# Tigilanol Tiglate is a naturally occurring small molecule oncolytic that effectively ablates tumors via intratumoral injection and can enhance response to immune checkpoint blockade

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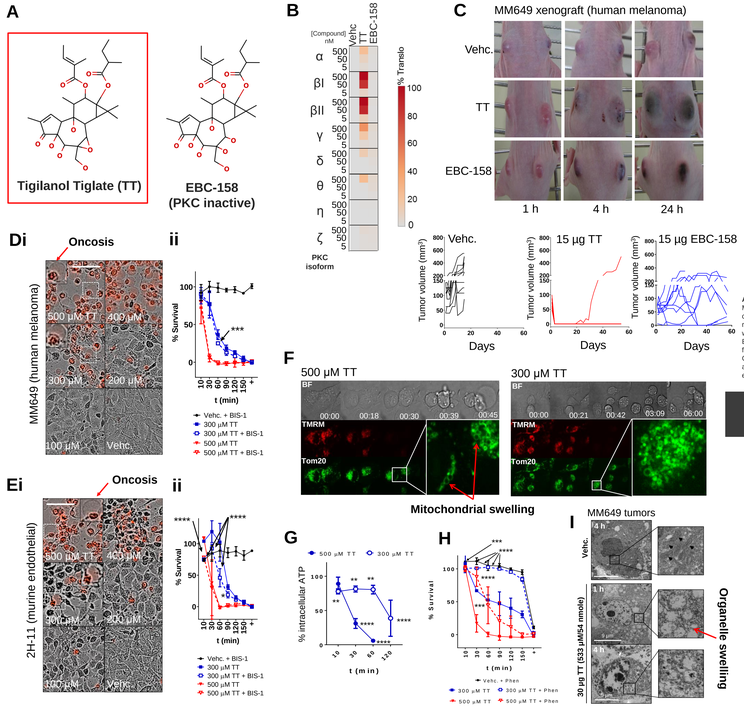
## Abstract

**Background:** Tigilanol Tiglate (TT) is a novel small molecule in development for the local treatment of solid tumors via intratumoral (IT) injection. TT is a protein kinase C (PKC)/C1 domain activator that disrupts tumor vasculature leading to haemorrhagic necrosis of the lesion. Strikingly, in both preclinical murine models and canine cutaneous/subcutaneous tumors presenting in the veterinary clinic, IT injection of TT induces a complete response with nil recurrence in >70% of injected tumors[1,2,3]. TT has completed a Phase I/IIa dose-escalation trial in 22 patients (ACTRN12614000685617), where the disease control rate (CR/PR/SD) was 100% when tumors received the full treatment rate. Two patients also showed signs of an abscopal effect, suggestive of the development of a systemic anti-tumor immune response[4]. However, the underlying mechanism of action (MOA) of TT, together with its immunotherapeutic potential in oncology, is not fully understood.

**Methods:** A combination of microscopy, immunofluorescence, immunoblotting, subcellular fractionation, intracellular ATP assays, phagocytosis assays and mixed lymphocyte reactions were used to probe the MOA of TT *in vitro*. TT-mediated damage associated molecular pattern (DAMP) release/externalization was assessed using luciferase (ATP), ELISA (HMGB1), flow cytometry and immunohistochemical (calreticulin) approaches. *In vivo* studies with TT utilized MM649 xenograft, CT-26 and immune checkpoint inhibitor (ICI) refractory B16-F10-OVA tumor bearing mice, with or without anti-PD1/anti-CTLA4 mAb treatment.

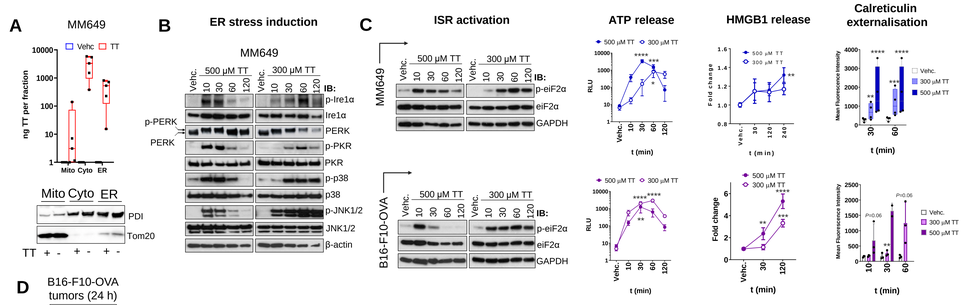
**Results:** Our data demonstrates that therapeutic concentrations of TT induce the death of cancer and endothelial cells, both *in vitro* and *in vivo*, via oncosis. Whilst largely PKC-independent, PKC/C1 domain signaling appears necessary for timely oncolysis *in vitro* and efficacious tumor ablation *in vivo*. Data also show that TT binds to ER membranes, causing ER stress with subsequent activation of the integrated stress response (ISR). This is followed by mitochondrial membrane potential loss, ATP depletion, organelle swelling, oncosis and terminal necrosis. We also found that TT treatment promoted the release/externalization of DAMPs (HMGB1, ATP, calreticulin) from cancer cells *in vitro* and *in vivo*, characteristics indicative of immunogenic cell death (ICD). Rechallenge of CT-26 tumor bearing mice showed that TT induced the production of tumor-specific T cells, preventing distal tumor growth. TT also reduced tumor volume, induced immune cell infiltration, as well as improved survival in B16-F10-OVA tumor bearing mice when combined with immune checkpoint blockade.

