

# Improved efficacy of a novel anti-angiogenic drug combination (TL-118) against colorectal-cancer liver metastases; MRI monitoring in mice

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**BACKGROUND:** The poor prognosis of patients with colorectal-cancer liver metastases (CRLM) and the insufficiency of available treatments have raised the need for alternative curative strategies. We aimed to assess the therapeutic potential of TL-118, a new anti-angiogenic drug combination, for CRLM treatment, in a mouse model.

**METHODS:** The therapeutic potential of TL-118 was evaluated and compared with B20-4.1.1 (B20; anti-VEGF antibody) and rapamycin in CRLM-bearing mice. Tumour progression and the vascular changes were monitored by MRI. Additionally, mice survival, cell proliferation, apoptosis and vessel density were evaluated.

**RESULTS:** This study demonstrated an unequivocal advantage to TL-118 therapy by significantly prolonging survival (threefold) and reducing metastasis perfusion and vessel density (ninefold). The underlying mechanism for TL-118-treatment success was associated with hepatic perfusion attenuation resulting from reduced nitric-oxide (NO) serum levels as elucidated by using hemodynamic response imaging (HRI, a functional MRI combined with hypercapnia and hyperoxia). Further, systemic hepatic perfusion reduction during the initial treatment phase by adding NO inhibitor has proven to be essential for reaching maximal therapeutic effects for both TL-118 and B20.

**CONCLUSION:** TL-118 harbours a potential clinical benefit to CLRM patients. Moreover, the reduction of hepatic perfusion at early stages of anti-angiogenic therapies by adding NO inhibitor is crucial for achieving maximal anti-tumour effects.

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Colorectal cancer is the third most common cancer in the United States (Siegel *et al*, 2012), where colorectal-cancer liver metastases (CRLM) are the main cause of mortality among patients presenting advanced stages of the disease (McLoughlin *et al*, 2006). Despite improvements in surgical techniques and developments of new treatments, the prognosis of patients diagnosed with CRLM is still poor.

Angiogenesis is essential for solid tumour growth (Folkman, 1990) and critical to the metastatic cascade of CRLM development (Meyers and Watson, 2003). Therefore, anti-angiogenic therapy may offer an additional treatment option for this highly aggressive cancer (Warren *et al*, 1995; Meyers and Watson, 2003). Recently, several new anti-angiogenic agents have become available for advanced colorectal cancer treatment, but have failed to induce enduring clinical responses (Miller *et al*, 2005; Bergers and Hanahan, 2008). TL-118 is a novel drug combination that takes advantage of the multi-faceted nature of the angiogenic process; thus, it is composed of four agents that complement each other in their anti-angiogenic effect. In essence, each drug in this composition is aimed at a different aspect of the angiogenic

process: (i) Low-dose high-frequency cyclophosphamide, which drives tumour endothelial cell apoptosis as was shown by Browder *et al* (2000), (ii) The non-steroidal anti-inflammatory drug – diclofenac, which targets inflammatory cells, monocytes in particular, which has a pivotal role in early stages of angiogenesis (Grunewald *et al*, 2006; Mayorek *et al*, 2010), (iii) Sulfasalazin is an NF-kappaB inhibitor (Weber *et al*, 2000) that inhibit angiogenesis (Li *et al*, 2009), probably via vascular smooth muscle cell inhibition (Chung *et al*, 2009) and (iv) Cimetidine, a histamine type 2 (H2)-receptor blocker, demonstrated an anti-angiogenic activity probably via the inhibition of downstream targets of mast cells, which are known to have a role in the angiogenic process (Sorbo *et al*, 1994; Natori *et al*, 2005; Kubecova *et al*, 2011). This final composition was selected based on a systematic screening of multiple candidate agents. Those drugs that enhanced each other's anti-angiogenic activity in a tumour-bearing mouse model were further optimised with respect to their relative dose. All TL-118 components are approved drugs, and their safety profiles have been fully established (Hellstrand, 2002; Warner and Mitchell, 2004; Pasquier *et al*, 2010). TL-118 drug combination was recently approved by the FDA for an anti-cancer clinical trial (Tiltan Pharma Ltd, 2008, 2012).

The current study aimed to assess the therapeutic potential of TL-118 for CRLM eradication in mice. The therapeutic efficacy was compared with B20-4.1.1 (B20), the human- and

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mouse-VEGF-binding monoclonal antibody (Liang *et al*, 2006; Singh *et al*, 2010), and the mTOR pathway inhibitor rapamycin (RAPA) (Guba *et al*, 2002), two well established anti-angiogenic agents. B20-4.1.1 is an anti-VEGF antibody, which has previously been used as a surrogate for preclinical modelling of bevacizumab activity, because it has affinity toward VEGF similar to bevacizumab, yet effectively blocks both the human and murine ligands (Liang *et al*, 2006; Singh *et al*, 2010). RAPA, besides of its immunosuppressive effect, has been shown to exert antiproliferative and anti-angiogenic effects both *in vitro* and *in vivo* (Guba *et al*, 2002; Gridelli *et al*, 2008).

Hemodynamic response imaging (HRI) is a functional MRI (fMRI) method combined with brief exposure to hypercapnic and hyperoxic challenges that was previously demonstrated, enabling monitoring changes in liver perfusion and hemodynamics without the need for contrast agents (Barash *et al*, 2007). The HRI method has been proved sensitive to changes in vessel density, blood flow, oxygen delivery and vessel maturation during tumour progression and anti-angiogenic therapies (Abramovitch *et al*, 1999, 2004). An additional aim of the current research was to evaluate if this method presents a feasible means of determining hemodynamic biomarker profiles indicative of response to anti-angiogenic therapies in CRLM.

