Tanshinone II A improves distribution and anti-tumor efficacy of pegylated liposomal doxorubicin via normalizing the structure and function of tumor vasculature in hepa1-6 hepatoma mice model

Zhang Ying, Tie Minghui, Bi Feng, Wang Ke

Abstract

OBJECTIVE: To investigate whether the Tanshinone II A could improve the distribution and anti-tumor efficacy of Pegylated Liposomal Doxorubicin (PLD) via normalizing the structure and function of vasculature in Hepa1-6 hepatoma mice model.

METHODS: Hepa-1-6 hepatoma-bearing mice were treated with Tanshinone II A for 14 d. Distribution and anti-tumor efficacy of PLD, and the structure and function of the tumor vasculature were evaluated using various techniques.

RESULTS: Tanshinone II A significantly reduced the micro-vessel density (MVD). After Tanshinone II A treatment, the tumor vascular walls were better structured, as the increased coverage of the pericytes and the promoted contact of the basement membrane and endothelial cell. Functional tests showed that tumor hypoxia was improved and the exudation amount of Evans blue in the parenchyma of the tumor decreased. In addition, mice treated with Tanshinone II A had greater PLD penetration distance intratumorally. Furthermore, combined therapy of Tanshinone II A and PLD significantly inhibited tumor growth.

CONCLUSION: This study suggests that Tanshinone II A helps normalizing the tumor vasculature and has therapeutic potential in increasing the distribution of chemotherapy drug in the tumor.

INTRODUCTION

It is generally accepted that the growth and metastasis of solid tumors are dependent on angiogenesis. In 1971, Folkman suggested a hypothesis that “starve” a tumor to death, that is, anti-angiogenesis was an effective way to avoid cancer. Over the past years, these findings have yielded very significant results, which have brought numbers of anti-angiogenic agents into pre-clinically or clinical trials. However, these agents do not have any significant effect in patients with cancer when used alone. The comprehensive treatment that is combined therapy of anti-angiogenesis with chemotherapy and radiotherapy often produces a synergistic effect. Then the mechanism underlying anti-angiogenic agents’ action in these patients became the scope of research.

Tumor angiogenesis is not simply the increased in the number of blood vessels. Firstly, tumor vessels are unordered, tortuous and saccular.
Hepatoma cell line (Chinese Academy of Sciences) was cultured in RPMI-1640 medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA) and kept at 37 °C in 5% CO₂. Female, C57/BL/6J mice of 5-6 weeks of age and weighing approximately 20 g were purchased from the Laboratory Animal Center of Sichuan University (Chengdu, China). Hepa-1-6 Hepatoma xenograft model was established by subcutaneous injection of 2 x 10⁶ Hepa-1-6 cells into the left armpit of the mice. Tan II A (Sulfotanshinone Sodium Injection, 5 mg/mL), were purchased from the first Biochemical Pharmaceutical Co., Ltd., Shanghai, China. Avastin (Bevacizumab injection, BV), commercially available from Roche Pharma (Shanghai) Ltd., Shanghai, China. PLD (pegylated pipoosomal doxorubicin injection, 10 mg/mL), were purchased from Shanghai Fudan-zhangjiang Bio-Pharmaceutical Co., Ltd., Shanghai, China.

