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RESEARCH ARTICLE

# Use of Ixolaris as a Diagnostic Tool for Cancer Models

**Sergio Augusto Lopes de Souza[1], Thiago Barboza[1], Bianca Gutfilen[1], Robson de Queiroz Monteiro[2].**

[1] Universidade Federal do Rio de Janeiro, Faculdade de Medicina, Departamento de Radiologia, Rio de Janeiro, Brasil
[2] Universidade Federal do Rio de Janeiro, Instituto de Bioquímica Médica Leopoldo de Meis, Laboratório de Trombose e Câncer, Rio de Janeiro, Brasil

* sergioalsouza@gmail.com

## ABSTRACT

Ixolaris is a tick salivary protein that binds to Factor Xa or Factor X as a scaffold for inhibition of the Tissue Factor (TF)/Factor VIIa (FVIIa). TF is not normally expressed in cells that are in direct contact with blood. However, TF is abnormally expressed under several pathological conditions, including inflammation, infectious diseases and cancer. It is known for decades that TF is a risk factor for metastasis, and in mouse models, TF drives metastasis in a coagulation-dependent manner. For these reasons, TF has recently been described as a potential target that can be exploited to image aggressive tumors. Ixolaris has been succcesfully used to treat some pre-clinical cancer models. It also has been shown its' radiolabeling for diagnostic and therapeutic purposes. The aim of this article is to show that Ixolaris could be an interesting tool to diagnose cancer, and also treat it in a near future. Some key findings presented here are that TF has recently been described as a potential target that can be exploited to image aggressive tumors, once it is often associated with migration, invasion, proliferation, metastasis, the inhibition of apoptosis and the production of several pro-aggressive factors.

**Ixolaris**

Ixolaris is a tick salivary 140-amino acid protein containing ten cysteines and two Kunitz-like domains identified in Ixodes scapularis that presents sequence homology to TFPI (tissue factor pathway inhibitor)[1]. Ixolaris has 2 Kunitz like domains and binds to Factor Xa or Factor X as a scaffold for inhibition of the Tissue Factor (TF)/Factor VIIa (FVIIa)[1]. However, Ixolaris does not bind to the active site cleft of FXa. Instead, complex formation is mediated by the FXa heparin-binding exosite. A remarkable feature that differentiates Ixolaris from the human tissue factor pathway (TFPI) is the ability to interact with FX, the zymogen form of FXa, possibly through a precursor state of the heparin-binding exosite. In addition to TFPI and other physiologic inhibitors of blood coagulation (e.g., antithrombin III), several exogenous coagulation inhibitors from the salivary gland of blood-sucking invertebrates have been characterized[2,3].

**Expression**

Ixolaris was first obtained by inducing partially engorged adult female Ixodis scapularis to salivate into capillary tubes using the modified pilocarpine induction method [4]. Further, recombinant protein was expressed in high five insect cells[5], and more recently in E. coli sed[6]. De Paula et al described an efficient strategy to obtain recombinant Ixolaris as a fusion protein with Thioredoxin (TRX) in E. coli to generate non-glycosylated protein for an NMR study. The pET32 vector uses TRX as the fusion partner, which can increase expression level, stability and solubility.

**Biological context**

The blood coagulation cascade is a major mechanism for maintaining homeostasis in the body. Several studies have shown that cancer patients may experience changes in blood clotting conducting to a pro-thrombotic state, which consists in one of the main causes of death in these individuals[7]. In addition, local and systemic blood coagulation activation favors several steps of tumor progression[7]. Due to its potent and long-lasting antithrombotic activity, Ixolaris was proposed as a promising agent for anticoagulant therapy. Following vessel injury, the exposure of membrane-bound tissue factor (TF) is a crucial step in the initiation of blood coagulation[8]. TF is not normally expressed in cells that are in direct contact with blood. However, TF is abnormally expressed under several pathological conditions, including inflammation, infectious diseases and cancer [10–12]. The expression of TF is often associated with the increased production of pro-angiogenic factors, intimately related to a group of G protein-coupled receptors known as protease-activated receptors, which elicits a vast number of cellular responses in cancer cells, including migration, invasion, proliferation, metastasis, the inhibition of apoptosis and the production of several pro-aggressive factors[13,14]. For these reasons, TF has recently been described as a potential target that can be exploited to image aggressive tumors.

