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Targeted Delivery of 5-FU-loaded Quantum Dots Based System into Cancer Induced Mice using Receptor-based Chemistry; Effect on Adaptive Immune Responses

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Abstract

Adequate understanding of the basic chemistry involved in the etiology of cancer has unravelled the doggedness of cancer cells and paved ways for new strategies in cancer therapy and diagnosis. In response to this doggedness, cancer cellular interaction, and associated signaling components are recently utilized as a key underlining components for effective targeted cancer therapy. Anticancer drugs such as 5-Fluorouracil (5-FU), despite its anticancer property, are characterized to have some limitations associated to drug resistance, and still remains a significant limitation to it clinical use. The folate expressing potential of cancer cells leads to the development of folate affinity compounds in this study to aid in enhancing the uptake of cytotoxic drugs such as 5-FU and ameliorate its possible resistance/or toxicity index. To this end, Folate-receptor based chemistry was proposed in this work to effectively deliver a cytotoxic drug (5-FU) in to breast cancer cells *in vivo*. The 5-FU drug was loaded onto Mn-ZnS quantum dots (QDs) encapsulated with chitosan (CS) biopolymer and conjugated with folate receptor targeting ligands, folic acid (FA) following wet chemical method. Based on the results presented in this study, the immunophenotyping profiles (CD3, CD4, CD8 and NK-T) in the splenic tissues of cancer induced Balb/c mice were observed to have increased significantly ($p<0.05$) in the groups treated with the folate based nanocomposites compared to the events recorded in the groups treated with the free 5-FU anticancer drugs. The results further shows that, the as-synthesized 5-FU@FACS-Mn:ZnS nanocomposite demonstrated an increase in the population of cytokines (IL-2 and IFN- γ) when compared to the pattern observed for the groups treated free 5-FU. However, a different phenomenon was observed in the activity of IL-Iβ cells, showing a decrease in the biomarker population in the groups treated with 5-FU@FACS-Mn:ZnS when compared to free 5-FU treated groups. The result suggest that, FA-FRs mediatory role enhances the tumor uptake of the 5-FU-folate conjugate thereby promoting the stimulation of tumor defense mechanism and same time suppressing the possible toxic effect from the 5-FU anticancer drugs.

Keywords: Cancer; Cytokine; Immunophenotyping; Folic acid; Folate Receptors; 5-Fluororaucil,

Introduction

Broadly speaking, the animal body could be defined as a perfect system, with each component units harmoniously exchanging its vital chemistry at an anatomical level (Ong *et al.*, 2018). In this perfect form, the cells are well coordinated, guided by intra/extra cellular biological processes that allows the cells to undergo phase transition and clone self (Greaves and Maley, 2012; Hanahan and Weinberg, 2011; Ong., 2018). However, eventual disruption of these transitions could alter the molecular-chemistry, disrupt the normal function of the immune systems and hence the entire molecular structure of the cells, leading to mutant cells generation. (Hanahan and Weinberg, 2011; Ong., 2018; Weinberg, 2013).

Though, number of cytotoxic drugs were reported to slow down cancer progression, research also reported series of toxic induced effect associated with the use and application of chemo-drugs (Bwatanglang *et al.*, 2017; Bwatanglang., 2016a; Bwatanglang *et al.*, 2016b). In order to ameliorate the nonselective toxic effect of cytotoxic cancer drugs, we captured in our previous study, the beauty of folate receptor based chemistry to selectively deliver 5-FU anticancer drugs into breast cancer cells *in vitro* and *in vivo*. The study was conducted to subtly deliver toxic anticancer (5-FU) drugs selectively into cancer cells without hampering or causing injury to neighbouring healthy normal cells. The targeting ligands used in the study is a B-vitamin (folic acid) needed by the fast dividing tumor cells for the replication and synthesis of purine and thymidine. The folic acid has a strong binding affinity towards cysteine-rich cell-surface glycoproteins (folate receptors) widely expressed by cancerous cells compared to normal healthy cells (Bwatanglang *et al.*, 2017; Bwatanglang *et al.*, 2016a).