**Histology and immunohistochemistry (IHC)**

When tumor volume reached approximately 200 mm³, mice were randomly allocated to three groups (n = 8 in each group), and received daily intraperitoneal injection NS, Tan II A (5 mg·kg⁻¹·d⁻¹) or BV (5 mg·kg⁻¹·d⁻¹) for 14 d, respectively. Animals were sacrificed by cervical dislocation for tumor tissue at 14 d after injection of the drugs. The tumors were fixed in 4% paraformaldehyde and embedded in paraffin. Then, adjacent sections with 3-5 μm thicknesses were cut from each tumor, mounted on glass slides and stained with hematoxylin and eosin. For immunohistochemistry, the endothelial cells were stained by rat anti-mouse CD31 monoclonal antibody (1:100, BD Biosciences, San Diego, CA, USA), the pericytes were identified with rabbit anti-mouse α-SM/CD105 (1:400, Abcam, Burlington, MA, USA), basement membrane was stained by rabbit type IV Collagen polyclonal antibody (1:2000, Millipore, Burlington, MA, US). After routine immunohistochemistry staining, the sections were observed under an optical microscope and images were taken. The images were analyzed by Image-Pro Plus 6.0 (Media Cybernetics Image Technology Co., Austin, TX, USA). The microvessel density (MVD) of the tumor was measured using methods described previously. Primarily, MVD was calculated by counting the number of CD31-positive-stained vessels in the image X400. The positive area of α-SAM and the CD31-positive vessels on the same area were calculated according to a magnification of x400. Then, the ratio of α-SAM/CD31 was calculated. The method for calculating the ratio of Collagen IV/CD31 was same as above. In addition, under the microscope with 10-20X high-powered (200X), 5 sections of each sample in 5 random sights were observed.

**Immunofluorescence (IF) of hypoxic tissue**

The condition of tumor-bearing mice and grouping was as mentioned above in IHC. Then, a Hypoxyprobe™-1 Kit (Hypoxyprobe, Inc., Burlington, MA, USA, was used to identify the effect of Tan II A on tumor vasculature and hypoxia, as well as the probable synergistic anti-tumor effect when combined with PLD.

**METHODS**

**Ethics approval**

Animal care and experimental protocols were approved by the Sichuan Medical Experimental Animal Care Commission.

**Cell lines, animal tumor model, and drug**

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containing of 100 mg solid pimonidazole HCI and 1.0 mL mouse IgG, monoclonal antibody (Mab1) against protein adducts of pimonidazole formed when the tissue pO2 was below 10 mm Hg) was applied in the tumor hypoxia examination. In brief, tumor-bearing mice were given pimonidazole (60 mg/kg, dissolved to 15 mg/mL in 0.9% saline) intraperitoneally 20 min before sacrifice. Then, tumor tissue was excised from mice and immediately frozen in liquid nitrogen and stored at −70 °C. For immunofluorescence, frozen tumors were cut into 10 µm thick sections with frozen section machine, six sections per tumor with 3 tumors in each group. The primary antibody to pimonidazole (1∶100) and the routinely stained with immunofluorescence was used. The sections were observed under a fluorescence microscope and the images were analyzed by Image-Pro Plus 6.0. Five randomly selected regions per slide were observed. The tumor tissue hypoxic area (%) was defined as the pimonidazole-positive area under a magnification of ×200.

**Tumor vascular permeability**

To further study the effect of Tan II A on tumor vascular, another set of tumor-bearing mice was used. Four animals in each group were studied for vascular permeability using an Evans blue dye method. In brief, 200 µL 2% Evans blue dye solution (20 mg/kg dissolved in saline, Sigma-Aldrich, Shanghai, China) was injected into the tail vein after the drugs of each group being continuously administrated for 14 d. The Evans blue in the vascular was washed by 15 mL saline through the lavage of heart after the dye circulated for 20 min. Thereafter, the mice were sacrificed and the tumor tissues were excised. The tissues were cut into small pieces and weighed, and the small pieces were extracted in 1 mL of formamide for 72 h. Level of the dye was detected by a spectrophotometer at a wavelength of 610 nm.

**Distribution of Pegylated Liposomal Doxorubicin (PLD) in tumor**

Tumor-bearing mice were divided randomly into three groups with 8 mice in each group, and received 0.2 mL Tan II A (5 mg/kg), BV (5 mg/kg) and NS when the mean tumor diameter was 8 to 10 mm. PLD (8 mg/kg) was injected into the tail vein after the other drugs of each group being continuously administrated for 6 d. Animals were killed and tumors excised at 24 h following injection of PLD. Tumor tissues were embedded immediately in OCT compound, frozen in liquid nitrogen, and stored at −80 °C for tissue sectioning and immunofluorescence staining. Three cryostat sections with 10 µm thicknesses were cut from each tumor, mounted on glass slides, and allowed to air-dry.

