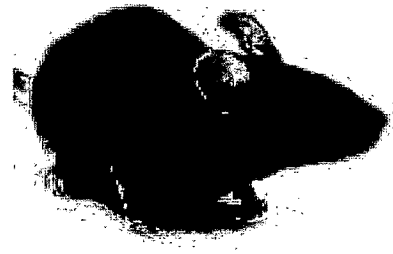
Chapter - 9

In-vivo Tumor Regression & Angiogenesis Study



9.1. Introduction

The targeting of chemotherapeutics to tumor blood vessels, using ligands that bind to specific molecules which over express in the tumor vasculature is a major research area (Kolonin et al., 2001; Trepel et al., 2002). The tumor growth can be inhibited by attacking the vascular supply of the tumor. Undeniably, the host endothelial cells are believed to play a central role in tumor growth, progression, and metastasis, acting as the primary building blocks of the tumor microvasculature (Folkman, 1995). As the solid tumors are angiogenesis dependent (Folkman, 1995), the selective inhibition or destruction of the tumor blood vessel using either antiangiogenic or antivascular treatments could trigger tumor growth inhibition, regression, and/or a state of dormancy and thereby offer a novel approach to cancer treatment. To date, preclinical studies have convincingly validated the guiding principles of this concept (Folkman, 1995; Boehm et al., 1997).

Vascular targeting offers therapeutic promise for the delivery of drugs (Arap et al., 1998; Curnis et al., 2002), radionuclides (Sipkins et al., 1998), and genes (Hood et al., 2002; Niethammer et al., 2002). This approach has the advantage that the delivery vehicle, once in the blood stream, should have direct access to the target endothelial cells. One of the newest and most promising strategies in molecularly guided cancer pharmacology is the development of techniques that can modify the kinetic features of these drugs by encapsulating them at considerable concentrations in high molecular order lipidic vesicles known as liposomes (Allen, 2002). Targeted liposomes [lipid vesicles bearing covalently conjugated antibodies (immunoliposomes) or other targeting moieties like specific peptides] have several advantages over simple antibody drug conjugates for specific drug delivery (Allen, 2002). Use of internalizing ligands for targeting liposomes allows the encapsulated contents to be delivered to the cytosol through the endosome/lysosome pathway (Allen, 2002; Sapra and Allen, 2002).

Endothelial cells in the angiogenic vessels within solid tumors express several proteins (reviewed by Schliemann et al., 2007) including neuropilins (Soker et al., 1998; Miao et al., 2000; Ellis, 2006) and that are absent or barely detectable in established blood vessels.

Neuropilins (NRP 1 and NRP 2) are membranous receptors capable of binding two disparate ligands, class 3 semaphorins (SEMA 3A) and vascular endothelial growth factors (VEGF-A165), and regulating two diverse systems, neuronal guidance and angiogenesis. NRP1 is expressed by a wide variety of human tumour cell lines and diverse human neoplasms, and are implicated in mediating effects of VEGF and Semaphorins on the proliferation, survival and migration of cancer cells. NRP1 is expressed in patient specimens from lung, breast, prostate, pancreatic and colon carcinomas. NRP1 has also been found in several other tumours including melanoma, astrocytoma and neuroblastoma. These findings taken together with the expression of NRPs in diverse neoplasms, suggests a possible role for this molecule in tumour invasion

and metastasis in addition to its involvement in tumour vascularisation (Bielenberg et al., 2006; Pellet et al., 2008).

Neuropilin-1 receptors are up-regulated in tumor vasculature and thus it may be possible to develop targeted chemotherapy strategies that are based on selective expression of these receptors in tumor vasculature. Thus we hypothesize that the PEGylated liposomes, as a carrier of anticancer drug, conjugated with anti-neuropilin-1 antibodies (Intact/Fab' fragments), as a targeting ligand, have A]. damage angiogenic blood vessels and indirectly the tumor cells that these vessels support, B]. accumulate in the tumor interstitial space and function as a sustained release system, resulting in direct cell kill, including cytotoxicity against cells that are at the tumor periphery and are independent of the tumor vasculature and C]. rapidly taken by the tumor cells, as compared to the non targeted liposomes, by the process of endocytosis through neuropilin-1 receptors which over express on the cancer cells. Therefore this combined strategy has the potential to overcome some major limitations of conventional chemotherapy.

