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The Cooperative Anticancer Effect of Dual Styrenemaleic Acid Nano-Miceller System against Pancreatic Cancer

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Abstract

Pancreatic tumors remain one of the most formidable cancer types to treat. Patients with locally advanced or metastatic disease, which collectively represent over 80% of patients, rarely survive beyond one year. Pancreatic cancer is especially difficult to treat because of the uniqueness of its histology, with few vasculatures that can be used to supply anticancer agents. In this work we test a new system made of two styrene maleic acid (SMA) Nanomiceles encapsulating the phototosensetizer Zinc Protoporphyrin (ZnPP) or anticancer agent 4'-O-tetrahydropyranyl-doxorubicin (THP/Pirarubicin). Our hypothesis is that, local inflammatory response in tumor vessels induced by photosensitization can improve anticancer drug delivery and hence the anticancer activity. Two pancreatic tumor cell lines (Panc-1 and ASPC-1) were used to test the synergism of cytotoxic effect vitro. PANC-1 animal model in SCID mice were utilized to test the anticancer effect and the safety of the dual system *in vivo*. The photosensitizer activity of ZnPP nanomicelles followed by light irradiation and the administration of the anticancer micelles resulted in a synergetic antitumor effect in SCID mice *in vivo* but not *in vitro*. In conclusion, the dual Nanosystem of a photosensitizer and anticancer agent were well tolerated in animal model of pancreatic cancer and resulted in a synergistic anticancer effect. To our knowledge this is the first report of using a dual nanosystem of a photosensitizer and anticancer agent to treat pancreatic cancer.

Introduction

Pancreatic cancer has the worst mortality rate and the lowest overall survival in all cancers. Only 10% of patients are suitable for potentially curative surgery [1]. Even among those qualified for surgery, aggressive metastasis often occurs after surgery, which is highly resistant to chemotherapy and radiation therapy [2]. Chemotherapy is still the only option in metastatic pancreatic cancer treatment with minimal impact on survival. Gemcitabine (2′-2′-difluorodeoxycytidine) is the standard chemotherapy for all stages of pancreatic cancer. However, neither gemcitabine alone nor gemcitabine-based combinational chemotherapy achieves a favorable outcome in advanced disease [3].

One major limiting factor to the efficacy of anticancer drugs in pancreatic tumors is the presence of dense stromal component, challenging the diffusion and penetration of chemotherapeutic agents to the core of the tumor mass [4]. Further, pancreatic tumors tend to be hypo-vascular compared to other adenocarcinomas [5]. New strategies for treatment of pancreatic treatment are thus, highly needed. In this respect, the collaborative use of two Nano-micelles, each with different cellular target may provide efficacy against these inherently chemo resistant tumors.

We prepared two micelles based on styrene maleic acid (SMA) block copolymer encapsulating either the anthracycline; 4'-O-tetrahydropyranyl-doxorubicin (THP/Pirarubicin) or Zinc Protoporphyrin (ZnPP).

Pirarubicin(THP), a semi-synthetic derivative of doxorubicin, is a reactive oxygen species (ROS) generating agent with significant tumor growth inhibiting properties together with other molecular mechanisms for cell killing [6]. However, being a low molecular weight compound of 627 Da, it readily diffuses across normal as well as tumor blood vessels non-selectively. Its accumulation in the cardiac tissues results in ROS associated cardiac tissue damage. In contrast, Nanosize drug formulations can preferentially leak out of tumor vessels that are hyperpermeable. We have previously demonstrated that micellar formation of SMA copolymer encapsulating a high pay load of pirarubicin, up to 60% w/w [7]. SMA-THP have proven efficacy against various tumor models with excellent safety profile, due to its concentration in tumor tissues of more than 13 fold compared to native drug. ZnPP has inhibitory activity against heat shock protein 32 (HO-1) which is over expressed by pancreatic cancer cells and crucial to their survival [8]. ZnPP inhibits epidermal growth factor tyrosine kinase (EGFR-TK), associated with tumor progression and metastasis [9]. Further, and most relevant to current study approach, ZnPP poses photo sensitizer effect suited for photodynamic therapy (PDT) [10]. To enhance further the targeting capability of SMA-ZnPP micelles, we chemically conjugated glucosamine to the malate group in SMA backbone. Glucose conjugation can enhance tumor cell internalization through receptor-mediated endocytosis utilizing glucose receptor (GLU1), which is overexpressed by pancreatic tumor cells. The current work aims at examining the efficacy and safety of a new collaborative strategy involving dual SMA micelles against pancreatic tumors and if synergistic therapeutic advantages could be achieved by the combination therapy.

