The Use of Styrene Maleic Acid Nanomicelles Encapsulating the Synthetic Cannabinoid Analog WIN55,212-2 for the Treatment of Cancer

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Abstract. *Synthetic cannabinoid WIN55,212-2 (WIN) has shown a promise as an anticancer agent but causes psychoactive side-effects. In the present study, nano-micelles of styrene maleic acid (SMA)-conjugated WIN were synthesized to reduce side-effects and increase drug efficacy. SMA–WIN micelles were characterised and their in vitro cytotoxic effect was compared to that of free WIN against triple-negative breast cancer (MDA-MB-231), hormone receptor-positive breast cancer (MCF-7) and castration-resistant prostate cancer (PC3) cell lines. SMA–WIN micelles were synthesised with a ~15% loading, 132.7 nm average diameter, −0.0388 mV charge, and pH-dependent release rate. A dose-dependent inhibition of cell growth was observed in all three cell lines treated with both free and micellar WIN, with both formulations demonstrating equal cytotoxicity. Conclusion: SMA–WIN demonstrated characteristics theorized to improve in vivo drug biodistribution. Potent cytotoxicity was found against breast and prostate cancer cells in vitro, showing promise as a novel treatment against breast and prostate cancer.*

Cancer is among the leading causes of death worldwide. Breast cancer is the most common cancer in women, contributing to 25% of all cancers diagnosed in 2012 (1), while prostate cancer is the second most common cancer in men, accounting for 15% of cancer diagnosed in men in 2012 (2). Triple-negative breast cancer (TNBC) accounts for approximately 15-20% of all breast cancer (3), and is associated with a younger age of disease onset, larger tumor size, increased lymph node positivity, high risk of metastasis, and decreased overall survival. This particular subtype of breast cancer lacks all three receptors [oestrogen (ER), progesterone (PR) and human epidermal growth factor 2 (Her2/neu)] exploited by currently available hormonal and targeted therapies (4). Similarly in the prostate, the androgen receptor mediates cell growth. Androgen-independent prostate cancer does not respond to androgen-deprivation therapy. Hence, chemotherapy remains the mainstay of treatment for these hormone-resistant cancer types (5).

Traditional chemotherapeutic agents exert their anticancer effects *via* inhibiting the growth of rapidly dividing cells. The non-specific nature of these drugs means that they also affect the growth of normal rapidly-dividing cells, such as those found in the bone marrow and the gastrointestinal mucosa, resulting in side-effects such as myelosuppression, hair loss, nausea and vomiting (6). Therefore, a safer and more targeted approach for the treatment of TNBC is desperately needed.

Cannabinoid is a term given to a class of chemical compounds that act on cannabinoid receptors and includes the endogenous cannabinoids anandamide and 2-arachidonyl glycerol (7). Cannabinoids potentially have anticancer effects in models of breast, prostate, and pancreatic cancer, glioma, and lymphoma (8-10). The broad efficacy of cannabinoids is paired with the observation that the cannabinoid receptors CB1 and CB2 show increased expression in some types of tumor cells when compared to normal tissue, implying a selectivity of the drug for these cancer types (9, 10).

Although the medicinal use of cannabis and its associated compounds have been known since antiquity, its place in modern medicine has remained controversial, due in part to the psychoactive effects of cannabinoids, mediated by CB1 receptors found in the central nervous system (CNS) (11).

The incorporation of cannabinoids into a micellar system is a strategy to improve drug solubility, and reduce CNS side-effects by increasing the drug diameter sufficiently such that it is unable to pass through the blood–brain barrier (12) to act on central CB1 receptors.