In the present study, TL-118 treatment demonstrated a remarkable therapeutic effect with increased animal survival, reduced tumour perfusion and progression as compared with B20 and RAPA, in a well-established mouse model of CRLM. Liver-HRI maps revealed significant attenuated hepatic blood flow during the early phase of TL-118 therapy, illustrating the utility of this technology as indicative biomarker for treatment response. The underlying mechanism for the improved therapeutic effect was associated with hepatic perfusion attenuation resulting from reduced nitric-oxide (NO) serum levels. Previous studies have described the direct impact of decreased NO concentrations on tumour growth, because of its critical role in tumour blood vessel development (Fukumura *et al*, 2001; Camp *et al*, 2006). Conversely, a number of works have suggested that administration of vasopressors (including NO inhibitors), together with anti-tumour drugs, can induce concomitant blood shunting and improve drug delivery to the tumour (Hemingway *et al*, 1992; Shankar *et al*, 1999), regardless of the role of NO in the angiogenic process. Thus, we further reduced the systemic hepatic perfusion during the initial treatment phase of both TL-118 and B20 by adding NO inhibitor and proved that it has a synergistic effect, resulting in improved outcome of both treatments.

## MATERIALS AND METHODS

### Mouse model of CRLM

All experiments were performed in accordance with the guidelines and approval of the Animal Care and Use Committee of the Hebrew University, which holds the NIH approval (OPRR-A01-5011). CT-26-murine colorectal adenocarcinoma cells ( $10^4$  cells in  $300 \mu\text{l}$  per mouse) were intrasplenically injected to 7–8 week-old, anaesthetised male CB6F1 mice (Harlan, Ein-Kerem, Israel) as previously described (Edrei *et al*, 2011). In this model, 1–5 hepatic nodules per mouse were detected by MRI, 13–17 days after cell inoculation.

**Table 1** TL-118 treatment composition and regimen

	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
Cyclophosphamide ( $\text{mg kg}^{-1}$ )	60			60		
Diclofenac ( $\text{mg kg}^{-1}$ )	30			30		
Sulfasalazine ( $\text{mg kg}^{-1}$ )	50	150	150	50	150	150
Cimetidine ( $\text{mg kg}^{-1}$ )		60	60		60	60

### TL-118

The TL-118 drug combination (Tiltan Pharma Ltd., Jerusalem, Israel) (Ben-Sasson, 2008) is composed of a low-dose cytotoxic agent (cyclophosphamide) (Browder *et al*, 2000), a COX1/2 inhibitor (diclofenac) (Grunewald *et al*, 2006), a H2 receptor antagonist (cimetidine) (Natori *et al*, 2005; Kubecova *et al*, 2011) and an NF- $\kappa$ B inhibitor (sulfasalazine) (Weber *et al*, 2000; Chung *et al*, 2009; Li *et al*, 2009). The TL components were intraperitoneally administered according to the schedule outlined in Table 1. The optimisation process included examination of the effect of different concentrations on mice weight as a measure of toxicity. In the optimisation process, we found that in the cytotoxic days (Sunday and Wednesday) it is necessary to reduce the dose of sulfasalazine in order to prevent animal weight loss. The optimal doses determined in mice have been translated into doses suitable for human, based on a body surface area, and were found to be within the acceptable range for clinical use. Moreover, in a phase I clinical trial it was found that this combined protocol composition is well tolerated by the patients.