Materials and Methods

Materials

SMA-ZnPP and SMA-THP was prepared and kept as lyophilized

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powder as described previously [11]. Samples were dissolved in phosphate buffered saline (PBS) and passed through 0.45 uM filters before usage. Panc-1 and ASPC-1 human pancreatic cell line was kind gift from Inha university hospital, South Korea**.** Both cell lines were cultured in Advanced Roswell Park Memorial Institute (RPMI) 1640 media (Invitrogen, Carlsbad, CA) supplemented with 4 μM-glutamine and 10 % (v/v) fetal bovine serum (FBS).

Preparation of SMA-ZnPP-GLU micelles

To prepare SMA-GLU; 2.2 mol glucosamine and 2 mol sodium methoxide were dissolved in DMSO under completely dry conditions. After 3h the resultant methanol was removed under vacuum at 20°-60°C. Then 1mol SMA was added and the solution stirred for 12h. For dialysis 3.5 KDa cut off membranes were used to remove the unreacted glucosamine. Then SMA-GLU was used at various feeding ratios to obtain SMA- ZnPP-GLU (Figure 1).

To measure the glucosamine content of SMA-ZnPP micelles, the samples (SMA-glucosamine, SMA-ZnPP-glucosamine) were dissolved in water to a final concentration of 1 mg/ml. 25μl of each sample were mixed with 575 μl HCl 2N. Then they were heated in a boiling water bath for 16 h. The samples and references were neutralized with 385 μl Na_2CO_3 and 15 μl Triethylamine to bind the salt of the solution. 500 μl of a 2% acetyl acetone in 1.5M Na_2CO_3 solution were added and the mixture was heated in a boiling water bath for 20 min. The mixture was cooled down and transferred into a more voluminous vial. 1 ml Ethanol and 500 μl Ehrlich's reagent were added. After 15 min the produced color was read at 530nm. Size measurement of the micelles was carried out by Dynamic light scattering (DLS) using Zeta sizer (nano series) from Malvern instruments (Malvern, UK) at a concentration of 1mg/ml.

In vitro anticancer activity

In vitro cytotoxicity of SMA micelles was determined by means of the 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with Panc-1 and ASPC-1 human pancreatic cancers. Cells were plated in 96- well culture plates (3000 cells/well). Cells were then cultured overnight in RPMI medium with 10% fetal calf serum. The cells were then incubated in the presence of different molar concentrations of THP, and ZnPP micelles for 3 days. Cell viability was measured by absorbance at 570 nm with 620 nm as reference using a multi-well plate reader. Toxicity was quantified as the fraction of cells surviving relative to untreated controls.

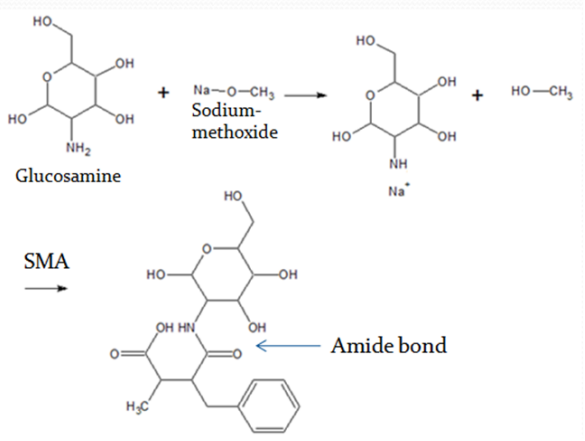

Figure 1: Chemical reaction of glucosamine with SMA by using sodium methoxide to form SMA-GLU miceller backbone. Different feeding ratio of the SMA-GLU was used to form SMA-ZnPP-GLU micelles.

