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RESEARCH ARTICLE

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The effects of Cernitin® on inflammatory parameters and benign prostatic hyperplasia: An in vitro study

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The pollen extract Cernitin® is widely used for treatment of benign prostatic hyperplasia (BPH) and non-bacterial chronin prostatitis. However, little is known about the underlying molecular mechanisms to explain the clinical effects of Cernitin®. In this study, we sought to investigate the cellular mechanisms by which Cernitin® induces its effects on human prostatic cell lines BPH-1 and WPMY-1 and primary human peripheral blood mononuclear cells (hPBMCs) in vitro. We examined the effects of Cernitin® formulas T60 and GBX on the protein expression, proliferation, and cytokines production. Results revealed that Cernitin® upregulated antiinflammatory cytokine interleukin (IL)-10 and its receptors IL-10RA and IL-10B in addition to the upregulation of tumour necrosis factor-related apoptosis-inducing ligand in hPBMC. Interestingly, the levels of proinflammatory cytokines IL-6 and IL-8 were also increased. Furthermore, Cernitin® had significantly increased the level of IL-10 in BPH-1 and WPMY-1 cells. The level of IL-6 was also significantly increased in these cells although both T60 and GBX inhibited STAT-3 phosphorylation. Moreover, Cernitin® formulas had significantly reduced androgen receptor and prostate-specific antigen protein expression in stromal cells ($p < .05$). Treatment with GBX and T60 had significantly inhibited proliferation of BPH ($p < .001$) and stromal cells ($p < .05$), in a dose-dependent manner. Taken together, treatment with Cernitin® showed to regulate cytokines level in both prostatic cell lines and hPBMCs and it was associated with decreased androgen receptor and prostate-specific antigen levels WPMY-1 cells.

KEYWORDS

benign prostatic hyperplasia, Cernitin®, cytokines, prostatitis

1 | INTRODUCTION

Administration of plant-based medicine, including Cernitin®, for treatment of benign prostatic hyperplasia (BPH) and chronic prostatitis (CP) is rapidly growing worldwide (Dutkiewicz, 1996; Latil, Pétrissans, Rouquet, Robert, & de la Taille, 2015; Shoskes, 2002; Wagenlehner et al., 2009), especially important in the treatment of nonbacterial chronic inflammation of the prostate (CP-NIH category III) where antimicrobial option is not beneficial. In addition, it contributes to serious overuse of antibiotics and development of microbial resistance (Magistro et al., 2016). The recognized Cernitin T60 and GBX pollen extract (identified as active pharmaceutical ingredients and hereinafter referred to as Cernitin®). Cernitin® is one of the herbal pharmaceutical drugs widely used in the treatment of CP/chronic pelvic pain syndrome (CPPS) in Japan and some European countries (Iwamura et al., 2015; Wagenlehner, Bschleipfer, Pilatz, & Weidner, 2011). Cernitin®

is developed and manufactured by AB Cernelle, Sweden. The substance contains Cernitin® T60 (water-soluble fraction) and Cernitin® GBX (fat-soluble fraction). Numerous clinical studies have shown that Cernitin® treatment significantly reduced prostatic volume in patients presenting with BPH (Buck, Cox, Rees, Ebeling, & John, 1990; Preuss et al., 2001). Furthermore, these clinical studies confirmed the efficacy of this drug by the relief of CP/CPPS symptoms and suppression of prostate-specific antigen (PSA) (Togo et al., 2018). In addition to clinical reports, the efficacy of Cernitin® in the inhibition of proliferation and inflammation has been validated in preclinical studies (Asakawa et al., 2001; Kamijo, Sato, & Kitamura, 2001; Talpur, Echard, Bagchi, Bagchi, & Preuss, 2003). The antiinflammatory role of Cernitin® is mainly attributed to its inhibitory effect on inflammatory mediators like proinflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β (Asakawa et al., 2001). Further investigations revealed that Cernitin® GBX inhibited production of 5-lipoxygenase and cyclooxygenase activity by 50% and the inhibition was comparable to that of diclofenac. Cernitin® T60 on the other hand inhibited stromal proliferation in association with an enhanced apoptosis (Loschen & Ebeling, 1991).