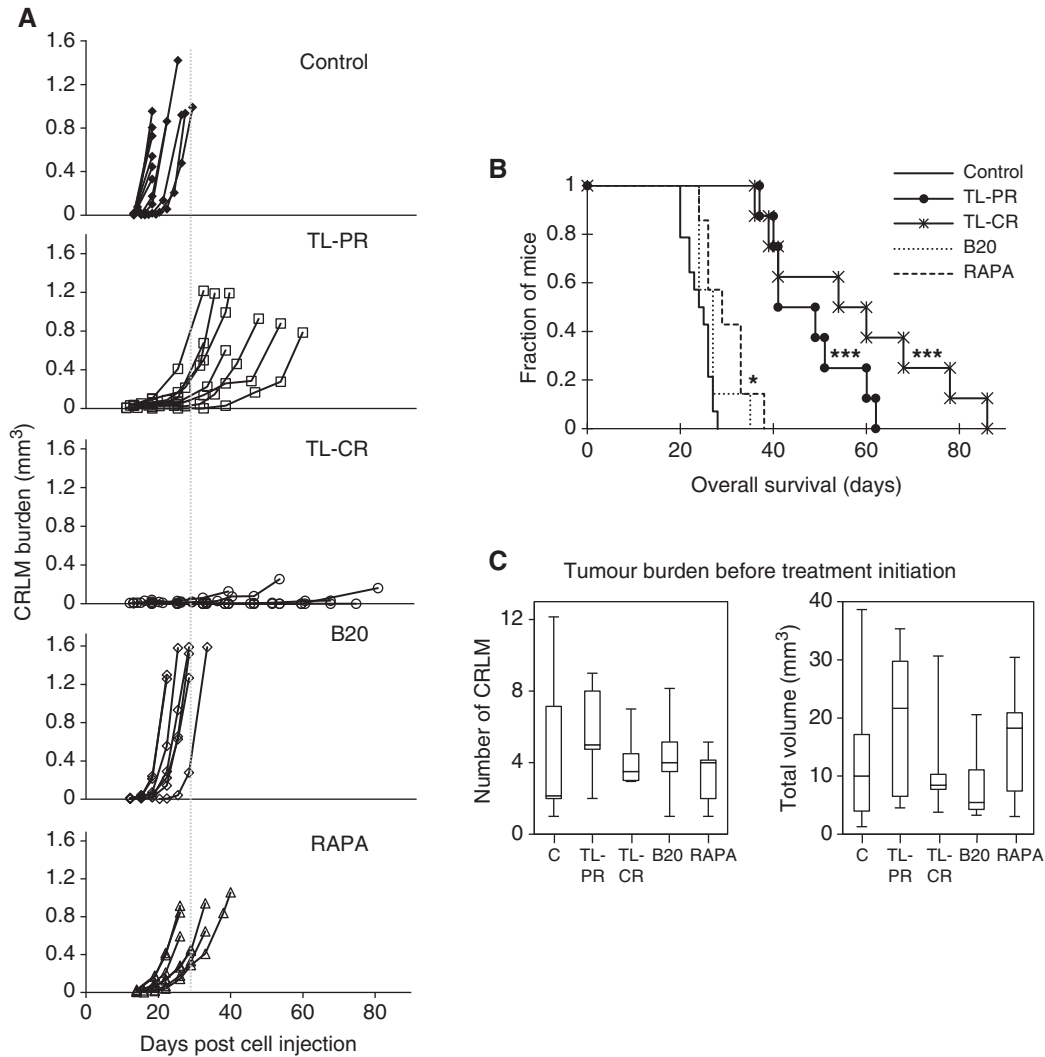
### Treatments

All the treatments started only after CRLM detection by MRI (day  $14 \pm 2$ ). Mice presenting similar CLRM burden, as detected by MRI, were randomly assigned to the different treatment groups (Figure 1C and D). The CRLM-bearing mice were treated, via intraperitoneal (IP) injection, with TL-118 at a volume of  $8 \text{ ml kg}^{-1}$  per day (days 1–6 according to the treatment protocol, as described in Table 1,  $n = 16$ ), B20 anti-mouse VEGF antibody (Genentech Inc., San Francisco, CA, USA) at a dose of  $5 \text{ mg kg}^{-1}$  three times per week ( $n = 10$ ), RAPA (Fermentek Ltd, Jerusalem, Israel) at a dose of  $2 \text{ mg kg}^{-1}$  per day ( $n = 8$ ) or an inert vehicle (control group) at a volume of  $8 \text{ ml kg}^{-1}$  per day ( $n = 14$ ). The treatments were continued until the tumour load reached ethical restrictions ( $1 \pm 0.2 \text{ mm}^3$ ) or up to 2 months in mice, which demonstrated tumour disappearance. Additional groups of naive mice (without tumours) were treated with TL-118 ( $n = 10$ ) or B20 ( $n = 5$ ) with the same protocol regimen as described above for 21 days. Additional groups of CRLM-bearing mice were implanted subcutaneously with micro-osmotic pump (Alzet model 1002; DURECT Corporation, Cupertino, CA, USA) containing NO inhibitor (N-Nitro-L-arginine methyl ester hydrochloride (L-NAME); Sigma Aldrich, St Louis, MO, USA) at a dose of  $20 \text{ mg kg}^{-1}$  per day for 2 weeks from the day of tumour detection and the treatment was combined with either TL-118 ( $n = 6$ ), B20 ( $n = 6$ ) or inert vehicle ( $n = 6$ ) at the standard regimen for 14 days (due to the osmotic pump limitations).

### Imaging

Anatomical and fMRI scans were performed on a horizontal 4.7T Biospec spectrometer (Bruker Medical, Ettlingen, Germany) with a 3.5-cm birdcage coil.

**Anatomical MRI** Mice were anaesthetised with isoflurane (Nicholas Piramal India Ltd, London, UK; 2% in a mixture of 30:70  $\text{O}_2:\text{N}_2\text{O}$ ) and placed in a supine position. Tumours were assessed twice a week using  $T_2$ -weighted ( $T_2W$ ) fast spin-echo



**Figure 1** The effects of TL, B20 and RAPA treatments on tumour growth and mouse survival in a mouse model of CRLM. **(A)** The total metastatic volume (mm<sup>3</sup>) for each individual mouse, as measured from T<sub>2</sub>W MRI as a function of time post cell injection (days) in control- (*n* = 14), TL-118- (TL-PR, *n* = 8; TL-CR, *n* = 8), B20- (*n* = 10) and RAPA- (*n* = 8) treated mice. The dashed line marks the maximal survival day of the control-treated group. **(B)** Kaplan–Meier survival analysis for each of the treated groups. \**P* = 0.006 and \*\*\**P* < 0.0001 compared with control. It should be taken into account that the longer the survival, the higher is the risk for tumour-unrelated death due to the repeated pentobarbital anaesthesia for imaging purposes (80%). **(C)** Tumour load prior to treatment initiation. Box and Whiskers plots of the mean number of metastatic foci per mouse (left) and the mean tumour burden (mm<sup>3</sup>) per mouse (right), as measured from T<sub>2</sub>W MRI prior to therapy initiation, for the mice randomly assigned to each treatment group. The differences between the groups were insignificant.

images (repetition time = 2000 ms; echo time = 37 ms; in plane resolution = 117 μm; and slice thickness = 1 mm).

**Hemodynamic response imaging** Hemodynamic changes were evaluated by HRI on pentobarbital anaesthetised mice (CTS group, Hod-Hasharon, Israel; 30 mg kg<sup>-1</sup>, IP) once a week during the treatment period. Images were acquired using T<sub>2</sub>\*-weighted gradient echo images (repetition time = 147 ms; echo time = 10 ms; field of view = 3 cm; in plane resolution = 117 μm; slice thickness = 1 mm; two averages; and 37 s per image), under normoxic, hyperoxic and hypercapnic conditions. The hypercapnic- and hyperoxic-reactivity maps are given as the percentage change of signal intensity (ΔS) (Barash *et al*, 2007).

**ΔR<sub>2</sub>\* maps** Multi-gradient echo sequence was used to quantify the transverse relaxation rate R<sub>2</sub>\* (repetition time = 200 ms; initial echo time = 5 ms; echo time spacing = 5 ms; five echo times; flip angle α = 40°; two averages; and total imaging time ~ 3.5 min).

R<sub>2</sub>\* maps were acquired under either normoxic, hypercapnic or hyperoxic conditions.

