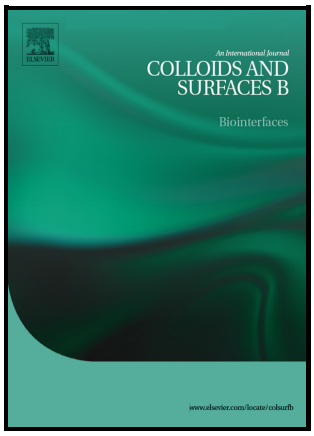

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Ivana Z. Kuzminac, Andjelka S. Ćelić, Sofija S. Bekić, Vesna Kojić, Marina P. Savić, Nenad L. Ignjatović

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Hormone receptor binding, selectivity and cytotoxicity of steroid D-homo lactone loaded chitosan nanoparticles for the treatment of breast and prostate cancer cells

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Keywords: D-homo lactone, androstane, nanocarrier, cancer, estrogen and androgen receptors

Highlights:

- Two anti cancer steroid D-homo lactones were encapsulated in a chitosan (Ch) nano carrier.
- Steroid-loaded Ch display selectivity toward MDA-MB-231 and PC-3 cell lines.
- Lack of estrogenicity and androgenicity was observed for Ch-encapsulated forms D-homo lactones.
- Ch-encapsulated steroid lactones are good candidates for the design of anticancer treatments.

Abstract:

Chemically modified steroids have a long history as anti-neoplastic drugs. Incorporation of a lactone moiety in the steroid nucleus, as in previously obtained 3β-acetoxy-17-oxa-17a-homoandrost-5-en-16-one (**A**) and 3β-hidroxy-17-oxa-17a-homoandrost-5-en-16-one (**B**), often results in enhanced anticancer properties. In this work, chitosan-based (Ch) nanoparticles were created and loaded with potent anticancer steroidal compounds, **A** and **B**. Changes to hormone receptor binding and cytotoxicity were then measured. In agreement with our previous results for **A** and **B**, **A**- and **B**-loaded Ch displayed cytotoxic properties against cancer cell lines. Both **A**-Ch and **B**-Ch showed activity toward estrogen negative breast cancer (MDA-MB-231) and androgen negative prostate cancer cell lines (PC-3). Greater selectivity toward cancer cells versus healthy lung fibroblast (MRC-5) was observed for **B**-Ch particles. Cell viability and cytotoxicity

measurements after a recovery period indicate more robust recovery of healthy cells versus malignant cells. Compounds **A** and **B** or their Ch-encapsulated forms were shown to have negligible affinity for the ligand binding domain of estrogen receptor β or the androgen receptor in a fluorescent yeast screen, suggesting a lack of estrogenicity and androgenicity. Steroid-loaded chitosan nanoparticles display strong cytotoxicity towards MDA-MB-231 and PC-3 with a lack of hormone activity, indicating their safety and efficacy.

1. Introduction

From the early 1900s until today, research focused on steroids has significantly expanded from compound isolation, structural characterization and biosynthetic pathway determination, to include chemical modifications and applications [1-3]. The first confirmation of the importance of these compounds came early, with seven Nobel prizes awarded for steroid research, and later with medical applications for natural and synthetically modified steroids. The high structural diversity of steroids led to identification of a large number of physiological activities and applications for the treatment of a wide range of diseases. Steroids are used or being developed for the treatment of allergies [4], asthma [5], Alzheimer's disease [6], muscle atrophy [7-9] and contraception [10, 11], etc. More recent findings suggest that the well-known corticosteroid, prednisolone, can accelerate recovery from severe drug induced liver injury [12]. During the present global health crisis, respiratory syndrome coronavirus 2 (SARS-CoV-2) coronavirus disease-19 (COVID-19), application of steroidal anti-inflammatory drugs increased, once again showing the importance of these compounds [13, 14]. Recent findings also suggest the protective effects of androgen deprivation therapy in prostate cancer patients against COVID-19 [15, 16]. One of the most important applications of structurally modified steroids is in the treatment of various types of cancers, such as breast [17, 18] and prostate [19] cancers. Thousands of new steroid structures have been isolated from natural sources [20] or are being synthesized each year in order to test their antitumor activity. Modern trends in medicinal chemistry enable careful structure-based or target-based molecular design prior to the synthesis of new compounds. For example, Testolactone is a well-known inhibitor of the enzyme aromatase and was used in the treatment of estrogen-dependent breast cancer for many years [21]. Its structure was a starting point for the design of a number of biologically active steroid D-homo lactones [22-29].These compounds show good cytotoxicity against multiple cancer types.

With a large number of physiological activities and medical applications, different modes of steroid administration have been developed: oral, parenteral, inhalation, topical, intra-articular, ocular, nasal, rectal, intravaginal, etc. [30-32]. All of these application methods have been developed in order to increase the bioavailability of steroid drugs and hence their bioactivity. These applications, as well as more targeted delivery were also the main goals of drug encapsulation research [33-37]. In addition to steroids, many other compounds have been subjected to encapsulation in order to gain more selectivity, higher activity, and diminish unwanted side effects through targeted delivery [38-43]. Using targeting drug carriers, active

compounds are guided toward specific organs, cells, or even receptors, and are accumulated in the target tissue, enhancing their effectiveness while lowering side effects [44-45]. A broad range of carriers have been used in order to enable selective targeting, including: liposomes, carbon nanotubes, gold nanoparticles, dendrimers, meso-porous silica nanoparticles, polymeric nanoparticles, emulsions, etc. Polymers have more therapeutic benefits and because of that nanoparticles commonly used in medicine are polymers of lipids [46]. Because only several loaded nanoparticles have passed clinical trials and are now on the market, targeted delivery remains a very active of area of research.