Moreover, patients with CP/chronic pelvic pain syndrome showed higher levels of proinflammatory cytokines such as IL-6 and IL-8 compared with controls (Penna et al., 2007). However, IL-10 has a potent antiinflammatory effect and the activation of the IL-10 receptor (IL-10Rs) results in the inhibition of the synthesis of several cytokines and blocking activities normally induced by these cytokines (Penna et al., 2007). IL-10 has also reported to strongly up-regulate TNF-related apoptosis-inducing ligand (TRAIL) receptors and consequent activation of a caspase cascade that ultimately leads to apoptotic cell death (Fiorentino et al., 1991). These clinical results along with previous preclinical studies prompted us to examine the effects of the Cernitin® at the cellular and molecular levels to increase our understanding of the mode of action of this medicament. In this study, we aim to elucidate the mechanisms of action of each of the Cernitin® formulas independently, in terms of androgen receptor (AR) and PSA expression level and proliferation as well as its role in the cytokines regulation.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culturing

The human prostatic stromal cell line WPMY-1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA), and the BPH-1 cell line was purchased from (DSMZ, Brauschweig, Germany). Authenticity of the cell lines was confirmed by Eurofins Genomics (Ebersberg, Germany) before use. Cell lines were routinely grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 20 nM testosterone, 1% penicillin–streptomycin-neomycin (Life Technologies, Paisley, UK), and 2 mM L-glutamine (Life Technologies).

Human peripheral blood mononuclear cells (hPBMCs) from a healthy anonymous blood donor were isolated from leukocyte concentrate using Ficoll Paque Plus (GE Healthcare). The cells were seeded in round bottom culture plate at a density of 0.5×10^6 cells per well in complete RPMI (10 U/ml penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate, 1% Hepes, and 0.1% mercaptoethanol) with 2% human serum (Sigma Aldrich). The cells were then stimulated with 50 ng/ml TNF-alpha to induce a proinflammatory response. Subsequently, cells were treated with 0.01 to 2.5 mg/ml of each of the Cernitin® formula cultures mentioned, $n = 5$ in each treatment group. dH_2O control was used for T60 formulas, and DMSO was used for GBX formulas as 0.0 mg/ml control. Cells were kept in culture for 24 hr in 37°C with 5% CO_2.The cell medium was then collected and stored in −80°C until further analysis.

2.2 | Cell treatments

Cernitin® formulas, Cernitin® T60 (T60) and Cernitin® GBX (GBX), were tested in this study. T60 was dissolved in water, and GBX was dissolved in DMSO; accordingly, H_2O is used for T60 controls and DMSO for GBX controls. For proliferation assay, cells were grown in phenol red-free RPMI-1640 medium containing 5% fetal bovine serum for 24 hr prior to treatment with 0.05 to 2.5 mg/ml of the Cernitin® formulas (AB Cernelle, Sweden) or with 20 μM 5-alpha-reductase inhibitor (finasteride; AstraZeneca) for 48 and 72 hr. In controls, cells were left untreated or exposed to DMSO. For the detection of phosphorylated Signal Transducer and Activator of Transcription (STAT)-3 (p-STAT3), cells were treated with 0.05, 0.5, and 2.5 mg/ml of each of the Cernitin® formula for 24 hr. Thereafter, cells were exposed to 50 ng IL-6 for 1 hr to stimulate STAT3 activation before harvest.

2.3 | O-link assay

This assay was used to measure an array of cytokines secreted from the hPBMCs. Inflammatory markers in the medium were analyzed by Proximity Extension Assay technique using Proseek Multiplex Inflammation reagent Kit (O-link Bioscience, Uppsala, Sweden) performed by Clinical Biomarker Facility (Science of Life Laboratory, Uppsala). In brief, oligonucleotide-labelled antibody probes bound to their respective targets present in the cell supernatant. Addition of DNA polymerase resulted in an extension and joining of the two oligonucleotides and formed a polymerase chain reaction template. Subsequently, universal primers were used to preamplify the DNA template in parallel. Finally, individual sequences of DNA were detected and quantified using specific primers by microfluidic real-time quantitate polymerase chain reaction (96.96, Dynamic Array IFC, Fluidign Biomark). The chip was then run with a Biomark HD instrument, and data analysis was performed by processing normalization procedure using O-link Wizard for GenEx. All data are represented as arbitrary units.

2.4 | Meso Scale Discovery assay

To measure an array of cytokines with accuracy, an enzyme-linked immunosorbent assay-based Meso Scale Discovery (MSD) U-plex platform (K15067L-1) was performed according to manufacturer's instruction. In brief, cells were grown on 48 wells at approximately 20,000 cells/well, such that they were confluent the next day. To induce a proinflammatory response, cells were exposed to 50 ng/ml lipopolysaccharide for 4 hr before treatment with the compounds for further 24 hr at concentrations indicated in the figure. Diclofenac at concentration of (10 μM) was used as positive control. Media was aspirated, centrifuged, and kept at -80°C until use. The 96-well plate was coated with the linked cytokines antibodies overnight at 4°C. Thereafter, the calibrators, which are containing a known concentration, were added in duplets to the wells and incubated at room temperature with shaking for 1 hr. Read buffer was added to each well, and the plate was analyze on an MSD instrument, a division of Meso Scale Diagnostics (LLC, Rockville MD).