### Image analysis and statistics

The number of tumours per liver and their volumes were assessed from the T<sub>2</sub>W images using Analyze-7.0 (BIR, Mayo Clinic, Rochester, MN, USA). Survival curves were constructed according to the Kaplan–Meier method and statistical significance was determined by the log-rank test. The HRI maps and ΔR<sub>2</sub>\* maps were generated as reported previously (Barash *et al*, 2007; Barash *et al*, 2008; Edrei *et al*, 2011) using interactive data language (ITT Visual Information Solutions, Boulder, CO, USA). For healthy mice, the selected regions of interest (ROI) covered the entire liver. For CRLM-bearing mice, tumour ROI included the entire lesion and liver ROI included representative liver tissue distant from the detected tumours, as defined by analysis of the T<sub>2</sub>W images using the Analyze-7.0 software. Tumours that reached

a size of  $0.15 \text{ mm}^3$  are considered as progressed tumours. Mean  $\Delta S$  values of these ROIs were calculated by including only pixels with a statistical threshold of  $P < 0.05$  (active pixels), as calculated by one-sided Mann–Whitney  $U$ -test. The percentage of active pixels was calculated for each gas challenge. All of these values are expressed as means and 95% confidence intervals (CIs). The difference between groups was analysed by two-sided Mann–Whitney  $U$ -test. Statistical analyses were performed with the Instat Biostatistics software (GraphPad Software Inc., San Diego, CA, USA). A  $P$ -value of  $< 0.05$  was considered statistically significant.

### Histology and immunostaining

Mice were killed after the final MRI scans and their livers were fixed in 4% formaldehyde. Several samples of CRLM and liver tissues were taken from each mouse and further embedded in paraffin. These liver segments were cut into  $4 \mu\text{m}$  slices and stained with hematoxylin and eosin (H&E) or subjected to immunohistochemical analyses. Quantification of necrosis was performed using the Ariol image analysis system (Genetix, San Jose, CA, USA) by scanning H&E-stained slides (12 lesions per group) with an automated scanning microscope. BrdU staining was performed using a Cell Proliferation Kit (GE Healthcare, Amersham, Buckinghamshire, UK), according to the manufacturer's protocol. Apoptosis was assessed through the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay (Cell Death Detection Kit POD, Roche Diagnostics Corp., Mannheim, Germany), according to the manufacturer's protocol. Nuclei were stained with DAPI, tumour blood vessels were detected using anti-PECAM-1 antibodies (CD31; Biocare Medical, Concord, CA, USA), mature tumour blood vessels were detected using  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibodies (dilution 1 : 300; Sigma Chemical Co., St Louis, MO, USA) and bile ducts within CRLMs were detected using a rat anti-A6 antibody (kindly provided by Valentina Factor). All the immunostainings were evaluated in 10 randomly selected high-power microscopic fields of viable tumour regions only (magnification  $\times 400$ ), and the mean value and 95% CIs of positive cells or vessels were calculated. The difference between groups was analysed by two-sided Mann–Whitney  $U$ -test.

### Nitric oxide assay

Blood samples ( $300 \mu\text{l}$ ) were collected from mice tail veins on the specified days, in the presence of heparin, and the plasma was further separated by centrifugation (1200 r.p.m. for 10 min). Plasma NO concentrations were determined by measuring nitrate and nitrite using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions.

## RESULTS

### Impact of anti-angiogenic therapies on tumour progression and mouse survival

MRI-based assessment of the impact of the three anti-angiogenic therapies on CRLM progression (Figure 1A) demonstrated exponential tumour-growth kinetics in control-treated mice, whereas delayed progression with a slight influence on overall animal survival was observed for both B20- and RAPA-treated mice (Figure 1A and B). Only TL-118-treated mice showed significant tumour-growth delay, which could be categorised as either partial response (PR) or complete response (CR). In this animal model, we considered tumours that reached a size of  $0.15 \text{ mm}^3$  as progressed tumours. In TL-PR mice ( $n = 8$ ), an approximate 15-day period of delay in tumour progression was observed, which eventually burst into exponential growth.

Furthermore, tumour progression was fully blocked in the TL-CR group ( $n = 8$ ), and in half of the cases, tumour regression with full disappearance of the tumours was observed (see an example in Supplementary Figure 1). Moreover, TL therapy prolonged mean animal survival of CRLM-bearing mice by twofold in the PR group and threefold in the CR group, when compared with control-treated mice (Figure 1B;  $P < 0.0005$ ). It should be taken into account that in the TL-CR group, 80% of the mice died due to the pentobarbital repeated anaesthesia unrelated to tumour burden.

### Impact of anti-angiogenic therapies on tumour morphology

Detailed histological examination of control-treated CRLMs revealed distinct tumour borders, high cell proliferation and a low percentage of apoptotic cells (Figure 2A, F and G). In contrast, TL therapy induced massive necrosis (Figure 2B and E;  $P < 0.0005$ ), significantly inhibited cell proliferation, particularly at the tumour centre (Figure 2F; 77% reduction,  $P < 0.0005$ ), and induced a 20-fold elevation in the number of apoptotic cells (Figure 2G;  $P < 0.0005$ ). In addition, tumour borders appeared indistinct with hepatocytes infiltration in the TL-PR mice (Figure 2B) and appearance of enlarged bile ducts integrated within tumour tissue and vessel cooption were detected (Supplementary Figure 2A–C). Tumours of both B20- and RAPA-treated mice, retained distinct borders and had only few necrotic regions (Figure 2C–E). Although these therapies inhibited cell proliferation (Figure 2F; 35% and 30% reduction, respectively,  $P < 0.05$ ), it also significantly reduced the number of apoptotic cells (Figure 2G;  $P < 0.0005$  and  $< 0.05$ , respectively). Finally, although no metastases could be detected by MRI in the TL-CR mice during the study period, histological examination of liver specimens performed subsequent to experiment completion, 3 month after cell inoculation, revealed residual tumour cells in these livers (Supplementary Figure 2D–I).

