# Histamine's vasoactive properties improving response rates in solid tumor treatment

De vasoactive eigenschappen van Histamine lijdt tot een verbeterde response bij de behandeling van solide tumoren

**Flavia Brunstein**

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# Histamine's vasoactive properties improving response rates in solid tumor treatment

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# Contents

# Chapter 1

## General introduction

## &

## Aim of the thesis

Isolated limb perfusion (ILP) was first described by Creech and Krementz in 1958 [1] as an heroic attempt to save the limb of a patient bearing melanoma metastasis. The ILP procedure allows the administration of high doses of chemotherapeutic agents, up to 25 times higher than the systemically used doses. The use of the alkylating agent melphalan became the golden standard with complete responses (CR) in the range of 50% [2,3]. However, ILP with chemotherapeutic drugs alone failed in the treatment of large tumors, such as advanced extremity soft tissue sarcomas. This situation was radically changed by the introduction of tumor necrosis factor α (TNF-α) in combination with melphalan, and interferon γ (IFN-γ) in the ILP with complete remission indexes of more than 80% in locoregionally advanced melanomas and soft tissue sarcomas [4]. A multi-center study involving eight cancer centers and 246 patients followed, leading to the approval of TNF by the European Medicine Evaluation Agency (EMEA) for the treatment of high grade advanced extremity sarcomas and opened a new era in the management of locally advanced tumors[5,6].

The ILP technique involves cannulation of the major vessels, clamping of the collaterals and application of a tourniquet. Its rational is the advantage of a leakage free system allowing as high as 15-20 times local drug concentrations without systemic complications. TNF-α works through tumor-associated vasculature (TAV) targeting, first leading to a higher melphalan uptake and secondly to the destruction of this vasculature making irressectable tumors ressectable and accounting for a limb salvage index of circa 71% [4-8].

Besides giving new impulse to the further development of regional therapy TNF also led to renewed interest in organ perfusion, mainly liver and lung. Despite the encouraging results seen in the ILP, the use of TNF-α in Isolated Hepatic Perfusion (IHP) was hampered by severe toxicity [9,10]. Thus the idea of exploring other inflammatory agents, which could also modulate TAV, as alternatives to TNF-α seemed tempting. In this sense Histamine (Hi) and Interleukin-2 (IL-2) appeared as good candidates. The classical effect of Hi on fine vessels is the formation of 1) edema by an increase in the flow of lymph and its protein content to the extracellular space and 2) gaps between endothelial cells, increasing transcapillary vesicular transport [11]. Potentially, this could translate into a higher drug concentration in the oncological scenario. As for IL-2, the choice was mainly based on the enthusiastic results seen in the systemic setting mainly for melanoma and renal cell carcinoma, while the dose-limiting problem could be easily manageable in an isolated perfusion setting

(ILP). Besides, IL-2 is also associated with the capillary leakage syndrome, which could potentially translate into higher local drug concentrations. [12-14].

A rat model for ILP, which closely resembles the clinical setting was developed in the laboratory for experimental surgical oncology in Rotterdam in 1996 [15,16]. Based on this reliable model the mechanisms of action of TNF were extensively explored as well as new ways to further improve the efficacy of ILP [17]. We showed that TNF increased tumor concentration of melphalan but not muscle one, further supporting its specific TAV targeting property. The same observations were valid for TNF in doxorubicin-based ILP [7,18]. Moreover, also in organ perfusion, i.e. in the experimental Isolated Hepatic Perfusion (IHP) rat model, a dramatic increase in melphalan tumoral concentration was observed when TNF was administered. Interestingly enough, best response rates correlated with the degree of tumor vascularisation, with best indexes seen for the highly vascularised BN-175 soft tissue sarcoma as compared to ROS-1 osteosarcoma and CC531 coloncarcinoma, both of which present a low microvascular density (MVD).[10]

## Aim of the study and thesis outline

The aim of this study was to explore the potential use of Histamine in the regional setting as an alternative for TNF-α, using our well-established experimental models of ILP and IHP in rats.

In **Chapter 2** the use of Histamine alone and in combination with melphalan in the ILP is evaluated with *in vivo* and *in vitro* studies exploring its effect and the mechanism of action. Next, **Chapter 3** reports on the effects of another vasoactive drug, Interleukin-2 (IL-2) in the ILP both alone and in combination with melphalan. Then, **Chapter 4** describes the attempt to further improve response rates by combining these two agents in the ILP. Despite the promising synergistic effects of each drug on melphalan-based ILP, and the reported positive synergy in the systemic setting, in fact the combination of Hi and IL-2 led to decreased response-rates.