In the study, based on the in vitro study, the incorporated targeting ligands, folic acid (FA) in the drug formulations were observed to enhance the therapeutic efficacy and selectivity of the anticancer (5-FU) drugs to the folate overexpressing breast cancer cells (Bwatanglang, *et al.*, 2016b). The Folic-folate receptor (FA-FRs) based chemistry that was used to selectively deliver the anticancer drugs were further brought into light

in our *in vivo* study using breast cancer challenge mice. In the *in vivo* study, the tumor inhibitory effect of the FA-drug loaded nanocomposite were observed to have augmented the therapeutic efficacy of the 5-FU anti-cancer drug in the targeted formulation over the non-targeted free drug (5-FU) (Bwatanglang *et al.,* 2016a). From the outcome of the histological examination, the subjects receiving the targeted drug loaded nanocomposite (5-FU@FACS-Mn:ZnS) exerted intense remission in metastasis of the selected organs compared to non-targeted drug (Bwatanglang et al., 2017). These findings further reaffirmed the role of FA-FRs chemistry towards enhancing the tumor cellular uptake of the folate-5-FU, thus inhibited the proliferating propensity of the 4T1 cancer cells.

One striking properties of cancer cells is the overwhelming effects of pro-inflammatory component, which according to some findings and depending on the individual physiochemistry and susceptibility are enhanced by most cytotoxic drugs such as 5-FU and other endogenous substances (Arias, 2008a; Longley *et al.,* 2003). These chemistry with the help of some associated white blood cells (leukocytes, lymphocytes and macrophages), promotes the local synthesis of growth factors, growth hormones and associated small proteins like cytokines [(Haltiwanger, 2010; Lu *et al.,* 2012; Multhaupt 2016; Oskarsson, 2013; Reichardt and Tomaselli, 1991; Xiong and Xu, 2016), to either act as pro-cancer associates *et al.*, or generate further active ingredients to fight cancer proliferation *(Kubota et al.,* 1999; Oft, 2014). Therefore, in this present study, we investigate the immunophenotyping profiles (CD3, CD4, CD8 and NK-T) in the splenic tissues of cancer induced Balb/c mice and same time investigated the activity of some selected cytokines, interleukin 2 (IL-2, IL-1β) and interferon-γ (IFN-γ), towards supporting or suppressing cancer growth and proliferation *in vivo*. The study was intended to investigate the efficacy of the folate-based formulation (5-FU@FACS-Mn:ZnS) with that of the pure or free cytotoxic anticancer (5-FU) drugs on the activity of the small proteins and arsenal T-cells components respectively.

Material and Methods

Synthesis of 5-FU loaded FACS-Mn:ZnS

The detailed procedure for the preparation of 5-FU@FACS-Mn:ZnS nanocomposite was presented in our previous study (Bwatanglang *et al.,* 2016b). Briefly, the composite was synthesized by first preparing a stable FACS-Mn:ZnS nanocomposite by reacting FACS solution with the colloidal solution of Mn:ZnS synthesized by adding 1.5 mL of $MnSO_4$ to 10 mL of $Zn(CH_3COO)_2$ solution under ultrasonication followed by drop-wise addition of Na_2S solution. Finally, the 5-FU@FACS-Mn:ZnS composite was prepared by dispersing the 5-FU anti-cancer drug in methanol to aqueous colloidal solution of FACS-Mn:ZnS under sonication followed by stirring at 4°C for 8 h. The 5-FU@FACS-Mn:ZnS composite was collated by centrifugation at 4000 rpm for 10 min.

Animal study and treatment

A total of twenty (20) female Balb/c mice aged between 5-6 weeks old and weighing between 18-20 g obtained from Universiti Putra Malaysia (UPM) animal resource unit were used for this study. The mice were acclimatized for two weeks prior to the commencement of the experiment. The experiment was conducted in accordance with the regulations set by the UPM animal ethics committee's guidelines for the care of laboratory animals (IACUC approval RO53/2015).