**Action mechanism**

It is known for decades that TF is a risk factor for metastasis, and in mouse models, TF drives metastasis in a coagulation-dependent manner. TF also serves as a cellular receptor to drive primary tumor growth and tumor angiogenesis, regulates tumor cell dormancy, is associated with lancer stem cell behavior, epithelial-to-mesenchymal transition, and dictates establishment of the tumor cell premetastatic niche, but there are no clear ideas on the exact molecular pathways that are initiated by TF. Experimental evidence points out that the linking pin between the blood coagulation cascade and cancer is the glycoprotein TF. Simultaneously to TF-FVIIa complex formation, several coagulation proteases activate the protease-activated receptors (PARs) to further facilitate efficient coagulation and repair. TF is upregulated in a variety of cancer types. Many studies have attempted to associate TF overexpression by tumor cells with clinical parameters, and most patient-based studies found that TF expression correlates with higher grade, reduced survival and invasive/metastatic behavior in epithelial cancers [15]. Blockade of TF function using specific antibodies or downregulation of TF expression generally results in strongly diminished tumor expansion in various mouse models for cancer[16]. TF-FVIIa dependent signal transduction induces the production of MMP that breakdown the basal membrane, allowing tumor cells to escape and invade the surrounding tissue. TF also plays a role in invasion by stimulating cell migration, once intravasated TF+ tumor cells activate

coagulation locally to a form a fibrin/platelet shield that protects the cell against NK cells [15]. Ixolaris, a non-immunogenic protein, and an anticoagulant derived from tick saliva, has extensive homology with the inhibitor of the TFPI and has anti-tumor activity probably derived from its ability to interact with tumor-expressed TF and the subsequent inhibition of PAR2 signaling[17]. Ixolaris treatment aims to target tf and treat cancer, avoiding its metastatization. There are many studies showing the potential of targeting TF as an anti-cancer treatment. Scaffner and Ruf showed that fITF-mediated signaling affects different aspects of cancer biology[13]. Monoclonal antibody anti-fITF antibody reduced tumor growth in aggressive breast cancer anti-fITF antibody 5G9 mediated blocking of coagulation inhibited hematogenous metastasis. HU et al showed the use of FVII/Fc immunoconjugates directed against TF in a human melanoma mouse xenograft model (in vivo) resulting in reduced tumor growth[18]. Blocking coagulation activity by either monoclonal anti-TF antibodies or TFPI has been reported to inhibit experimental lung metastasis. Amarzguioui tested short interfering RNA (siRNA) in order to prove evidence that TF drives pulmonary metastasis in melanoma models (B16F10) [19]. In this study, it has been shown that transient knockdown of TF mRNA in the B16 cells in vitro by 70% to 80% dramatically reduced the number of pulmonary tumors on days 10, 15, and 20 after the injection of siRNA transfected cells; confirming that the observed delay in tumor development was indeed due to the reduction in the level of TF expression in target cells rather than a result of some unspecific effects of siRNA. There is also data to state that TF promotes melanoma metastasis by a pathway independent of blood coagulation[20,21]. Bromberg compared two sets of TF lines regarding there metastatic potential by injecting human melanoma cell lines (Yu-sit1, Yu-tac7, Yu-zaz6) into the tail vein of SCID mice. Metastatic tumors were detected in 86% of the mice injected with low-TF lines, indicating that a high-TF level promotes metastasis of human melanoma in the SCID mouse model.TF effect on metastasis occurs with iv-injected melanoma cells, suggesting that TF acts at a late stage of metastasis after tumor cells have escaped from the primary site and entered the blood. Considering it, Ixolaris would act well in patients with advanced disease regarding therapy and/or could be an early diagnostic marker of metastasis in newly diagnosed patients. Because death from cancer is most often caused by metastatic rather than primary tumors, understanding the metastatic process is a principal concern of cancer research. Amirkhosravi using a murine experimental model found that iv injection of recombinant murine tissue factor pathway inhibitor immediately before the introduction of tumor cells reduced metastasis by 83%. No difference in primary tumor growth was observed between TFPI+ and control cells. It seems that the relation is only with metastasis growth, the primary tumor growth suffers no influence [22]. At least three distinct events are required in order to generate pulmonary nodules. These include tumor cell residency, tumor cell implantation, and tumor cell growth within the lung parenchyma. It is not difficult to imagine a role for TF in tumor cell adhesion and implantation given that the extracellular, factor VIIa binding domain has been implicated in mediating interaction with matrix-associated TFPI and in generating protease activity required for tumor cell invasion [23]. It is also important to look at the proteolytic activity of TF/FVIIa. Wang et al. showed that the proteolytic activity of TF/FVIIa is important for TF-dependent metastasis in a murine model. This was shown by the injection of cells transfected with a mutated TF gene, in which FVIIa can no longer bind to TF [24]. Ixolaris also plays a role in this kind of metastasis, once whether TF can't bind FVIIa nothing happens [25], Ixolaris specifically binds to FXa heparin-binding exosite, This interaction also results in a decrease in the productive recognition of prothrombin as substrate by FXa either in the absence or in the presence of FVa. Complex formation between ixolaris and FX strongly decreases the zymogen activation by the intrinsic tenase complex (FIXa/FVIIIa). At this point ixolaris probably impairs FX-FVIIIa interactions, thus decreasing the productive recognition of the zymogen as substrate by the intrinsic tenase complex. The use of TF/FVIIa inhibitors block angiogenesis and tumor growth through a nonhemostatic mechanism [26]. Hembrough showed that both TFPI and nematode anticoagulant protein rNAPc2, inhibit both primary and metastatic tumor growth in mice. Clinical studies demonstrating potential antitumor effects of coagulation inhibitors have generated renewed interest in developing anticoagulants as antitumor agents. Lee et al