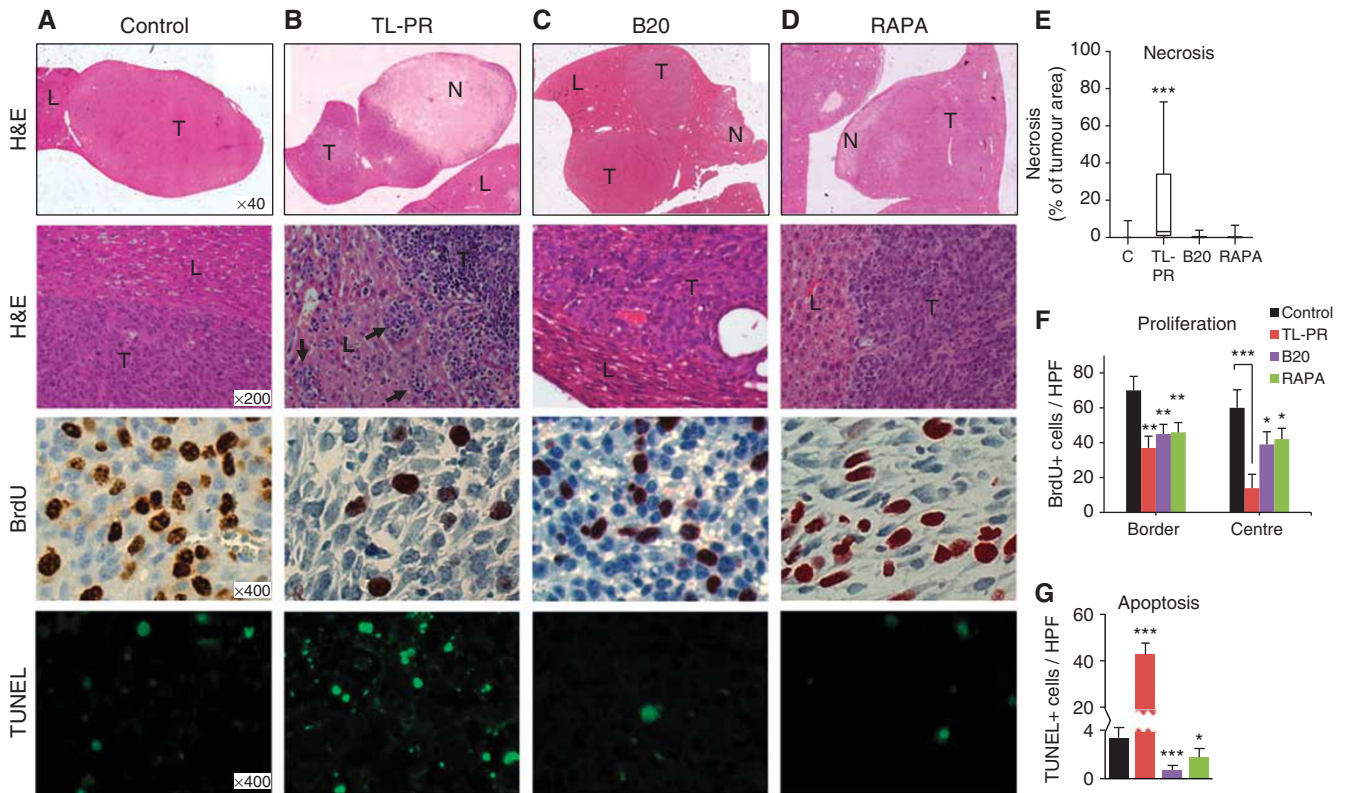
### Impact of anti-angiogenic therapies on tumour vascularisation

Immunohistological analysis revealed significantly reduced  $\text{CD31}^+$  intratumoural vessel density in all the treated tumours, with the most prominent decrease observed in the TL-PR subgroup (Figure 3G; ninefold,  $P < 0.005$ ). When analysing the vessel maturation status, most of the vessels were  $\alpha$ -SMA $^+$  (Figure 3). Morphological changes in vessel integrity were observed in TL-PR mice, where vessels appeared collapsed (Figure 3B). Moreover, numerous blood vessels in B20-treated and RAPA-treated CRLM were extremely widened and  $\alpha$ -SMA $^+$  (Figure 3C and D). The HRI-based assessment of the vascular and hemodynamic changes occurring in the treated tumours demonstrated a significant decrease in tumour-HRI values of TL- and RAPA-treated mice (Figure 3E and F;  $P < 0.005$  and  $< 0.05$ , respectively). The percent reduction in tumour-HRI values was in good correlation with treatment success as measured by the delay in tumour-growth progression and mice survival (Figure 1).

### Anti-angiogenic therapies affect liver perfusion

During the early stages of TL treatment, HRI values of the entire liver parenchyma, in CRLM-bearing mice, were significantly lower compared with control-treated mice (Figure 4A and E;  $P < 0.0005$ ) with the most prominent decrease observed in the TL-CR subgroup. Additionally, the liver-HRI values of RAPA- and B20-treated mice were also reduced significantly (Figure 4E;  $P < 0.0005$ ). As seen with CRLM-bearing mice that were treated with anti-angiogenic therapies, a significant decrease in liver reactivity was also observed in naive treated mice, already after 10 days of treatment. The reduced values remained low for at least





**Figure 2** Effects of anti-angiogenic therapies on CRLM morphology. Representative histological sections of control- (A), TL-PR- (B), B20- (C) and RAPA- (D) treated mice. Slides were stained with H&E (first and second rows), BrdU (third row) and TUNEL (fourth row) to allow for respective quantitative assessment of necrosis (E, box-and-whiskers plot;  $n = 15$  tumours per group), tumour cell proliferation (F, BrdU-positive cells; calculated from 10 fields of each of the nine tumours analysed per treatment group) and apoptosis (G, TUNEL-positive cells;  $n = 10$  high-power microscopic field (HPF) per mouse; four mice per group). Original magnification is indicated on the left image of each row.  $*P < 0.05$ ,  $**P < 0.005$  and  $***P < 0.0005$ . The histological sections were taken at the end of the experiments when the tumour load per mouse reached ethical limits ( $1 \pm 0.2 \text{ mm}^3$ ). Abbreviations: T = tumour tissue; L = liver tissue; and N = necrotic areas.