Cell propagation

For establishing xenografts, PANC-1 cells were cultured in RPMI 1640 medium in 3 surface nunclon cell culture flasks (Nunclon, Roskilde, Denmark). Each flask have the yield of 30 x 10^6 Panc-1 cells, a total of 35 flasks were used to propagate enough cells for animal studies.

Light irradiation

Light irradiation was performed utilizing ultraviolet lamp (osram Ultera-Vitalux 300 W-Italy). The lamp emits light wavelength in the range of 270-420 nm. Both cells and animal were exposed to 50.000 Lux for 5 minutes to allow for interaction with SMA-ZnPP micelles. Light intensity was measured at the center of irradiation field by digital illuminometer (DX-200, INS, Taiwan).

Animals

6-8 male ddY mice were used to demonstrate the enhancement of drug delivery following SMA-ZnPP PDT. To test the efficacy and safety of the dual miceller system in pancreatic tumor model, 6 weeks male SCID mice were obtained from Korean research institute of bioscience and technology (KRIBB). Mice were kept in 12 hour light/dark cycles under sterile conditions and were used in accordance with the Institutional Animal Care and Use Committee (IACUC) of Inha University.

Demonstration of nanosize tumor drug enhancement in response to SMA-ZnPP

To test the efficacy of SMA-ZnPP mediated PDT on improving the drug concentration in tumor tissue we used the putative Nanosize Evans blue dye (EBD) in S 180 tumors described as above. EBD was dissolved in DW and administered at 10mg/kg of mice weight in 0.2 ml, as the tumor reaches the diameter of 7 mm. Thirty minutes after injection of SMA-ZnPP animals were irradiated with light as described earlier. At 6 hours following this treatment, Animals were humanly euthanized and the dorsal skin having the tumor was exposed and tumor extracted from the base using surgical scissor, weighed and added to 3 ml formamide. Tumors from animals given EBD (+/-) SMA-ZnPP PDT were harvested similarly. The tumors then were incubated at 60°C water bath with shaking for 48 hours to extract EBD. EBD concentrations were then quantified by absorbance at 620 nm, and reading was converted into μg EBD/mg tissue using standard curve of EBD.

In vivo studies

To demonstrate the enhancement of drug delivery in response to SMA-ZnPP PDT, mouse fibrosarcoma model was used. Briefly; 2 million cells of mouse sarcoma (S-180) were implanted subcutaneously (s.c.) in the dorsal skin of the ddY mice. Animals were followed daily, and when tumors had reached an average diameter of 7 mm with no necrotic areas, treatment commenced. For evaluation of the collaborative anticancer efficacy of the dual miceller system, pancreatic cancer xenografts were established. Briefly, After tumor cell propagation described as above, cells were collected from Nunclon flasks by trypsinization and counted, then concentrated in ice cooled PBS at concentration of 5 million cells per 0.2 ml. Mice were then anesthetized using 4% isoflurane mixed with oxygen, by subcutaneously injecting 5×10^6 Panc-1 cells bilaterally on the flank of each mouse. Tumors were allowed to grow for three weeks to reach average volume of 100

mm^3, then treatment commenced. Groups of five animals each with a total of ten tumors were randomly assigned to the study group. Table 1 shows the design of the study. Light irradiation was carried out for selected groups as described above. Animal were weighed and tumor size was measured with digital calipers biweekly. The estimated tumor volume (V) was calculated using the longitudinal cross section (L) and transverse cross section (W) according to the formula: $V = (L \times W2)/2$ (expressed in mm^3). The day of start of the treatments was designated as day 1. To evaluate the MTD of SMA-THP, 3 mice were injected with doses up to 80 mg/kg with follow up of animal's weight for 2 weeks. The animal study design is shown in Table 1.

Statistical analysis

Differences between means were assessed by student t. test using Microsoft excel software. Data ware expressed as the mean ± SE. Significant differences ware defined as $P < 0.05$.