9.2. Tumor regression and angiogenesis study

Animals

Female C57BL/6 mice (6-8 weeks old), ranging from 18 to 22g were provided by Animal Care Facilities, ACTREC, TATA Hospital, Mumbai. All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee. All care and handling of animals were performed with the approval of institutional review board of animal experiments.

Method

Intradermal tumor model (Dua et al., 2007): 6-8 week old C57BL/6 female mice were shaved on the ventral side and challenged intradermally with 1 million B16F10 cells/50ul of PBS. Mice were randomly assigned into 4 groups (5 mice/group): (A) control (PBS), (B) Taxotere (Sanofi Aventis), (C) DTX loaded PEGylated liposomes and (D) Anti-neuropilin-1 (Fab' fragments) conjugated PEGylated immunoliposomes. The tumor regression and anti-angiogenesis performance of the all formulations (Taxotere, PEGylated liposomes and PEGylated Immunoliposomes) was determined at the normal human dose of 2mg/kg. The PEGylated and PEGylated immunoliposomes equivalent to 2.7±0.2μM of total phospholipids per dose was injected. The PLs and PILs having mean particle size of 125±6nm (PDI: 0.216±0.024) and zeta potential of -54±3mV were used in the study. The totals of three doses were injected through tail vein. The first dose was injected when the tumor size ranged from 21-55mm^3 (at 7th day after tumor implantation). The second and 3rd doses were injected at 10th and 12th day after tumor implantation, respectively. The mice were monitored at every second day for the evidence of weight loss, tumor development, quantification of tumor size, and evidence of tumor-associated morbidity during the experiment. On 14th day the mice were sacrificed and actual tumor volumes were measured using digital vernier calliper and

tumor volume was calculated using the below mentioned formula (Ebos et al., 2009, see supplemental data).

Tumor Volume (TV) = $0.5(ab^2)$

Where, 'a' is the length and 'b' is the breadth of the tumor assuming the depth of tumor 'C' = b/2.

The same model was used to determine the effect of drugs on angiogenesis (micro-vessel density). The mice were sacrificed by cervical dislocation and the skin containing solid tumor was collected, spreaded on whatman paper, stapled, photographed and the number of angiogenic vessels (micro-vessel density) around the tumor were counted manually using the photographs.

9.3. Results and Discussion

9.3.1. Tumor regression performance of formulations

The tumor inhibitory activities of Taxotere and liposomal formulations were evaluated in C57BL/6 mice bearing B16F10 melanoma tumor (Figure 9.1). The DTX formulations were effective in preventing tumor growth compared to the treatment with saline (Figure 9.2). The treatment with PILs displayed stronger tumor inhibition than the treatment with PLs and marketed Taxotere injection. The anti-neuropilin-1 (Fab' fragments) immunoliposomes caused more suppression of tumor growth as compared to control ($p<0.05$, $p=0.016$), PLs, and marketed Taxotere for the data points of day 14 (Table 9.1 and Figure 9.2). The Taxotere injection suppressed the tumor growth significantly as compared to control and PLs as like PILs but after the third dose, the Taxotere could not able to suppress the tumor growth further while, PILs decreased the tumor size further significantly.

The accumulation of ligand-modified liposomes or non-modified liposomes into solid tumors is governed by the process of extravasation and passive diffusion. Previous studies (Unezaki et al., 1996; Uster et al., 1998; Xiong et al., 2005) have shown that the extravasation of PEG-modified liposomes from the vascular compartment into the tumor interstitium was dependent on liposomes size and tumor type and the diffusion rate of the liposomes into tumor tissue. Because of smaller particle diameter, the prepared PLs and PILs (125±6nm) accumulated in the solid tumor mainly *via* EPR effect. Once accumulated in the tumor tissue, Fab' fragment function as an accelerator for the cellular uptake of anti-neuropilin-1 (Fab') immunoliposomes by the neovascularity and tumor cells. In this sense, the Fab' fragment contributed in controlling intra-tumor disposition of liposomes, after they reached tumor tissue by EPR effect, but cannot target tumor tissue while in systemic circulation (Hatakeyama et al, 2007). Hence, it can be concluded that the anti-neuropilin-1 antibody Fab' fragments is a potent ligand

against neuropilin-1 receptor, which accelerate the cellular (cancer and neovascular cells) accumulation of DTX encapsulated sterically stabilized liposomes.