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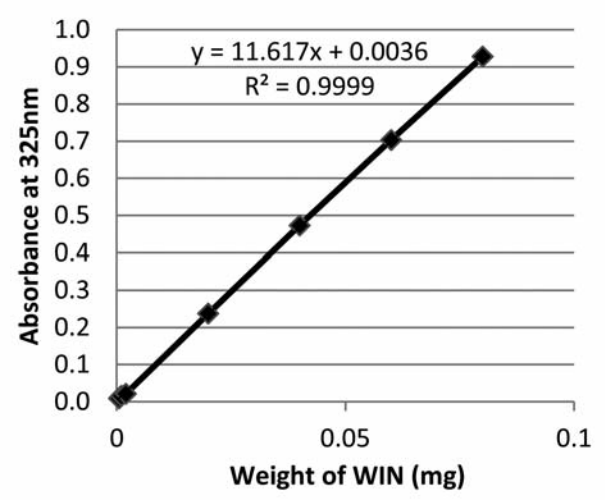

Figure 1. *Standard curve for free WIN55,212-2 (WIN) dissolved in 100% dimethyl sulfoxide.*

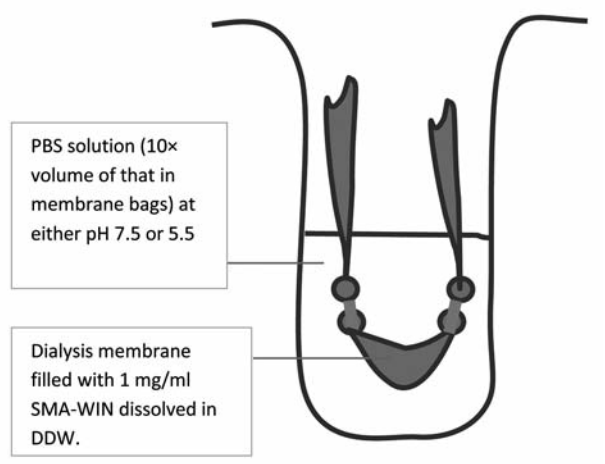

Figure 2. *Experimental setup for determining rate of release of free WIN from SMA–WIN micelles in aqueous solution. SMA-WIN: Styrene maleic acid; DDW: double-distilled water; PBS: phosphate buffered saline.*

Polymer micelles are composed of amphipathic co-polymers self-assembling into spherical nanostructures consisting of a hydrophobic core containing the lipid-soluble drug, and a hydrophilic corona. These macromolecules are unable to traverse through the narrow endothelial junctions of normal blood vessels or the kidney but show enhanced permeability through the defective tumor vasculature. As lymphatic drainage of tumor tissue is also often impaired, the drug is selectively retained in the tumor tissue. Together, these two parameters form the basis of the enhanced permeability and retention (EPR) effect, which allows an increased delivery of drug to the target site while reducing systemic toxicity (13). Clinical studies of micellar constructs of anticancer drugs paclitaxel, cisplatin and epirubicin have demonstrated increased drug efficacy and reduced adverse effects (14).

The synthetic cannabinoid WIN-55,212-2 (WIN) has previously been synthesized into a nanomicellar drug with poly(styrene)-co-maleic anhydride (SMA) (15), although its toxicity towards cancer cells has not been examined. Hence, with this preliminary background, this study aimed to replicate the synthesis and characterisation of SMA–WIN micelles and compare the cytotoxicity of the micellar drug with that of its free drug counterpart against breast and prostate cancer cells. For this study, MDA-MB-231 cells were used as a model for TNBC, lacking ER, PR and HER2 receptors, while MCF-7 was used as a comparison for ER- and PR-positive breast cancer (16). PC3 prostate cancer cells, with the absence of androgen receptor (17) were used as a model for androgen-independent prostate cancer.