**Chapter 5** shows that synergistic effects of Hi were also present when combined to another chemotherapeutic agent, doxorubicin known as the best single agent for systemic therapy of sarcoma, with activity in more than 20% of the treated patients. Furthermore, the vasoactive properties of Histamine, namely its antivascular effect on TAV and its ability to increase tumor drug uptake are documented by various histologic studies including HE, CD-31 and Perls staining (for hemorrhage). Also included are confocal images of thick frozen sections,

taking advantage of the natural red fluorescence of doxorubicin combined with a green vessel staining (FITC-lectin). Images were further worked in an image program for quantification of drug distribution and vessel density.

Supported by the enthusiastic results seen with Histamine combined to different chemotherapeutic drugs in the ILP, the next step was to explore Histamine use in a different setting. **Chapter 6** describes the studies done in melphalan-based IHP for the treatment of soft tissue sarcoma (BN-175) liver metastasis. It includes response rate curve and regional and systemic toxicity grading with histologic evaluation.

Finally, in **Chapter 7** a broad review of the literature starting on Coley's toxin, which led to the isolation of TNF-α and the first attempts to use it systemically before the crucial description of its regional application, is presented. Then, the history of ILP is shortly described. Next, normal and tumor endothelium properties are reported. Finally we go over the potential role and physiologic properties of Histamine, mainly focusing on its vasoactives properties and discuss the future of antivascular biochemotherapy strategies.

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# Chapter 2

## *Synergistic antitumor activity of histamine plus melphalan in the isolated limb perfusion (ILP) – preclinical studies*

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## Abstract

We have previously shown how tumor response of isolated limb perfusion (ILP) with melphalan was improved when tumor necrosis factor alpha (TNFα) was added. Taking into account that other vasoactive drugs could also improve tumor response to ILP, we evaluated histamine (Hi) as an alternative to TNF-α. We used a rat ILP model to assess the combined effects of Hi and melphalan (n=6) on tumor regression, melphalan uptake (n=6), and tissue histology (n=2) compared with Hi or melphalan alone. We also evaluated the growth of BN-175 tumor cells as well as apoptosis, necrosis, cell morphology, and paracellular permeability of human umbilical vein endothelial cells (HUVECs) after Hi treatment alone and in combination with melphalan. The antitumor effect of the combination of Hi and melphalan *in vivo* was synergistic, and Hi-dependent reduction in tumor volume was blocked by $H_1$ and $H_2$ receptor inhibitors. Tumor regression was observed in 66% of the animals treated with Hi and melphalan, compared with 17% after treatment with Hi or melphalan alone. Tumor melphalan uptake increased and vascular integrity in the surrounding tissue was reduced after ILP treatment with Hi and melphalan compared with melphalan alone. *In vitro* results paralleled *in vivo* results. BN-175 tumor cells were more sensitive to the cytotoxicity of combined treatment than HUVECs, and Hi treatment increased the permeability of HUVECs. In conclusion, Hi in combination with melphalan in ILP improved response to that of melphalan alone through direct and indirect mechanisms. These results warrant further evaluation in the clinical ILP setting and, importantly, in organ perfusion.

## Introduction

Isolated limb perfusion (ILP) is a treatment method in which high concentrations of drugs were administered to a limb containing an unresectable tumor that is temporarily isolated from the rest of the body's circulatory system by the use of an extracorporeal perfusion circuit and a tourniquet placed at the root of the limb. ILP with tumor necrosis factor alpha (TNFα) and melphalan is associated with synergistic antitumor effects against melanoma (1), large soft tissue sarcomas (2,3) and various other tumors in the clinical setting (4-6). We have previously shown that the basis for the synergy is both a substantial enhancement of tumor-selective melphalan uptake (7) and the complete destruction of tumor vasculature

(2). The enhanced tissue uptake of different cytotoxic agents, when combined with TNFα, shown in various limb and liver tumor models in our laboratory (7-12) prompted us to investigate a number of vasoactive substances for similar effects.

Histamine (Hi) is an obvious candidate to enhance tissue uptake of cytotoxic agents during ILP. It is an inflammatory mediator that is formed and stored mainly in the granules of mast cells and basophils, but it has also been identified in epidermal cells, gastric mucosa, neurons of the central nervous system, as well as in cells in regenerating or rapidly growing tissues. Its effect on fine vessels is to cause edema by increasing the flow of lymph and lymph proteins into the extracellular space and also by promoting the formation of gaps between endothelial cells, thus increasing transcapillary vesicular transport (13). The same mechanism that causes edema in fine vessels could potentially be used to increase drug concentrations in tumor tissues.

In this study, we performed ILP in a rat model by using combinations of Hi and melphalan to determine if Hi would increase the effects of melphalan. To determine the *in vivo* mechanisms involved, we measured melphalan uptake and performed histologic analysis after treatment. In addition, cultured sarcoma (14) and normal endothelial cells were treated *in vitro* with Hi, melphalan, or a combination of the two, and cytotoxicity, necrosis, apoptosis, and paracellular permeability were assayed.