Application of chitosan polymers in steroid drug delivery is the research subject of many groups, including our own [33, 35, 38, 41, 47]. This is due to the biocompatibility, lack of toxicity, and biodegradability of this polysaccharide and the possible application of steroid derivatives in the treatment of multiple cancers. Besides possessing anticancer properties, steroid hormones and their derivatives can bind to hormone receptors and exhibit hormone activities that are occasionally linked with a number of side effects. Furthermore, there are results that indicate the possibility of receptor-based targeting in tumor cells by nanocarriers [48]. Therefore, the aim of the present study was to investigate the effects of chitosan encapsulation of steroid derivatives on cell cytotoxicity and steroid receptor binding properties. We chose two steroidal D-homo lactones with small differences in structure, because of their good cytotoxicity toward cancer cells, and their applicability in drug design and synthetic organic chemistry. Using these compounds, loaded chitosan-based nanoparticles were created and their cytotoxicity, selectivity, and cytotoxicity after a recovery period were investigated, as well as cell viability. Last but not least,, to test for the possibility of estrogenic or androgenic hormone activity and associated side effects, the relative binding affinity of these particles to the ligand-binding domain of estrogen and androgen receptors was measured.

2. Experimental

2.1. Synthesis of A and B

Detailed synthetic procedures for compounds **A** and **B** (**Figure 1**) can be found in our previous papers [23, 24, 49]. Cytotoxicity was previously evaluated using the MTT assay [27].

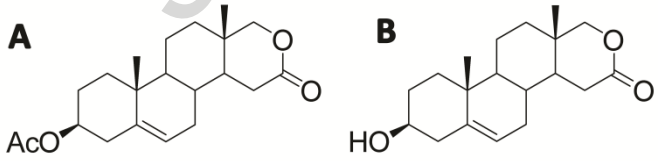

Figure 1. Structures of steroidal D-homo lactone derivatives **A** ($C_{21}H_{30}O_4$) and **B** ($C_{19}H_{28}O_3$)

2.2. Synthesis of A-loaded Ch (A-Ch) and B-loaded Ch (B-Ch)

Chitosan (Ch) with a medium molecular weight (Sigma-Aldrich, deacetylation > 80%), was dissolved in acetic acid (1 wt%), and mixed with an ethanol solution of **A** (or **B**) in a weight ratio

of Ch : **A** (**B**) = 8 : 2, while stirring with a magnetic stirrer at 400 rpm. The obtained mixture of **A** (or **B**), and Ch was slowly poured into a solution of sodium triphosphate pentabasic (STP, Sigma-Aldrich) 0.1 wt% in H_2O, while stirring at 21000 rpm for 30 min (IKA, ULTRA-TURRAX T 25). The obtained mixture was then centrifuged at 5000 rpm and 5 ^oC for 1 h, and the resulting precipitate was subjected to lyophilization (Freeze Dryer, Christ Alpha 1-2/ LD Plus) at temperatures ranging from -10 to -60 ^oC and pressures ranging from 0.37 mbar to 0.1 mbar for 1 h to 5 h. The obtained powder was washed with distilled water three times, centrifuged at 1000 rpm and dried again. The final products were dry powders composed of **A** loaded Ch (**A**-Ch) and **B** loaded Ch (**B**-Ch) particles.

2.3. Product characterization

Fourier transform infrared spectroscopy (FT-IR) was conducted using a Nicolet iS10 FT-IR Spectrometer equipped with an attenuated total reflectance (ATR) accessory in the spectral range from 400 to 4000 cm^{-1}. Field Emission Scanning Electron Microscopy (FE-SEM) was performed on a Tescan MIRA 3 XMU microscope. The particle size distribution (PSD) was measured on 10 mg/ml of powders dispersed in water using a Mastersizer 2000 (Malvern Instruments Ltd.) and a HydroS dispersion unit for liquid dispersants. High-performance liquid chromatography HPLC (Ultimate 3000 HPLC, Thermo Fisher Scientific, Woltham, USA) was applied to measure the encapsulation efficiency of **A** and **B** and the *in vitro* drug release profile. The percent encapsulation efficiency was calculated using the following equation: The percentage of drug capture efficiency = (actual drug content / theoretical drug content) X 100 was 90%.
The release rate of **A** and **B** *in vitro* in phosphate buffered saline (PBS) at 37 ^oC and in the presence of 0.1 wt% sodium lauryl sulfate (SLS) was determined by HPLC. The detection of **A** and **B** was carried out using a UV detector at wavelength of 230 nm. Concentrations of **A** and **B** in PBS were calculated using the following equation:

$$C_{A(B)}\,(\%) = [(A \times V_{standard} \times C_{standard})/(A_{standard} \times V_{steroid} \times C_{steroid})] \times 100,$$

where $A_{steroid}$ and $A_{standard}$ (mAU min) is the area of released steroid in HPLC chromatograms, $V_{steroid}$ and $V_{standard}$ are injection volumes of steroid and standard (100%, pure steroid) solutions, respectively, and $C_{steroid}$ and $C_{standard}$ are concentrations of steroid and standard solutions, respectively.

