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Near-infrared mediated tumor destruction by photothermal effect of PANI-Np *in vivo*

L E Ibarra[1], E I Yslas[1,2], M A Molina[2], C R Rivarola[2], S Romanini[3], C A Barbero[2], V A Rivarola[1] and M L Bertuzzi[1]

[1] Departamento de Biologìa Molecular, Universidad Nacional de Rìo Cuarto, Agencia Postal Nro3, X580NY A Río Cuarto, Argentina
[2] Departamento de Quìmica, Universidad Nacional de Rìo Cuarto, Agencia Postal Nro3, X580NY A Río Cuarto, Argentina
[3] Departamento de Patología, Universidad Nacional de Rìo Cuarto, Agencia Postal Nro3, X580NY A Río Cuarto, Argentina

E-mail: eyslas@exa.unrc.edu.ar and vrivarola@exa.unrc.edu.ar

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Abstract
Photothermal therapy is a therapy in which photon energy is converted into heat to kill cancer. The purpose of this study is to evaluate the *in vivo* efficacy of photothermal therapy, toxicity and hepatic and renal function of polyaniline nanoparticles (PANI-Np) in a tumor-bearing mice model. The *in vivo* efficacy of nanoparticles, following NIR light exposure, was assessed by examining tumor growth over time compared to the untreated control. Signs of drug toxicity and the histopathology and morphology of tumor and tissues, after intratumoral injection treatment, were examined or monitored. Excellent photothermal therapy efficacy is achieved upon intratumoral injection of PANI-Np followed by near-infrared light exposure. These results suggest that PANI-Np could be considered as an effective photothermal agent and pave the way to future cancer therapeutics.

(Some figures may appear in colour only in the online journal)

1. Introduction

Photothermal therapy is a minimally invasive, harmless, and highly efficient therapeutic technique treatment in which photon energy is converted to thermal energy sufficient to induce thermal cell death [1]. A number of *in vivo* model studies have been published using various near-infrared absorbing particles for tumor ablation, including nanoparticles, gold nanorods, and nanoshells [2, 3].

Nanotechnology has recently gained attention as one of the critical research endeavors of the 21st century [4]. The application of nanotechnology for cancer therapy is receiving considerable attention these days. Cancer nanotechnology is emerging as a new field of interdisciplinary research and is expected to lead to major advances in cancer detection, diagnosis and treatment [5–8]. Photothermal therapy (PTT) based on nanomaterials that can be activated by skin-penetrating near-infrared (NIR) irradiation has been recently proposed as a strategy for the next generation of cancer treatments. In this study, we conducted an *in vivo* cancer photothermal study of polyaniline nanoparticles (PANI-Np) in a mouse model. Among conducting polymers, polyaniline (PANI) has been one of the most widely studied due to its unique thermal stability, high conductivity, environmental stability, and reversible electrochemical and physical properties, controlled by its oxidation and protonation state [9–12]. The properties of the aniline polymers make them potentially useful in energy storage devices, optoelectronic devices, display devices, electrodes and sensors, and for corrosion protection of metals and drug delivery [13, 14]. PANI nanoparticles have been

synthesized by dispersion polymerization in the presence of polymeric stabilizers. Recently, several kinds of polymeric stabilizers have been reported, including poly(vinylalcohol), poly(*N*-vinylpyrrolidone), poly(vinyl methyl ether), and methyl cellulose [15–17].

The purpose of this study is to evaluate the *in vivo* efficacy of photothermal therapy, toxicity and hepatic and renal function of PANI-Np in a tumor-bearing mice model.

2. Materials and methods

2.1. Animals

Female BALB/c mice from 6 to 8 weeks old (20–22 g body weight) were obtained from The Foundation Balseiro (Buenos Aires, Argentina) and maintained in standard cages with free access to a pellet diet and tap water *ad libitum*. All animals received good care according to the guidelines established by the ANMAT Disposition N 6344/96, pp 1–7 for Human Care of Experimental Animals. The mice were closely monitored daily for signs of pain and distress by evaluating their appetite, hydration status and activity level.