The mice in the untreated and the respective treatment groups were first inoculated subcutaneously on the upper portion of the right hind thigh with 1×10^5 of 4TI cell lines in RPMI media under anaesthesia and randomly separated and distributed into 4 groups of 5 mice each. Group I received 100 ml/kg of PBS, group II and III 4.8 mg/kg of Mn:ZnS, and 5-FU respectively. While 8 mg/kg of 5-FU@FACS-Mn:ZnS was administered to group IV mice via intraperitonial injection. The treatment was conducted thrice/week for four cycle. At the end of the treatment days, after sacrificing the mice by terminal bleeding under anaesthesia, the blood were collected and spleen harvested for further assessment.

Serum Detection of IL-2, IFN-γ and IL-1β Cytokines

To measure the levels of cytokines expression of the treated mice; interleukin 2 (IL-2, IL-1β) and interferon-γ (IFN-γ), were analyzed using enzyme-linked immunosorbent assay kits (R and D Systems, USA) (Abu *et al.,* 2015). Briefly, 96-well plates were coated with the designated capture anti-bodies and left overnight in 4°C. The following day, the plates were washed three times with PBS-0.5% Tween 20 and the serums obtained from the mice were incubated in the 96-well plates for 2 hours on a rocking platform at room temperature. Following this procedure, subsequent washing of the plates was conducted thrice. After the washing, the plates were then incubated with the detection antibody for 2 hours on a rocking platform at room temperature. At the end of the incubation time, the plates were washed three times and then incubated in streptavidin–HRP for 30 minutes. Further washing of the plates was repeated four times, follow by loading of 3,3′,5,5′-tetramethylbenzidine substrate into the plates. One nundred (100) µL stop solution were added to the plates as soon as

the color developed and the reading were measure using the μQuant™ microplate reader (Bio-Tek Instruments, Winooska,VT,USA).

In vivo Immunophenotyping of Splenocytes

The immunophenotyping profiles of CD3, CD4, CD8 and NK-T in the splenic tissues harvested were determined by meshing the harvested spleen with a 70 μm strainer. The single-cell suspensions were washed twice with ice-cold PBS and centrifuged at 2000 xg for 5 min. The red blood cells were then removed by incubating the splenocytes in lysis buffer (8 g NH_4Cl, 1 g Na_2HCO_3, EDTA, 0.1 g KH_2PO_4 at pH 7.4) for 15 minutes at 4°C. After that, the cells were further washed again with PBS twice and then stained with appropriate fluorochrome-conjugated antibodies (Abcam, USA) for 2 hours. Later, the cells are washed with PBS and fixed in 4% paraformaldehyde. A week later, the stained cells were then run through the FACSCalibur™ flow cytometry (Becton Dickinson, USA) (Abu *et al.,* 2015).

Statistical analysis

The data was expressed as Mean ± SD of three individual experiments and the statistical analysis was conducted using Graph pad-prism software (version 6.0). One-way ANOVA and the student t-test were performed with the significance set at $p < 0.05$, highly significance set at $p < 0.01$, and very highly significance as $p < 0.001$.

Results & Discussion

Up to date, cancer therapy using antineoplastic drugs assumed a quantitative success rather than qualitative due to its non-specific killing spree on both normal and cancerous cells (Mišković et al., 2013). From the pharmacokinetics study reported by some researchers (Chen and Li, 2006; Duan *et al.,* 2012; Guimarães *et al.,* 2015; Ma *et al.,* 2014), the administration of free anti-cancer drugs lacks the inherent ability to selectively target only the cancer cells and has the propensity to exerts strong side effects on healthy tissues. In addition, free cytotoxic drugs suffer premature clearance from circulation owing to their low molecular weight and precipitate readily in aqueous media. Furthermore, as a results of possible degradation *in vivo*, patients are usually subjected to schedule administrations to meets the required therapeutic dosage which more or less could induces drug resistance (Longley *et al.,* 2003).