2.5 | Western blot analysis

To measure the levels of proteins of interest, a western blotting technique was performed as described before (Dizeyi, 2019). Briefly, cells were treated with Cernitin® formulas as indicated in the figures. Afterward, the cells were lysed in RIPA buffer (Invitrogen Carlsbad, CA, USA). Total proteins (30–40 μg) were electrophoretically separated and transferred into polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The proteins of interest were detected using primary antibodies: antibodies anti-androgen receptor (AR) (PG-21) and anti-p-STAT3 life science (Thermo Fisher, Inc.), anti-PSA (Dako, Glostrup, Denmark), and anti-B-actin (Sigma-Aldrich), at 4°C overnight. Thereafter, membranes were washed and incubated in secondary antibodies HRP-conjugated anti-mouse IgG and anti-rabbit IgG (GE Healthcare, Stockholm, Sweden) for 1 hr at room temperature. Bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL), and images were acquired using the Bio-Rad Western workflow (BioRad). Densitometric quantification of immunoblots was performed by the ImageJ Image Analysis Software (NIH, Baltimore, MD) and represented as fold change relative to control, normalized relative to β-actin bands.

2.6 | Proliferation assay

Proliferation of the cells was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymetoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) assay (Promega Biotech, Nacka, Sweden), according to the manufacturer's protocol. Approximately 4×10^3 cells/well were cultured in 96-well plates for 24 hr before treatment for 48 and 72 hr, and H_2O or DMSO-treated cells served as a control for T60 and GBX, respectively. Cells were washed; thereafter, 80 μl new medium containing 20 μl MTS was added to each well. Cells were incubated at 37°C for 2 hr and measured for optical density values at 490 nm on Milenia Kinetic Analyser (Diagnostic Products Corporation, DPC, LA). Wells containing medium only served as blank control. Three independent experiments were carried out.

2.7 | Statistical analysis

Statistical significance was determined with unpaired Student's *t* test and one-way analysis of variance analysis of variance and Sidak's multiple comparison test to identify significant differences between the control group and treated group. Results were expressed as the mean ± standard deviation, where $p < .05$ is considered significant.

3 | RESULTS

3.1 | Effect of Cernitin® on cytokines production in hPBMC

In order to validate the effects of Cernitin® treatment in the inflammation process, we examined a panel of proinflammatory and antiinflammatory cytokines in hPBMC. The results from O-link assay revealed that T60 but not GBX had significantly increased the level of the antiinflammatory cytokine IL-10 in a dose-dependent manner. The higher concentration of T60, 2.5 mg/ml, resulted in a significant increase in IL-10 production by hPBMC ($p < .001$; Figure 1a). However, there was a trend but no significant increase in IL-10 production by GBX (Figure 1b). Moreover, the results demonstrated that both T60 and GBX formulas showed a significant increase in the level of TRAIL (Figures 1c and 1d). TRAIL is a protein that is associated with apoptotic cell death, and it is strongly up-regulated by IL-10. Cernitin T60 concentrations of 0.01–2.5 mg/ml resulted in a marked increase of TRAIL ($p < .001$; Figure 1c). The GBX formula showed similar effect at concentrations used ($p < .05$ to $p < .01$; Figure 1d).

We also examined the production of IL-10 receptors in response to T60 and GBX treatment. T60 significantly increased the production of IL-10RA and IL-10RB in a dose-dependent manner as can be seen in Figure 2a. The production of IL-10RB was evident at both low and high concentrations ($p < .01$ and $p < .001$, respectively), whereas significantly high level of IL-10RA was found at lower concentrations (0.01, 0.05, and 0.5 mg/ml; $p < .05$ and $p < .01$) but not at 2.5 mg/ml concentration (Figure 2b). The GBX increased production of IL-10RA, which was statistically significant at all concentrations ($p < 0.01$; Figure 2c). However, the production of IL-10RB was not statistically significant in the cells treated with GBX (Figure 2d). Paradoxically, T60 but not GBX treatment had also resulted in a significant increase in the levels of IL-6 ($p < .01$) and IL-8 ($p < .05$) in hPBMC.

3.2 | Effects of Cernitin® on cytokines production in prostatic cells

In the next study, we examined the effects of Cernitin treatment on a number of cytokines in BPH-1 and WPMY-1 cells. The results from MSD assay revealed that there is a great difference in the