Materials and Methods

Preparation of SMA–WIN. The method for synthesis of SMA–WIN micelles was as previously described by Linsell (15). WIN, hydrolysed SMA solution (10 mg/ml, Mr 1700) and *N*-Ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) were obtained from Sigma-Aldrich, St. Louis, MO, USA. The weight ratios were 1:3 for WIN:SMA and 1:1 for SMA:EDAC. WIN and EDAC were dissolved in 100% dimethyl sulfoxide (DMSO) and double-distilled water, respectively. Hydrolysed SMA was brought to a pH of 5 using HCl under stirring conditions. WIN and EDAC were added simultaneously to the stirring SMA solution, which became cloudy as the pH rose. The pH was returned to and stabilised at pH 5.0, then brought to pH 11 using NaOH. The solution was stirred for a further 25 min. The pH was then subsequently lowered to 7.4 and the solution filtered by means of an Amicon ultrafiltration system (YM-10 membrane, cut-off molecular size 10 kDa; Merck Millipore, Auckland, New Zealand) four times to achieve a solution with 99.99% purity. The solution was frozen at −80˚C for 24 h before being lyophilized.

Recovery. Recovery was calculated as the weight of the micelles after lyophilisation divided by the initial weight of WIN and SMA used, multiplied by 100.

Loading. A standard curve was prepared by serial dilution of WIN in 100% DMSO and drug quantification was carried out using UV/Vis spectrometry at 325 nm. SMA–WIN micelles were disrupted in 100% DMSO and the absorbance measured at 325 nm. The weight of drug was determined from the standard curve (Figure 1). Loading was calculated as the average weight of WIN, divided by total weight of SMA–WIN, multiplied by 100.

Drug release. SMA–WIN micelles were prepared at a concentration of 1 mg/ml in distilled water. Using a dialysis bag with a 12-kDa

Table I. *Cytotoxicity of free WIN55,212-2 (WIN) and styrene maleic acid-WIN55,212-2 (SMA-WIN) on cancer cell lines (expressed as mean±SD of 3 independent repeats). IC_{50}: Half- maximal inhibitory concentration.*

Cell line	Maximum inhibition (10 μM)		IC_{50} (μM)		*p*-Value
	Free WIN	Micellar WIN	Free WIN	Micellar WIN	
MDA-MB-231	90%	88%	3.820±0.163	4.211±0.305	0.913
MCF-7	95%	94%	4.776 ± 0.047	5.866 ± 0.183	0.493
PC3	82%	70%	6.069±0.349	6.342±0.379	0.670

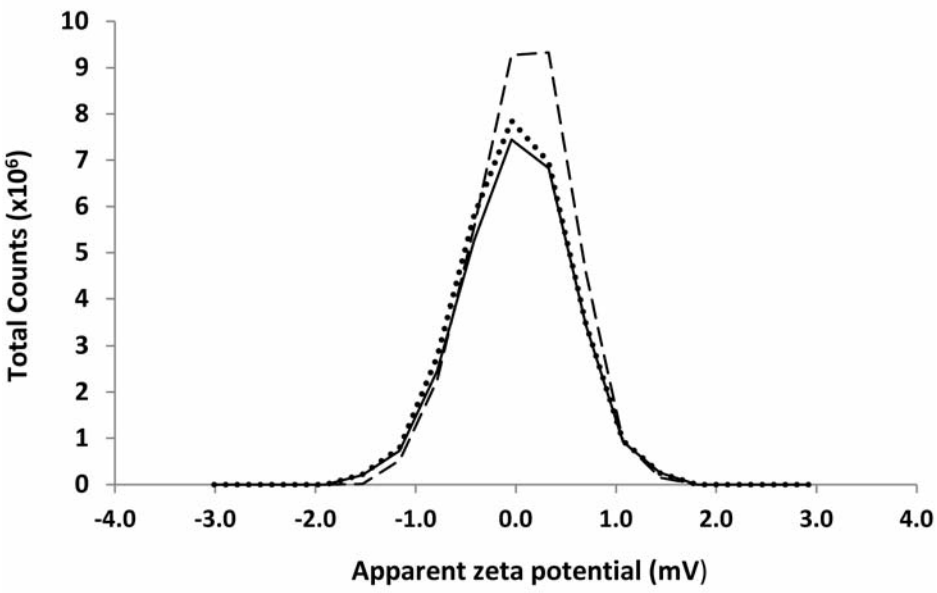

Figure 3. *Charge distribution of SMA–WIN micelles dissolved in DDW as measured by the Malvern Zeta Sizer. Blue, green and red lines correspond to the three replicate runs measuring a minimal negative charge of –0.0391mV, -0.0288 mV and –0.0484mV, respectively.*