2.4. Biological assays

2.4.1. *In vitro* cytotoxicity tests

Cells and culturing

Particle cytotoxicity measurements were carried out on three cell lines: human lung fibroblasts MRC-5 (ATCC CCL 171), human prostate adenocarcinoma PC-3 (ATCC CRL 1435) and human estrogen receptor negative (ER-) breast carcinoma MDA-MB-231 (ATCC HTB-26). All cells were grown attached to a plate (Costar, 25 cm^3) in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 4.5 g/L glucose and 10% FCS (fetal calf serum, Sigma). The medium contained an antibiotic and antimycotic solution (Sigma). The cell lines were maintained under standard conditions: at a temperature of 37 °C in a humidity saturated atmosphere with 5% CO_2 (Heraeus incubator). Cells were passaged twice a week, and only viable cells in the logarithmic growth phase between the third and the tenth passage were used in experiments. The number of cells and their viability were determined by the dye exclusion test (DET) with 0.1% trypan blue.

DET - dye exclusion test

Cells were centrifuged (10 min / 200 ×g) and counted in 0.1% trypan blue. Viable cells were seeded in Petri dishes (Center well, Falcon) containing the examined substance / disc at a concentration of 2×10^5 / mL. Control samples did not contain the test substance. Petri dishes with seeded cells were left at 37 °C, with 5% CO_2 for 48 hours. After incubation, cell counting was performed using an inverted microscope (REICHERT Biostar) in counting chambers. Cytotoxicity (CI) was expressed as a percentage according to the formula:

$$CI = (1 - Ns / Nk) \cdot 100$$

where Nk is the number cells in the control samples, and Ns is the number of cells in the samples with the tested substance [50].

Colorimetric test with tetrazolium salts (MTT test)

The cells were harvested in the logarithmic growth stage by centrifugation (10 min / 200 ×g) and counted in 0.1% trypan blue. Viable cells were seeded in Petri dishes (Center well, Falcon) containing the tested substances / discs at a concentration of 2×10^5 / mL. The control samples did not contain the test substances. Petri dishes with seeded cells were incubated in a thermostat at 37 °C, with 5% CO_2 for 48 h. Viable cells were seeded (5×10^3 / 100 µL) in quadruplicate in 96- well microplates. The plates with seeded cells were left in a thermostat at 37 °C, with 5% CO_2 for 48 h. MTT solution, prepared immediately prior to addition, was added to all wells in the 10 µL / volume and incubation was continued for 3 h (in a 37 °C thermostat with 5% CO_2). After 3 h, 100 µL of 0.04 mol/L HCl in isopropanol was added to each well. The absorbance was read immediately after the incubation period using a microplate reader (Multiscan Ascent, ThermoLabsystems) at a wavelength of 540 nm and with a reference wavelength of 690 nm. The wells on a plate that contained only medium and MTTserved as blank [51]. Cytotoxicity was expressed as a percentage according to the formula:

$$CI = (1 - As / Ak) \times 100$$

where Ak is the absorbance of the control samples, and As is the absorbance of the samples treated with test substance.

2.4.2. Yeast based fluorescence assay

The relative binding affinities of compounds **A** and **B** for the ligand-binding domain (LBD) of estrogen receptor β (ERβ) or androgen receptor (AR) were measured using a fluorescent assay in yeast, following a procedure previously optimized for testing steroid derivatives [52, 53, 54, 29]. Ch-encapsulated forms of compounds **A** and **B** were also tested for ERβ binding affinity. Plasmids (pRF4-6-ERβ-LBD-YFP and pRF4-6-AR-LBD-YFP) and the yeast strain (*S. cerevisiae* FY250 MATα, *ura3-52, his32Δ00, leu2Δ1, trp1Δ6*) used in the present study were generously provided by dr. Blake Peterson. Yeast cells were transformed using a standard lithium acetate/polyethylene glycol method [55] and transformants were selected on drop-out agar plates in the absence of tryptophan. Isolated single yeast colonies were then grown to saturation at 28 ^{o}C, in selective liquid medium lacking tryptophan supplemented with 2% raffinose. After 40 hours of incubation in a Biosan orbital shaker-incubator ES-20/60, an aliquot of recombinant yeast cells from late exponential phase was transferred to fresh medium and growth was monitored spectrophotometrically at 600 nm. Expression of LBD-ERβ/AR-YFP is under the control of a GAL1 promoter, and was induced by addition of galactose (final concentration of 20 g/L) at an optical density (OD600) of 0.4-0.6. Simultaneously, test compounds or control ligands, freshly dissolved in dimethyl sulfoxide (DMSO), were also added. The final concentration of steroid derivatives and positive control ligands for each steroid receptor (estradiol for ERβ and testosterone for AR), as well as their Ch-loaded forms, was 10 μM. Several control probes were included in the experiment: cells where protein expression was not induced, cells treated only with solvent, and cells treated with empty chitosan carrier. After treatment, recombinant cells were grown at room temperature in the dark for 14-16 hours. Fluorescence intensity was then quantified using a Fluoroskan Ascent FL fluorometer equipped with a 485/538 nm excitation/emission filter. All measurements were performed in triplicate at room temperature. Imaging experiments were performed using a Olympus BX51fluorescence microscope equipped with a FITC filter for visualization of YFP fluorescence changes following ligand binding induced dimerization of the receptor LBD-YFP probe. Binding affinity was expressed as the fold fluorescence change between cells treated with test compound and cells treated with solvent, with error bars representing propagated standard errors of the mean. Graphs were created and analyzed using Origin Pro 8 software.