To deal with the aforementioned limitations in the use of cytotoxic drugs for cancer therapy, scientist over the years proactively combine the basic fundamental properties of nanomaterials and biomolecules with the existing anti-cancer drug to conveniently administer chemotherapeutics with certain degree of efficiency (Bharali and Mousa, 2010; Dhankhar *et al.,* 2010; Wang and Thanou, 2010). The incorporation of engineered nanomaterials in the management of cancer has significantly extended research in clinical oncology as most formulation demonstrated sufficient solubility in aqueous media, encouragingly reduces the associated adverse cytotoxic effects of the free anti-cancer drugs and interestingly allows systemic drug release and accumulation to be achieved (Guimarães *et al.,* 2015; Kim *et al.,* 2010; Ma *et al.,* 2014; Wang and Thanou, 2010; Yuan *et al.,* 2010). For example, the administration of free 5-FU induces some associated side effects ranging from neural to hematological and gastrointestinal disorders. Other side effects reported are myelosuppression and dermatological adverse side effects (Longley *et al.,* 2003), but of greater concern is the development of resistance by the tumor cells toward 5-FU showing only ~10% response rate for the treatment of colorectal cancer with slight improvement to about ~45% response rate in combination form with other anti-cancer drugs (Arias, 2008b). And similarly, report indicated that prolonged exposure to 5-FU induces thymidine synthase overexpression, that ends up inducing 5-FU resistance in cancer cells; thus limiting its application in cancer treatment (Vinod *et al.,* 2013). Therefore, one-way to overcome the associated tumor-resistance to 5-FU is by exploring targeted delivery approaches. Engineered nanoparticles (NPs) with selective targeting characteristics are reported to be an effective approach towards improving the anti-cancer properties and selective bioavailability of 5-FU (Blanco *et al.,* 2011; Ma *et al.,* 2014; Ngernyuang *et al.,* 2016).

Cancer cells are characterized by deficit in antigen-specific immunity and intratumoral CD8+ T-cells. Based on this findings, recent advance in oncology reported that the expression of the arsenal T-cells agents and anti-inflammatory cytokines plays a key role in the fight against cancer cells; acting as a stimulator of anti-tumor immunity agents and inhibitor of tumor associated inflammation (Oft, 2014). Some anti-cancer drugs such as 5-FU were observed to promote the activation of immune effectors, sensitizing tumor cells to T-cells dependent cytotoxicity and same time help towards reducing the levels of immunosuppressive-derived cells from invading the immunity responsive cells (Ghiringhelli and Apetoh, 2015). Consequences to this actions, same 5-FU was reported having the potential to trigger the activation of suppressor cells thereby favoring tumor progressions (Bruchard *et al.,* 2013). Thus, Fig-1 relates the activities of some selected effectors cells toward cancer chemotherapy. As could be seen on the Figure the 5-FU@FACS-Mn:ZnS increased the

populations of T-cells (commonly called T-helper cells) markers and NK1.1 (commonly called natural killer cells) populations. The percentage of CD3+/CD4+, CD3+/CD8+ T-cells and that of NK 1.1/CD3+ were observed to have increase in the groups treated with 5-FU@FACS-Mn:ZnS by 16.72 ± 0.22 %, 14.49 ± 0.11% T-cells and 3.54 ± 0.01% NK respectively when compared to those treated with the free 5-FU (11.11 ± 0.3, 10.22 ± 0.27 and 2.43 ± 0.10 %) respectively in that order. When compared to the untreated groups, the administration of 5-FU was observed to have suppressed the expression of the T-cells (CD3+/CD4+, CD3+/CD8+). The 5-FU may have introduced toxic competent into the mice. However, due to the high binding affinity of FA to the FRs expressed on the cancer cells, the folate containing 5-FU drug composite (5-FU@FACS-Mn:ZnS) was observed to enhance the expression of these arsenal T-cells. These T-cells and the NK cells were reported to be associated to process elimination of cancer cells by the lysing tumor cells (Abu *et al.*, 2015). The result suggest that, FA-FRs mediatory role enhances the tumor uptake of the 5-FU-folate conjugate thereby promoting the stimulation of tumor defense mechanism and same time suppressing the possible toxic effect from the 5-FU anticancer drugs.