## Materials and Methods

### *ILP*

Male inbred Brown Norway rats weighing 250-300g were obtained from Harlan-CPB (Austerlitz, the Netherlands), and were fed a standard laboratory diet *ad libitum* (Hope Farms Woerden, the Netherlands). The studies were done in accordance with protocols approved by the Animal Care Committee of the Erasmus University Rotterdam (Rotterdam, the Netherlands. Small fragments (diameter = 3 mm) of the spontaneous, nonimmunogenic, syngeneic BN-175 sarcoma (14) were inserted subcutaneously in the right hind legs of the rats, as previously described (8). Tumor growth was measured daily with a caliper, and tumor volume was calculated using the formula $0.4(A^2xB)$ (where B represents the largest tumor diameter and A is the diameter perpendicular to it). When tumor diameter exceeded 25 mm or at the end of the experiment rats were anesthetized and killed by cervical dislocation.

The treatment consisted of the experimental ILP, previously described (8,11). In brief, 7-10 days after tumor fragments were inserted (when they reached a diameter of 12-15 mm) rats were anesthetized by intraperitoneal ketamine and intramuscular hypnomidate. An incision parallel to the inguinal ligament was made, and the inguinal vessels were cannulated and connected by way of a low-flow roller pump (Watson Marlow, Falmouth, U.K.) to an oxygenated reservoir where drugs were added, in bolus, to the perfusate (total volume = 5 mL). A groin tourniquet was used to occlude collateral vessels, allowing a proper isolation of the limb. The temperature of the limb was maintained at 38$^{o}$C using a warm-water blanket.

The perfusate consisted of Haemaccel alone (sham) (Boehring Pharma, Amsterdam, the Netherlands); Haemaccel plus 40 μg of melphalan (Alkeran® Wellcome, Beckenham, United Kingdom); Haemaccel plus 40 μg of melphalan and 1000 μg of Hi (kindly provided by Maxim Pharmaceuticals Inc., San Diego, CA) or Haemaccel plus 1000 μg of Hi.

To evaluate the role of the different Hi receptors in the Hi-based ILP, the Hi receptor blockers promethazine ($H_1$-R) (Centrafarm, Etten-Leur, the Netherlands) and famotidine ($H_2$-R) (Sigma, Zwijndrecht, the Netherlands) were added to the perfusate (200 and 50 μg/mL, respectively) and allowed to circulate into the limb for 5 minutes before melphalan and Hi were added.

Tumor dimensions were measured every day and used to monitor tumor volume. Volume on day 9 was compared with that on day 0, and response was classified as follows: progressive disease (PD) increase of more than 25%; no change (NC) volume between -25% to +25%; partial remission (PR), decrease between –25% and –99%, or complete response (CR), no palpable tumor.

Limb function was clinically observed as the ability to walk and stand on the perfused limb after ILP. On a scale from grade 0 to 2, grade 0 is severely impaired function in which the rat drags its hind limb; grade 1 is slightly impaired function (can not use it in a normal way, but can stand on it), and grade 2 is an intact function (normal walking and standing pattern) (8).

***In vivo Melphalan uptake***.

To evaluate melphalan distribution, we killed 11 rats (six treated with Hi plus melphalan and 5 treated with melphalan alone) immediately after ILP was performed. Tumors and muscle from the limb were removed, snap-frozen in liquid nitrogen, and stored at –80$^{o}$C. Tissues were homogenized in 2 mL acetonitrile (PRO 200 homogenizer, Pro Scientific, Oxford, CT) and centrifuged at 2500g and 4oC. Melphalan concentration (reported as nanograms of melphalan per gram of tissue) was measured by gas chromatography-mass spectrometry on at least three different pieces of similar final weight per sample, as described previously (7,15). Given the tumor and muscle values for melphalan uptake, the tumor-to-muscle ratio was calculated, considering the amount of melphalan measured in muscle as 100% and calculating the tumor value in comparison with it.

***Histologic evaluation***.

Two animals from each treamtent group were killed by cervical dislocation directly after ILP; tumors and a piece of muscle from the limb were excised and cut in half. One half was fixed in 4% formaldehyde solution, embedded in paraffin, and stained with hematoxylin and eosin. Images of stained samples were taken on a Leica DM-RXA microscope (Leica Microsystems, Rijswijk, the Netherlands) with a Sony 3CCD DXC camera (Sony Netherlands, Badhoevedorp, the Netherlands).

***Cell culture***.

BN-175 cells (spontaneous rapidly growing and metastasizing soft tissue sarcoma) (14) were grown in RPMI-1640 medium (Life Technologies, Leiden, the Netherlands) supplemented with 10% fetal calf serum (FCS) and 0.1% penicillin-streptomycin (Life Technologies, the Netherlands). For growth assays, BN-175 cells were plated in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA, USA) at $10^5$ cells per well (in 100 μl) 24 hours before treatment and allowed to grow to confluence. Next, the cells were incubated at 37$^{o}$C in 5% $CO_2$ for 72 hours in the presence of medium alone or medium plus various concentrations of melphalan and Hi. Hi concentrations ranged from 0 to 200 μg/mL. Melphalan concentrations ranged from 0 to 8 μg/mL.