1 month (Figure 4B–D and F;  $P < 0.0005$ ), demonstrating a CRLM-independent effect of TL or B20 therapies on liver perfusion.

The decrease of liver-HRI reactivity was proven previously to be associated with reduced liver perfusion (Barash *et al*, 2008). Because  $\Delta R_2$  maps reflect changes in tissue oxygenation, with no effect from flow rate (Howe *et al*, 2001), we compared HRI and  $\Delta R_2^+$  maps of naive- vs TL-treated livers. Although the liver-HRI maps were significantly attenuated in the treated mice, the corresponding  $\Delta R_2$  maps were highly similar (Supplementary Figure 3). Thus, we concluded that both TL and B20 treatments led to reduced hepatic blood flow. In line with these findings, both treatments were found to significantly decrease serum NO levels when compared with naive untreated mice (Figure 4G;  $P < 0.005$ ). Thus, we hypothesised that NO levels during early treatment stages inversely correlated with therapeutic potential.

### Reduced systemic NO level improves anti-angiogenic therapy

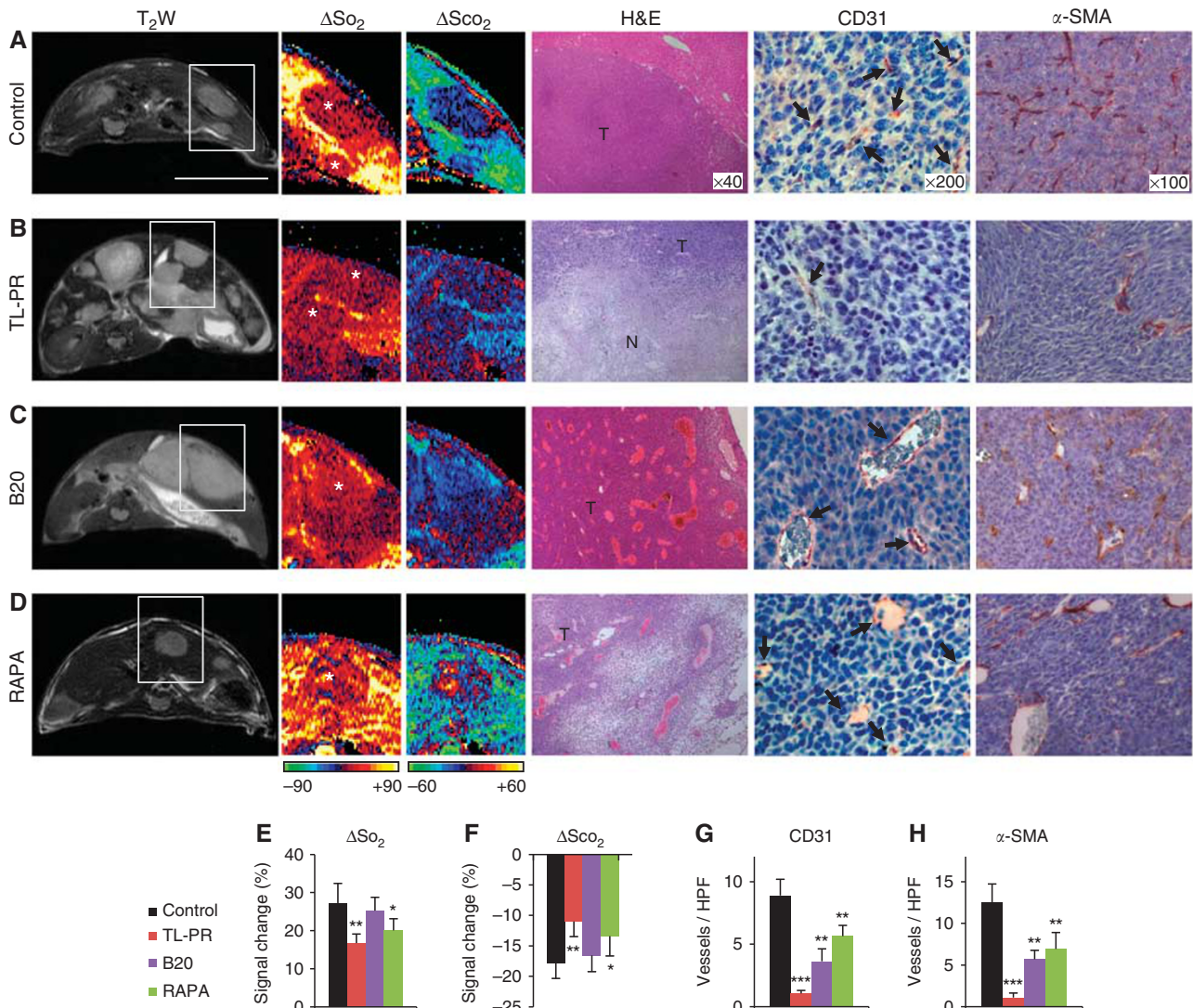
In order to verify the suspected association between the reduced serum NO levels and the anti-angiogenic therapeutic outcome, we evaluated the synergistic effect of NO inhibitor on both TL and B20 therapies. Mice with clearly identifiable CRLM were treated with L-NAME (during the first 2 weeks due to the osmotic pump limitations) combined with the standard TL or B20 regimen. Liver metastases were fully eradicated in the TL + L-NAME-treated mice (Figure 4H, top), whereas in the B20-treated mice, only moderate improvement was observed; yet, in two of these mice the tumours were completely eliminated (Figure 4H, bottom). Treatment with

L-NAME alone had a subtle effect on tumour progression (data not shown). These results suggest that reducing blood flow to the liver at early stages of the anti-angiogenic treatment is crucial for achieving maximal anti-tumour effects.