2.2. Cell line

The mouse mammary adenocarcinoma cell line (LM2) was obtained from Hospital Roffo (Buenos Aires, Argentina). The LM2 cell line was maintained in a humidified 5% CO_2 atmosphere at 37 °C using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin solution and L-glutamine 1%.

2.3. Animal and tumor model

One million LM2 cells suspended in 100 μl of phosphate buffer saline (PBS, pH 7) were inoculated subcutaneously into the lower right flanks of the depilated dorsal region of syngeneic 6–8-week-old female BALB/c mice. When the tumor size reached 0.7 cm in outer diameter, tumor propagation was carried out by extracting 2 mm of tumoral tissue, which was subcutaneously re-implanted into the dorsal region of the required number of mice for each experiment.

Seven days after implantation, when tumors measured around 4–5 mm, the animals were used for phototherapeutic experiments. Spontaneous necrosis was minimal and absent for these tumor sizes. Animals were anesthetized when required, using an intraperitoneal injection of a mixture of ketamine hydrochloride (Ketaject; Phoenix Pharmaceutical, St. Joseph, MO, USA; 50 mg kg^{-1} body weight (bw)), acepromazine maleate (Acedan, Holliday-Scott, Sa, Buenos Aires, Argentina; 17 mg kg^{-1} bw) and xylazine hydrochloride (Bayer, Shawnee Mission, KS, USA; 5 mg kg^{-1} bw).

2.4. Hepatic and renal function

In order to determine the toxicity of PANI-Np, physiological tests were performed employing the following diagnostic kits (obtained from Weiner Laboratories, Saic, Rosario, Argentina): direct creatinine, uremia and glutamic–pyruvic transaminase GPT200. Mice were sacrificed by cervical dislocation at 1 ($n = 6$), 3 ($n = 6$) and 7 days ($n = 6$) after intravenous injection of PANI-Np (25 mg kg^{-1} bw), or alternatively 10 days after PTT of mice treated with PANI-Np intratumoral injection (6 mg kg^{-1} bw). The blood was extracted to obtain the serum for the functionality tests. To evaluate hepatic function, the levels of serum enzyme glutamic–pyruvic transaminase (GPT) were measured, since high levels in the serum of GPT are generally associated with hepatotoxicity. Kidney function was monitored by measuring the serum levels of creatinine and urea.

2.5. Histopathology

After intratumoral injection (IT) with a dose of 6 mg kg^{-1} bw after a period of ten days, the animals were euthanized. The vital organs (liver, kidney and spleen) were collected from the mice, fixed with a 10% formalin neutral buffer solution, embedded in paraffin, and cut into 5 μm-thick sections. Sections were stained with hematoxylin and eosin (H&E) staining for general histology.

The histopathological analysis was carried out in the Animal Pathology Department of Agronomy and Veterinary Faculty, UNRC, under the supervision of Silvia Romanini.

2.6. Tumor histological examination

Tumor samples from the tumor-regressed mice and control tumor-bearing mice (tumor samples after 10 days PTT) were excised and fixed in 10% formalin for routine histological preparation. The representative tissues were dehydrated in ascending grades of alcohol, embedded in paraffin wax, and sections (3–4 μm) were obtained. The tissue sections were stained with H&E and examined under a microscope (Axiovert 135; Zeiss, Germany). The images were recorded using a digital color camera (Axiocam; Zeiss) and Motic software.

Tumor sections were stained with H&E to identify the areas of viable and necrotic tissue. All experiments were repeated at least three times.

2.7. In vivo photothermal destruction of tumors

The mice were divided into five groups ($n = 8$). Animals of group 1 were a control group that received an IT injection of PBS; group 2 were only irradiated (irradiation control); group 3 received an IT injection of PVP (dispersant control) and were then irradiated; group 4 received an IT injection of PANI-Np (6 mg kg^{-1} bw) but were not irradiated and group 5 received an IT injection of PANI-Np (6 mg kg^{-1} bw) and were then irradiated.