PLD auto-fluorescence was detected using Nikon Eclipse CI inverted fluorescence microscopy 475 to 485 nm excitation and 575 to 585 nm emission filters, and images of section were captured with a Nikon DS-U3 camera. Blood vascular in the tissue section were recognised by the expression of CD31 on endothelial cells. Following PLD imaging, tissue section was stained with a rat anti-mouse CD31 monoclonal antibody (1∶100, BD Biosciences, San Diego, CA, USA) for 1 h in 4 °C and then stained with goat anti-rat IgG (1∶400, Jackson ImmunoResearch Laboratories, Inc., Philadelphia, PA, USA) secondary antibody. Finally, sections were cover-slipped with a mounting medium containing anti-fluorescein quencher. Anti-CD31 fluorescence representing endothelial cells was visualized using 510 to 560 nm excitation and 590 nm emission filters.

Composite images of PLD and CD31 were generated using Image Pro Plus (version 6.0) software (Media Cybernetics Image Technology Co., Austin, TX, US). The process of synthesis must ensure that the image displaying anti-CD31 staining was overlaid with the corresponding field of view displaying PLD fluorescence image, and the procedure using the method introduced by Primeau et al. Penetration of PLD was measured using the Matrix Laboratory software application. Briefly, the composite images of PLD and CD31 were separated firstly, certainly, those two images from the same field. Then, the segmented blood vessel image was masked, so that all the blood vessels can be identified and be converted to a black and white binary image. In this 8 bit image, white color with an intensity of 255 determined the blood vessel density and the remaining pixels in this image had an intensity of 0. Then the image was formed to a new image by using the function of distance filter. In this image, the gray value of each point represented the distance of the point to the nearest vessel. Similarly, the PLD image was also switched to the binary image in which each PLD positive spot represented as its center point. The distance of each PLD spot from the nearest blood vessel was calculated by using the minimum function of Matlab. Every slide was took three horizons after the areas of necrosis and staining artifact were excluded and the noise from tissue auto-fluorescence be minimized. Each picture captured from the corresponding horizons was working out an average as the PLD penetration distance of the field. The mean penetration distance of the nine fields from three slides was taken as the final PLD penetration distance for each animal.

**Treatment protocol**

Hepa-1-6 Hepatoma xenograft model was again set up as described above. When tumor volume reached approximately 200 mm³, mice were randomly divided to six groups of five. Group 1 received normal saline (NS); group 2 received 5 mg/kg sulfotanshinone sodium injection intravenously daily for 14 d (TNA); group 3 received 5 mg/kg bevacizumab injection intravenously daily for 14 d (BV); group 4 received 8 mg/kg pegylated liposomal doxorubicin injection intravenously only once on day 7 (PLD). On day seven after the administration of sulfotanshinone sodium injection...
and bevacizumab injection treatment, 8 mg/kg pegylated liposomal doxorubicin injection was combined intravenously only the day and these groups were defined as group 5 (combined 1), group 6 (combined 2), respectively. All animals were sacrificed on day 15 and tumor diameters were measured every other day using calipers. The tumor volume (V) was calculated by the formula: $V = W^2 \times Y / 2$; where W and Y are the smaller and larger perpendicular diameters, respectively.

Statistical analysis
Data were presented as mean ± standard error of mean. All data were analysed with SPSS 13.0 (SPSS Inc., SPSS for Windows, Version 13.0, Chicago, IL, USA) and GraphPad Prism5. One-way analysis of variance was conducted to test the differences between groups. A $P < 0.05$ was the significant level.

RESULTS
Tumor MVD, pericyte coverage and contact of basement membrane in tumor vessels
On day 14 after Tan II A and BV administration, the MVD was reduced to 76.14% and 67.95% of NS group, respectively. There was significantly difference in the MVD between the Tan II A and NS groups (Figure 1A, 1B). MVD of the BV group was less than that in the Tan II A group, while there was no statistical significance between them ($P > 0.05$).

