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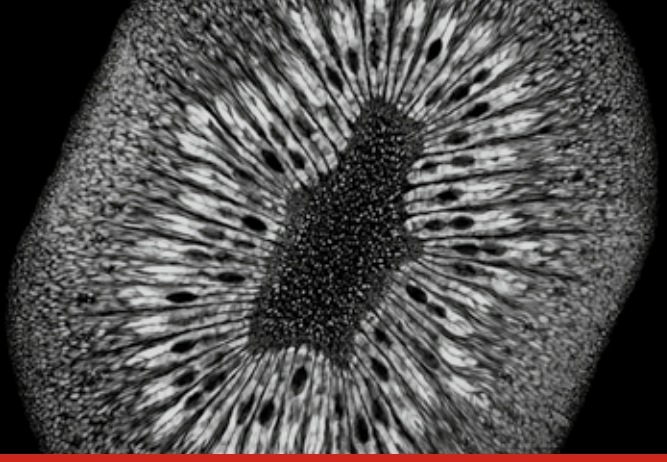

Monitoring of tumor vascularization

Longitudinal µCT tumor angiography by ExiTron™ nano 12000

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Introduction

Angiogenesis is the biological process through which new blood vessels are formed from pre-existing vessels. It is a complex, multi-step process involving endothelial cell proliferation, migration and differentiation and plays a vital role in the normal course of growth as well as in wound healing[1]. Angiogenesis, however, also plays a fundamental role in the growth and malignancy of tumors[2]. Through secretion of angiogenic activators (e.g. *vascular endothelial growth factor, VEGF*), tumors induce angiogenesis, which in turn is believed to enable the tumor tissue to acquire oxygen and nutrients and/or to eliminate metabolic waste. Consequently, tumor angiogenesis allows tumors to thrive and grow. Besides enabling tumor growth, angiogenesis is also responsible for the transformation of tumors from a benign to a malignant state[3]. Via migration of single cancer cells from a solid tumor into the tumor vascular network, the cancer cells can be carried to various locations throughout the body, where they can implant, leading to metastasis formation. Indeed, the vascularization level of a solid tumor is thought to be an excellent indicator of its metastatic potential.
In the area of oncology, a strong focus is currently being placed on the development of agents that act as angiogenesis inhibitors, suppressing the formation of new tumor blood vessels so as to reduce tumor growth and limit malignancy potential[4]. In our study we use micro-computed tomography (µCT) and the Viscover™ contrast agent ExiTron™ nano 12000, to longitudinally image the ability of a peptide, termed TAX2[5], to suppress the growth and vascularization of tumors in two different experimental murine cancer models.

Materials and Methods

Peptides
The TAX2 peptide (CEVSQLLKGDAC), and the scrambled peptide used as a control (LSVDESKAQGIL) were synthesized and purified by Genecust (Dudelange, Luxembourg).

Animals
Subcutaneous melanoma tumor model - For the subcutaneous melanoma tumor model, suspensions of 2.5×10^5 B16F1 cells were subcutaneously injected into the left flank of 8-week-old female C57BL/6J mice (n = 8-10 per group), as previously described[6]. Intraperitoneal administrations of a 100 µM solution of either TAX2 or control peptides in physiological saline were performed at a dose of 10 mg peptide/kg bodyweight at days 3, 5 and 7 after melanoma cell injection. ***Human pancreatic carcinoma xenograft model*** - For the human pancreatic carcinoma xenograft model, 3×10^6 MIA PaCa-2 cells were implanted subcutaneously into the left flank of 8-week-old female BALB/C nu/nu mice (n=9 per group). TAX2 (100 µM in physiological saline) at a dose of 10 mg peptide/kg bodyweight or control (vehicle, physiological saline) were injected intraperitoneally 3 times a week during 4 weeks starting at day 10 after inoculation of tumor cells.