Results and Discussion

Synthesis and charcterixzartion of SMA-ZnPP-GLU micelles

Tumor cells usually show a higher expression of glucose-transporter-protein (GLUT). GLUT-1 protein was found to be highly expressed in pancreatic cell culture as well as in isolated human pancreatic tumors [12,13]. By modification of SMA with glucosamine, we aimed at targeting this overexpression of transport proteins to increase the uptake of the micelle into the tumor cell. SMA-glucosamine was synthesized as described in the methods. Figure 1 shows SMA-glucosamine chemical reaction. Using this reaction, we successfully attached 27.3 wt. % glucosamine to SMA. As shown in Figure 2, the sizes of the micelle with different drug content and with or without conjugated glucosamine were close. The drug content and the attachment of glucosamine have no or only a very small influence on the size of the micelle. As true for other micelles, the size presented here is related to experimental condition (concentration, pH, and time after preparation), verifying this parameter can result in different micelle conformation and thus different size [14]. Apart from slight difference in molecular size and charge, SMA-glucosamine micelles were similar to plain micelles prepared and described in [15]. The only significant difference between plain SMA-ZnPP and SMA-ZnPP-GLU was the higher release rate of SMA-GLU micelles. SMA-ZnPP and SMA-ZnPP-GLU release rate were 1.3% and 6% per day respectively at buffered phosphate saline with pH 7.0. The modification of SMA with glucosamine could have resulted in decreased stability of the micelle and lead to a higher release rate. A reason for that could be the core/shell ratio change, or the hydrophilic hydrophobic shift towards the hydrophilic side, both factors might have contributed to this improved release profile, however, these remains to be investigated. The current improvement of release profile can prove to be beneficial in delivering the active ZnPP to tumor cells that cannot be reached through the vascular access. Drug released from micelle and diffusing into tumor cells can further the photodynamic effect of ZnPP photosensitization deeper into tumor tissues. This might be even more advantageous with tumor of special nature such as the pancreatic tumors that have limited vascular access.

Treatment	Dose mg/kg	N	Total
Control	NA	5	5
SMA-ZnPP (15%) + light	4,8	5	10
SMA-ZnPP (15%) Glu	4,8	5	10
SMA-ZnPP (15%) Glu + light	4,8	5	10
SMA-THP	10,20	5	10
SMA-THP + SMA-ZnPP + light	4, 10	5	5
SMA-THP + SMA-ZnPP	4, 10	5	5
SMA-THP + SMA-ZnPP (Glu) + light	4,10	5	5
Total 60			

Table 1: Animal study groups.

In vitro activity of the dual SMA miceller system against panceritic tumor cell lines

Panceriatic tumors are knowen for its inherent resistance for chemotherapy [2]. Gemcitabine (2′, 2′-difluorodeoxycytidine) is the standard treatment for patients with advanced pancreatic cancer. However, gemcitabine has shown modest response rates (partial and complete responses) of 6–11% with disease stabilization occurring in a further 19–32% [16,17]. This challenging resistance of pancreatic tumors warranted the proposed combination therapy evaluated in this study. The SMA-ZnPP photosensitizer was used prior to the administration of the chemotherapeutic cytotoxic anthracyclin micelles. As shown in Figure 3, ZnPP micelles showed higher anticancer activity against Panc-1 cells compared to ASPC-1, further , in both cell lines, light irradiation for 5 minutes at 50000 Lux improves the cytotoxicity of micelles. Even though the improvement of cytotoxicity with PDT was 15-20 % over no light irradiation, this limited improvement noticed in this study on tested pancreatic cell lines is reproducible and in accordance with previous studies both *in vitro* and *in vivo* [10,11].