The results showed that vessels in Tan II A and BV group were more likely to cover by pericyte (Figure 1C). However, loss of pericyte was common in the vessels of the NS group (Figure 1C). Quantification of the α-SAM/CD31 ratio showed significantly more pericyte-covered vessels in the Tan II A group than those in the NS group, while there was no significant differ-

![Figure 1](image-url)

Figure 1 Effect of Tan II A on tumor microvessel density, pericyte and basement membrane
A-C: the morphologic changes of CD31-positive vessels, endothelial cells were stained by anti-CD31 antibody, respectively for NS, TAN, BV (IHC, × 400); J: IHC analysis microvessel density; D-F: the morphologic changes of pericyte-covered vessels, pericyte cell stained by anti-α-SAM, respectively for NS, TAN, BV (IHC, × 400); K: the ratio of α-SAM/CD31 in Tan II A and BV showed a increase of pericyte-covered vessels ($P < 0.01$); G-I: the morphologic changes of basement membrane enveloped vessels, basement membrane stained by type IV Collagen antibody, respectively for NS, TAN, BV (IHC, × 400); L: the ratio of Collagen IV/CD31 in Tan II A and BV group were reduced compared with NS group ($P < 0.01$; $P < 0.05$), while, the less has significance difference between the Tan II A and BV group ($P < 0.01$). TAN, BV vs NS, $P < 0.01$; TAN vs NS, $P < 0.05$. NS: daily intraperitoneal injection normal saline (2 mL/d) for 14 d; TAN: daily intraperitoneal injection Sulfotanshinone Sodium Injection (5 mg · kg$^{-1}$ · d$^{-1}$) for 14 d; BV: daily intraperitoneal injection Bevacizumab injection (5 mg · kg$^{-1}$ · d$^{-1}$) for 14 d. TAN, BV vs NS, $P < 0.01$. IHC: histology and immunohistochemistry.
ence between Tan II A group and BV group (Figure 1D). We also found that the basement membrane was thicker in NS group than in Tan II A and BV group, especially in BV group (Figure 1E). There was a significant difference in the Collagen IV/CD31 ratio between the Tan II A and NS group (Figure 1F) and this significant differences was also found between the Tan II A and BV group (Figure 1F).

**Hypoxic area**

In Figure 2A, we found that the distributed of hypoxic areas mainly on the edge of necrosis tissues. The hypoxic areas in the Tan II A group were at a low level compared to the control. However, from the figures, the hypoxic areas in the Tan II A group was higher than in BV group, while the difference did not reach statistical significance ($P > 0.05$) (Figure 2B).

**Tumor vessel leakiness**

In the study, intratumoral vascular permeability were also evaluated after drug treatment. The results showed that Tan II A and BV induced a significant reduction in tumor vessel permeability. On day 14 after the administration of Tan II A and BV, Evans blue dye extravasation into the tissue of tumors decreased to 54.94% (20.39 ± 0.26 vs 37.12 ± 0.31, $P < 0.01$), 49.53% (18.39 ± 0.20 vs 37.12 ± 0.31, $P < 0.01$, respectively), while the dye level in Tan II A group is higher than in BV group.

**Distribution of PLD**

In Figure 3A, 3B, all of the images from three groups show that PLD distributed around tumor blood vessels and decreased with distance from the blood vessels (Figure 3C). The fluorescence intensity of PLD is also decayed with distance from the blood vessels, so some regions of tumors had a detectable PLD. In addition, there were also some CD31-positive blood vessels without surrounding PLD.