HUVECs were prepared by collagenase treatment of freshly obtained human umbilical veins and cultured in human endothelial serum-free medium-RPMI medium (Cambrex

Bioscience, Verviers, Belgium) supplemented with 10% heat inactivated human serum (Invitrogen Life Technologies, Breda, the Netherlands), 20% FCS, human epidermal growth factor, human basic fibroblast growth factor, and 0.1% penicillin-streptomycin (Life Technologies). For growth assays, HUVECs were plated 24 hours before treatment at 6x104 cells per well and cultured for 48 hours with various concentrations of Hi (0 to 200 μg/mL) and melphalan (0 to 200 μg/mL).

***Cell growth***

Growth of BN-175 cells and HUVECs was measured using the Sulphorhodamine-B (SRB) assay (16). In brief, cells were washed with phosphate buffered saline, incubated with 10% trichloroacetic acid for one hour at a 4$^{o}$C, and washed again with phosphate buffered saline. Cells were stained with SRB (0.5% SRB in 1% acetic acid) for 15 to 30 minutes, washed with 1% acetic acid and air-dried. Protein-bound SRB was dissolved in Tris base (10mM, pH 9.4). Absorbance at 540 nm was measured for each well, and tumor cell growth was calculated according to the following formula: percentage of tumor cell growth = (absorbance of test well/absorbance of control well) x 100%. The Hi concentration leading to 50% reduction in absorbance compared with control (i.e. 50% inhibitory concentration [$IC_{50}$]) was determined from the growth curve. Each experiment was performed four times in duplicate. The mean of all values and the 95% confidence intervals (CIs) were determined and reported.

***HUVEC morphology and necrosis-apoptosis assays***

HUVECs were plated 24 hours before treatment at 6x10$^{4}$cells per well in flat-bottomed 12-well plates (Costar, Cambridge, MA, USA) in a final volume of 900 μl per well and grown to confluence. Cells were then incubated at 37$^{o}$C in 5% $CO_2$ with various concentrations of Hi for various times. At each time point, medium was discarded and replaced with 500 μl of HUVEC medium plus 0.05% YO-PRO for detection of apoptotic cells (Molecular Probes) or propidium iodide to detect necrotic cells (Sigma). Cells were incubated for 30 minutes in the dark, at 37$^{o}$C and pictures were taken with a Zeiss AxioVert 100M inverted microscope supplied with a AxioCam camera (Carl Zeis, Sliedrecht, the Netherlands).

Cells were cultured and treated using the time points above with the Vybrant Apoptosis assay kit #3 (Molecular Probes) for both adherent and detached cells. In brief, cells were

treated with various concentrations of Hi alone (0 to 200 μg/mL), melphalan alone (0 or 8 μg/mL), or with combinations of the drugs for 15 or 30 minutes. Culture medium containing floating cells was removed from the wells and transferred to 5-mL tubes. Adherent cells were washed with RPMI medium, trypsinised with 300 μl of trypsin-EDTA (Biowhitaker), neutralized with 100 μl of HUVEC medium containing 20% FCS, and added to the 5-mL tubes. Tubes were centrifuged for 5 minutes at 250g, and the supernatant was discarded. Cells were then incubated in 200 μL of annexin binding buffer alone, or with annexin V, or with propidium iodide (both reagents from the Vybrant Apoptosis assay kit) at room temperature for 15 minutes in the dark and evaluated by flow cytometry with a FACscan (Becton Dickinson, Alphen aan den Rijn, the Netherlands) flow cytometer. Data was processed with Winmidi software 2.7 (J. Trotter; Salk Institute, San Diego, CA). Experiments were done three times in duplicate, and the mean and 95% CIs of the percentage of living, apoptotic, and necrotic cells were reported.

***Endothelial cell monolayer permeability assay.***

HUVECs were plated 48h before treatment at $6x10^4$ cells per well in a monolayer on a fibronectin-coated polycarbonate membrane (diameter = 6.5 mm; pore size = 0.4 μm) in a transwell device (Costar). HUVEC medium (1 mL) was added to the lower compartment. Aproximately 24 hours after the cells reached confluence, medium in the upper chamber was replaced with 50 μl of fluorescein-isothiocyanate-bovine serum albumin (FITC-BSA) (1 mg/mL; Sigma) plus 250 μL of HUVEC medium containing various concentrations of Hi. At the same time, medium in the lower chamber was replaced by 700 μL of HUVEC medium. Fifty-microliter samples were taken from the lower chamber at various times, and FITC fluorescence was measured with a fluorescence photospectrometer (Victor$^2$ FSR; Perkin Elmer, Bucks, U.K.) at 490 nm excitation and 530 nm emission. Values were compared with a standard curve based on known concentrations of FITC-BSA

Next, to evaluate whether melphalan would have any effect on endothelial cell permeability, directly or in conjunction with Hi, the HUVEC monolayer was exposed to the composition of 250 μL of HUVEC medium alone (control), melphalan at 8 μg/mL or Hi at 100 μg/mL with or without melphalan (8 μg/mL). Permeability was assayed as described

above. Experiments were done three times in duplicate. The data were reported as the mean and 95% CIs of all values.