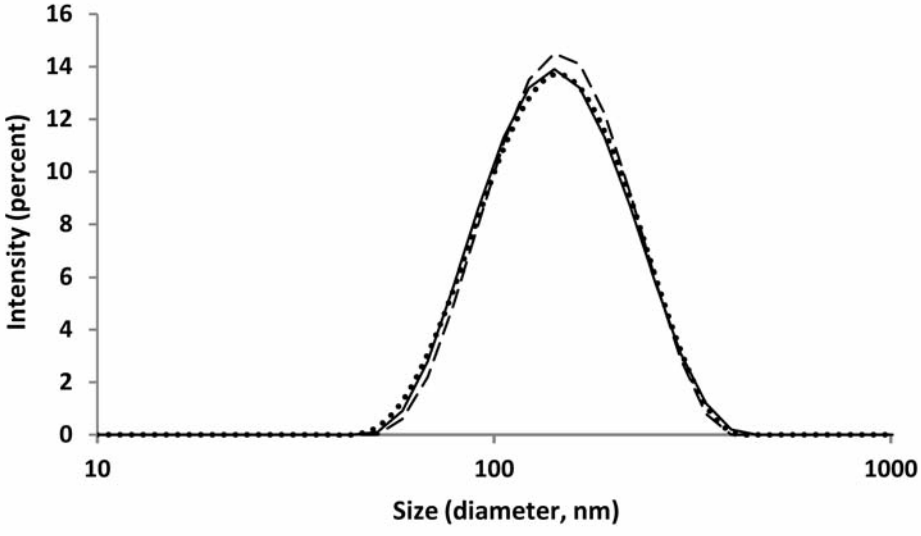

Figure 4. *Size distribution of SMA–WIN micelles dissolved in DDW as measured by the Malvern Zeta Sizer. Red, blue and green lines correspond to the three replicate runs measuring an average diameter of 133.9 nm, 132.0 nm and 132.2 nm respectively.*

molecular weight cut-off, 1.5 ml of SMA–WIN solution was dialyzed against 15 ml of distilled water at pH 7.5 or pH 5.5 (Figure 2). pH 7.5 was chosen to model physiological pH (and hence reflect the release rate in circulation) and pH 5.5 to mimic the acidic environment of lysosomes upon uptake of the micelle (14). At specified time points, 1 ml of sample outside the dialysis bag was removed and the absorbance was measured at 325 nm. The percentage release was determined by the ratio of the absorbance (ABS) of the solution outside the bag at defined time points and that within the bag at t=0

$$\frac{\text{ABS} - \text{ABS (time 0)}}{\text{(stock)}} \times 100$$

Determination of size and charge: Size and charge were determined by Malvern Zeta Sizer ZEN3600 (Malvern instruments Inc., Westborough, MA, USA). SMA–WIN micelles were dissolved in ~8 ml of 0.1 M $NaHCO_3$ or double-distilled water to determine size or charge, respectively. All measurements were repeated in triplicates.

Determination of cytotoxicity. All cell lines were obtained from American Type Culture Collection, Manassas, Virginia, USA and maintained and cultured in Advanced Dulbecco's Modified Eagle Medium (DMEM), 5% foetal bovine serum (from Life-TechnologiesTM, Carlsbad, CA, USA). Cells were seeded into 96-well plates (8000 cells/well for MDA-MB-231 and PC3; 6000 cells/well for MCF-7) and incubated for 24 h before being treated with free WIN or SMA–WIN (at equivalent WIN concentrations), at the concentrations between 0 and 10 μM for 72 h. Treatment were prepared by dissolving free WIN in 100% DMSO and SMA-WIN PBS, and diluting with 0.1% bovine serum albumin. Cell numbers were determined by a sulforhodamine B (SRB) assay (18) at the conclusion of the treatment period. Cytotoxicity was quantified as the fraction of cells surviving relative to that of untreated controls. Experiments were conducted in triplicate with three independent replications.