showed that the antitumor activity of rNAPc2 was sent doses significantly higher than necessary for anticoagulant activity, this identification of this nonhemostatic activity of TF/FVIIa provides a way to completely separate the protumor and procoagulant activities of TF/FVIIa [27].

**Ixolaris studies**

Ixolaris has been used in different pre-clinical models. Carneiro-Lobo et al showed that blocking of TF activity with the tick anticoagulant Ixolaris interfered with glioblastoma progression[28]. TF was identified in U87-MG cells by flow-cytometric and functional assays (extrinsic tenase). It was shown that Ixolaris is highly efficient in inhibiting the U87-MG-assembled extrinsic tenase complex. Therefore, the antitumor effect of Ixolaris is likely to be attributable to the suppression of tumor-associated FVIIa/TF complex activity. This is supported by other studies showing that: (i) xenograft models employing a specific anti-human TF showed that suppression of tumor- but not host-derived TF coagulant activity is sufficient to impair primary tumor growth; (ii) low-TF mice exhibit unaltered growth of TF-expressing tumor cell lines, as compared with wild-type mice.

Oliveira et al showed that Ixolaris reduced primary tumor growth and experimental metastasis in a murine model of melanoma[29]. The effect of Ixolaris on the metastatic potential was further estimated by intravenous injection of B16F10 cells in C57/BL6 mice. Ixolaris (250 µg/kg) dramatically decreased the number of pulmonary tumor nodules compared to the control group. Furthermore, a significant decrease in tumor weights was observed in primary tumor growth assays. Immunohistochemical analyses also showed that inhibition of melanoma growth by Ixolaris is accompanied by a significant downregulation of both VEGF expression and microvascular density in the tumor mass.

Regarding Breast cancer, Ixolaris blocks the TF coagulation initiation complex on breast cancer cells (MDA-MB-231mfp). Ixolaris potently inhibited the procoagulant activity of human MDA-MB-231mfp or murine PyMT breast cancer cells. Ixolaris blocked signaling by the ternary TF–FVIIa–FXa complex, and, surprisingly, at higher concentrations also the binary TF–FVIIa complex on MDA-MB-231 cells. In this study Carneiro-Lobo et al. suggested that Ixolaris may block tumor growth of human cell models with ectopic FVIIa expression through inhibition of direct TF–FVIIa–PAR2 signaling as its anticoagulant activity[17].

Another role for Ixolaris was presented by Barboza et al. in a study radiolabeling Ixolaris with Tc-99m in order to develop a new diagnostic radiopharmaceutical. Here, we evaluated the ability of 99mTc-Ixolaris to target tumor-derived TF using an orthotopic GBM model in mice[30]. No 99mTc-Ixolaris uptake was observed in the brain of tumor-free mice, independently of the integrity of brain–blood barrier. In contrast, the presence of TF-expressing brain tumor masses determined a significant 99mTc-Ixolaris uptake. Figure 1 shows an example of 99mTc-Ixolaris as a diagnostic tool in a model of melanoma. Later we've studied Ixolaris as a substrate for the development of a Theranostic radiopharmaceutical [31]. To evaluate the anti-metastatic ability of 131I-Ixolaris, animals were divided in three experimental groups: D0, receiving only one dose of 131I-Ixolaris, D15, which received one dose of 131I-Ixolaris fifteen days after melanoma induction, and D1-D15, which received two therapeutic doses of 131I-Ixolaris. The best strategy was D1-D15.The results obtained with 131I-Ixolaris demonstrate its ability to recognize TF-expressing tumors, which concentrates the emission of β-particles from the radionuclide into the tumor microenvironment. In fact, by targeting TF, 131I-Ixolaris may directly interfere with tumor progression by impairing TF-mediated signaling, which has been associated with several pro-tumoral responses, including angiogenesis and production of cytokines such as interleukin-8 and VEGF.