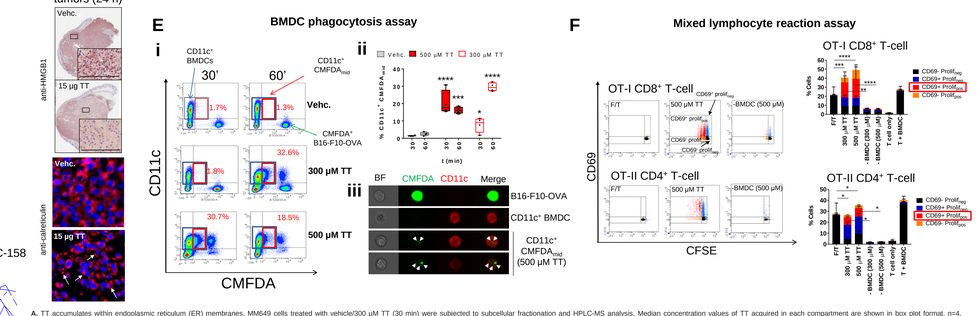
## TT induces oncosis in endothelial and tumor cells at therapeutically relevant concentrations



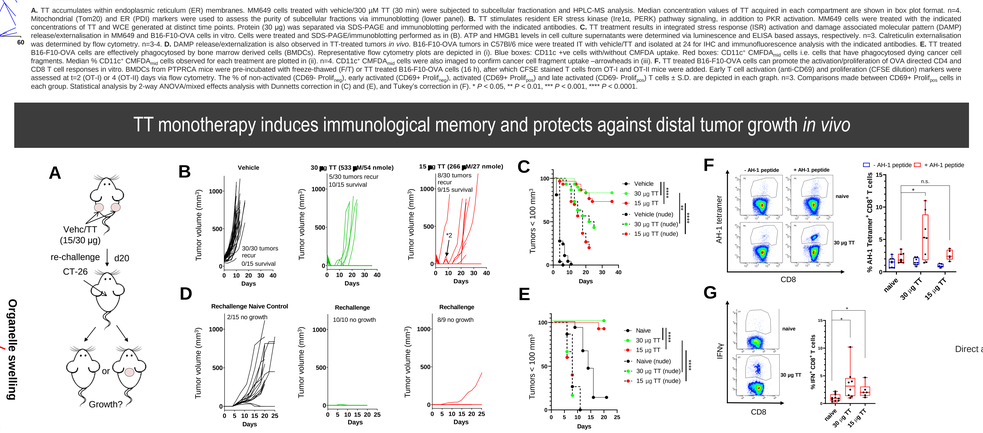

**A.** TT accumulates within endoplasmic reticulum (ER) membranes. MM649 cells treated with vehicle/300 µM TT (30 min) were subjected to subcellular fractionation and HPLC-MS analysis. Median concentration values of TT acquired in each compartment are shown in box plot format. n=4. Mitochondrial (Tom20) and ER (PDI) markers were used to assess the purity of subcellular fractions via immunoblotting (lower panel). **B.** TT stimulates resident ER stress kinase (Ire1α, PERK) pathway signaling, in addition to PKR activation. MM649 cells were treated with the indicated concentrations of TT and WCE generated at distinct time points. Protein (30 µg) was separated via SDS-PAGE and immunoblotting performed with the indicated antibodies. **C.** TT treatment results in integrated stress response (ISR) activation and damage associated molecular pattern (DAMP) release/externalisation in MM649 and B16-F10-OVA cells in vitro. Cells were treated and SDS-PAGE/immunoblotting performed as in (B). ATP and HMGB1 levels in cell culture supernatants were determined via luminescence and ELISA based assays, respectively. n=3. Calreticulin externalisation was determined by flow cytometry. n=3-4. **D.** DAMP release/externalization is also observed in TT-treated tumors *in vivo*. B16-F10-OVA tumors in C57Bl/6 mice were treated IT with vehicle/TT and isolated at 24 hr IHC and immunofluorescence analysis with the indicated antibodies. **E.** TT treated B16-F10-OVA cells are effectively phagocytosed by bone marrow derived cells (BMDCs). Representative flow cytometry plots are depicted in (i). Blue boxes: CD11c +ve cells with/without CMFDA uptake. Red boxes: CD11c$^{+}$ CMFDA$_{high}$ cells i.e. cells that have phagocytosed dying cancer cell fragments. Median % CD11c$^{+}$ CMFDA$_{high}$ cells observed for each treatment are plotted in (ii). n=4. CD11c$^{+}$ CMFDA$_{high}$ cells were also imaged to confirm cancer cell fragment uptake –arrowheads in (iii). **F.** TT treated B16-F10-OVA cells can promote the activation/proliferation of OVA directed CD4 and CD8 T cell responses in vitro. BMDCs from PTPRCA mice were pre-incubated with freeze-thawed (F/T) or TT treated B16-F10-OVA cells (16 h), after which CFSE stained T cells from OT-I and OT-II mice were added. Early T cell activation (anti-CD69) and proliferation (CFSE dilution) markers were assessed at t=2 (OT-I) or 4 (OT-II) days via flow cytometry. The % of