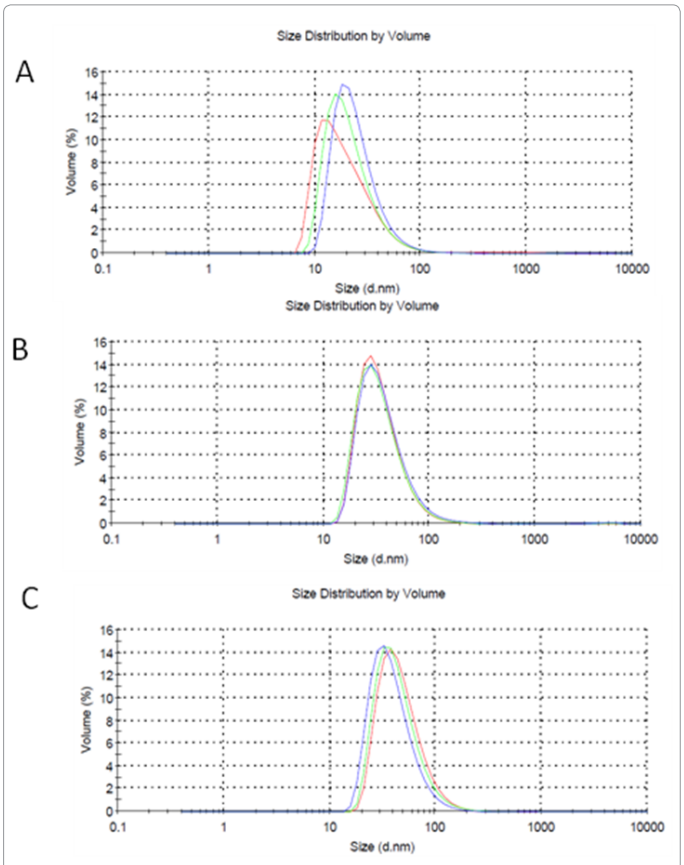

Figure 2: The size distribution of different SMA-ZnPP micelles.Three independent measurements were done for each loading at the concentration of 1 mg/ml in DW within 10 min after dissolution. The average size of A. SMA-ZnPP 15% is 54.6 nm; B. SMA- ZnPP-GLU 15% is 54.7 nm; and C. SMA-ZnPP30% is 62.0 nm.

The presence of anticancer activity in the dark in response to SMA-ZnPP can be attributed to the Heme oxygenase-1 (HO-1) inhibition activity of ZnPP. HO-1 has been repeatedly reported to be overexpressed in human pancreatic tumors especially in response to gemcitabine or radiation [8]. Further HO-1 over expression in animal pancreatic models, were shown to accelerate tumor aggressiveness, by increasing tumor growth, angiogenesis and metastasis in mice. The mere inhibition of HO-1 with SMA-ZnPP thus can account for the significant cytotoxic effect noted in the pancreatic cell lines in the absence of the PDT effect [18]. The enhancement of the cytotoxicity of SMA-ZnPP by light irradiation in both pancreatic cell lines is a further proves of the suitability of ZnPP micelles for Photodynamic therapy as previously demonstrated [11]. Both cell lines show relative resistance against SMA-THP anthracyclein micelle, though comparable to that of gemcitabine. Combination of SMA-THP and SMA-ZnPP micelles did not show added benefit to the efficacy of either of them (data not shown). The reason of this result *in vitro* was expected, as the main purposed mechanism of SMA-ZnPP enhancement was thought to be related to the enhancement of the delivery of the second agent (SMA-THP) through the resultant local inflammation on the tumor vessels that support the growing tumor. With such element lacking from the *in vitro* experimental conditions, verification of our research hypothesis was only possible with the *in vivo* animal experiments. SMA-ZnPP-GLU showed similar cyctoxic activity compared to the plain SMA-ZnPP micelles (data not shown). Possible explanation of such result could be related to the *in vitro* cytotoxicity assay condition where the glucose level in the culture media is several folds of the normal glucose level in mice or human plasma in biological environment. The presence of high glucose level can thus compete with SMA-GLU for the GLUT-1 receptors efficiently *in vitro*. In contrast, *in vivo* tumor environment usually have limited glucose availability, this render targeting tumor cells with this strategy a plausible strategy.

Enhancement of nanomedicine drug delivery after SMA-ZnPP PDT

In photodynamic therapy, typically a photosensitizer is administrated systemically or locally followed by light photoactivation, leading to the generation of cytotoxic reactive oxygen species in the presence of oxygen.