µCT
In vivo µCT was performed on isoflurane-anaesthesized tumor-bearing mice at days 7, 10 and 14 after melanoma cell inoculation and at days 24, 31 and 38 after pancreatic carcinoma cell inoculation. CT images were acquired on a dedicated small animal µCT scanner (Skyscan 1076, Bruker, Kontich, Belgium) while continuously rotating the camera by 180° with the following parameters: 50 kV, 0.5 mm Al filter, 200 µA source current, 35 µm isotropic resolution, 180 ms exposure time, 4 projection images per 0.7° rotation step and a retrospective synchronization. The projections were reconstructed using a filtered backprojection algorithm using Skyscan software (NRecon, Skyscan). Tumor angiography was performed after tail vein injection (5 ml/kg bodyweight) of the CT contrast agent, ExiTron™ nano 12000 (Viscover™, *nanoPET Pharma* GmbH, Germany). ExiTron nano 12000 is an alkaline-earth metal-based nanoparticulate contrast agent exhibiting a very high blood half-life of

up to 4 hours in mouse. Mice were imaged during the first 30 min post imaging agent application, a time period during which no reduction in contrast was observed. Analysis of reconstructed images and quantification of the vascular network were performed using Amira 5.4.3 software (Visualization Sciences Group, Burlington, MA, USA).

Results and Discussion

Subcutaneous melanoma tumor model - To investigate the ability of the TAX2 peptide to suppress tumour vascularization, melanoma-bearing mice were assigned to therapeutic regimens and longitudinal µCT tumor angiography using ExiTron nano 12000, was performed[7]. Whereas tumor vascular structures were easily visualized in control mice at day 7 after melanoma cell injection, blood vessels were undetectable within tumors of TAX2-treated mice (Fig. 1A, left panel). Intratumoral vascularization remained significant within growing tumors of control mice, while it appeared restricted to tumor periphery under TAX2 treatment at day 10 (Fig. 1A, middle panel). At day 14, a central vascular network within tumors of TAX2-treated mice was still lacking and a haemorrhagic necrotic zone was observed (Fig. 1A, right panel). 3D reconstructions 14 days after tumor cell inoculation revealed a mature peritumoral and intratumoral vascular network in control mice (Fig. 1B, upper panel). In contrast, after TAX2 treatment, tumor vasculature appeared highly discontinuous, located only at the tumor surface (Fig. 1B, lower panel). Segmentation and quantification of the tumor-associated vascular network revealed that the TAX2 peptide induces a 2-fold reduction in blood vessel mean volume through a significant reduction of both the length and diameter of blood vessels (Fig. 1C). These findings were corroborated by histological analysis (data reported elsewhere[5]), implying that the TAX2 peptide suppresses tumor vasculature, thus acting as an antiangiogenic agent.

Human pancreatic carcinoma xenograft model - To further confirm efficacy of TAX2 as an antiangiogenic agent, the study was extended to include a second, less aggressive mouse cancer model with increased clinical relevance[8],