3. Results and Discussion

3.1. Synthesis

3β-Acetoxy-17-oxa-17a-homoandrost-5-en-16-one (**A**) and 3β-hidroxy-17-oxa-17a-homoandrost-5-en-16-one (**B**) were synthesized according to a known procedure, starting from dehydroepiandrosterone [23, 24, 49]. These compounds were encapsulated in chitosan carrier by emulsification and lyophilization.

Figure 2. shows the FTIR spectra of **Ch**, **A**, **A**-Ch, **B** and **B**-Ch in the frequency range from 4000 to 400 cm^{-1}. FTIR spectra of pure derivative **A** display characteristic peaks at: 2942 cm^{-1} assigned to the C-H vibration and 1726 cm^{-1} due to the C=O vibration. These characteristic bands are also present in the FTIR spectra of **A**-Ch, and new bands are noticeable. The peaks at 1320 cm^{-1} and 892 cm^{-1} are CN (amide III) and OH bands of Ch [56]. In contrast to compound **A**, the **B** derivative has a characteristic band at 3475 cm^{-1}, which is due to the HO group (**Figure 1b.**). The IR spectrum of **B**-Ch is additionally characterized by absorption bands arising from **B** and Ch: HO at ~3475 cm^{-1}, CH at ~1726 cm^{-1}, C=O at ~1726 cm^{-1} (**B**) and CN at ~1320 cm^{-1} and OH at ~892 cm^{-1} (Ch). The presence of the characteristic bands of pure derivatives **A** and **B** in **A**-Ch and **B**-Ch powder, respectively, suggest that derivatives **A** and **B** were successfully entrapped in the Ch polymer.

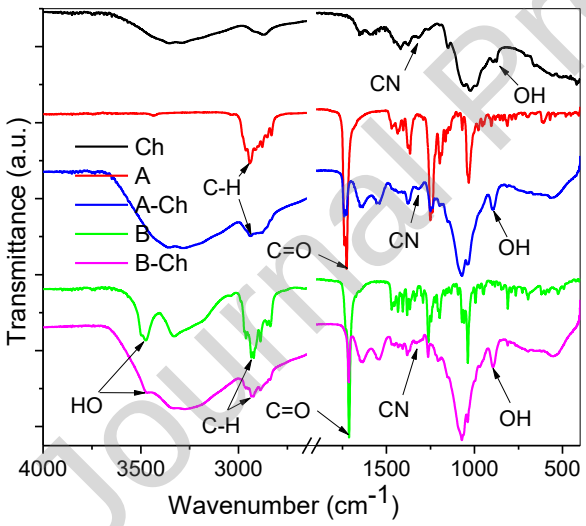

Figure 2. FTIR spectra of **Ch, A**, **A** loaded Ch, **B** and **B** loaded Ch

Surface morphologies and volume-based particle distribution diagrams for **A**-Ch and **B**-Ch particles are shown in **Figure 3**. Both powders contained spherical particles, as expected. Processing of polymer systems based on Ch in a centrifugal field leads to the formation of spherical morphologies for the resulting processed particles [36]. **A**-Ch particles had a distribution of sizes, with a d50 parameter equal to 98 nm. The powder also contained smaller particles, correspondingly d10 = 20 nm (**Figure 3a**). Similar results were observed for **B**-Ch powder (**Figure 3b**). **B**-Ch particles had the following size distribution values: d50 = 93 nm and d10 = 25 nm. The possibility of obtaining bimodal particle size distribution profiles after using

the emulsification-evaporation method has been observed in the literature [57, 58]. It is most likely that heterogeneous colloidal clusters were formed during emulsification of a suspension of a binary mixture of Ch and **A** (or **B**) in aqueous medium (Experimental part). Moreover, subsequent processing bimodal droplet clusters in a centrifugal field could also lead to the formation of a bimodal size distribution of dry particles.