Neuropilin-1 receptors are over-expressed on tumor vascular endothelial cells and are involved in tumor angiogenesis also (Soker et al., 1998; Miao et al., 2000; Ellis, 2006). The significant tumor growth suppression observed with PIL in our study, than with PLs and Taxotere, suggests that the neuropilin-1 antibody Fab' fragment modified PLs might have delivered more DTX to angiogenic endothelial cells, resulting in vascular damage which is more serious for the survival of the tumor cells (Pastorino et al., 2003; Asai et al., 2002; Oku et al., 2002).

In addition, the mice weights were also measured as an indicator of drug toxicity during the study (Table 9.2 and Figure 9.4). There was no significant change observed in the mice weight during the treatment with formulations at 2mg/kg dose. However, on 10th day (immediately after 2nd dosing) the decrease in body weight in case of control group (1.5%), Taxotere treated group (4.2%), PLs treated group (1.2%) and PILs (1.9%) was observed. This indicates the more toxic nature of marketed Taxotere as compared to DTX loaded liposomes. Also, on 14th day we observed better recovery of body weights with control groups (1.4% increase), Taxotere (3% increase), and PILS (5.2% increase) as compared to 10th day. But the PLs treated group showed further decrease in body weight (0.6% decrease) at 14th day as compared to 10th day. Therefore, the above results indicate that the PILs suppressed the tumor growth more significantly with minimum toxicity as compared to marketed Taxotere and PLs. This is might be due to increased circulation (due to specific neuropilin-1 binding) time and uptake of PILs by both tumor endothelial cells and tumor cells *via* neuropilin-1 receptors which over express on both the cells. No tumor associated death was observed during experiments.