The mice of each group were intratumorally injected with 50 μl of each solution (6 mg kg^{-1} bw PANI-Np dispersion, 2% PVP, PBS) and 3 h post-injection irradiated with a continuous NIR laser at the corresponding group (785 nm at a power density of 500 mW cm^{-2}) to the tumor region for 15 min under anesthesia. The efficacy of the treatment was

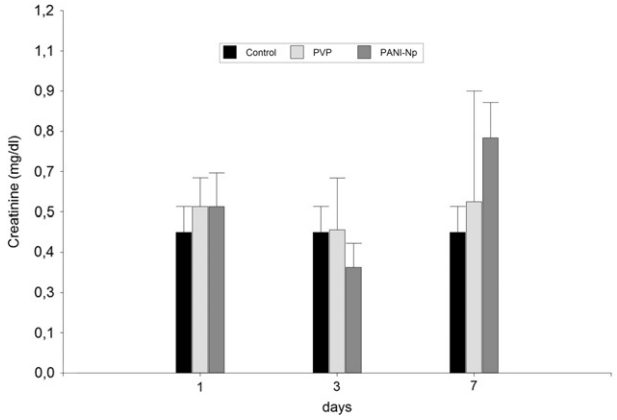

Figure 1. Serum concentration of creatinine in the control group, 1, 3 and 7 days after PANI-Np (25 mg kg^{-1} bw) or PVP injection. Data represent the means ± SEM ($n = 6$). $^{*}p < 0.05$.

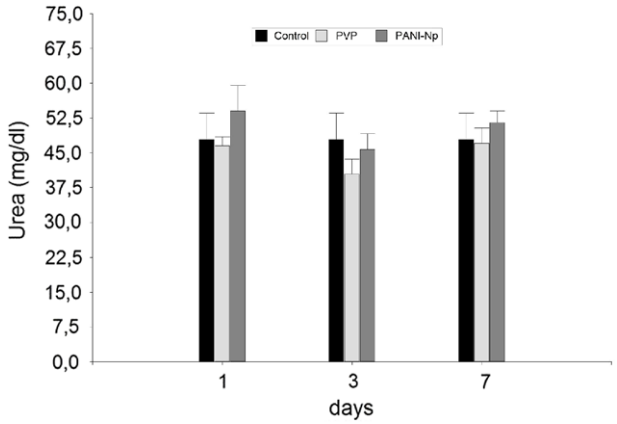

Figure 2. Serum concentration of urea in the control group, 1, 3 and 7 days after PANI-Np (25 mg kg^{-1} bw) or PVP injection. Data represent the means ± SEM ($n = 6$). $^{*}p < 0.05$.

evaluated by comparing the tumor growth as a function of post-irradiation time. The size of the tumor of each mouse was monitored daily, and thereafter three bisection diameters of each tumor were measured with an electronic slide caliper to determinate the tumor volume (mm^3). The tumor volume (V) was determined by the following equation:

$$\text{Volume } (\text{mm}^3): \ V = L \times W \times H \times 0.5636$$

where L is the length, W is the width and H is the height of the tumor [18, 19].

No spontaneous regression of the tumor was observed during our investigations.

2.8. Statistical analyses

Statistical comparisons were performed using ANOVA for the experiment of hepatic and renal function. Data are expressed as mean ± SE, and differences between means were considered statistically significant at $p < 0.05$.

In addition, a general linear mixed model based on maximum likelihood estimation (MLE), correlating the data temporally and modeling the heterogeneity of variance was used to compare the tumor volume of different groups according to the criteria Akaike information criterion and Bayesian information criterion. The Di Rienzo, Guzmán and Casanoves (DGC) test was used when an appropriate post-hoc comparison was possible, and a value of $p < 0.05$ was considered significant [20]. Data are expressed as mean ± SEM.