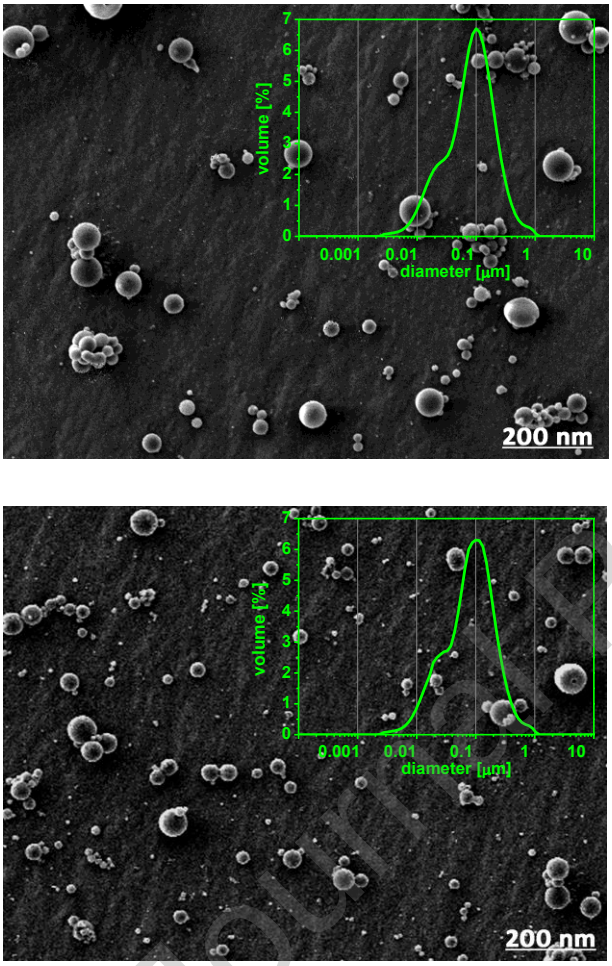

Figure 3. FE-SEM and PSD of (a) **A**-Ch, (b) **B**-Ch

The ratio of Ch and **A** (or **B**) was 8 : 2 (Experimental part), since the encapsulation efficiency was 90%, the share of entrapped **A** and **B** was 18 wt%. In accordance with these results, the cumulative curve of release of **A** and **B** in percents in PBS at 37 ^{o}C under static conditions was defined (**Figure 4**). Over 50% of pure compounds **A** and **B** were released in the first 12h; in 24h approximately 70% were released. The cumulative release curves for **A** and **B** were close, with a characteristically rapid release of up to 12h. After 72h it was confirmed that there was complete release (100%) of **A** and **B**. The complete release of various compounds from chitosan nanoparticles after 72 h was also noted in studies by other authors [59, 60]

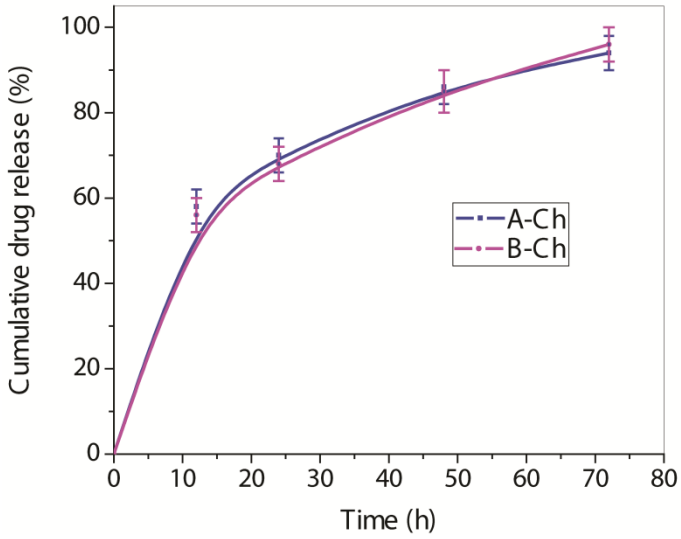

Figure 4. Comparative cumulative release curves for **A** and **B** in PBS at 37 ^{o}C over the degradation period.

3.2. Biological assays

3.2.1. *In vitro* cytotoxicity tests

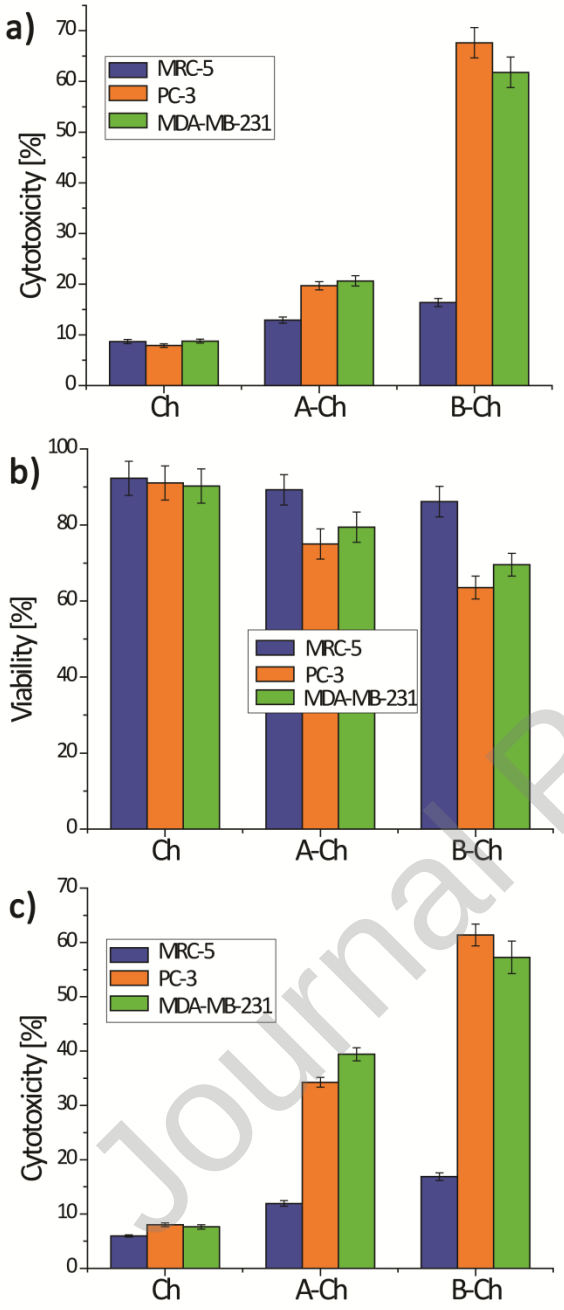

Figure 5. *In vitro* cytoxicity and cell viability measurements for **A**-Ch and **B**-Ch against three cell lines: human lung fibroblasts MRC5, human prostate cancer PC-3 and human breast cancer MDA-MB-231: a) cytotoxicity and b) viability was assessed by the dye exclusion test (DET); c) cytotoxicity after a recovery period of 72 hours using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test.