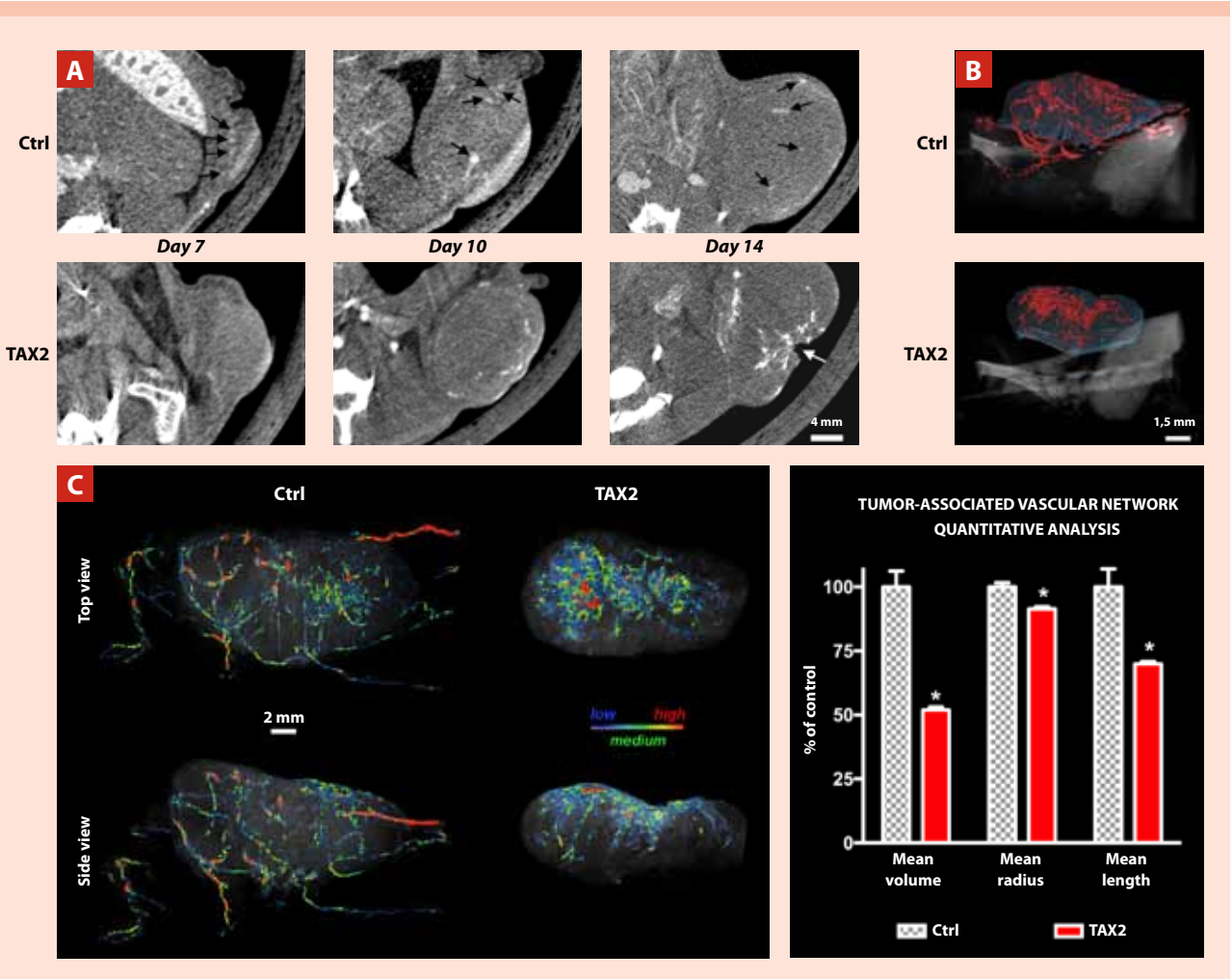

Figure 1: TAX2 peptide impedes tumor angiogenesis in a subcutaneous mouse melanoma tumor model.
A. Longitudinal µCT tumor angiography performed using the CT contrast agent, ExiTron nano 12000 at days 7, 10 and 14 after inoculation of tumor cells in control (Ctrl) and TAX2-treated mice (TAX2). Black arrows illustrate contrast enhancement of intratumoral blood vessels; white arrow indicates tumor necrosis (representative of n=5). **B.** 3D reconstructions of tumors at day 14, showing tumor (blue) and its associated vascular network (red). **C.** Color-coded representation of tumor-associated blood vessel network depending on structure thickness. Histogram displays quantification of tumor mean blood vessel volume, radius and length expressed as a percentage of control. Data is expressed as the mean±SE (*p<0.05, t test).