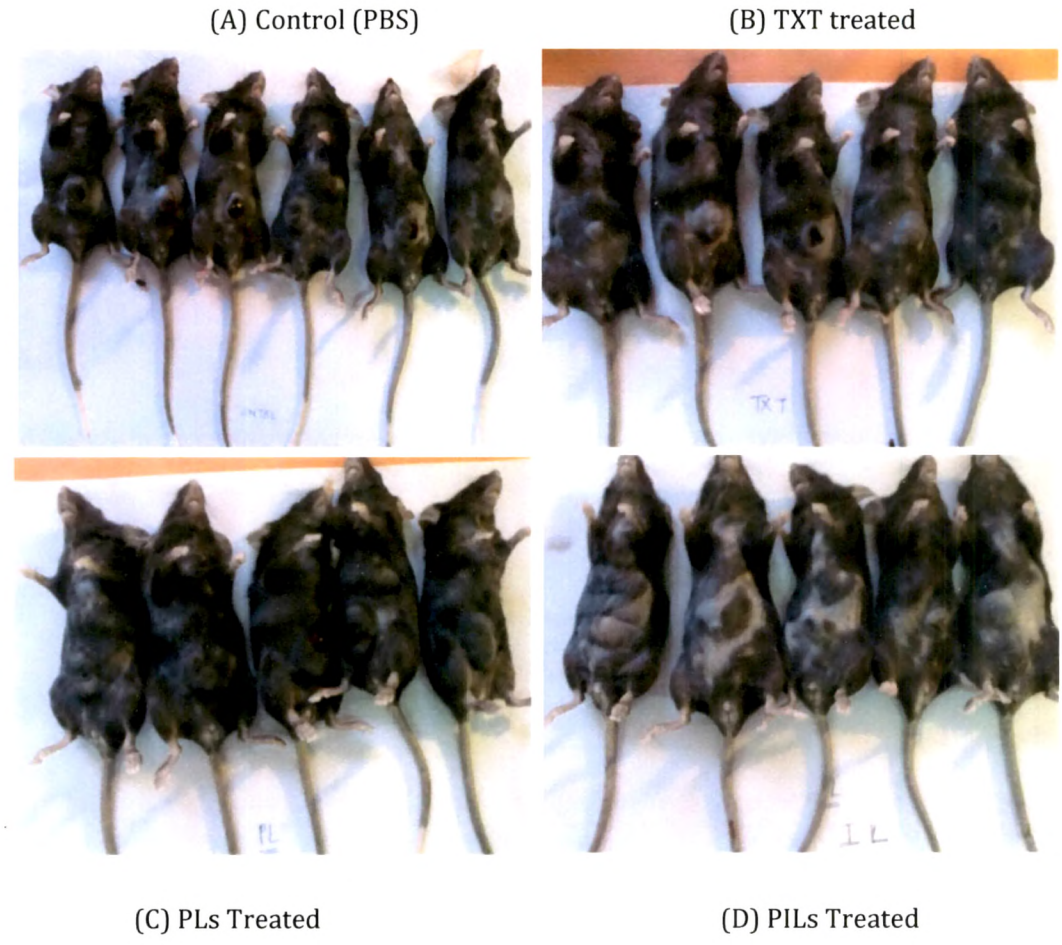


Figure 9.1. (A) PBS, (B) Taxotere (TXT), (C) PEGylated liposomes (PLs) and (D) PEGylated Immunoliposomes (PILs) treated C57BL/6 mice bearing B16F10 melanoma tumor at 14th day of experiment.

Figure 9.2. Solid tumors of control (PBS), Taxotere (TXT), PEGylated liposomes (PLs) and PEGylated immunoliposomes (PILs) treated groups after separation from the mice.

Table 9.1. Tumor volumes of control and formulations treated groups measured during the experiment

Days following tumor implantation	Tumor Volumes (in mm^3)			
	Control (PBS)	Taxotere	PLs	PILs
0*	0	0	0	0
5	9.177±4.133	7.013±2.533	14.802±5.265	5.173±2.505
7	30.501±16.276	22.778±12.113	55.684±21.134	21.023±6.82
9	113.62±47.4	68.771±45.75	91.653±41.501	38.173±12.248
11	203.485±56.239	71.246±34.973	132.55±29.902	55.778±37.472
13	351.878±66.324	109.7895±64.547	240.061±24.309	107.216±69.599
14	524.976±161.443	253.354±135.892	404.854±101.548	101.937±63.647

0*: tumor volume on day when cells were injected. Values are Mean±SD, n=5. PBS: phosphate buffer saline; PLs: PEGylated liposomes; PILs: PEGylated immunoliposomes. On 11^{th} day both Taxotere ($p<0.05$, $p=0.025$) and PILs ($p<0.05$, $p=0.013$) significantly suppressed the tumor growth as compared to control group. On 13^{th} day both Taxotere and PILs significantly suppressed the tumor growth as compared to control group ($p<0.01$, $p=0.006$). At the end of the experiment (14^{th} day) only the PILs suppressed the tumor volume significantly as compared to control group ($p<0.05$, $p=0.016$).