Figure 5 shows the results of viability and cytotoxicity tests after incubation of healthy (MRC-5) and cancer cells (PC-3 and MDA-MB-231) with drug-free Ch and **A**-Ch and **B**-Ch, which were tested using DET and MTT assays, respectively. The results of comparative cytotoxicity tests for Ch, **A**-Ch and **B**-Ch particles against MRC-5, as well as against PC-3 and MDA-MB-231cells are shown in **Figure 5a**. Compound free Ch particles showed negligible cytotoxicity toward all three tested cell lines. Particles containing **A** or **B** in the Ch carrier showed stronger cytotoxicity against both cancer cell lines. The cytotoxic activity of **B**-Ch was significantly higher for cancer cells compared to **A**-Ch. Compared to cancer cells, the cytotoxicity against healthy cells (MRC-5) was lower for particles with both derivatives **A** and **B** (approximately two fold lower for **A** and three fold lower for **B**). By comparing with our previous results, it can be observed that loading compound **B** in chitosan carrier significantly improves its cytotoxic activity against the tested cancer cell lines, especially PC-3 [24, 27]. Cell viability measurements were obtained after 48 h of incubation with **A**-Ch and **B**-Ch particles, washing and counting in 0.1% trypan blue (**Figure 5b**). Cell viability decreases with derivative B in the carrier. Based on the results shown in **Figure 5b**, it is clear that the viability of healthy cells (MRC-5) is always higher compared to both malignant cell lines (PC-3 and MDA-MB-231) in all three tested systems containing both derivatives **A** and **B**. The viability of healthy cells (MRC-5) was greater than 80% for particles **A**-Ch and **B**-Ch (89.25% and 86.16%, respectively), while for cancer cells (PC-3 and MDA-MB-231) it was less than 80% (69.67 and 62.12%, respectively). After 48 h of incubation with particles **A**-Ch and **B**-Ch, all examined cell lines (MRC-5, PC-3 and MDA-MB-231) were allowed to recover for 72 hours and the obtained cytotoxicity results are shown in **Figure 5c**. After recovery, cytotoxicity values were more pronounced in malignant cells (PC-3 and MDA-MB-231) than in healthy ones (MRC-5), indicating that healthy cells recovered better than malignant cell lines. Cytotoxicity results show that MRC-5 cells are less sensitive than PC-3 and MDA-MB-231. The action of **B**-Ch is stronger than **A**-Ch, which reduces the chances of recovery.

3.2.2. Fluorescence measurements of relative binding affinities for estrogen receptor β or androgen receptor in yeast cells

Because estrogenicity or androgenicity may be an undesirable side effect of the use of steroid derivatives for the treatment of hormone-dependent cancers, in the present study the relative binding affinities of compounds **A** and **B** for ERβ/AR-LBD were quantified using a fluorescent assay in yeast. This assay was previously used by our group for identification of steroid derivatives with potential to modulate steroid receptors signaling pathways, which are often disrupted in hormone-sensitive cancers of breast, prostate and other reproductive organs [29, 52, 54]. In the present study, fluorescence assay conditions were first optimized for measurements in the presence of chitosan. Although it is known that chitosan lacks optical properties [61], we tested whether these particles interfere with the detection of fluorescent signal. As shown in **Figure 6**, addition of chitosan (*) to recombinant yeast cells treated with DMSO or positive control ligands did not result in significant changes to fluorescence intensities measured by fluorimetry. In addition, uninduced recombinant

yeast cells display no background fluorescence in the presence or absence of chitosan. These results confirm that chitosan does not interfere with fluorescence measurements at the wavelengths used in our assay.

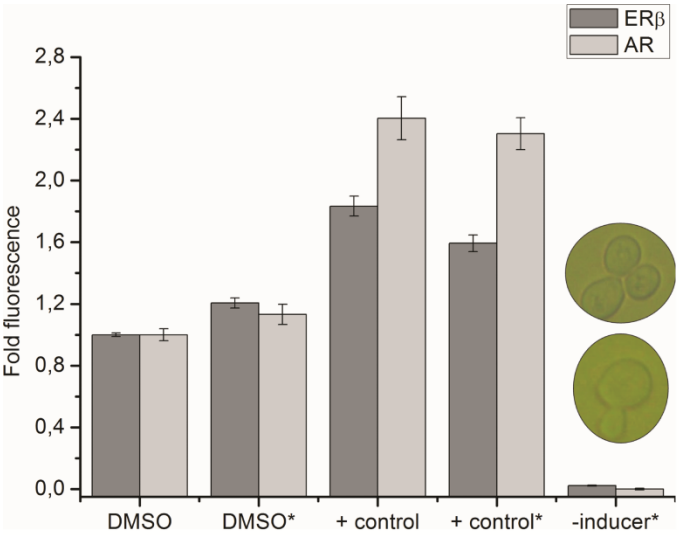

Figure 6. Fold fluorescence changes between ligand-treated and solvent-treated cells expressing LBD-ERβ-YFP or LBD-AR-YFP detected by fluorimetry. Recombinant yeast cells were treated for 14-16 h with 1% solvent (DMSO, normalized to 1) or a known high-affinity ligand (estradiol for ER and testosterone for AR, + control) at 10 μM. Results for cells treated with chitosan in DMSO are labeled with an asterix (*). Measurements for uninduced cells treated with chitosan are labeled (-inducer*). Microscopy images represent uninduced cells transformed with pRF4-6-ERβ-LBD-YFP (above) and pRF4-6-AR-LBD-YFP (below) following exposure to chitosan. Error bars represent propagated standard errors of the mean. Fluorescence intensity is proportional to ligand binding affinity.