Table 1 and Figure 4 summarizes the PLD penetration distance in three groups. Tumor in the group Tan II A and BV showed a significant increase in PLD penetration distance compared with the group NS ($P = 0.037$ and 0.011, respectively). The PLD penetration distance in Tan II A group was lower than that in the BV group, however, there were no significant differences between them. The calculation of PLD-specific fluorescence spot among the three groups showed a trend of change similar to the trend seen in PLD penetration distance (Table 1). The calculation of PLD fluorescence in Tan II A and BV groups was significantly greater than that in the NS group ($P = 0.036$ and 0.021). Furthermore, no significant differences were found between group Tan II A and BV in drug fluorescent count.

**Anti-tumor efficacy of PLD**

From the data above, we found that Tan II A can normalize the structure and function of the tumor vasculature and improve the tumor microenvironment in a
significant. So, the results indicated that Tan II A promoted normalization of the tumor vasculature and function to some extent. In addition, Tan II A can have a synergic action with PLD, and the effect was associated with augmented PLD penetration distance inside the tumor.

In the previous study, Wang et al.18 found that Tan II A did not inhibit MVD but improve microvessel integrity (MVI). Our study differs from their found in MVD. This difference may be attributed to the use of different animal model and observation times. Wang et al.18 established a double-tumor xenograft of hepatocellular carcinoma model and administration of Tan II A after palliative resection, while we established subcutaneous cancer models in C57/BL/6J mice by inoculation of mice hepatoma cell line Hepa-1-6 and administration of Tan II A through intraperitoneal injection daily. Interestingly, we are identical in our views that Tan II A could improve microvessel integrity. In addition, in our findings, Tan II A could inhibit MVD, which is in agreement with the results of Xing et al.22 Hypoxia is an important characteristic of the microenvironment of solid tumors. It is well known to aid tumor progression, metastasis and to increase the resistance to various treatments.16 In our study, we found that Tan II A decreases tumor hypoxia, which is consistent with the results of Wang et al.16 in their study, a significantly alleviated residual tumor hypoxia was found after administration of Tan II A. As the mechanisms of the decreased tumor hypoxia after Tan II A therapy, some studies considered this is the consequences of a inhibited expression of HIF-1α.21 However, another report believed that it resulting in a normalized vasculature. Our results confirm the view of latter.21 The anti-angiogenic effect of Tan II A has been verified by precious work.22,23 The dose of anti-angiogenic agents is important for anti-tumor activity.21 Increase the dose of anti-angiogenic agents often results in excessive vascular regression, which may reduce the effect of anti-tumor activity because it compromises the delivery of drugs and oxygen.23 Furthermore, increased doses of anti-angiogenic agents can have adverse effects on normal tissues.23 Indeed, in the study to evaluate the
certain extent. In order to verify the effect of combination therapy of Tan II A and chemotherapy, we designed an experimental protocol. We found that except for PLD [(863 ± 212) vs (1057 ± 88) mm³, P = 0.028], monotherapy with either Tan II A or BV had no different effect on tumor inhibition compared with that in the NS group [(986 ± 134) or (946 ± 150) vs (1057 ± 88) mm³, P > 0.05]. On day 6, after Tan II A and BV administration, addition of PLD significantly inhibited tumor growth [(452 ± 50), (353 ± 86) mm³, respectively; P < 0.001]. Furthermore, either combined group 1 or combined group 2 had more effect on tumor inhibition compared with corresponding monotherapy with PLD group. The synergistic effect of combined group 1 was better than that in combined group 2, while the difference did not reach statistical significance (P > 0.05).

DISCUSSION

In this study, we established subcutaneous cancer models in C57/BL/6J mice by inoculation of mice hepatoma cell line Hepa-1-6. The Administration of Tan II A remodeled the structure and function of the tumor vasculature, and changed the anti-tumor efficacy of PLD through enhancing its penetration distance inside the tumor.

In this study, we found that the contact of the basement membrane and endothelial cell was promoted and the tumor vessel leakiness were reduced in both the BV group and Tan II A group, but BV group was significantly better than Tan II A group in both of these measures. Meanwhile, reduced MVD, increased coverage of pericyte, augmented PLD penetration distance inside tumor, and the therapeutic synergy when combined with PLD were found in both the BV group and Tan II A group, but the differences were not statistically significant. So, the results indicated that Tan II A promoted normalization of the tumor vasculature and function to some extent. In addition, Tan II A can have a synergic action with PLD, and the effect was associated with augmented PLD penetration distance inside the tumor.