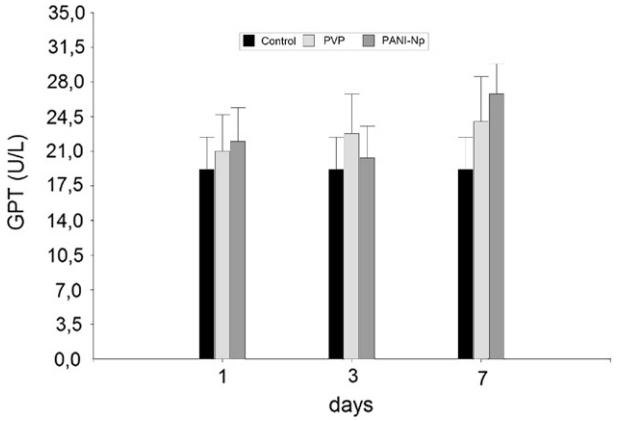

Figure 3. Serum concentration of GPT in the control group, 1, 3 and 7 days after PANI-Np (25 mg kg^{-1} bw) or PVP injection. Data represent the means ± SEM ($n = 6$). $^{*}p < 0.05$.

3. Results and discussion

3.1. Serum biochemistry analysis

While histology provides microscopic visual evidence, it is difficult to make a quantitative assessment of the PANI-Np-induced *in vivo* toxicity. We thus measure various typical biochemical markers: GPT, urea and creatinine, which are commonly used serum indicators to quantitatively assess liver and kidney function and hepatocellular injury.

No functional damage was observed in the liver and kidney of mice treated with PANI-Np (25 mg kg^{-1} bw). Also, there were no statistically significant differences in creatinine, urea and GPT serum concentration from those of the control mice, 1, 3 and 7 days post-injection of PANI-Np (25 mg kg^{-1} bw) (figures 1–3, respectively). These results suggest that PANI-Np does not cause adverse effects in mice.

Furthermore, 10 days after PTT of mice treated with a concentration of PANI-Np of 6 mg kg^{-1} bw by intratumoral injection there were no differences in creatinine, urea and GPT serum concentration from those of the control mice. These results suggest that PANI-Np does not cause adverse effects in mice (figure 4). The data agree with those obtained through histopathological analysis.

3.2. Histology results

In order to investigate the toxicity of PANI-Np (6 mg kg^{-1} bw), a histological analysis of organs was performed to determine whether or not the PANI-Np themselves cause tissue damage, inflammation, or lesions.