Next we tested whether chitosan-bound steroid ligands of estrogen and androgen receptors can still enter yeast cells and induce a fluorescent signal. Among the limited number of studies concerning the delivery of chitosan-coated steroids, Wang and coworkers investigated intranasal administration of Ch-loaded estradiol in rats and found that chitosan enhances permeation [33], while chitosan nanoparticles were found to be capable of transporting testosterone *in vitro* [47]. In the present study, chitosan encapsulation did not prevent steroid ligands from entering yeast cells and did not change their affinity for the cognate receptor. Positive control ligands, testosterone for recombinant yeast cells expressing LBD-AR-YFP and estradiol for recombinant yeast cells expressing LBD-ERβ-YFP, showed high affinity for cognate receptors as expected, with fold fluorescence enhancements of 2.7 and 1.2 relative to DMSO controls (**Figure 7. a** and **c**). Chitosan encapsulated steroids, testosterone-Ch and estradiol-Ch, produced nearly identical fluorescence intensities as their chitosan-free counterparts, indicating that chitosan conjugation does not affect binding affinity. Moreover, in our experiments, chitosan had no effect on the growth rate of the *Saccharomyces cerevisiae* at the tested concentration (150 μg/ml), although

Zakrzewska *et al*. observed loss of viability and cell membrane disruption even at lower concentrations [62].

Since we have shown that chitosan-encapsulation does not interfere with our fluorescence measurements, we tested the relative binding affinities of compounds **A** and **B** and their chitosan-encapsulated forms for the ERβ ligand binding domain, and chitosan-free **A** and **B** for AR-LBD. Both **A** and **B** displayed negligible affinity for AR-LBD, while binding to the LBD of ERβ was very weak. (**Figure 7. a and c**). Fluorescence microscopy confirmed results obtained fluorimetrically (**Figure 7. b and d**). Furthermore, the relative binding affinities of chitosan encapsulated compounds **A** and **B** for ERβ LBD were also negligible; although the fluorescent signal in the case of **A**-Ch was higher compared to its unencapsulated form, it was not above solvent only controls (**Figure 7. a**). Thus, chitosan encapsulation of steroid compounds **A** and **B** does not increase their estrogenicity. Altogether, our results indicate that compounds **A** and **B** lack estrogenicity and androgenicity, which suggest that non-hormonal mechanisms may be responsible for their antiproliferative activity against estrogen-independent breast cancer cells and prostate cancer cells. The lack of measurable binding affinity of compound **B** for ERβ and AR, coupled with its strong cytotoxicity against breast and prostate cancer cell lines, makes it a promising candidate for the design of new treatments.

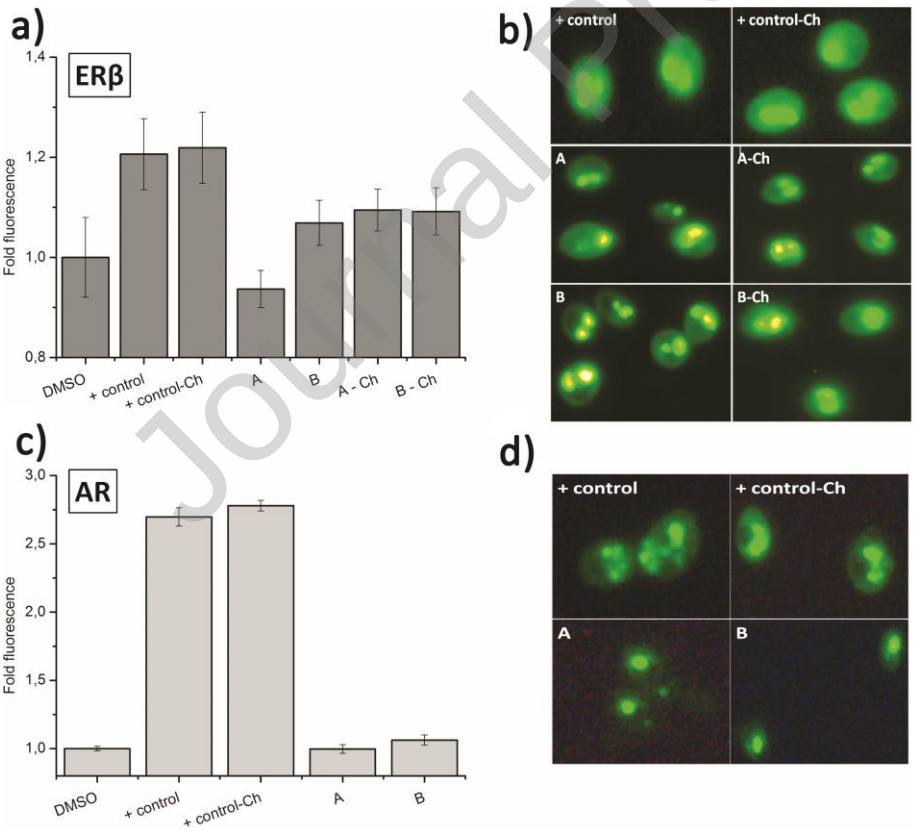

Figure 7. **Panel a)** Fold fluorescence changes between ligand-treated and solvent-only treated recombinant yeast cells expressing LBD-ERβ-YFP detected by fluorimetry. Cells were treated for 14-16h with 1% solvent (DMSO, normalized to 1), 10 μM estradiol (+ control), 10 μM chitosan-encapsulated