PDT as such has been linked to enhanced tumour vascular permeability [19,20]. In one study, it was found that the concentration of 2000-kDa FITC-dextran were 5-folds higher in orthotropic Mat-Lylu rat prostate tumors treated with vascular-targeting photodynamic therapy verteporfin, at 15min following light irradiation, compared to non-irradiated control group [21]. The permeability enhancement verteporfin photosensitization was attributed to its effect on endothelial cell morphology, and cytoskeleton. Photosensitization was found to causes endothelial cell microtubule depolymerisation and induces the formation of actin stress fibres. Thus endothelial cells were found to retract, leading to the formation of intercellular gaps, which result in enhanced vascular permeability. In addition, endothelial cell damage leads to the establishment of thrombogenic sites within the vessel lumen and this initiates a physiological cascade of responses including platelet aggregation, the release of vasoactive molecules, leukocyte adhesion and increases in vascular permeability [20]. Based on the previous studies, we tested and quantified the enhancement of EBD delivery to S-180 tumors in ddY mice. EBD instantly complex with plasma albumin resulting in a Nanosize molecular complex, which can simulate the concentration of related Nanosize molecules in tumour tissues. As shown in Figure 4, the administration of SMA-ZnPP followed by light irradiation resulted in three folds higher increase in the concentration in EBD in similar tumour volume compared to animals which did not receive prior PDT. In addition, in Figure 4b, the local inflammatory response in vasculature in response to PDT could be clearly noticed.

In vivo anticancer activity of SMA –micelles against Panc-1

Pirarubicin (THP) is a pyranyl derivative of doxorubicin, its mechanism of action involves the generation of reactive oxygen species. THP is characterized by faster intracellular uptake and more potent antitumor activity than doxorubicin *in vivo* due to its pyranyl group and lipophilic properties. However, it exerts poor tumor targeting properties as is common to other low molecular weight anticancer agents. Once SMA-THP micelles reach the target tumor tissue, the micelles will release free pirarubicin slowly in a sustained manner in the vicinity of tumor cells that can even further into the

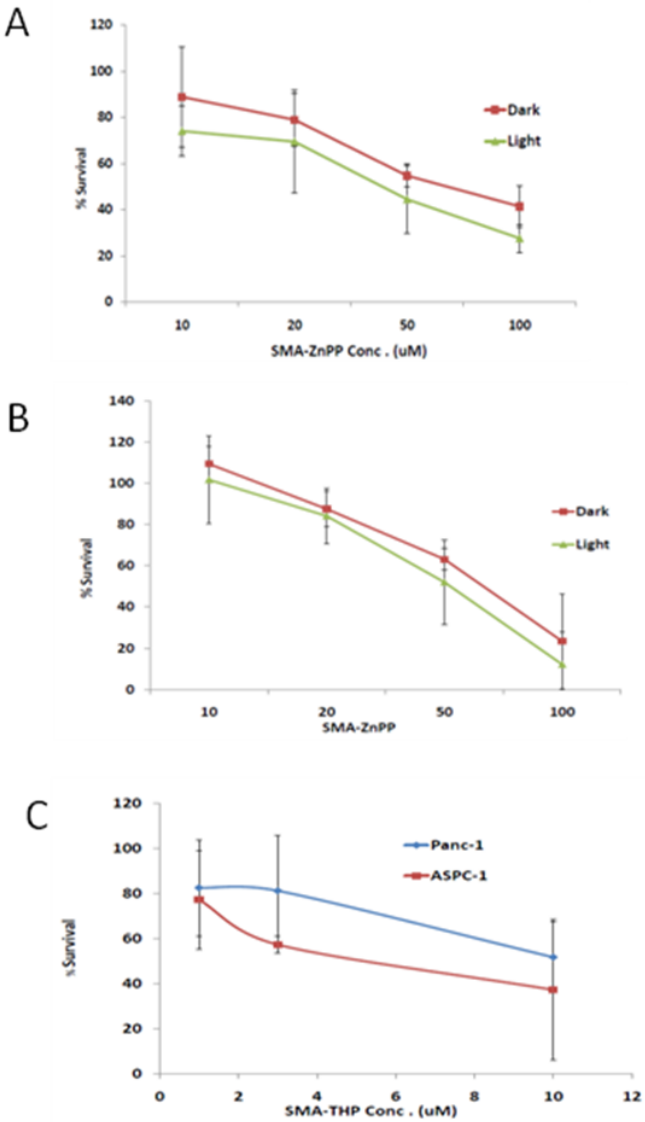

Figure 3: *In vitro* anticancer activity of SMA–micelles against Panc-1, and ASPC-1. (A) The effect of SMA-ZnPP against ASPC-1 with/without 50000 Lux irradiation for 5 minutes (B) The effect against PANC-1 pancreatic cell line. C. The effect of SMA-THP micelles in both cell lines