non-activated (CD69- Prolif$_{high}$), early activated (CD69+ Prolif$_{high}$), activated (CD69+ Prolif$_{med}$) and late activated (CD69- Prolif$_{med}$) T cells ± S.D. are depicted in each graph. n=3. Comparisons made between CD69+ Prolif$_{low}$ cells in each group. Statistical analysis by 2-way ANOVA/mixed effects analysis with Dunnetts correction in (C) and (E), and Tukey's correction in (F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

## TT promotes ER stress, ISR induction and immunogenic cell death in cancer cells

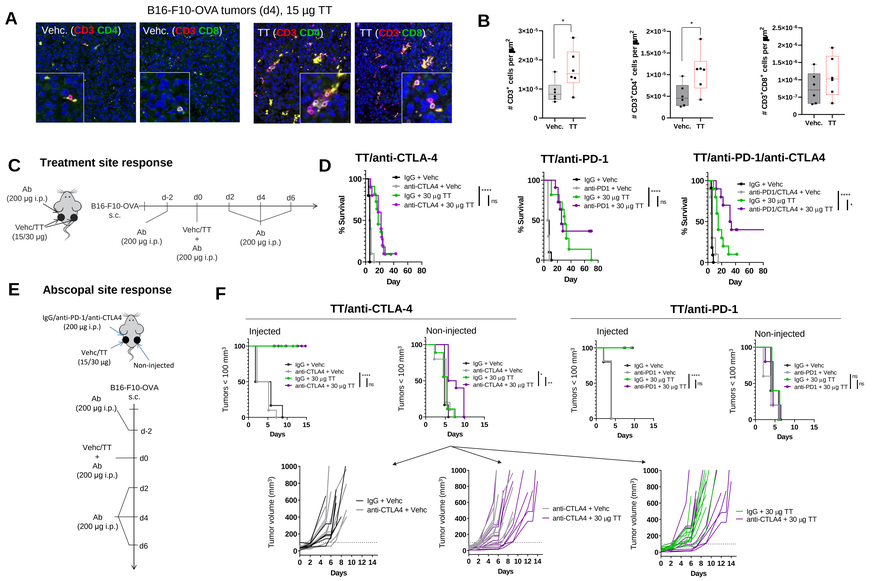



**A.** Experimental design. BALB/c immunocompetent mice with CT-26 tumors were treated with vehicle/TT (15 or 30 µg/tumor), n=15 mice per group, 30 tumors per group. Following ablation of injected tumors, cured mice were then rechallenged with CT-26 cells (day 20) at a distal site. **B.**TT at two dose levels ablates CT-26 tumors in BALB/c immunocompetent mice. Mice with 2 distinct CT-26 tumors were injected with the indicated concentrations of TT. Tumors that recurred (* indicates n) were then re-injected with TT. **C.** TT cells are required to prevent recurrence of TT injected tumors in mice. Kaplan-Meier analysis of individual CT-26 tumors (% tumors <100 mm3) in immunocompetent and immunodeficient (nude) mice injected with the indicated concentrations of TT. **D, E.** TT promotes the development of anti-tumor immunity. Naïve and TT cured mice were re-challenged with CT-26 cells at a distal site. Tumor growth curves are depicted for each condition. Vehicle: 15 mice. 30 µg TT: 10 mice. 15 µg TT: 9 mice. **E.** Protection against tumor cell rechallenge requires T cells. Kaplan-Meier analysis of individual CT-26 tumors observed after rechallenge (% tumors <100 mm3) in immunocompetent and immunodeficient (nude) mice previously treated with the indicated concentrations of TT. **F, G.** TT treatment leads to the development of tumor-directed T cells in the periphery. Splenocytes isolated from naïve and re-challenged mice detailed in (D, E) were incubated with AH-1 peptide, stained with AH-1 directed tetramers (F) or anti-IFNγ (G), together with LIVE/DEAD Aqua, anti-CD3, CD4, CD8 and analyzed via flow cytometry. Representative flow cytometry dot plots, together with box plots (median values) of replicate data (% AH-1 tetramer+ CD8+ or % IFNγ+ CD8+ T cells) are shown. Vehicle: n=9; 30 µg TT: n=8; 15 µg TT: n=6. Statistical analysis by Log-rank (Mantel-Cox) test in (C) and (D). Statistical analysis by Student's *t* test in (F) and (G). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