**Conclusion**

Taken together, the data shown here elicits that Ixolaris may directly interfere with tumor progression by impairing TF-mediated signaling. These results also shows the potential to translate the use of Ixolaris for a clinical trial for both diagnostic and therapeutic purposes.

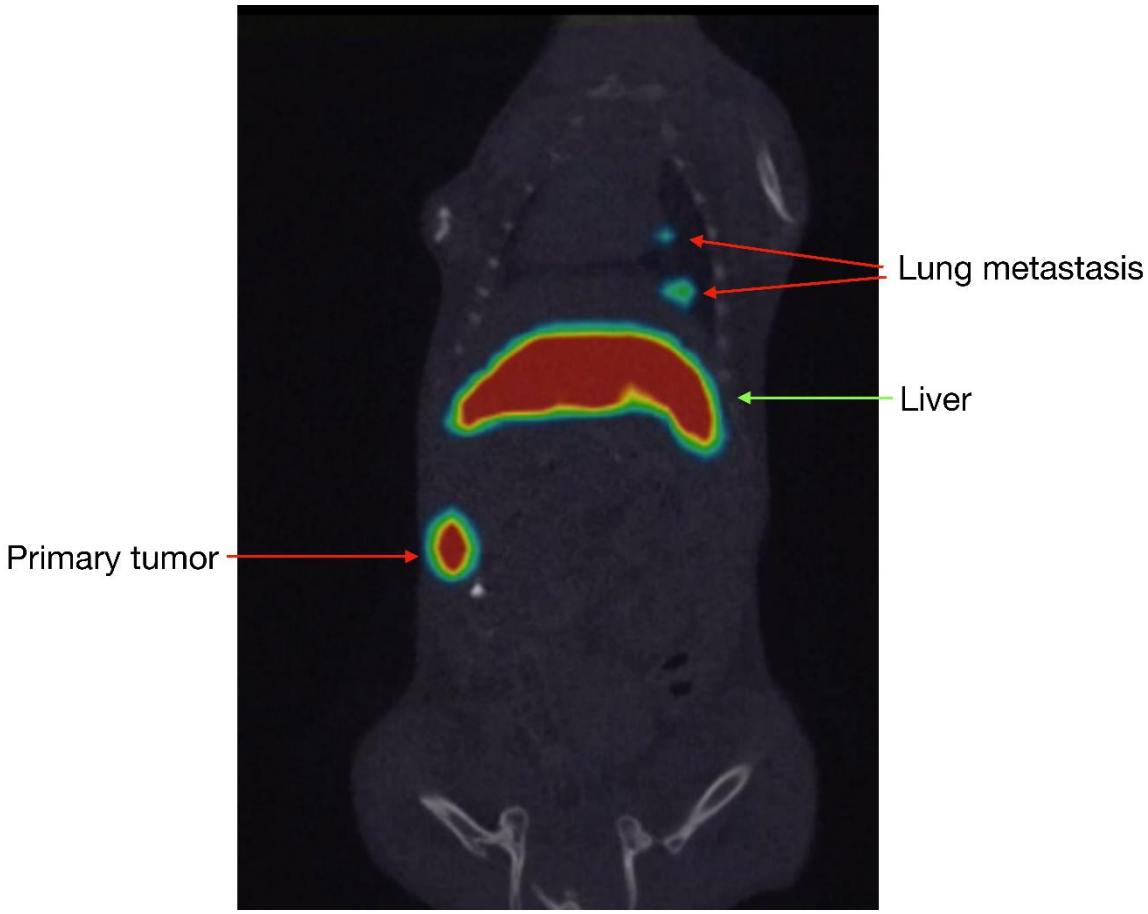


**Figure 1:** 99mTc-Ixolaris microSPECT/CT showing liver uptake (normal biodistribution) and the primary and tumor and lung metastasis one hour after intravenous administration of the radiopharmaceutical.

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