## DISCUSSION

The considerably poor prognosis of patients presenting CRLMs and the insufficient efficacy of available treatment options have called for the development of alternative curative strategies. Although, VEGF-targeted therapies have shown some promising results, new anti-angiogenic approaches are still required to fully curb distal growth of tumour mass. By careful selection of mechanistically unrelated agents, each targeting a different aspect of angiogenesis, the TL-118 cocktail offers a multi-faceted mean of intervening with the complex angiogenic process by providing a synergistic composition.

The comparative analysis of TL, B20 and RAPA treatment outcomes demonstrated an unequivocal advantage to TL therapy, as demonstrated through the significant increase in mean animal survival, the reduced vessel density and tumour perfusion, increased apoptosis and necrosis and decreased proliferation within the tumour core when compared with control mice. Although some of these phenomena were also observed in B20- and RAPA-treated mice, the therapeutic potential of the TL-118 treatment was significantly superior. More specifically, in cases of CR, TL tripled the mean mouse-survival period and completely abolished MRI-detectable metastatic foci, resulting in



**Figure 3** Effects of anti-angiogenic therapies on CRLM vasculature and perfusion. Representatives axial T<sub>2</sub>W images (first column from left, Bar = 1 cm), and the corresponding enlarged ( $\times 2$ ) HRI maps (second and third columns) of the indicated tumour region (white box, tumours are marked by white asterisks). Representative histological slides were stained with H&E (fourth column), with the endothelial cell marker CD31 (fifth column; arrows point to vessels) and  $\alpha$ -SMA (sixth column) of control- (**A**), TL-PR- (**B**), B20- (**C**) and RAPA- (**D**) treated mice. In TL-PR mice, vessels appeared collapsed (**B**), whereas in B20- and RAPA-treated mice, numerous blood vessels were extremely widened (**C, D**). Mean  $\Delta S_{O_2}$  (**E**) and mean  $\Delta S_{CO_2}$  (**F**) values with 95% CIs were calculated from 13 tumours per treatment group during tumour progression. (**G**) Quantification of CD31-positive vessels per HPF; (**H**) Quantification of  $\alpha$ -SMA-positive vessels per HPF. The quantitative data (**G, H**) were calculated from 10 fields for each of the nine tumours analysed per treatment group. Original histological magnification is indicated on image **A** of each row. **\*\*** $P < 0.005$  and **\*\*\*** $P < 0.0005$ . The histological sections were taken at the end of the experiments when the tumour load per mouse reached ethical limits ( $1 \pm 0.2 \text{ mm}^3$ ). Abbreviations: T = tumour tissue and N = necrotic areas.

complete remission. Moreover, in the TL-CR group most of the mice died due to the pentobarbital repeated anaesthesia unrelated to tumour burden.

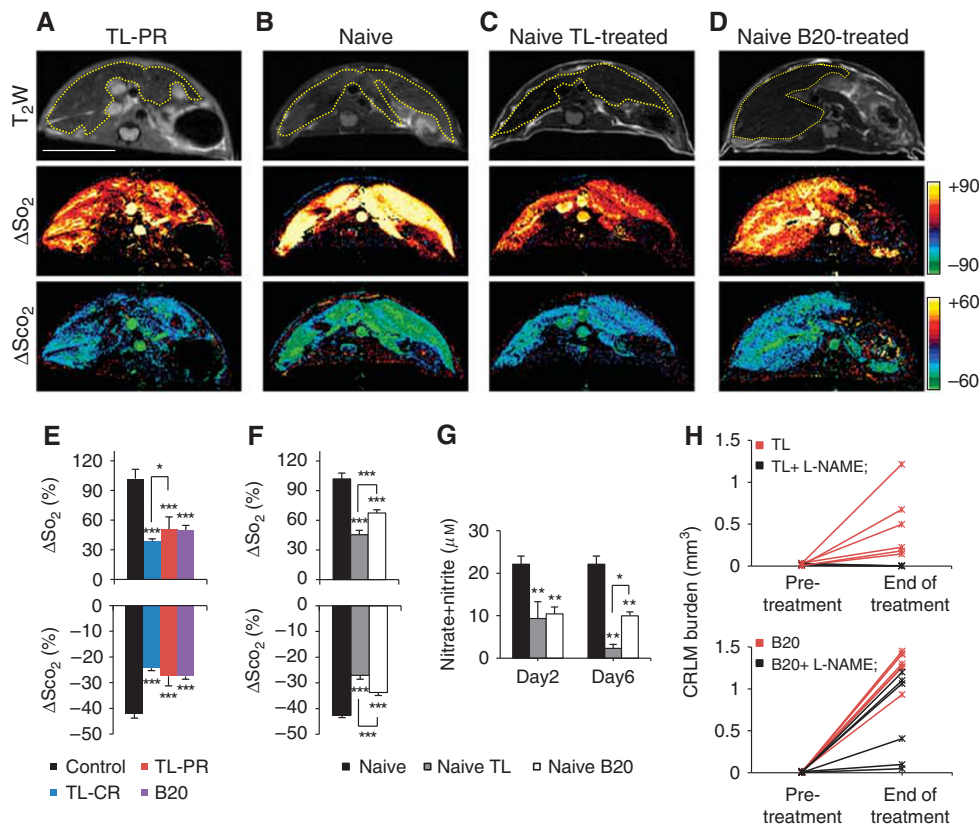
Numerous studies on xenograft tumour models have demonstrated the marked ability of VEGF-blocking agents to suppress tumour progression, with occasional tumour regression (Kim *et al*, 1993; Rowe *et al*, 2000a, 2000b; Huang *et al*, 2003). However, none of these agents have successfully cured animals, even when combined with other therapies. When considering the relatively advanced stage of metastases in the present model, the remarkable therapeutic capacity of TL-based therapy is further highlighted. This disparity in therapeutic impact is most likely rooted in the nature of the multitasking TL-118 cocktail, better suited to combat the multi-faceted angiogenic process. It has been suggested that the effective therapeutic window for anti-VEGF therapy precedes vessel maturation (Benjamin *et al*, 1999). Analysis of the CRLM

vessel-maturation status demonstrated a high degree of  $\alpha$ -SMA-positive intratumoural vessels both before (data not shown) and during B20 and RAPA therapies. These findings elucidate the origin of B20 and RAPA therapeutic failure, while strengthening the powerful potential of TL treatment at advanced disease stages.