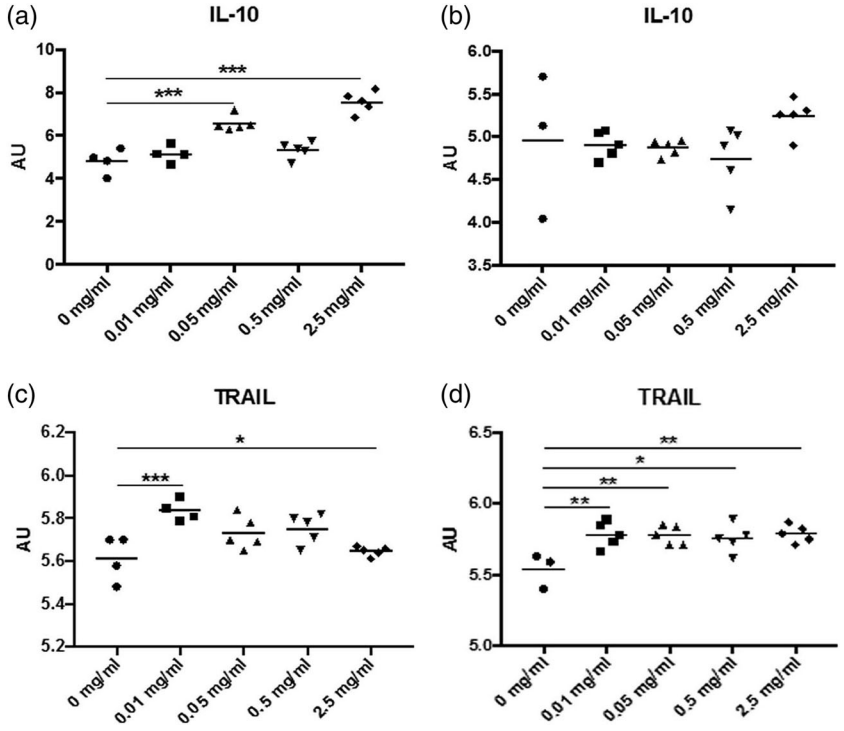

FIGURE 1 Effects of Cernitin® on IL-10 and tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) production in human peripheral blood mononuclear cell. Supernatants from human peripheral blood mononuclear cells stimulated with 50 ng/ml TNF-α to induce a proinflammatory response for 24 hr in the presence or absence of increasing concentrations of T60 or GBX and levels of cytokines determined using O-link assay. Effect of (a) T60 and (b) GBX on the expression of IL-10. Effect of (c) T60 and (d) GBX on the expression of TRAIL. One way analysis of variance analysis of variance and Sidak's multiple comparison test to identify significant differences between the control group and treated group. Results were expressed as the mean ± standard deviation (*SD*), where $p < .05$ is considered significant. $^{*}p \leq .05$; $^{**}p \leq .005$

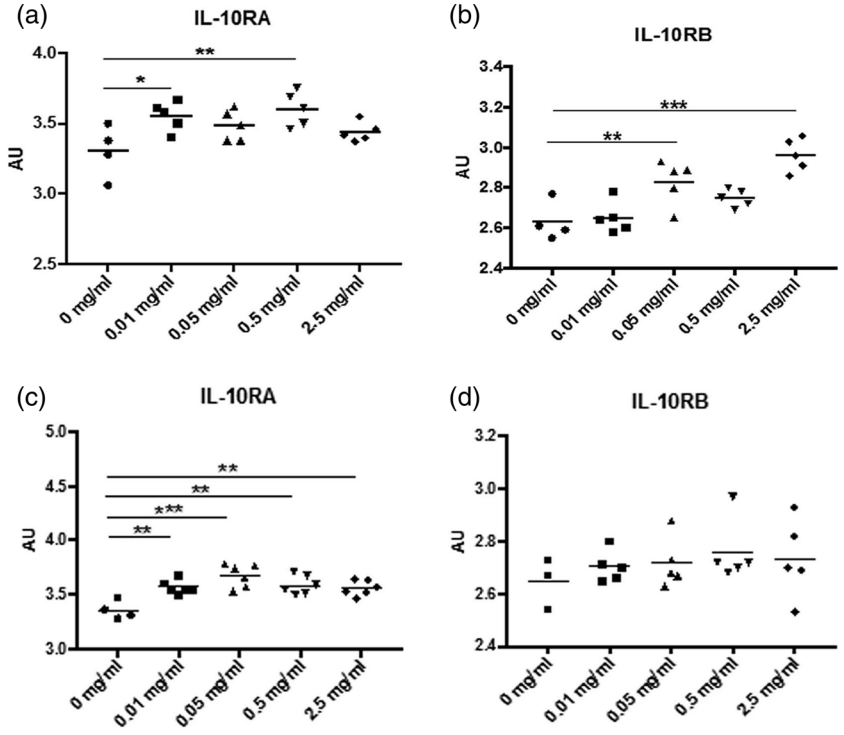

FIGURE 2 Effects of Cernitin® on IL-10RA and IL-10RB production in human peripheral blood mononuclear cell. Supernatants from human peripheral blood mononuclear cell stimulated as mentioned in Figure 1 analyzed for effects of T60 on the (a) IL-10RA and (b) IL-10RB, GBX on the (c) IL-10RA and (d) IL-10RB. One way analysis of variance analysis of variance and Sidak's multiple comparison test to identify significant differences between the control group and treated group. Results were expressed as the mean ± standard deviation (*SD*), where $p < .05$ is considered significant. $^{*}p \leq .05$; $^{**}p \leq .005$