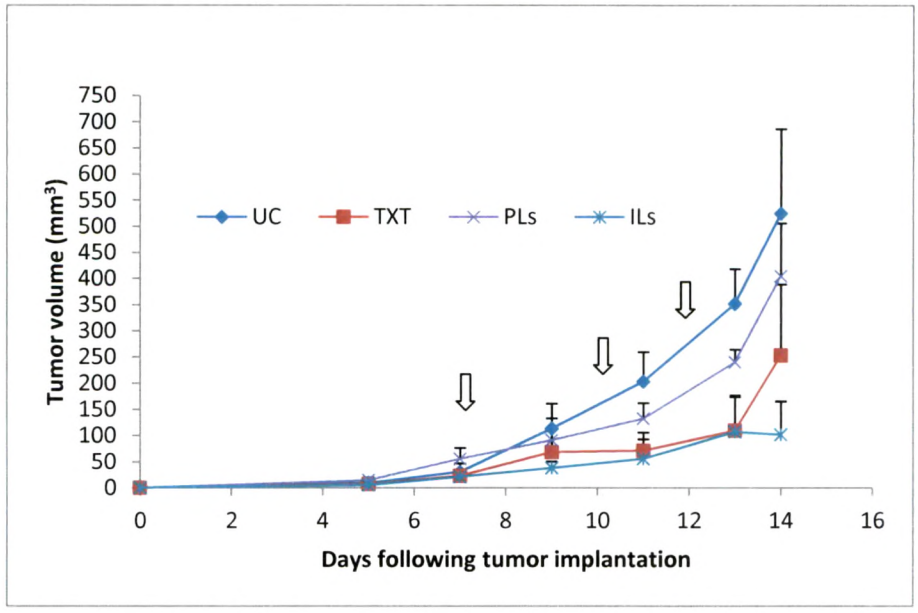


Figure 9.3. Tumor growth inhibition by multiple injections of Taxotere and DTX loaded PLs and PILs in tumor bearing C57BL/6 mice. Formulations were injected at dose of 2mg/Kg. Data are presented as the mean tumor volume (mm^3) with SD bars. The only plus bars are shown to maintain the clarity of figure. Arrows shows the day of treatment.

Table 9.2. Control and formulations treated group mice body weight measured during the experiment

Days following tumor implantation	Mice weight measured during the experiment (in grams)			
	Control (PBS)	TXT	PLs	PILs
0*	23.14±1.93	22.01±1.59	21.98±1.12	22.78±2.1
2	23.26±1.84	22.56±1.8	22.15±1.31	23.11±1.84
4	23.26±1.23	22.4±1.63	21.72±1.02	23.28±1.83
6	23.21±1.04	22.33±1.66	22.08±1.73	23.13±1.84
8	23.43±1.52	22.53±1.45	21.96±1.24	23.35±1.77
10	23.08±1.22	21.58±1.77	21.7±1.22	22.9±1.62
12	23.1±1.11	22.61±1.34	21.8±1.41	23.05±1.78
14	23.41±1.42	22.27±1.33	21.56±1.55	24.11±1.22

0*: animals weight on day when the cells were injected. PBS: phosphate buffer saline; TXT: Taxotere; PLs: PEGylated liposomes; PILs: PEGylated immunoliposomes

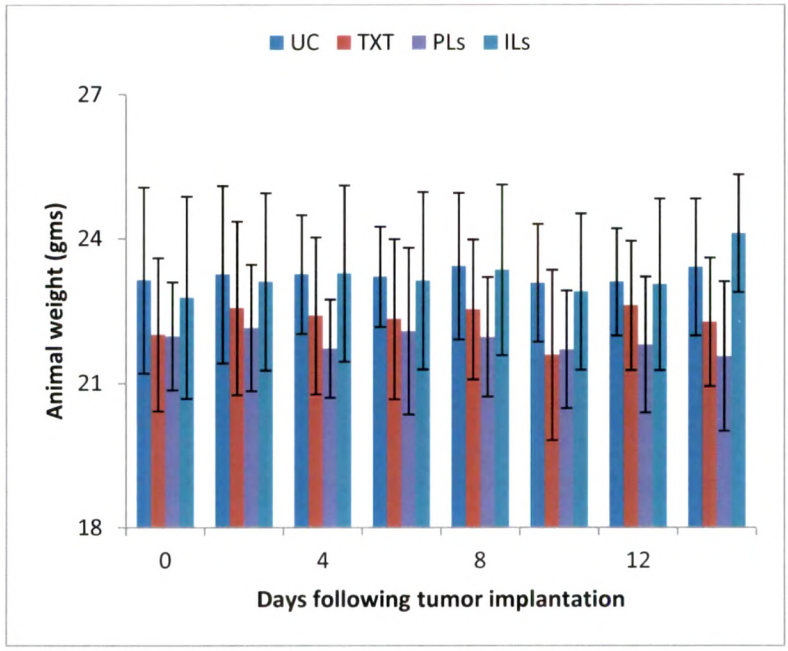


Figure 9.4. Control and formulations treated group mice body weight measured during the experiment

9.3.2. Anti-angiogenesis performance of formulations

The efficient tumor suppression performance of anti-neuropilin-1 (Fab′ fragment) antibody conjugated immunoliposomes can be attributed to delivery of DTX to both tumor endothelial cells (leading to vascular damage) and tumor cells *via* the over expressing neuropilin-1 receptor on both the cells. Hence, to confirm the vascular damage performance of PILs the same tumor model was used in which the skin containing solid tumor was collected, spreaded on whatman paper, stapled, photographed and the number of angiogenic vessels around the solid tumor were counted manually using the photographs (Figure 9.5). The Figure 9.6 shows the micro-vessel density around the solid tumors treated with Taxotere and liposomal formulations.