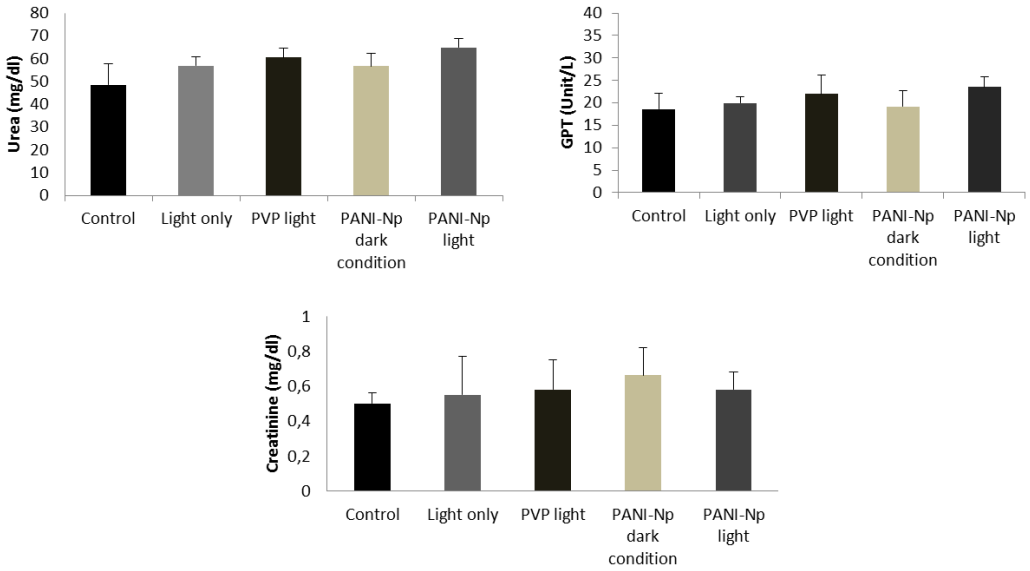

Figure 4. Serum concentration of creatinine, urea and GPT in the control group, PVP irradiated, PANI-Np non-irradiated, ten days after PANI-Np-PTT (6 mg kg^{-1} bw). Data represent the means ± SEM ($n = 5$). $^{*}p < 0.05$.

Tissues 10 days after intratumoral photothermal therapy were obtained immediately after euthanasia for histopathologic examination. The potential toxicity of polyaniline nanoparticles, 10 days post PTT, to vital organs such as the liver, spleen, and kidney was evaluated.

In the liver of PANI-Np irradiated and non-irradiated groups (figure 5 (Li) C and D), histopathological changes were identified as small regions with sinusoidal congestion and the presence of mononuclear inflammatory cells, which suggest mild inflammation. More macrophages than usual were observed. A minor degree of dilatation of sinusoids was shown and a few hepatocytes were slightly deranged and swollen, while others showed organized vacuolar degeneration. However, there was no observed signal of necrosis, suggesting reversible damage to the liver. The control and control dispersant (PVP) groups did not show any of these changes.

In the spleen, no abnormal histopathological lesion was observed in any treatment groups. The sections show a normal splenic architecture with normal lymphoid follicles and sinuses (figure 5 Sp).

The histopathological changes of kidney are shown in figure 5 (Ki). Tissue sections from the control group show a normal kidney architecture with normal appearing glomerular tufts and tubules and normal renal papilla. These findings are still seen in the PVP group. Only cloudy swelling in renal cortical tubular epithelium was observed in the PANI-Np non-irradiated group. In the PANI-Np irradiated group, tubular lights were seen full of a protein substance. Slight macrophage infiltration was observed in the cortex.

3.3. Tumor regression

No differences in tumor volume were noted between the group control, control non-irradiated (injection of PANI-Np and in dark conditions), and control irradiated (injection of PVP and irradiation) with laser 785 nm at a power density of the 500 mW cm^{-2} during 15 min. Compared to the control groups, the growth of the tumors was significantly inhibited, with reduced tumor volumes after PTT. On the other hand, there were obvious differences between the PANI-Np-PTT and the control groups regarding tumor size throughout the observation period (figure 6(a)). Five out of eight irradiated tumors on mice injected with PANI-Np disappeared 7 days after laser irradiation, leaving black scars on the original tumor sites (figure 6(b)). These five tumors did not re-grow within four months post-treatment. The other three tumors experienced a partial regression and the tumor volume decreased by more than 50% compared to the control group. In marked contrast, tumors in the control untreated group, the irradiation only group (no PANI-Np injection), the non-irradiated PANI-Np group and the irradiated PVP group showed similarly rapid growth, demonstrating that NIR laser irradiation or PANI-Np or PVP injection alone did not affect tumor development.

3.4. Histology tumor

Histological examination of tumors treated with PANI-Np-PTT (figure 7(a)) when the nanoparticles were directly injected into the tumor and then exposed to a laser at an intensity of 500 mW cm^{-2} for 15 min showed severe cellular damage (areas with necrosis and some cells in apoptosis) as well as inflammatory cells, in comparison with a control (figure 7(b)) and cells treated with laser only (figure 7(c)), which did not show cell death. Together, our results demonstrate that the combination of PANI-Np and NIR light is a highly effective and feasible photothermal-ablation cancer therapy.