responsiveness between the cell lines and Cernitin formulations. There were also an intervariations depending on the concentration of the compounds (Figures 3a–3f). When the proinflammatory cytokines were analyzed, we found that the level of IL-6 was increased after T60 and GBX treatment in both cell lines (Figures 3a and 3b). The highest level of IL-6 was reached when WPMY-1 cells were exposed to T60 at concentration of 0.05 and 0.5 mg/ml ($p < .01$; Figure 3b). The GBX induced small or no effects on the induction of IL-6. Neither T60 nor GBX treatment at any concentrations used was shown to alter IL-8 levels (Figure 3d) in WPMY-1 cells and a non-significant trend was found in BPH-1 cells (Figure 3c). T60 but not GBX had significantly increased the level of the antiinflammatory cytokine IL-10 in BPH-1 and WPMY-1 cells in a dose dependent manner (Figures 3e and 3f). At nearly all concentrations T60 had

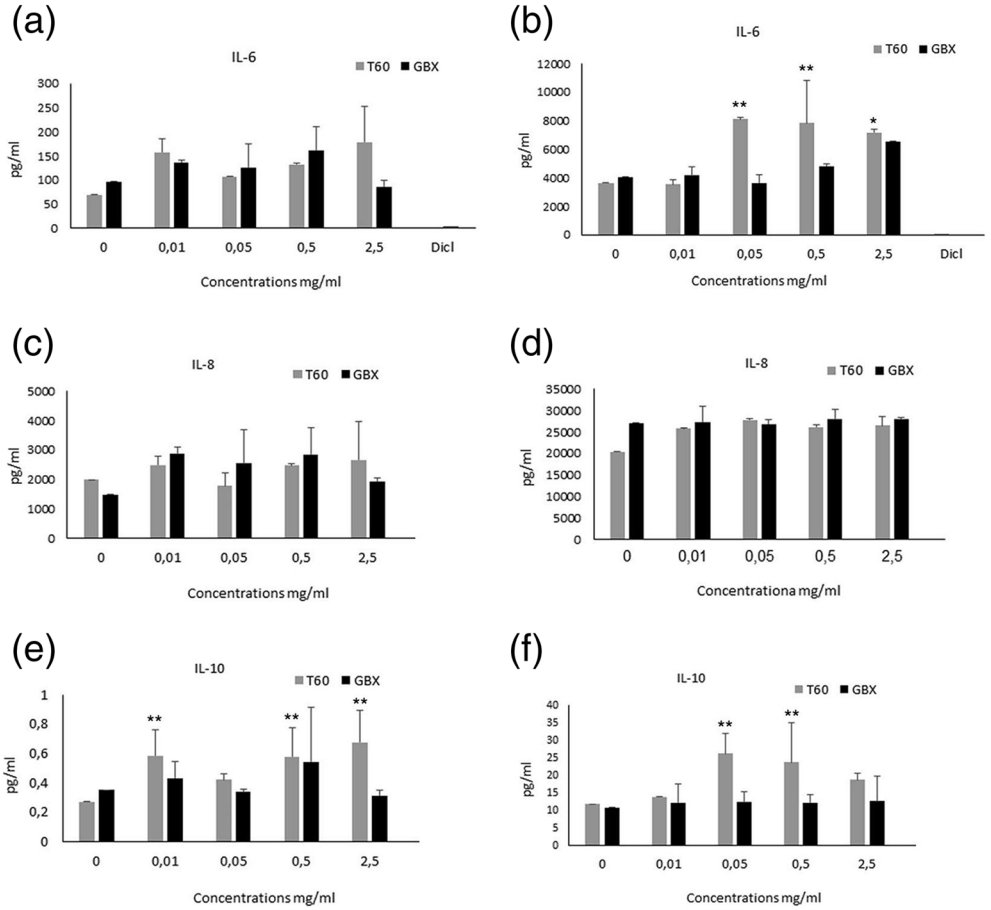

FIGURE 3 Effects of Cernitin® on cytokines production in prostatic cells. Cell culture supernatants from the cells in the presence or absence of increasing concentrations of T60 or GBX were examined for levels of cytokines using enzyme-linked immunosorbent assay-based MSD assay. Levels of (a) IL-6, (c) IL-8, and (e) IL-10 in BPH-1 cells and levels of (b) IL-6, (d) IL-8, and (f) IL-10 in WPMY-1 cells. The values are mean ± *SD* of two values determined by Student's *t* test. *p*-values lower than .05 are considered statistically significant. $^{*}p \leq .05$; $^{**}p \leq .005$.

significantly increased the level of IL-10 in BPH-1 cells ($p < .01$; Figure 3e), whereas in WPMY-1 T60 led to a significant increase in IL-10 production at concentration of 0.05 and 0.5 mg/ml ($p < .01$; Figure 3f).

