Combination Nanotherapeutics for the Treatment of Advanced Prostate cancer

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Abstract

Main challenges to docetaxel use in advanced prostate cancer treatment include resistance and toxicity. To overcome these challenges and improve therapeutic efficacy in the treatment of heterogeneous prostate cancer, the use of multiple agents that can destroy different subpopulations of the tumor are needed. Brusatol is a multitarget inhibitor which has been shown to exhibit potent anticancer activity and play an important role in drug response and chemoresistance. Thus, the combination of brusatol with docetaxel in nanoparticle platform for the treatment of prostate cancer is expected to produce synergistic effects and inhibit chemoresistance.

In this work, we report the development of polymeric nanoparticles for the delivery of brusatol and docetaxel in the treatment of prostate cancer. The one-factor-at-a-time method was used to screen for formulation and process variables that impact particle size. Subsequently, factors that had modifiable effects on particle size were evaluated using 2^4 full factorial statistical experimental design followed by optimization of drug loading. Optimization of blank nanoparticles gave a formulation with a mean size of 169.1nm±4.8nm in agreement with the predicted size of 168.333nm. Transmission electron microscopy showed smooth spherical nanoparticles. The drug release profile show that the encapsulated drugs were released over 24 hours. Combination index data show a synergistic interaction between the drugs. Cell cycle analysis, evaluation of caspase activity and immunoblots show differences in PC-3 and LNCaP prostate cancer cells response to the agents. The nanoparticles are suitable for the treatment of prostate cancer and may be used in cases of docetaxel resistance.

Introduction

Prostate cancer is the second leading cause of cancer death in American men.[1] Currently, it is estimated that about 12.9% of men will be diagnosed with the disease during their lifetime.[2]

Treatment is dependent on the stage and grade of cancer, and some men eventually develop metastatic prostate cancer with androgen deprivation therapy (ADT) as the standard of care.[3] Disease progression and resistance to ADT ultimately leads to the development of metastatic castration-resistant prostate cancer (mCRPC). mCRPC is defined as prostate cancer characterized by clinical, radiographic, or biochemical progression despite castration levels of serum testosterone.[4]

Docetaxel (Taxotere) is a potent, first-line chemotherapeutic drug approved for patients with castration-resistant prostate cancer (CRPC) that has progressed despite hormone therapy. However, a vast majority of patients treated with docetaxel develop resistance to the drug in addition to other reported toxicity.[5] Thus, to overcome the challenges associated with docetaxel use and to improve therapeutic efficacy in heterogenous prostate cancer, the use of agents that inhibit multiple targets or the use of multiple agents that can destroy different subpopulations of the tumor leading to greater antitumor efficacy have been exploited.

Brusatol, is a multitarget inhibitor which has been shown to exhibit potent anti-cancer activity as a protein synthesis inhibitor and as an inhibitor of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway.[6] The combination of brusatol with docetaxel in the treatment of prostate cancer is expected to produce synergistic effects as a result of its potent anticancer effects and its potential to inhibit the mechanisms that facilitate resistance to docetaxel.

Nanoparticle-based drug delivery systems have emerged as a promising approach for enhancing the efficacy and safety of anticancer drugs.[7] Thus, combination nanotherapeutics is expected to combine the advantages of nanotechnology with those of combination therapy.

Methodology

Preparation and optimization of stealth blank nanoparticles

A three-step process for the development of a polymeric nanoparticle platform for the delivery of brusatol and docetaxel in the treatment of prostate cancer was developed. Initial, one-factor-at-a-time approach was used to screen for formulation and process variables that impact particle size. From the initial eight factors screened, four factors and their interactions that had modifiable effects on particle size were evaluated using 2^4 full factorial statistical experimental design in the second step followed by optimization to achieve particle size minimization using Minitab® software. In the third step, the optimized solution was used in the preparation of brusatol- and docetaxel-loaded nanoparticles fabricated using the emulsification-solvent evaporation method.

The nanoparticles were prepared using the oil-in-water (o/w) emulsification solvent evaporation method using a modified published method.8 Briefly, the desired amount of mPEG-PLGA was dissolved in the selected organic phaseand emulsified in an aqueous solution of polyvinyl alcohol (PVA) in an icebath using a probe sonicator. Evaporation of the organic solvent in the emulsion obtained was carried out under a fume hood. Nanoparticles were obtained by centrifugation followed by lyophilization to obtain white powder.