Indeed, in the study to evaluate the

Table 1 PLD penetration distance and count of PLD fluorescence spot in three groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>PLD penetration distance (µm)</th>
<th>Count of PLD fluorescence spot</th>
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<tbody>
<tr>
<td>NS</td>
<td>6</td>
<td>40±14</td>
<td>391±213</td>
</tr>
<tr>
<td>TAN</td>
<td>6</td>
<td>72±35</td>
<td>590±275</td>
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<tr>
<td>BV</td>
<td>6</td>
<td>80±52</td>
<td>611±199</td>
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Notes: NS: daily intraperitoneal injected normal saline (2 mL/d) for 14 d; TAN: daily intraperitoneal injection Sulfortanshione Sodium Injection (5 mg·kg⁻¹·d⁻¹) for 14 d; BV: daily intraperitoneal injection Bevacizumab injection (5 mg·kg⁻¹·d⁻¹) for 14 d. PLD: pegylated liposomal doxorubicin. Data were presented as mean ± standard error of mean. TAN, BV vs NS, *P < 0.01.

Figure 4 Composite images of PLD and blood vessels (IF, × 400)
A: NS; B: Tan II A; C: BV. NS: daily intraperitoneal injection normal saline (2 mL/d) for 14 d; TAN: daily intraperitoneal injection Sulfortanshione Sodium Injection (5 mg·kg⁻¹·d⁻¹) for 14 d; BV: daily intraperitoneal injection Bevacizumab injection (5 mg·kg⁻¹·d⁻¹) for 14 d. Images shows the difference in PLD penetration between groups. IF: immunofluorescence.
safety and efficacy of bevacizumab in patients with breast cancer, Cobleigh et al.70 verified that higher doses of bevacizumab increases toxicity but do not result in significant survival benefit. In the previously mentioned studies, Tsai et al.71 used the CAM to study the in vivo effect of Tan II A on PMA-induced angiogenesis. The result of them found that addition of 1, 5 and 10 uM of Tan II A led to 15%, 53% and 64% reduction of the angiogenic index, respectively. In addition, Wang et al.72 administrated Tan II A daily in 1, 5, and 10 mg·kg⁻¹·d⁻¹ and the result showed that the greatest inhibitory effects were seen at a dose of 10 mg·kg⁻¹·d⁻¹. From above argument, we speculated that the high dose of Tan II A would lead to excessive vascular regression, so the middle dose (5 mg·kg⁻¹·d⁻¹) was selected in this study.

In our study, we found that the combination of Tan II A and PLD treatment led to even more pronounced synergistic anti-tumor activity. It was consistent with the results of Fu et al.69 which found that Tan II A ameliorates hypoxia-induced chemotherapy resistance to PLD in breast cancer cell lines, and the possible mechanism may be attributed to the down regulation of HIF-1α expression.

However, there are certain weaknesses in our study. In order to verify whether Tan II A could normalize the structure and function of the tumor vasculature, firstly, we did not explore the “time window” of Tan II A. Besides, interstitial fluid pressure, which serves as useful indicators for tumor vessel function and tumor microenvironment, had not been evaluated due to equipment limitations in our laboratory. In conclusion, Tan II A could normalize tumor vasculature in mice bearing hepatoma cell line Hepa1-6. Remodeling of the tumor vasculature resulted in reduced tumor vessel leakiness and improved the tumor microenvironment through reducing the tumor hypoxic. Meanwhile, those all resulted in improved delivery of PLD to the tumor and it is correlated closely with synergistic efficacy when Tan II A combined with PLD. Take together, Tan II A is under a huge potential in anti-tumor. In addition, the molecular mechanism underlying anti-tumor effect of Tan II A also needs to be further studied.

REFERENCES