***Statistical analysis***.

Tumor growth curves were plotted as means and 95% CIs of the data from all animals. We used repeated-measure analyses of variance on the three most representative days, taken from the growth curve patterns 4, 8 and 10 using SAS Software Release 8.2 for Windows 2000 (SAS institute Inc., Cary, NC, USA) using PROC MIXED. Main effects of treatment and day (three levels: day 4, 8 and 10) were included in the models, as was the interaction between treatment and day. For days in which response was statistically significant, interaction terms were further investigated by testing for differences following treatment on that day.

The data from HUVEC monolayer permeability assays was also analyzed as described above. The effects of treatment and time (5 levels: 0,15, 30, 45 and 60 minutes) were evaluated. Viability of HUVECs after Hi incubation data was presented and analyzed using the Kruskal-Wallis test with SPSS version 10.0 for Windows 2000.

Melphalan accumulation was shown both as mean values (with 95% CIs) of three measurements performed using different tumor areas and as a ration between tumor and muscle values, expressed in percentages of tumor versus muscle melphalan uptake. Data were analyzed using the Mann-Whitney U test with SPSS v10.0 for Windows 2000.

Synergism between Hi and melphalan was evaluated by determining whether tumor response after Hi alone or melphalan alone added together was different from the tumor response after Hi plus melphalan. First, the tumor response index was calculated by dividing the initial tumor volume by the tumor volume on a given day after treatment; then, the tumor response index of a rat from the Hi-treated group was randomly added to the tumor response index of a rat from the melphalan-treated group and compared with the tumor response index from the Hi-plus-melphalan group. Next, the data were analyzed with the Mann-Whitney U test (exact significance [2x(one-tailed significance)] using SPSS version 10.0 for Windows 2000. All statistical tests were two-sided. For all statistical tests, a P value less than 0.05 was considered statistically significant.

## Results

### *Tumor response after Histamine-based ILP.*

We previously showed that TNF-α improves the response to ILP by increasing the amount of melphalan delivered to tumor tissues (7). In this study, we used a similar model to test whether another vasoactive molecule, Hi, could also enhance melphalan uptake. A range of Hi concentrations were tested (20 to 200 μg/mL), and the concentration that led to optimal tumor regression was determined to be 200 μg/mL. Tumors grew exponentially in the Brown Norway rats after control ILP. However, the response to Hi plus melphalan ILP was striking, with a regression (more than 25% decrease in tumor volume) in four (66%) of the six treated animals, including two (33%) with no palpable tumors approximately 10 days after treatment ($p<0.01$). Perfusion with Hi alone or melphalan alone reduced or stabilized tumor growth - three stable (50%) and one regression (17%) (Fig. 1A and Table 1). .

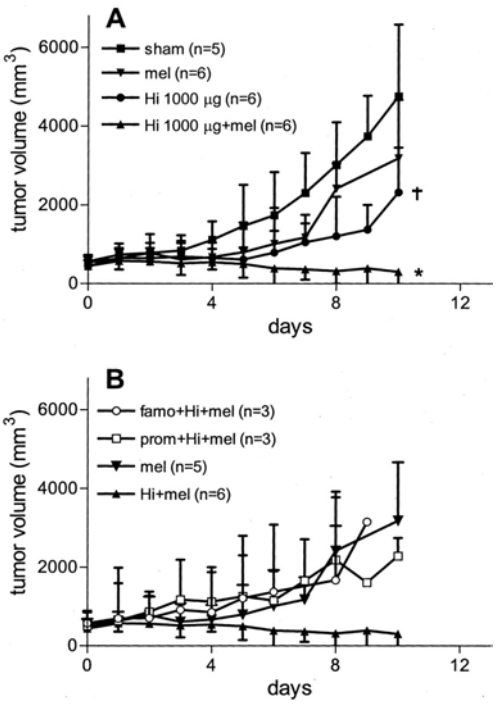


**Figure 1 A)** Tumor response after histamine-based isolated limb perfusion (ILP): small fragments of BN-175 soft tissue sarcoma were inserted in the right hind limb of Brown Norway rats (see "Materials and Methods"). After 7 to 10 days when tumors reached 12 to 15 mm in diameter, they were randomly submitted to ILP with perfusate alone (sham), 8 μg/mL melphalan (mel), 200 μg/mL histamine (Hi), or 200 μg/mL Hi plus 8 μg/mL melphalan. Tumors were measured daily with a caliper, and tumor volumes were calculated. When tumor diameter exceeded 25 mm, rats were killed. * $p<0.001$ on day 8 and 10 compared with sham; **+**$p=0.003$ on day 8 and $p<0.001$ on day 10 compared with sham (repeated-measure analyses of variance; two-sided).