Particle size, size distribution and morphological studies

The particle size and size distribution of the different batches of blank and drug-loaded nanoparticles were determined by Dynamic Light Scattering (DLS) using a 90 Plus particle size analyzer. Structural morphology of the nanoparticles was evaluated using transmission electron microscopy (TEM) after negative staining of the nanoparticle suspension followed by imaging of the dry grids.

Infrared spectroscopy analysis

Fourier transform infrared spectroscopy (FT-IR) analysis was carried out to evaluate potential interactions between each drug and polymer and to qualitatively evaluate the efficiency of encapsulation of both drugs.

Drug content determination and release profile analysis

The weight percent of brusatol and docetaxel in the optimized nanoparticle formulation was quantified by High-Performance Liquid Chromatography (HPLC) from standard calibration curves of pure drugs. For determination of release profile, the dialysis bag method was used. Freeze-dried NPs were dispersed in PBS and immersed in an Eppendorf tube containing PBS clamped to a rotator shaker at 37°C. Sampling of the release medium was done at different time intervals.

Cell culture and *in vitro* experiments

All cell culture studies were performed on LNCaP and PC-3 cells. Viable cell counts was done using an automated cell counter. *In vitro* cytotoxicity evaluations were carried out using XTT and MTS assays. Cell cycle arrest and caspase 3/7 activity analysis were measured using a flow cytometer and analysed using Flow Jo software. Specific protein expression was carried out by immunoblotting methods.

Results

	Docetaxel (mg)	Brusatol (mg)	Size (nm)	Docetaxel Loading (%)	Brusatol Loading (%)	Drug: Polymer
Blank	0	0	169.1±4.8	0	0	20%
1	7	3	201.87±1.40	7.84±0.17	0.87±0.17	20%
2	6	4	195.70±1.15	6.37±0.11	0.79±0.07	20%
3	5	5	198.27±2.78	6.32±0.11	1.14±0.03	20%
4	4	6	182.57±1.90	3.73±0.25	0.82±0.09	20%
5	3	7	194.97±0.75	2.49±0.13	0.79±0.08	20%
6	2.5	7.5	184.80±1.30	2.04±0.04	0.89±0.02	20%
7	2	8	188.80±0.80	1.41±0.17	0.99±0.15	20%
8	1	9	193.83±1.59	0.70±0.06	1.48±0.28	20%
9	4.5	10.5	185.07±3.55	3.33±0.96	0.89±0.19	30%
10	3	12	183.03±5.66	1.88±0.57	0.92±0.30	30%

Table 1: Showing amount of brusatol and docetaxel, mean particle size and percent loading of each drug using the optimized nanoparticle formulation (n = 3).

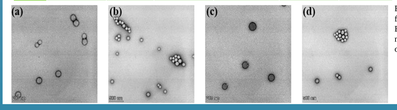

Figure 1. Typical TEM images of optimized formulation 1at different magnifications (A & B) and optimized formulation 2 at different magnifications (C & D) showing the structure of the nanoparticles.

Results Contd.

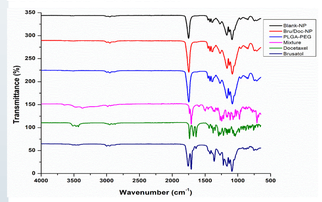

Figure 2. Overlay of FT-IR spectra of blank nanoparticles (black), brusatol- and docetaxel-loaded nanoparticles (red), pure brusatol (dark blue), pure docetaxel (green), PLGA-PEG polymer (purple), physical admixture (blue).

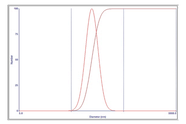

Figure 3: Typical particle size distribution data of nanoparticles

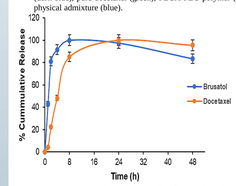

Figure 4: In vitro drug release profile of brusatol- and docetaxel-loaded nanoparticles in PBS at 37 ± 1 °C (n = 3; data represent mean ± SD).