4. Discussion

Our current work establishes targeted PANI-Np for *in vivo* photothermal ablation. In the synthesis of PANI-Np, PVP

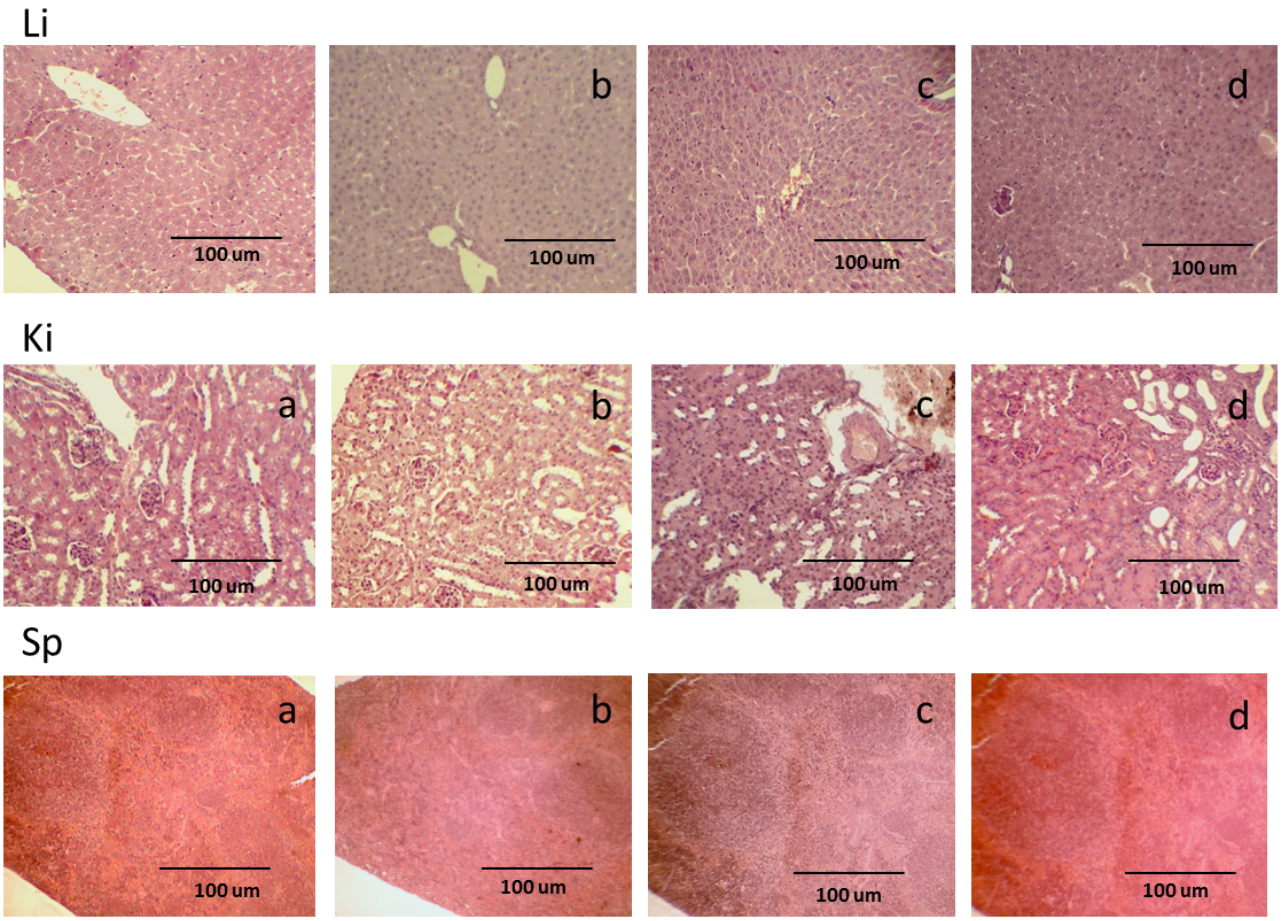

Figure 5. Representative organ histopathology for control and treated animals. Liver (Li), Spleen (Sp) and Kidney (Ki) of the irradiated-only group (a), PVP group (b), PANI-Np non-irradiated group (c) and PANI-Np irradiated group (d) are shown. Our analysis shows that PANI-Np groups exhibit signs of mild toxicity in liver and kidney.

was used as stabilizer, which has been reported as a capping agent to control the particle size and shape and to prevent aggregation [21]. PVP is a biocompatible, water-soluble polymer that can be found in various drug delivery systems, including microspheres, nanoparticles, liposomes, and polymer conjugates [22–27].