3.3 | Effects of Cernitin® on STAT3 phosphorylation

In the next experiment, we wanted to identify the signalling pathway exploited by Cernitin® formulas. Western blot analyses revealed that T60 partially inhibited the IL-6-induced phosphorylation of STAT3 in BPH-1 cells. At concentration of 2.5 mg/ml, the phosphorylation was inhibited to the basal level (Figure 4). Similar to T60, treatment of the cells with GBX inhibited phosphorylation of STAT3 to a basal level at concentrations of 0.5 and 2.5 mg/ml but not at low concentration (0.05 mg/ml).

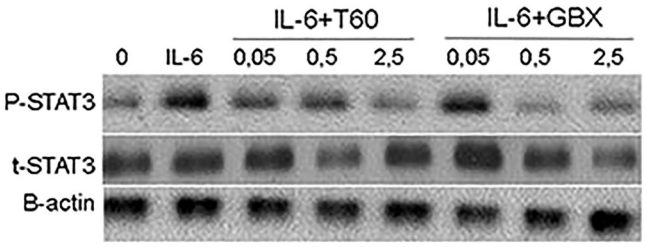

FIGURE 4 Cernitin® reduced Signal Transducer and Activator of Transcription 3 (STAT3) phosphorylation in benign prostatic hyperplasia (BPH) cells. Western blot analyses of protein extracts show the reduced level of phosphorylation of STAT3 (Ser^{703}) in a concentration-dependent manner in BPH-1 cells after treatment with T60 or GBX formulas. The expression of total STAT3 and β-actin was used as loading controls

3.4 | Cernitin® decreased the AR and PSA protein level

Treatment with T60 and GBX decreased the level of AR and PSA in WPMY-1 cells in a concentration dependent manner (Figures 5a and 5b). T60 induced a statistically significant decrease in AR level at concentration of 2.5 mg/ml ($p < .01$; Figure 5a). In contrast, the GBX resulted in a statistically significant decrease in AR and PSA levels at concentrations used ($p < .01$) with the lowest level at concentration of 0.05 mg/ml (Figure 5b). The lowest level of AR and PSA was

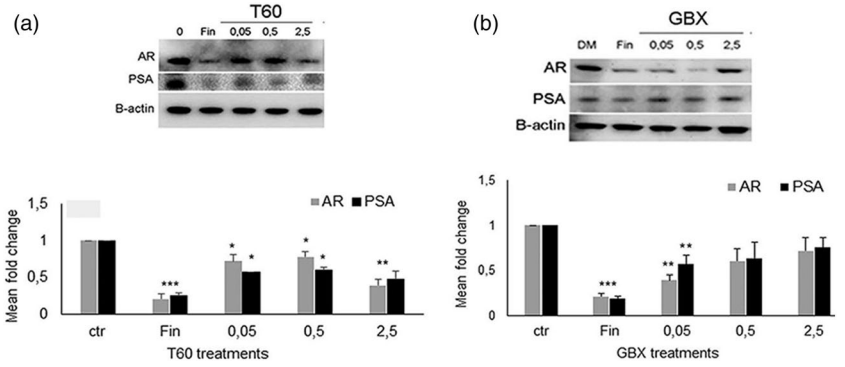

FIGURE 5 Androgen receptor (AR) and prostate-specific antigen (PSA) expression in cell lines. Western blots show the expression of AR and PSA in (a) T60- and (b) GBX-treated WPMY-1 cells. Cells were treated for 48 hr with Cernitin® formulas or Finasteride (Fin). In negative controls (ctr) H_2O or DMSO were used. β-actin was used as loading controls. Lower panels are densitometric analyses of Western blot analyses. The expression values were normalized to β-actin and determined by mean change fold. The values are mean ± *SD* of at least three independent experiments determined by Student's *t*-test. *p*-values lower than.05 are considered significant. $^{*}p \leq .05$; $^{**}p \leq .005$; $^{***}p \leq .001$

observed in the cells treated with finasteride ($p = .0001$). The *p* values were determined by densitometric analysis shown in lower panels.