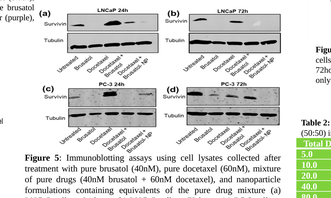

Figure 5: Immunoblotting assays using cell lysates collected after treatment with pure brusatol (40nM), pure docetaxel (60nM), mixture of pure drugs (40nM brusatol + 60nM docetaxel), and nanoparticle formulations containing equivalents of the pure drug mixture (a) LNCaP cells at 24 hours (b) LNCaP cells at 72 hours (c) PC-3 cells at 24 hours (d) PC-3 cells at 72 hours.

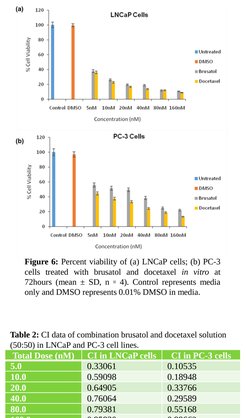

Figure 6: Percent viability of (a) LNCaP cells; (b) PC-3 cells treated with brusatol and docetaxel *in vitro* at 72hours (mean ± SD, n = 4). Control represents media only and DMSO represents 0.01% DMSO in media.

Table 2: CI data of combination brusatol and docetaxel solution (50:50) in LNCaP and PC-3 cell lines.

Total Dose (nM)	CI in LNCaP cells	CI in PC-3 cells
5.0	0.33061	0.10535
10.0	0.59098	0.18948
20.0	0.64905	0.33766
40.0	0.76064	0.29589
80.0	0.79381	0.55168
160.0	0.95830	0.99662

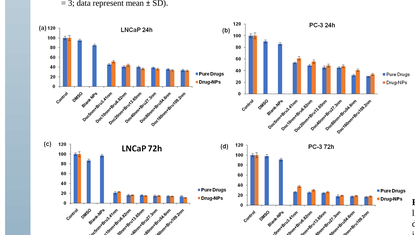

Figure 7: *In vitro* evaluation of cytotoxicity of combination drug solution and docetaxel- and brusatol-loaded nanoparticles containing equivalents of pure drugs (based on drug loading determination) and controls at different concentrations of drugs using the MTS assay. (a) LNCaP cells (24 hours), (b) PC-3 cells (24 hours), (c) LNCaP cells (72 hours), (d) PC-3 cells (72 hours). Data are mean ± SD (n=3).

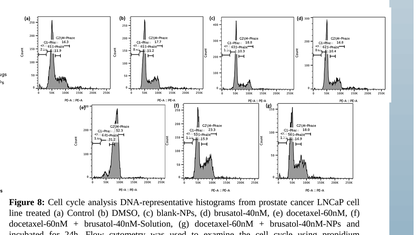

Figure 8: Cell cycle analysis DNA-representative histograms from prostate cancer LNCaP cell line treated (a) Control (b) DMSO, (c) blank-NPs, (d) brusatol-40nM, (e) docetaxel-60nM, (f) docetaxel-60nM + brusatol-40nM-Solution, (g) docetaxel-60nM + brusatol-40nM-NPs and incubated for 24h. Flow cytometry was used to examine the cell cycle using propidium iodide/RNAse staining. (n = 3)

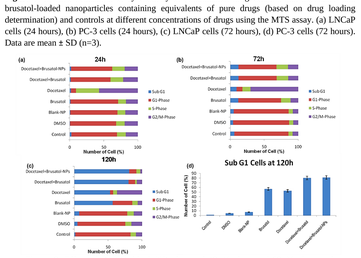

Figure 9: Effect of DMSO, blank-NPs, brusatol-40nM, docetaxel-60nM, docetaxel-60nM + brusatol-40nM-Solution, and docetaxel-60nM + brusatol-40nM-NPs in cell cycle distribution of LNCaP cell line post-treatment (a) 24hr, (b) 72hr, (c) 120hr, (d) showing the population of cells in subG1-phase at 120h post-treatment (n = 3).