**B)** Involvemente of histamine receptors in histamine-based ILP. Promethazine (H1 receptor inhibitor, 200 μg/mL) or famotidine (H2 receptor inhibitor, 50 μg/mL) were added in bolus to the perfusate and allowed to circulate for 5 minutesbefore Hi and melphalan were added. Mean tumor volumes and upper 95% confidence intervals are depicted in both graphs. The number of independent experiments (rats) for each treatment is shown in parentheses.

The combination of Hi plus melphalan showed a synergistic effect because the response index of the combination group was statiscally significantly greater than that when the response index from the Hi and melphalan alone groups was randomly added ($p = 0.043$, Mann-Whitney U test{exact significance [2x(one-tailed significance)]}. Perfusion with Hi, either alone or combined with melphalan, did not cause systemic toxicity. Only a transient, mild edema after Hi ILP, both with and without melphalan, was observed leading to a temporary grade 1 toxicity in two rats for each group. After two days, the edema disappeared and limb function returned to normal.

***Involvement of histamine receptors in histamine based ILP.***

To determine which Hi receptor ($H_1$-R or $H_2$-R) is involved in the effects observed above, specific Hi inhibitors were used during the treatment. Both pyrilamine, an $H_1$-R blocker and famotidine, an $H_2$-R blocker, could block the effect of Hi in the ILP setting, which means that either $H_1$, and $H_2$ receptors are involved (Fig. 1B).

**Table 1**. Tumor response after histamine-based ILP.

| Treatment | CR (%) | PR (%) | NC (%) | PD (%) |
|---|---|---|---|---|
| Sham (n=5) | - | - | - | 100 |
| Melphalan (n=6) | - | 17 | 17 | 66 |
| Hi (n=6) | - | - | 50 | 50 |
| Hi + melphalan (n=6) | 33 | 33 | 33 | - |

Volume on day 9 was compared with that on day 0, and response was classified as follows: PD = progressive disease, increase of more than 25%; NC = no change, volume between – 25% and + 25%; PR = partial response, decrease between –25% and –99%; CR = complete response, no palpable tumor. Values are expressed in percentage of animals per response per group

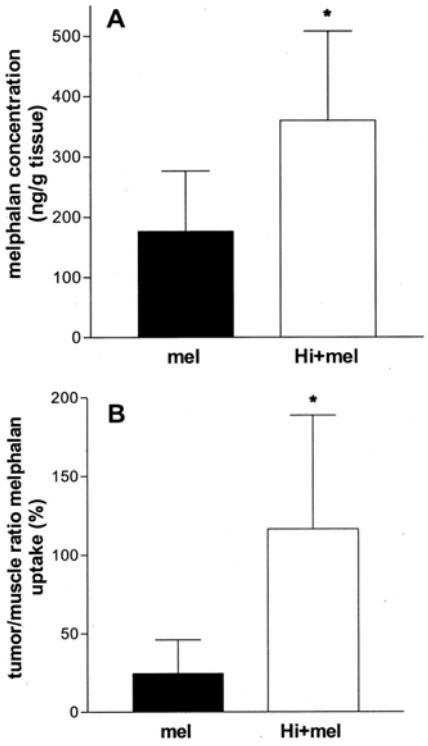


**Figure 2**. Better effect of Hi on the accumulation of melphalan in tumors treated with isolated limb perfusion (ILP). Both tumor and adjacent muscle were excised immediately after ILP and snap-frozen in liquid nitrogen. Melphalan was measured by gas chromatography-mass spectrometry on at least three different pieces per sample as described in "Materials and Methods". A) Tumor melphalan concentration. Closed bar, melphalan alone; open bar, Hi plus melphalan. * p=0.024 (Mann-Whitney U test, two-tailed). B) Ration between tumor and muscle melphalan uptake. Closed bar, melphalan alone; open bar, Hi plus melphalan. * p=0.02 (Mann-Whitney U test, two-tailed). Mean values with upper 95% confidence intervals are shown.

***Indirect effect of Hi on tumor melphalan uptake***.

We next evaluated whether Hi could indirectly affect tumor-associated vasculature, by increasing vascular permeability, which could cause more melphalan to accumulate in tumors than in normal tissue, as we previously showed using TNF-α combined with melphalan in ILP (7). To compare melphalan uptake in tumors and adjacent muscle, we excised tumors and muscle immediately after ILP with melphalan alone or melphalan combined with Hi and measured melphalan concentration. Hi addition not only led to a twofold increase in the amount of melphalan in tumor tissue (p=0.024) but also reduced melphalan concentration in the muscle. As a result, adding Hi increased the ratio of melphalan in the tumor to that in the adjacent muscle by four (p=0.02) (Fig. 2).