3.5 | Cernitin® inhibited proliferation of BPH and stromal cells

We examined the effects of T60 and GBX on the proliferation inhibition in BPH-1 and WPMY-1 cells. The results show that both formulas inhibited proliferation of cells albeit varying degree depending on the formula and concentration (Figures 6a–6d). After 48-hr exposure to the T60, there was a trend but no significant proliferation inhibition was found in BPH cells (Figure 6a). However, after 72 hr, a significant inhibition of BPH cells treated with T60 was found at concentration used ($p < .05$; Figure 6a). In contrast, GBX treatment resulted in a significant inhibitory effect already after 48 hr and continued until 72 hr (Figure 6b; $p < .01$). Furthermore, T60 resulted in a statistically significant inhibition of the WPMY-1 cell proliferation after 48 and 72 hr ($p < .05$; Figure 6c). GBX inhibited WPMY-1 cell proliferation in the similar manner as BPH cells ($p < .01$; Figure 6d). Intriguingly, GBX at concentration of 0.5 mg/ml seems to be less effective in inhibiting cell growth compared with T60 at the same concentration. Finasteride had significantly inhibited proliferation of both prostatic cell lines ($p < .001$; Figures 6a–6d).

4 | DISCUSSION

Although Cernitin® has been established as a safe and effective therapy before detailed mechanistic studies became possible and, despite its clinical use for 60 years, its molecular mechanisms of action are largely unknown. In this in vitro study, we performed a comprehensive analysis of the Cernitin® formulas, T60 and GBX, at the clinically relevant concentrations and tolerated by cell lines (Furusawa, Chou, Hirazumi, & Melera, 1995; 0.01–2.5 mg/ml) in terms of AR and PSA expression, proliferation, and regulation of cytokines in BPH-1 and WPMY-1 representing human prostatic epithelial and stromal cell lines, respectively. We also analysed the effects of Cernitin® formulas on the cytokines regulation in hPBMCs. The major findings of this study are that both formulations of Cernitin® induced a significant decrease in the AR and PSA protein levels of the WPMY-1 cells. A parallel proliferation inhibition was detected in both cell lines. Furthermore, Cernitin® demonstrated a significant increase in IL-10, IL-10Rs, and TRAIL in the hPBMC secretion and increase IL-10 in prostatic cells, all in a formulation and concentration-dependent manner.

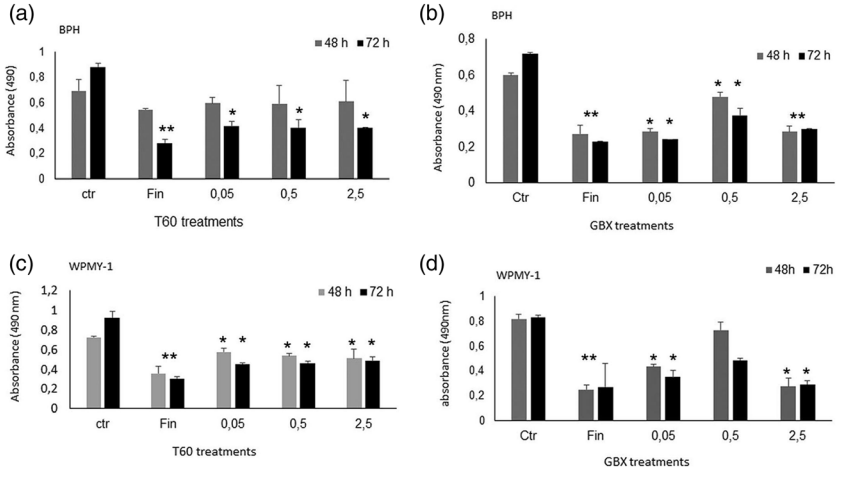

FIGURE 6 Cernitin® inhibited proliferation in cell lines. Graphs show MTS results in (a) T60- or (b) GBX-treated BPH-1 cells, as well as (c) T60 and (d) GBX-treated WPMY-1 cells. Finasteride (Fin). In negative controls (ctr) H_2O or DMSO were used. The values in MTS are mean ± *SD* of three independent experiments determined by Student's *t* test. *p*-values lower than.05 are considered statistically significant. $^{*}p \leq .05$; $^{**}p \leq .005$