As demonstrated by HRI of naive treated mice, both TL and B20 therapies affect liver perfusion independent of tumour presence. Losser *et al* (2010) reported significant hepatic artery vasoconstriction and subsequent reduction in arterial flow velocity upon NOS inhibition in healthy rabbits. Indeed, systemic downregulation of NO serum concentrations correlated with TL- and B20-stimulated hemodynamic responses and is suggested to form the mechanistic basis of reduced liver perfusion.

Nitric-oxide is known to mediate vasodilation and rapidly induce alterations in blood flow (Moncada *et al*, 1991). In contrast, NO-synthase inhibition induces sinusoidal vessel constriction,





**Figure 4** Hepatic perfusion attenuation during TL and B20 treatments. (**A–D**) Representative axial T<sub>2</sub>W MR images (top row) and the corresponding HRI maps of  $\Delta\text{SO}_2$  (middle row) and  $\Delta\text{SCO}_2$  (bottom row) obtained from a TL-PR-treated (**A**), naive (**B**), naive TL-treated (**C**) and naive B20-treated (**D**) mice. Liver borders are marked in yellow, Bar = 1 cm; (**E**) Mean hepatic  $\Delta\text{SO}_2$  and  $\Delta\text{SCO}_2$  values with 95% CIs of control- ( $n = 14$  mice), TL-PR- ( $n = 8$  mice), TL-CR- ( $n = 8$  mice) and B20- ( $n = 6$ ) treated CRLM-bearing mice. (**F**) Mean hepatic  $\Delta\text{SO}_2$  and  $\Delta\text{SCO}_2$  values with 95% CIs of naive ( $n = 10$  mice), naive TL-treated ( $n = 10$  mice) and naive B20-treated ( $n = 5$  mice) mice. (**G**) Nitrate plus nitrite serum levels with 95% CIs were analysed from blood samples obtained before ( $n = 10$  mice) and during TL and B20 treatments of naive mice ( $n = 5$  mice per time point). (**H**) Systemic NO inhibition improves TL and B20 therapies. The total metastatic volume for each individual mouse before and at the end of treatment in TL- (top; TL-PR,  $n = 8$  and TL + L-NAME,  $n = 5$ ) and B20-treated (bottom; B20,  $n = 10$  and B20 + L-NAME,  $n = 6$ ) mice. \* $P < 0.05$ , \*\* $P < 0.005$  and \*\*\* $P < 0.0005$ .

which decreases flow and results in hypertension. Some studies have suggested that vasopressor administration (including NO inhibitor) may lead to blood shunting into the tumour concomitantly with improved drug delivery (Hemingway *et al*, 1992; Shankar *et al*, 1999). Conversely, a number of works have described the direct impact of decreased NO concentrations on tumour growth, because of its critical role in tumour blood vessel development. They demonstrated that VEGF-mediated angiogenesis relies, at least in part, on the downstream effects of NO (Fukumura *et al*, 2001; Camp *et al*, 2006). Nevertheless, treatment with NO inhibitor combined with TL-118 in a subcutaneous tumour model failed to induce any additive effect compared with TL-118 alone (data not shown). The current findings suggest that although the reduced NO levels affect the systemic blood flow, only liver metastases were influenced from this strategy. Therefore, we suggest that reduced blood flow to the liver at early stages of the anti-angiogenic treatment is crucial for achieving maximal anti-tumour effects. The combination of NO inhibitor and B20 had only a moderate impact on tumour growth compared with the combination with TL. Because the reduction of the systemic NO level by TL was greater than that obtained by B20, we assume that an additional increase of the NO inhibitor dosage with B20 therapy may improve the therapeutic outcome.

Both B20 and RAPA treatment yielded similar morphological profiles of reduced vascularity with widened vessels and inhibition of cell proliferation, suggesting that both treatments act via the anti-angiogenic and anti-proliferative pathways, as previously

reported (Wang *et al*, 2009; Yang *et al*, 2010). Interestingly, fewer apoptotic cells were detected in the B20-treated group, when compared with control-treated mice. These results may explain the modest therapeutic effect of B20 compared with RAPA. Similar enhancement of vessel diameter and maturation was recently described following bevacizumab therapy of human colorectal adenocarcinoma liver metastases (Weisshardt *et al*, 2011). The increased vessel diameter and maturation following B20 therapy may contribute to improved therapeutic potency when combined with either radiation or chemotherapy, supporting the vessel normalisation mechanism (Jain, 2005).

Study limitations include the use of B20 (Liang *et al*, 2006), which may have contributed to the relatively mild response observed, when compared with those reported in the clinics. Although bevacizumab has been approved for the treatment of metastatic colorectal cancer in combination with the standard chemotherapy (Hurwitz *et al*, 2004; Kabbinavar *et al*, 2005), the present study considered the stand-alone efficacy of B20.

In the present study, TL displayed a remarkable therapeutic effect on CRLM-bearing mice, even when treatment was initiated at advanced metastatic stages, where the vasculature was highly covered with pericytes. The application of the HRI technique provided critical evidence regarding the mechanisms underlying the improved anti-tumorigenic effect of TL, the reduced hepatic perfusion. Indeed, the reduced hepatic perfusion was proven as essential for therapeutic success because the addition of the NO inhibitor during the initial treatment phase of both TL and B20

improved treatment outcome dramatically. Furthermore, HRI has been proven to be a sensitive and beneficial non-invasive tool for monitoring responses to anti-angiogenic therapies.

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