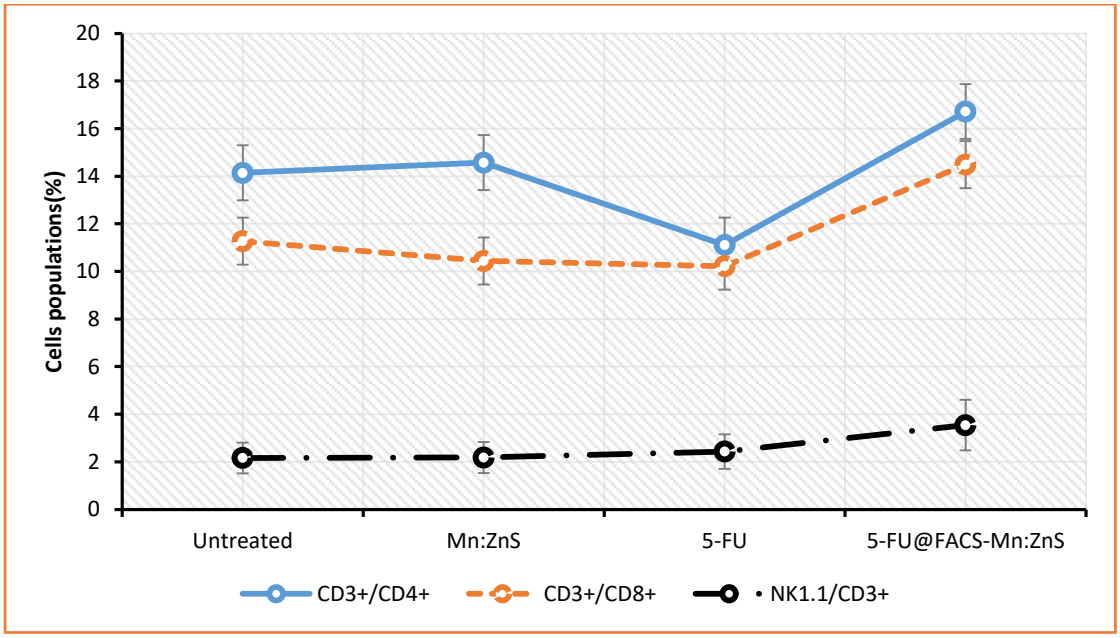

Figure 1: *In vivo* Immunophenotyping of splenocytes of 4T1 induced mice treated with the QDs, pure 5-FU anti-cancer drugs and the 5-FU@FACS-Mn:ZnS nanocomposites. The value represent mean ± SD. *Significant was set at $p< 0.05$ by comparing the treated groups to the untreated group.

Another immune-regulators that plays a crucial role in cancer chemotherapy are cytokines (Kubota *et al.*, 1999). Cytokines such as IL-2 interplay to enhance the activation of T-cells actors, signaling the mechanistic response of interferon (IFN- γ) to regulate the cytotoxic activity of T-cells such as CD8 and NK cells to act aggressively toward inhibiting cancer progression (metastasis) (Kubota *et al.*, 1999; Zaidi and Merlino, 2011). In Fig-2, the results shows that, the as-synthesized 5-FU@FACS-Mn:ZnS nanocomposite demonstrated an increase in the population of IL-2 (897.25 ± 11.45 %), IFN- γ (1050.27 ± 26.53 %) when compared to the pattern observed for the groups treated free 5-FU showing IL-2 (823.41 ± 36.38 %), IFN- γ (926.67 ± 20.53 %). However, a different phenomenon was observed in the activity of IL-Iβ cells, showing a decrease in the biomarker population in the groups treated with 5-FU@FACS-Mn:ZnS (654.41 ± 11.84 %) when compared to free 5-FU treated groups (739.33 ± 12.90). Recently, it was reported that 5-FU treatment could trigger the release of IL-Iβ from the immunosuppressive-derived suppressor cells which were observed to contribute in facilitating tumor progression. The activity of this cytokines where observed to be involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (Bruchard *et al.*, 2013). Thus, the reduction in the level of the IL-Iβ in groups treated with the 5-FU@FACS-Mn:ZnS could be a way of suppressing possible tumor progression (Abu *et al.*, 2015). The overall results suggest that, despite the responsive action of the free 5-FU anti-cancer drugs toward regulating the immune markers when compared to the untreated groups, the significant activity levels recorded in the groups treated with the 5-FU@FACS-Mn:ZnS could be conferred to the mediatory role the conjugated FA played in pumping in more of the

drug loaded conjugated at a rate far greater to the pure 5-FU uptake; thus increasing the responsive rate of the immune markers by raising their activity levels significantly when compared to the pure anti-cancer drugs.