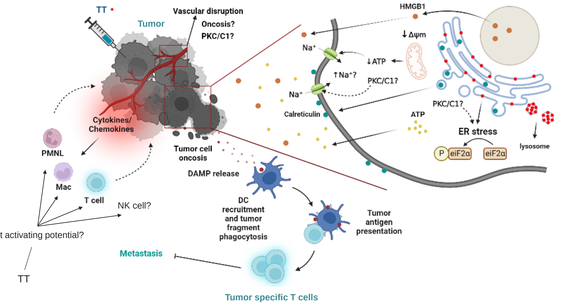



## TT enhances response to ICI blockade in an ICI-refractory model of murine melanoma




**A, B.** TT treatment promotes T cell recruitment to tumors. B16-F10-OVA tumors were isolated 4 days after IT treatment with vehicle (Vehc) or 15 µg TT and stained for CD3, CD4 and CD8 (see images in (A) for representative examples). The number of total CD3+, CD3+ CD4+ and CD3+ CD8+ cells per µm2 were determined for each tumor and condition (B). n=6. Median data expressed in box plot format. **C.** Schematic of the dual treatment regimen. Mice with two distinct B16-F10-OVA tumors on their hindquarters were administered IgG/anti-PD-1/anti-CTLA-4 or a combination thereof via i.p. injection prior to IT injection of both tumors with vehicle or TT (15/30 µg). Mice received antibody every 2 days after IT injection for a further 3 cycles. **D.** TT combines with checkpoint inhibitors to prevent tumor recurrence and improve overall survival. Kaplan-Meier analysis of overall mouse survival. 20 tumors treated per group. 10 mice per group. **E.** Schematic of the abscopal treatment regimen. Mice with two distinct B16-F10-OVA tumors on their hindquarters were administered IgG/anti-PD-1/anti-CTLA-4 or a combination thereof via i.p. injection prior to IT injection of a single tumor with vehicle or TT (15/30 µg). Mice received antibody every 2 days after IT injection for a further 3 cycles. **F.** TT combined with anti-CTLA-4 reduces the growth of non-injected B16-F10-OVA tumors. Kaplan-Meier analysis of individual tumors < 100 mm³, 10 mice per group. Tumor volume for non-injected tumors in mice treated with anti-CTLA-4 are shown. Statistical analysis by Student's *t* test in (A) and Log-rank (Mantel-Cox) test in (D) and (F). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

## Conclusions




**Conclusions:** Data show that tigilanol tiglate is an oncolytic small molecule that induces immunogenic cell death via oncosis and can promote the development of a systemic, anti-tumor immune response in murine models of cancer. Tigilanol tiglate also enhances anti-tumor responses and survival in mice treated with immune checkpoint inhibitors. Tigilanol tiglate is currently being tested in Phase I/II trials in head and neck cancers (ACTRN12619001407189), soft tissue sarcomas, and Stage IIIB to IV M1c melanoma as either a monotherapy (NCT05234437) or in combination with pembrolizumab (NCT04834973)[5].

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3. De Ridder TR *et al.* Randomized controlled clinical study evaluating the efficacy and safety of intratumoral treatment of canine mast cell tumors with tigilanol tiglate (EBC-46). J. Vet. Intern. Med. 2021; 35, 415-429.
4. Panizza BJ *et al.* Phase I dose-escalation study to determine the safety, tolerability, preliminary efficacy and pharmacokinetics of an intratumoral injection of tigilanol tiglate (EBC-46). EBioMedicine. 2019; 50, 433-441.
5. QBiotics Group Ltd. website. https://qbiotics.com

## TT monotherapy induces immunological memory and protects against distal tumor growth *in vivo*