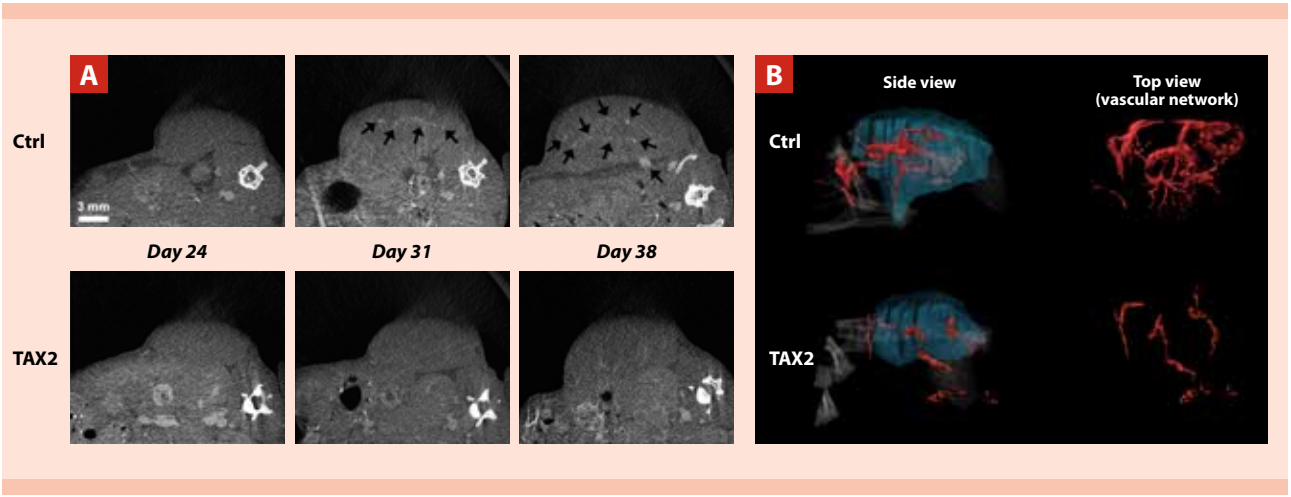

Figure 2: TAX2 suppresses tumor vascularization in a human pancreatic carcinoma xenograft model.
A. Longitudinal µCT tumor angiography performed using the CT contrast agent, ExiTron nano 12000 at days 24, 31 and 38 after inoculation of tumor cells in control (Ctrl) and TAX2-treated mice (TAX2). Black arrows illustrate contrast enhancement of intratumoral blood vessels (representative of n=5). **B.** 3D reconstructions of tumors at day 38 showing the human pancreatic carcinoma xenograft (blue) and the associated vascular network (red).

namely, human pancreatic carcinoma xenograft. The xenograft-bearing mice were assigned to therapeutic regimens and tumors were monitored longitudinally via contrast-enhanced *in vivo* µCT using ExiTron nano 12000. Compared to control animals, tumor angiography of TAX2-treated mice performed at days 24, 31 and 38 after pancreatic cell inoculation clearly showed that the peptide caused a significant decrease in tumor vascularization (Fig. 2A). Furthermore, 3D reconstructions at day 38 confirmed that TAX2 strongly inhibits growth of the tumor-associated vascular network (Fig. 2B).

Conclusion

In this study we use contrast-enhanced µCT to longitudinally monitor the ability of the TAX2 peptide to suppress tumor vascularization in two different murine cancer models. The results demonstrate that the applied contrast agent, ExiTron™ nano 12000, is highly optimized for angiography due to its prolonged high vessel contrast, presenting cancer researchers with an innovative tool for the longitudinal and quantitative monitoring of tumor vasculature *in vivo*.

Viscover™ Product	Order No.
ExiTron nano 6000, 5 injections	130-095-146
ExiTron nano 6000, 25 injections	130-095-147
ExiTron nano 12000, 5 injections	130-095-698
ExiTron nano 12000, 25 injections	130-095-700
ExiTron P, 5 injections	130-095-144
ExiTron P, 25 injections	130-095-145
ExiTron U, 5 injections	130-095-142
ExiTron U, 25 injections	130-095-143
ExiTron V, 5 injections	130-095-283
ExiTron V, 25 injections	130-095-284

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