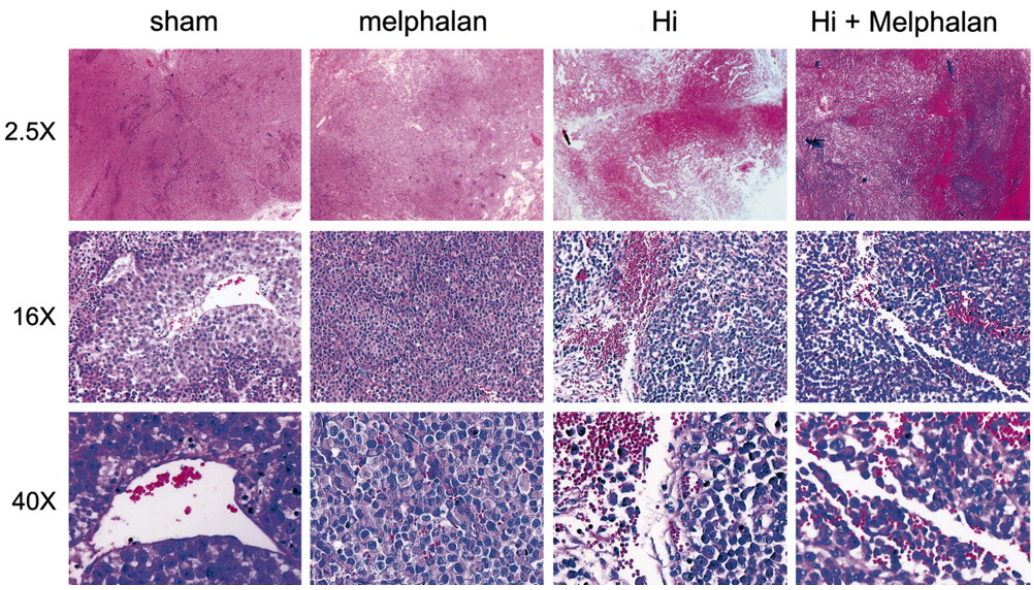


**Figure 3.** Histology of tumor after isolated limb perfusion (ILP). Tumors were excised immediately after ILP for each treatment, fixed in 4% formaldehyde solution and embedded in paraffin for hematoxylin-eosin staining. Perfusate alone (sham) ILP with intact vessels and normal tumor tissue; melphalan 8 μg/mL ILP with some spots of necrosis on tumor tissue but no vascular damage; Hi alone 200 μg/mL ILP showing vascular vasodilation, extravasation of red blood cells into the tumor and damage to the endothelial cell lining of tumor vessels; Hi plus melphalan (200 μg/mL and 8 μg/mL, respectively) ILP showing the damage to tumor vessels and massive hemorrhage. Pictures illustrate representative examples of each treatment)

***Histology.***

To evaluate both the direct and indirect effects of Hi-based ILP on the tumor and the tumor-associated vasculature, we histologically examined tissue sections taken right after ILP was performed. After ILP with 200 μg/mL Hi alone, scattered vascular damage was observed (Fig. 3). After ILP with 200 μg/mL Hi and 8 μg/mL melphalan, vascular damage became more pronounced. Perfusion with Hi alone resulted in vasodilatation of the tumor vasculature, extravasation of red blood cells into the tumor and damage to the endothelial cell lining of tumor vessels. After ILP with Hi and melphalan, most of the tumor vessels were severely damaged and massive haemorrhage was observed. Tumor vessels showed loss of integrity and extensive gap formation, indicating edema. Red and white blood cells observed in the tissue suggested extravasation. We hypothesize that the edema observed in

tumor tissue may indicate an augmented influx of melphalan from the blood stream into the tumor. In the muscle, however, no apparent changes in terms of haemorrhage, vasodilatation, or infiltrates after treatment, as above, were observed (data not shown).

These vascular effects were not observed when rats received sham ILP or melphalan via ILP (Fig. 3). After sham ILP, vessels were intact and tumor tissue was unaffected. When tumors were perfused with melphalan alone, some necrosis of the tumor tissue could be observed, but no vascular damage was seen. Together, these results indicate that Hi has tumor vascular-selective activity against the endothelial lining. This vascular effect was even more pronounced when Hi was combined with melphalan.

***Cytotoxicity of histamine***.

The direct cytotoxic effects of Hi on BN-175 tumor cells and HUVEC endothelial cells were evaluated by means of *in vitro* cytotoxicity assays. Cell growth was inhibited in a concentration-dependent manner for both cell lines evaluated. BN-175 tumor cells were more sensitive to Hi, with an $IC_{50}$ of 30 μg/mL. HUVEC appeared less sensitive to Hi with an $IC_{50}$ of approximately 100 μg/ml (Fig. 4). The cytotoxic effect of Hi combined with melphalan *in vitro* was not synergistic, it was only additive.

