptors: structure and function, Trends Cell Biol. 5 (10) (1995) 392–399.
[25] M.E. Kadin, Regulation of CD30 antigen expression and its potential significance for human disease, Am. J. Pathol. 156 (5) (2000) 1479–1484.
[26] P. Romagnani, F. Annunziato, R. Manetti, C. Mavilia, L. Lasagni, C. Manuelli, G.B. Vannelli, V. Vanini, E. Maggi, C. Pupilli, S. Romagnani, High CD30 ligand expression by epithelial cells and Hassal's corpuscles in the medulla of human thymus, Blood 91 (9) (1998) 3323–3332.
[27] C. Grossman, Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis, J. Steroid Biochem. 34 (1–6) (1989) 241–251.
[28] V. Morell, Zeroing in on how hormones affect the immune system, Science 269 (5225) (1995) 773–775.
[29] D. Verthelyi, Sex hormones as immunomodulators in health and disease, Int. Immunopharmacol. 1 (6) (2001) 983–993.
[30] M. Del Vecchio, E. Bajetta, S. Canova, M.T. Lotze, A. Wesa, G. Parmiani, A. Anichini, Interleukin-12: biological properties and clinical application, Clin. Cancer Res. 13 (16) (2007) 4677–4685.
[31] K. Liu, M. Catalfamo, Y. Li, P.A. Henkart, N.P. Weng, IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in CD8+ memory T cells, Proc. Natl. Acad. Sci. U.S.A. 99 (9) (2002) 6192–6197.
[32] Z. Liu, K. Geboes, S. Colpaert, G.R. D'Haens, P. Rutgeerts, J.L. Ceuppens, IL-15 is highly expressed in inflammatory bowel disease and regulates local T cell-dependent cytokine production, J. Immunol. 164 (7) (2000) 3608–3615.
[33] W.E. Carson, M.E. Ross, R.A. Baiocchi, M.J. Marien, N. Boiani, K. Grabstein, M.A. Caligiuri, Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro, J. Clin. Invest. 96 (6) (1995) 2578–2582.
[34] W.E. Carson, J.G. Giri, M.J. Lindemann, M.L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, M.A. Caligiuri, Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor, J. Exp. Med. 180 (4) (1994) 1395–1403.