estradiol (+ control-Ch), or test compounds **A**, **B**, **A**-Ch and **B**-Ch. **Panel c)** Fold fluorescence changes between ligand-treated and solvent-only treated LBD-AR-YFP expressing cells. Cells were treated for 14-16h with 1% solvent (DMSO), 10 μM testosterone (+ control), 10 μM chitosan-encapsulated testosterone (+ control-Ch), or test compounds **A** and **B**. Error bars represent propagated standard errors of the mean. Fluorescence intensity is proportional to ligand binding affinity. Fluorescence microscopy images of recombinant yeast cells expressing LBD-ERβ-YFP (panel **b**) and LBD-AR-YFP (panel **d**) confirm results obtained by fluorimetry, although differences in the localization of fluorescence intensities were observed for various ligands.

4. Conclusions

Previously synthesized compounds **A** and **B** are similar in structure, but differ with respect to the group on C3. In the structure of compound **A**, there is an acetoxy group, while a hydroxy group is present in this position in compound **B**. These structural distinctions did not lead to significant differences in cytotoxic activity between the two compounds: both were active against estrogen negative breast cancer (MDA-MB-231) and androgen negative prostate cancer cell lines (PC-3). Since these two types of cancers are very hard to treat, we have focused further research on the compounds' anticancer properties. Chitosan (Ch) was used as a carrier of **A** and **B**, and the nano particles were synthesized by emulsification and lyophilization. There were no significant differences in the physicochemical properties of **A**-Ch and **B**-Ch particles, but **B**-Ch displayed much stronger cytotoxicity against both tested cancer cell lines. This indicates that encapsulation of substance **B** in a chitosan carrier significantly improves its antitumor activities. Encapsulation of estradiol had no effect on fluorescence measurements, suggesting that chitosan-encapsulation does not prevent estradiol from entering yeast cells or interacting with the ligand binding domain of ERβ. Furthermore, fluorescence signals for cells treated with either encapsulated or unencapsulated forms of compounds **A** and **B** were in the range of solvent-only controls. In addition, based on results from fluorescence measurements in yeast cells expressing LBD-ERβ or LBD-AR, both compounds **A** and **B** displayed negligible affinity for the androgen receptor and ERβ; an important consideration in the design of new treatments, in order to avoid potential estrogenic or androgenic effects on hormone-sensitive cancers. Based on the present study, chitosan encapsulated compound **B** may represent a good candidate for the design of treatments of hormone-dependent cancers.

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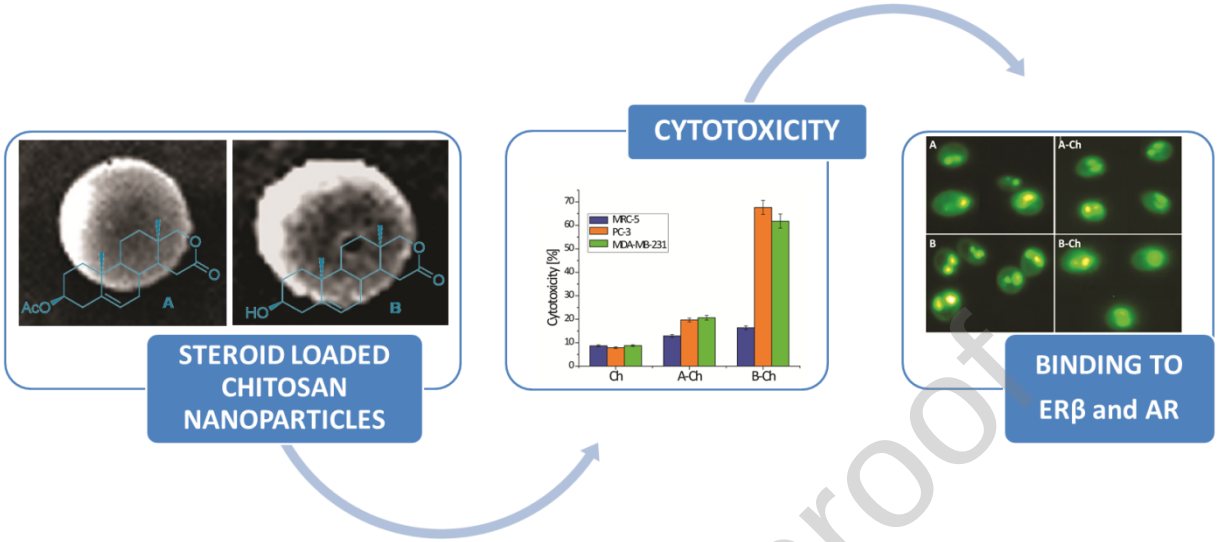
Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Graphical abstract

Highlights:

- Two anti cancer steroid D-homo lactones were encapsulated in a chitosan (Ch) nano carrier.
- Steroid-loaded Ch display selectivity toward MDA-MB-231 and PC-3 cell lines.
- Lack of estrogenicity and androgenicity was observed for Ch-encapsulated forms D-homo lactones.
- Ch-encapsulated steroid lactones are good candidates for the design of anticancer treatments.