The PLs treated group showed micro-vessels of 8.6±3.84 number around the solid tumor. The number of micro-vessel density was decreased significantly around the solid tumors treated with Taxotere (6.8±2.28; $p<0.05$, $p=0.027$) and PILs (6.6±2.64; $p<0.05$, $p=0.034$) as compared to control group (12.6±1.94). This significant decrease in micro-vessel density would be correlated to the strong tumor growth suppression by these Taxotere and PILs treated groups. The further significant suppression of tumor growth by PILs, as compared to Taxotere, would be attributed to long circulation time and rapid B16F10 melanoma uptake of PILs *via* neuropilin-1 receptor mediated endocytosis as compared to Taxotere which would rapidly cleared from the circulation and remain more in the tumor interstitial space. This combined strategy of tumor blood vessel

damaging and direct cancer cell killing *via* over-expressing neuropilin-1 receptors leads to significant tumor growth suppression as compared to conventional treatment.

In Figure 9.5 we can clearly distinguish the denser and well branched tumor blood vessels with control group as compared to Taxotere and PILs treated group. We observed very thick blood vessels in PLs treated group as compared to other treated groups including control group (Figure 9.5). Also, these thick vessels are well branched just near and around the tumor as compared to other groups and supply sufficient blood leading to decreased suppression of tumor growth during the treatment with PLs. Also, the PLs will not uptake by both endothelial and tumor cells like PILs, leading to no tumor blood vessel damage and more tumor growth. The delivery of liposomal Doxorubicin (DXR) to tumors by passive targeting (not expected to bind both endothelial cells and tumor cells directly) is the mechanism of action of the successful clinical liposomal drug, Doxil/Caelyx (Muggia and Hamilton, 2001). Thus the potential dual action of our neuropilin-1 receptor-targeted DTX loaded PEGylated liposomes may result in a higher and more sustaining anticancer effect than a target delivery system based on EPR effect only.

Although our studies have been performed in a mouse model, we expect the humanised anti-neuropilin-1 monoclonal (Fab' fragments) antibody conjugated immunoliposomes can target human vasculature as well as tumor cells which over express this protein. Most of the cancer cells do over express this protein hence it will be a common target for the treatment of all types of solid tumors in patients. In conclusion, the targeting of the tumor vasculature could be the basis of a new pharmacological approach for the treatment of malignancies by taking advantage of formulations that deliver cytotoxic drugs to both blood vessels located specifically at sites of disease and to the tumor cells. This could improve the therapeutic efficacy and reduce the side effects.