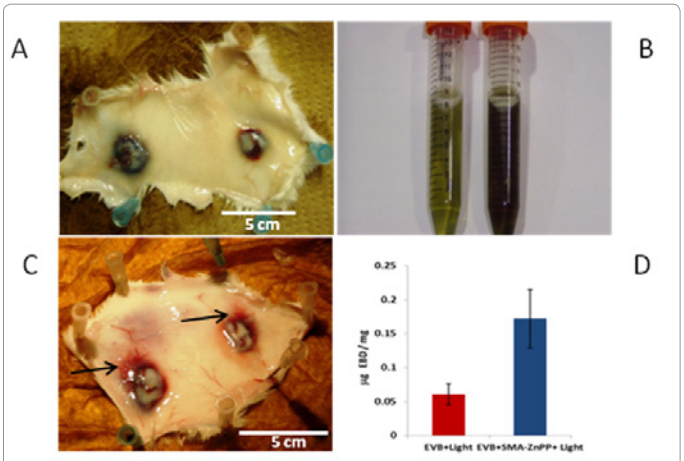

Figure 4: Enhancement of EBD tumor permeability in response to SMA-ZnPP photosensitization. (A) EBD concentration in S-180 murine sarcoma without PDT (B) After injection of 5mg/kg ZnPP photosensitizer, followed by 5 minutes light irradaition at 50.000 Lux. The arrows point to areas of vascular inflammation, scale bar: 5 cm. (C) Representative tubes of the extracted EBD from light irradiated and non-irradiated animals. (D) The difference between extracted EBD with light irradiation was almost 3 folds higher compared to no irradiation.

pancreatic tumour tissue owing to the vascular inflammation mediated by the PDT effect. In our previous work, we have shown significant therapeutic effect of SMA-pirarubicin accompanied by up to 13 fold higher concentration of the THP micelle in tumour tissues compared to free THP. Among the two pancreatic cell lines used in this study (i.e. with Panc-1 and ASPC-1), Panc-1 cells showed higher take and better tumor development in SCID mice in a limited trial. SMA-THP was dosed at either 10 or 20 mg/kg animal weight. As shown in Figure 5A, both concentration of administered SMA-THP micelle showed significant tumor inhibition which is dose dependent. In addition, adding SMA-ZnPP further increased the anticancer efficacy (contrary to the *in vitro* data). The difference between the treated groups and control group was statistically significant. Figure 5B shows the difference between the SMA-ZnPP micelles at 4, and 8 mg/kg (+/-) light irradiation. The micelle shows higher anticancer activity with light irradiation, the addition of glucose moiety showed enhanced activity at 8 mg/kg compared to the non-targeted micelles.

Safety of the dual miceller system *in vivo*

Animal weights were measured twice a week as an indicator of general toxicity during the efficacy study. Relative to untreated animals, animals treated with SMA- micelles showed no significant difference in normalized mean animal weight during the study. Matter of fact, the control group showed high weight reduction compared to all micelle treated groups, even considering the extra weight added due to tumour progression in control group. These results demonstrate clearly that SMA- micelles were well tolerated (Figure 6). It worth mentioning that in a small study involving 3 animals, the animals tolerated the injection of SMA-THP at 80 mg/Kg in sequential injections, with less than 10% weight loss over 2 week's duration (data not shown).

Thus our data proves that collaborative system used in this study, not only an effective strategy to tackle pancreatic cancer but a remarkably safe system as well.