It is well established that androgen via AR plays a crucial role in the regulation of the proliferation in prostatic epithelial and stromal cells (Izumi, Mizokami, Lin, Lai, & Chang, 2013). Consequently, targeting AR to manage BPH is the main therapeutic options (Clark et al., 2004). This is the mode of action of the current treatment of large prostatic glands with 5-alpha-reductase inhibitor (e.g. finasteride and dutasteride), used in this study as a comparative control. As a result, prostate volume is decreased and PSA suppressed. However, there are undesirable side effects associated with finasteride. Conversely, the plant based medicine is favourably used with basically few or no side effects. This aspect is very important to highlight, as low levels of side effects is correlated with high treatment compliance (Latil et al., 2015; MacDonald, Ishani, Rutks, & Wilt, 2001). The clinical impact of Cernitin® is confirmed in several clinical studies with positive outcome in patients with urinary tract symptoms relief instigated by BPH (MacDonald et al., 2001). The results presented in this study revealed that Cernitin® treatment was able to significantly reduce AR and PSA levels, albeit lesser than finasteride. The decrease of PSA value is also demonstrated in a recent clinical study (Togo et al., 2018). A simultaneous decrease in cell proliferation of both cell lines was found after treatment with Cernitin®. The inhibitory effect of Cernitin® was in line with the study conducted by Habib and coworkers (Habib, Ross, Lewenstein, Zhang, & Jaton, 1995). They reported that a specific component in the pollen extract induced a strong inhibitory effect on growth of the primary culture of prostate stroma and epithelial cells and prostate cancer cell line DU145 cells after 2 days of exposure (Habib et al., 1995). We found that GBX more efficiently inhibited proliferation compared with T60 (Figure 2). This may be due to the fact that GBX is fat soluble and could easily pass through the lipid layer of the cell membrane, whereas T60 is water soluble, which needs a carrier to enter the cell. Decreased AR level and PSA down regulation show that Cernitin may exert its function via the AR, but whether it blocks or inhibit AR similar to treatment with bicalutamide or enzalutamide or through 5-alpha-reductase inhibition (Clark et al., 2004) is yet to be explored. Another putative mechanism of Cernitin® effect is the interaction with the enzymes necessary for testosterone synthesis. It is noteworthy to mention that the BPH-1 cell line is reported to not express AR or PSA at both protein and mRNA level (Hayward et al., 1995). However, we detected a weak double band consistent to AR molecular size, but PSA protein was not detected (figure not shown).

To mimic inflammation, hPBMCs we stimulated the cells with TNF-α because TNF receptors are mostly expressed in immune cells but not in nonmalignant prostatic cells. We found that the hPBMC and prostatic cells similarly responded to Cernitin® treatment with variation in the responsiveness to the formulas. However, our results were not in line with the study conducted by Kamijo and coworkers (Kamijo et al., 2001). They reported that the dose-dependent, antiinflammatory action of Cernitin® in nonbacterial prostatitis in rats leading to decreased levels of IL-1b, IL-6, and a tumour necrosis factor decreases glandular inflammation (see review by Wagenlehner et al., 2011). In contrast, we did not find the decrease of these proinflammatory cytokines in our in vitro study. Inversely, the level of these cytokines was found to be increased or unchanged (Figures 4 and 5). On the other hand, Cernitin® resulted in the production of antiinflammatory IL-10 and its receptors besides increase in TRAIL, of which its production is correlated with IL-10 level. The discrepancy in these results is most likely related to the models used. The production and regulation of cytokines is strictly regulated in the living entities. Whereas in vitro we used supernatant and the cytokines might not be optimally regulated. It also raises the issue of a differential receptor expression in the different species cell types. Nevertheless, the increased IL-10 and TRAIL in hPBMC or IL-10 in prostatic cells may not be able to inhibit pro-inflammatory cytokines but may be able to inhibit proliferation caused by many cytokines. It has been shown that cytokines, including IL-6 through activation of the STAT signalling pathways, can activate transcription factors related to cell proliferation (Braun, Fribourg, & Sealfon, 2013). Importantly, we demonstrated that Cernitin® had inhibited the phosphorylation of STAT3 activated by IL-6, suggesting the role of this drug in the regulating inflammation process in the prostate.

Building on these results, it is important to further verify mechanisms of action, whether Cernitin® interacts directly with AR or if it interferes with testosterone metabolism. Lastly, it is difficult to extrapolate these results to the clinical scenario without further studies in animal models. An animal model is ongoing in our laboratory.

In conclusion, Cernitin® formulas affected prostatic cell types in similar fashion. However, there are minor differences, which are worth mentioning, for instance, water soluble formula T60 had effectively inhibited proliferation at higher concentrations, and GBX was effective at all concentration with the lower being more pronounced. Moreover, BPH-1 and hPBMC were more responsive to Cernitin® formulas than WPMY-1 cells, in regard to cytokines regulation. Cernitin® was able to upregulate TRAIL, which is important because monocytes/macrophages are uniquely susceptible to TRAIL-mediated apoptosis. In addition, Cernitin® induction of a significantly high IL-10 production in hPBMC and PBH cells suggested that Cernitin may be effective in stimulating IL-10 production, thus, can explain the antiinflammatory effects of Cernitin®.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors have the following conflicts of interest to disclose. The first author Dr. Dizeyi declares financial support from AB Cernelle for carrying out in a specialized laboratory the series of tests presented in the present document. The project falls into the frame of a larger preclinical research project. Author Mrs. Ramnemark is employed by AB Cernelle; Dr. Yao Mattisson has been given project related financial support for performing the O-link assay. Professor Abrahamsson and Associate Professor Grabe are advisors to the project. Prof. Abrahamsson is member of the European Urology Physicians guideline committee on Urology.

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