Analysis. Statistical analysis was conducted using Graphpad Prism®, Version 6.01. (San Diego, CA, USA) A two-tailed *t*-test was performed using Microsoft Excel comparing variations between data points at each concentration of free *versus* micellar WIN. The half maximal inhibitory concentration's (IC_{50}s) are expressed as means±Standard Deviation.

Results

Recovery and loading. Two successful batches of micelles were synthesized. Recovery of 53.8% and 69.5% was achieved for batches 1 and 2, respectively.

Loading of 15.3% and 15.5% was achieved for the first and second batches, respectively.

Charge and size. The average charge of the second batch of micelles was determined to be slightly negative at –0.0388 mV, with peaks at –0.0484 mV, –0.0288 mV and –0.039 mV for the three separate runs though the Malvern Zetasizer (Figure 3).

Three separate runs of the first batch of SMA–WIN micelles through the Malvern Zetasizer displayed a single peak at 155 nm, 154 nm, 153.9 nm, respectively, all with an intensity of 100%. The average micellar diameter was 133.9 nm, 132.0 nm and 132.2 nm respectively, giving an overall average diameter of 132.7 nm (Figure 4).

Release rate. The pH was found to impact upon the release rate of drug. Release was more rapid at pH 7.4 compared to pH 5.5 (~30% *vs*. ~17% , respectively, at 96 h).

Cytotoxicity. A dose-dependent inhibition of cell growth was observed for all three cell lines treated with both free and micellar WIN (Figure 5). The cytotoxic effect of SMA–WIN was similar to that of free drug after 72 h of treatment. A summary of the cytotoxicity results can be found in Table I.

Discussion

Synthesis of water-soluble SMA–WIN micelles was successful, with a loading of 15%. The micelle's average diameter of 132.7 nm exceeds the renal threshold for elimination by the kidneys (19), allowing for prolonged plasma half-life and restricting extravasation from healthy vasculature, while allowing extravasation from tumor vasculature (13). The slightly negative charge of the micelles of –0.0388 mV should prevent non-specific interaction with negatively-charged components of the blood vessels which would shorten the plasma half-life and lead to potential host toxicity (20).

A higher release was observed at the higher pH of 7.5 compared to 5.5, as consistent with the results obtained by Linsell (15). The slow release may not be a major factor in determining whether a sufficient dose of drug reaches the tumor tissue, as the drug can be released outside the cell to exert its effects on cell surface receptors and micelles can be internalized *via* endocytotic pathways and their active components released into the cell cytoplasm (21). The slow release at physiological pH, reaching only 30% after 96 h, indicates that the micelles are stable in aqueous solution and should not rapidly release the drug into the circulation before it reaches the tumor site. This should minimize systemic side-effects while maximizing the amount of drug that reaches the tumor site.

A dose-dependent inhibition of cell growth was found for all cell lines when treated with free and micellar WIN, as consistent with other studies (8-10). At least 70% cell growth inhibition was demonstrated by both free and micellar drug at 10 μM, proving the cytotoxic efficacy of both drug formulations. Statistical analysis indicated that SMA–WIN was as potent as free WIN against MDA-MB-231, MCF-7 and PC3 cells. This indicates that the current formulation,