In this work we determined whether the PANI-Np induced *in vivo* toxicity, through assay methods such as serum biochemistry analysis and histopathology. The histopathological changes observed in the organs of the Np-PANI groups may suggest a slight toxicity. These changes are considered mild reversible damage, and therefore do not alter the biochemical parameters tested for functionality. In summary, these results did not indicate any irreversible lesions or changes in the biochemical parameters associated with the nanoparticles.

We demonstrated that the treatment of tumors with the combination of PANI-Np and NIR results in rapid tumor regression and long-term survival in a mouse model. The tumor cells showed images indicating different forms of cell death and necrosis: karyopyknosis, karyorrhexis, cell lysis and inflammatory cell infiltration. Also we observed typical signs of apoptosis. Both apoptosis and necrosis reveal irreversible alterations in tumor cells. In marked contrast, tumors in the control untreated group, the irradiation-only group (no PANI-Np injection) and the non-irradiated PANI-Np group showed similarly rapid growth and tumor cells showed no histological signs of cell death, demonstrating that NIR laser irradiation or PANI-Np injection alone did not affect tumor development.

The tumor microenvironment pH is generally more acidic due to the increased glycolysis and the plasma membrane proton-pump activity of tumor cells, which make them produce more lactic acid than normal cells [28, 29]. These nanoparticles appear promising for cancer therapy due to the present properties, in which the acidic conditions of the tumor microenvironment favor the presence of a conductive state (emeraldine salt).

In a recent work, reported by Yang *et al* [30], polyaniline nanoparticles were used for *in vivo* photothermal therapy of cancer, showing tumor ablation effects in a mouse model where tissues presented a low NIR region radiation absorption after irradiation. We used a similar nanoparticle dose in this study and our results demonstrate and confirm that the combination of PANI-Np and NIR light is a highly effective and feasible photothermal-ablation cancer therapy.