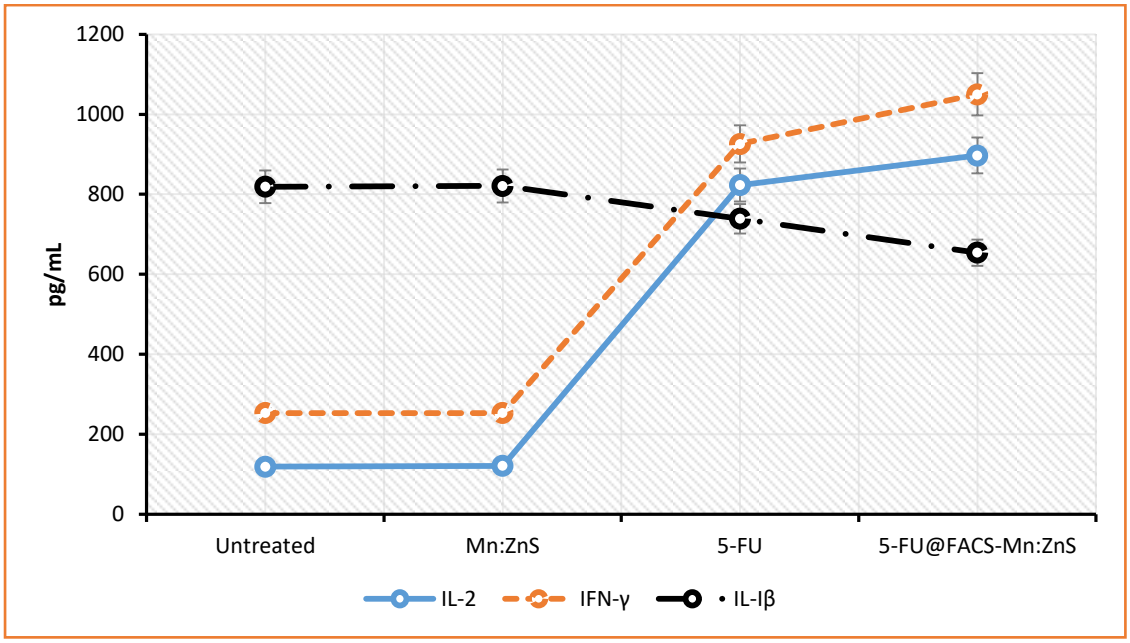

Figure 2: *In vivo* efficacy of the Mn:ZnS, the pure 5-FU anti-cancer drugs and the 5-FU@FACS-Mn:ZnS nanocomposite in the activity levels of IL-2, IFN-γ, and IL-Iβ cytokines in 4T1 induced mice. The value represent mean ± SD. *Significant was set at $p< 0.05$ by comparing the treated groups to the untreated group.

It will suffice to say that the folate based nanocomposite used in this study was able to enhanced the efficacy of the 5-FU in regulating the immune system. As described therein, the IL-2 is necessary for T-cells to develop into T helper cells and T cytotoxic cells. Thus, the observed increase in the IL-2 enhances the immune system of the cancer challenged mice by supporting the T-cell activity. Furthermore, the expression of IL-2 work hand-in-hand IFN-γ, toward regulating activity of CD8/CD3 T-cells (Abu *et al.*, 2016)] .Thus, the activation of the cytolytic CD8 T-cells and NK cells in addition to the expression of cytokines (IL-2 and IFN-γ) may actively contributed to inhibition of cancer cells in as observed in this study.

Conclusion

In conclusion, this site-specific delivery approach used in this study was observed to have reduced the systemic toxicity index of the cytotoxic drugs (5-FU) and hence improves its therapeutic efficacy. The combination of the CS-QDs based system with the specific cell targeting capability of FA, leads to the enhanced cellular uptake by the tumor and prolonged its bioavailability *in vivo*. These results further suggest that, the synthesized 5-FU@FACS-Mn:ZnS nanocomposite can be used for tumor cell-selective and non-invasive targeted therapy

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