Conclusion

A dual miceller system of SMA-ZnPP and SMA-THP was designed

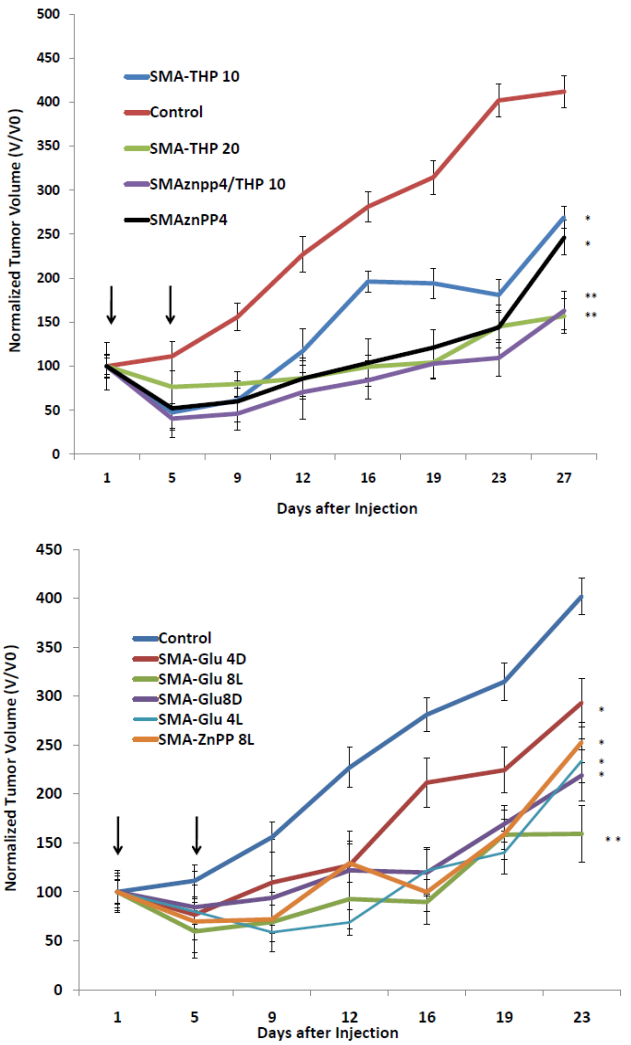

Figure 5: *In vivo* anticancer activity of SMA micelles against Panc-1 pancreatic cell lines in SCID mice. (A) The effect of SMA- THP at 10, and 20 mg/kg as well as in addition to SMA-ZnPP with 50.000 Lux irradiation for 5 minutes (B) Comparison between glucose targeted SMA-ZnPP micelles to plain micelles.

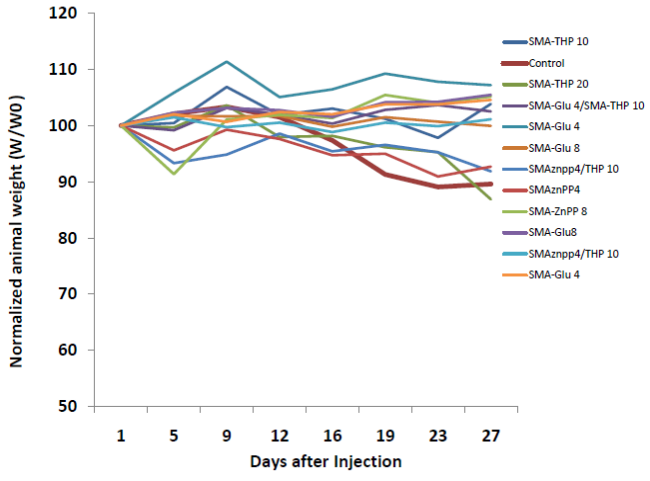

Figure 6: The effect of all study compounds on animal weigh. Arrow, days of injection/light irradiation, D; Dark, L; light. **, P value<0.01, * P <0.05.

and tested in treatment of pancreatic cancer animal model. The two miceller system proved synergistic effect against Panc -1 tumor pancreatic tumor model in SCID mice. Possible mechanism for the results obtained could be related to the vascular inflammatory response elicited by SMA-ZnPP photo irradiation, which clearly shown threefold increase in EBD concentration in tumor tissues. To our Knowledge, this is the first report that describes a dual collaborative Nano systems involving PDT for treatment of pancreatic tumors.

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