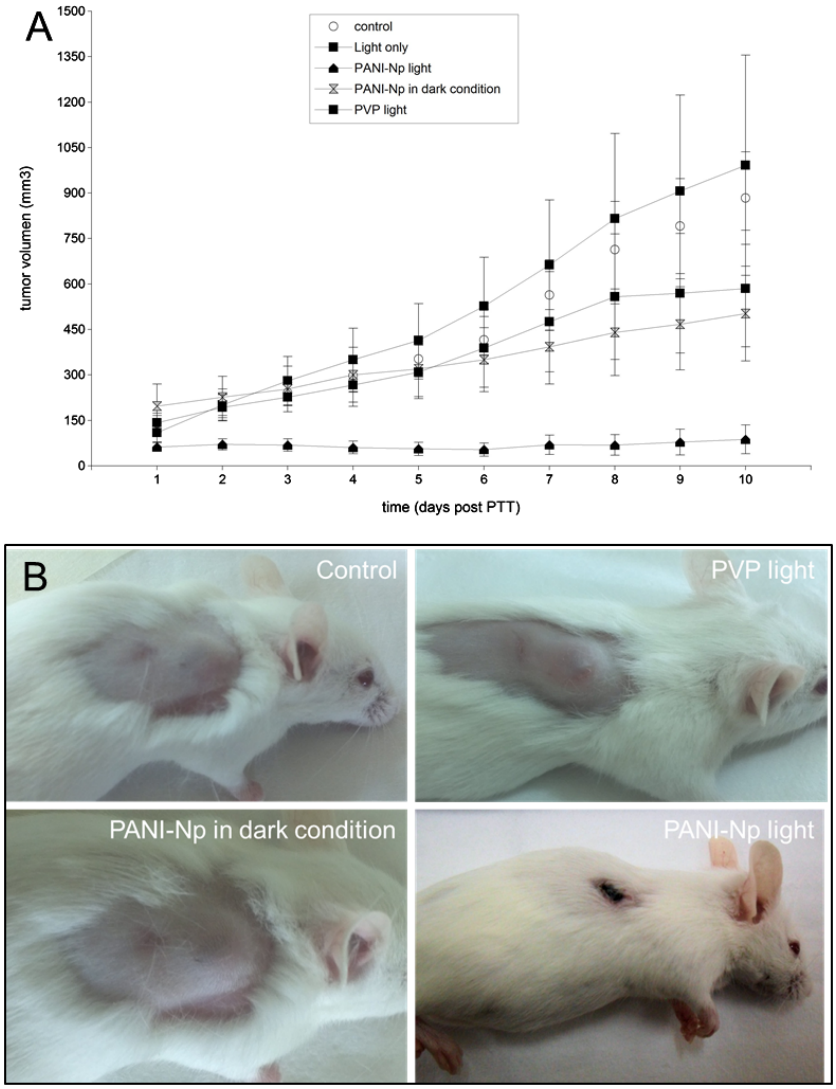

Figure 6. *In vivo* PTT treatment of tumor-bearing mice. (a) The growth of LM2 tumors on different groups of mice after the various treatments indicated. (b) Representative photos of mice on the seveth day after the treatments indicated.

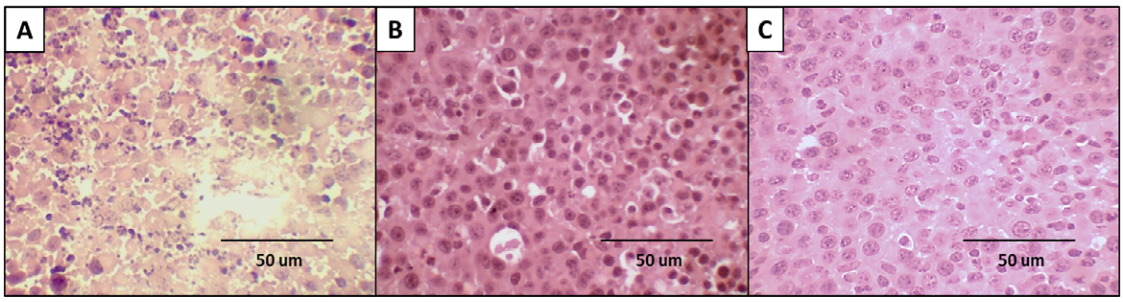

Figure 7. (A) H&E-stained micrograph of a tumor 7 days after PTT with 6 mg kg^{-1} bw PANI-Np by IT injection and 3 h post-injection irradiated with a continuous NIR laser (785 nm at a power density of 500 mW cm^{-2}). The micrograph (×400) shows a large area of cell death and ruptured vasculature. Tumor cells showed different forms of cell death and necrosis, with karyopyknosis, karyorrhexis and cell lysis. (B) H&E-stained tumor specimen obtained after 7 days from the untreated group (magnification ×400). The organization of the tumor tissue is well preserved and typical for this tumor type. (C) H&E-stained micrograph of a tumor 7 days after PTT with PBS IT injection and 3 h post-injection irradiated with a continuous NIR laser (magnification ×400). The organization of the tumor tissue is well preserved and there are no observed areas of cell death.

The success of the particle-assisted photothermal therapy depends on the particle accumulation and the appropriate laser dosimetry.

In conclusion, PANI-Np could serve as a very effective agent for cancer photothermal therapy with NIR light exposure. This study shows that PANI-Np belong to a

new class of optically active therapeutic agents due to their strong absorption of near-infrared light, with the subsequent localized heat generation promoting tumor cell death. Therefore, it is a promising candidate for clinical phototherapeutic applications. We will continue optimizing PANI-Np by dansyl chloride addition, which will assist us in our future research biodistribution and pharmacokinetic studies.

Acknowledgments

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