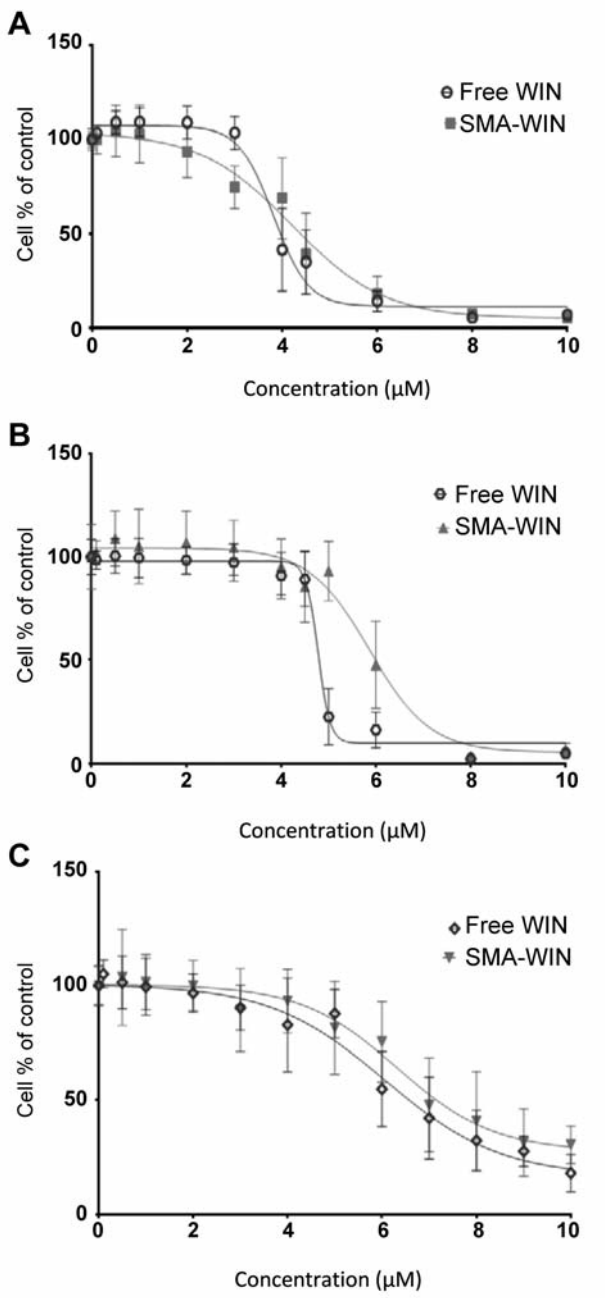

Figure 5. *Dose–response curve of free WIN and SMA-WIN for MDA-MB-231 (A), MCF-7 (B) and PC3 (C) cell lines as measured by Sulforhodamine B assay. The difference in cytotoxicity between free and miceller Win were not statistically significant.*

pursued to prevent unwanted side-effects, does not reduce the potency of the drug. Furthermore, *in vivo*, the micellar drug may become more efficacious than the free drug due to favourably altered biodistribution as a result of the EPR effect (13).

This study showed that the nano-micellar SMA–WIN exhibits similar potency to the free drug equivalent *in vitro*. These results are consistent with previous studies comparing free drug *versus* micellar drug *in vitro* models of cancer (22-26).

These results suggest that SMA–WIN is a promising option for the treatment of breast and prostate cancer. This is of particular relevance for the treatment of TNBC, which at present does not have a targeted- treatment option. The incorporation of synthetic cannabinoid WIN into a micellar formulation successfully transforms the drug characteristics such that its efficacy may be improved through utilizing the EPR effect and its systemic and CNS side-effects be mitigated.

Despite the psychoactive effects of cannabinoids, cannabinoid-based medicines have been shown to have minimal toxicity in various clinical trials (27-29).

Notably, the safety of Δ9-tetrahydrocannabinol on patients with recurrent glioblastoma multiforme was confirmed in a pilot clinical trial (30).

Acute fatal cases attributable to cannabis use in humans have not been substantiated, and the potential for lethal overdose is low (31). Furthermore, cannabinoids possess additional benefits as palliative treatment for cancer-related side effects such as pain, cachexia, nausea and vomiting (32).

Hence, these favourable characteristics of cannabinoids, coupled with potent antiproliferative effects and improved micellar drug formulation, as demonstrated by this study, provide an attractive prospect for SMA–WIN55,212-2 as an anticancer treatment.

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