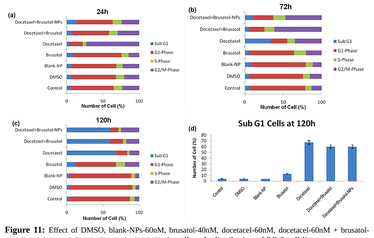

Figure 11: Effect of DMSO, blank-NPs-60nM, brusatol-40nM, docetaxel-60nM, docetaxel-60nM + brusatol-40nM, and docetaxel-60nM + brusatol-40nM-NPs in cell cycle distribution of PC-3 cell line post-treatment (a) 24h, (b) 72h, (c) 120h, (d) showing the population of cells in subG1-phase at 120h post-treatment.

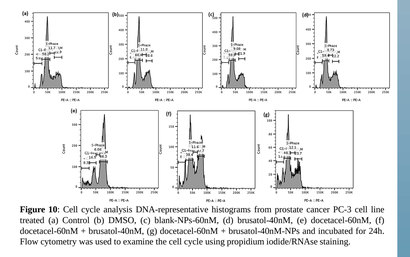

Figure 10: Cell cycle analysis DNA-representative histograms from prostate cancer PC-3 cell line treated (a) Control (b) DMSO, (c) blank-NPs-60nM, (d) brusatol-40nM, (e) docetaxel-60nM, (f) docetaxel-60nM + brusatol-40nM, (g) docetaxel-60nM + brusatol-40nM-NPs and incubated for 24h. Flow cytometry was used to examine the cell cycle using propidium iodide/RNAse staining.

Conclusion and Recommendations

We have successfully fabricated and characterized sub-200nm combination docetaxel- and brusatol-loaded nanoparticles and evaluated its potential for prostate cancer therapy *in vitro* using PC-3 and LNCaP prostate cancer cells.

Formulation and optimization data show that the experimental design is robust and can be used to accurately predict particle size.

Biological experiments using model cell cultures reveal that the drug combination shows synergistic cytotoxic effects and propose mechanisms responsible for the observed synergistic effects.

Results Contd.

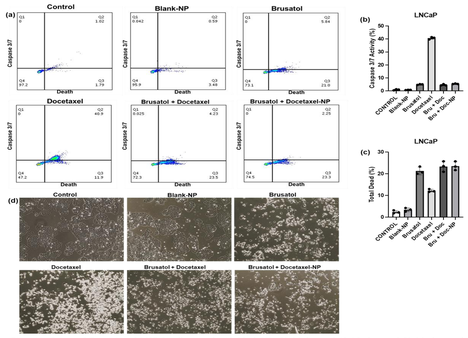

Figure12: Caspase 3/7 activity of LNCaP cells treated with blank-NPs-60nM, brusatol-40nM, docetacel-60nM, docetacel-60nM + brusatol-40nM, and docetacel-60nM + brusatol-40nM-NPs and incubated for 72h. (a) Dot plot of caspase 3/7 activity, (b) Percentage of caspase 3/7 activity, (c) Percentage of total death, (d) Phase contrast of cell morphology.

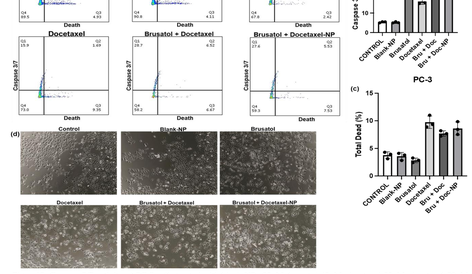

Figure13: Caspase 3/7 activity of PC-3 cells treated with blank-NPs-60nM, brusatol-40nM, docetacel-60nM, docetacel-60nM + brusatol-40nM-Solution, and docetacel-60nM + brusatol-40nM-NPs and incubated for 72h. (a) Dot plot of caspase 3/7 activity, (b) Percentage of caspase 3/7 activity, (c) Percentage of total death, (d) Phase contrast of cell morphology.

Conclusion and Recommendations Contd.

A biphasic cell cycle arrest profile is expected to prevent proliferation of cancer cells more efficiently and may contribute to the synergy observed with the cytotoxic agents.

Caspase 3/7 and immunoblotting results could be a significant finding, as it suggests that the nanoparticle formulation may be effective in reducing the survival of cancer cells and synergistically enhance the efficacy of docetaxel chemotherapy.

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