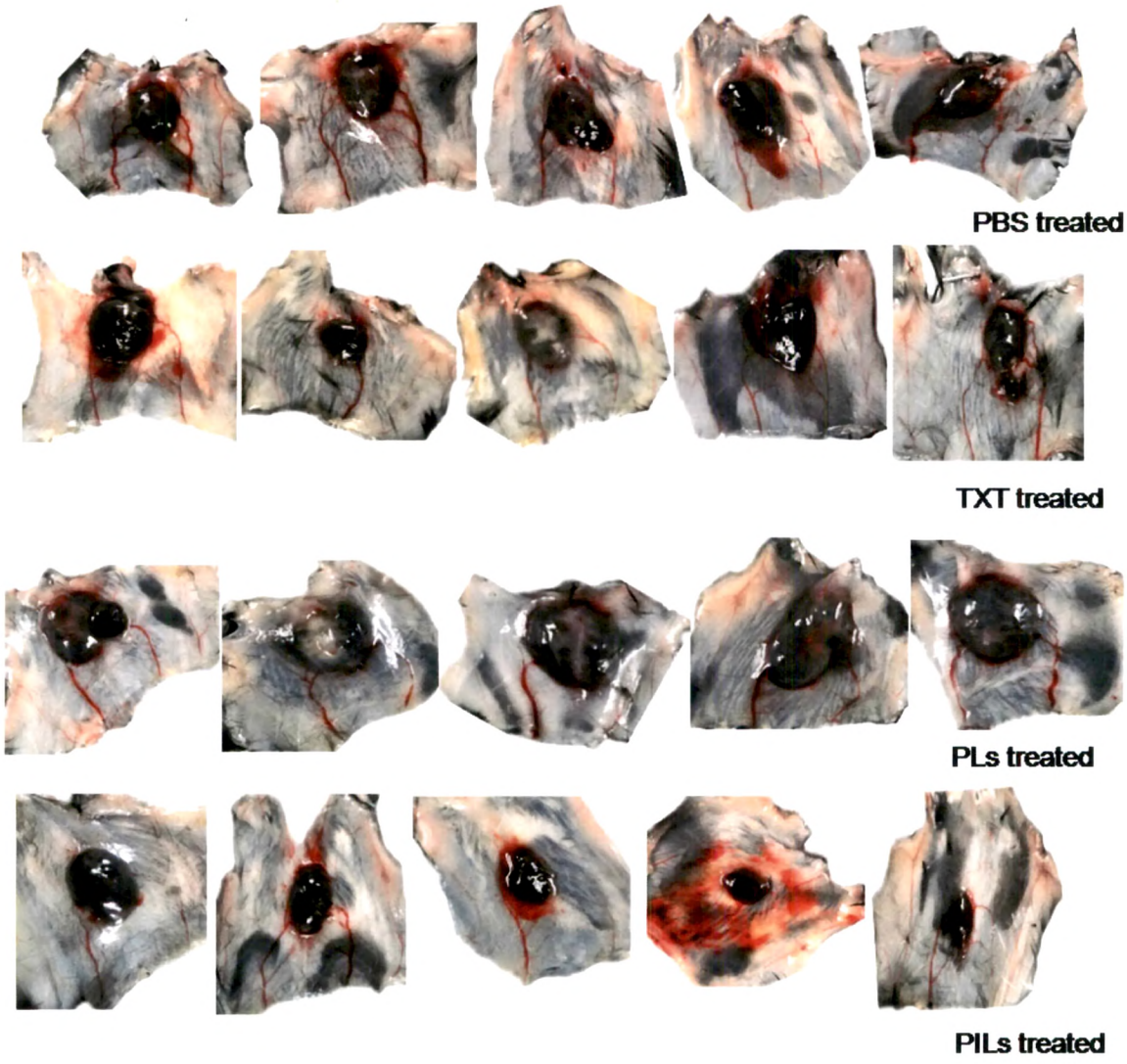


Figure 9.5. The mouse skin attached with solid tumor showing the blood vessels around the tumor.

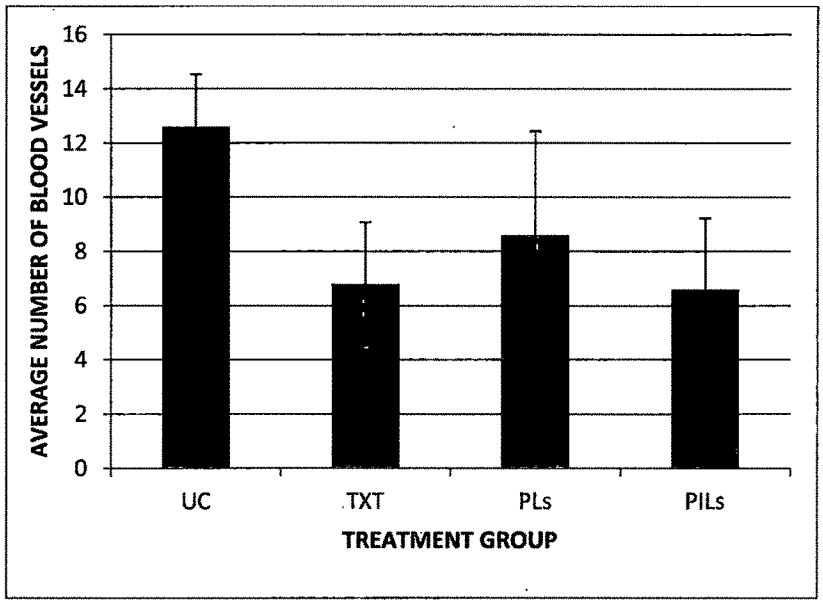


Figure 9.6. The micro-vessel density around solid tumors treated with different formulations. The Taxotere ($p<0.05$, $p=0.027$) and PILs ($p<0.05$, $p=0.034$) treated group showed significant decrease in micro-vessel density as compared to control group.

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