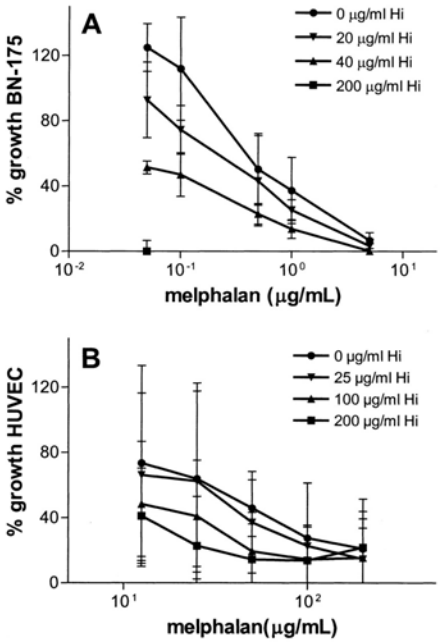


**Figure 4.** In vitro cytotoxicity of Hi according to percentage of tumor growth inhibition. Cells were incubated with different concentrations of Hi with or without melphalan, and cell growth was evaluated using the Sulphorhodamine B assay as described in "Materials and Methods". A) BN175 sarcoma (Hi 50% inhibitory concentration [IC50] of 30 μg/mL); B) Human umbilical vein endothelial cells (HUVECs) (Hi IC50 of 100 μg/mL). Each point represents an average of four independent experiments. Error bars show 95% confidence intervals of the mean.

***Direct effect on HUVEC: morphology and apoptosis assay***.

*In vitro* Hi was only slightly cytotoxic to HUVEC cells after long-term treatment (Fig 4B). Moreover, addition of Hi to melphalan did not enhance the sensitivity of HUVEC toward melphalan (Fig 4B). However, after ILP, a strong effect of Hi on the endothelial lining of tumor vessels was observed (Fig. 3). Therefore, we examined the morphology of HUVECs after short incubations (no longer than 60 minutes) with Hi plus melphalan. We observed a dose and time-dependent effect of Hi on HUVEC, starting with the appearance of gaps between the cells. As time progressed, some cells became rounded and others became extended. In the higher concentration range or after prolonged incubation, cell fragments were seen in the medium (Fig. 5). Cells exposed to medium alone did not show these morphologic changes.

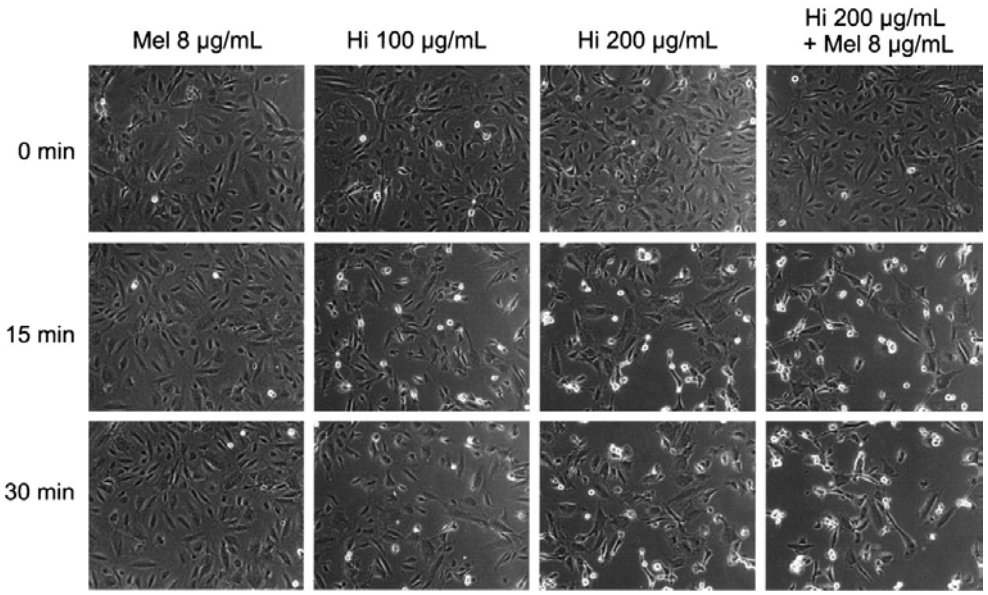


**Figure 5**. Direct effect of Hi on endothelial cells. Human umbilical vein endothelial cells (HUVECs) were incubated with medium alone; melphalan (8 µg/mL) alone, Hi (100 µg/mL or 200 µg/mL) alone or in combination for 15 and 30 minutes. Gap formation and morphologic changes can be observed already after 15 minutes incubation both with 100 and 200 µg/mL (a more pronounced effect for 200 µg/mL).

The observed differences in HUVEC morphology after Hi treatment prompted us to investigate whether these changes were irreversible, that is, whether they could lead to apoptosis or necrosis. With YO-PRO and propidium iodide to detect apoptosis and necrosis

of adherent cells, respectively, we found no differences in the number of apoptotic or necrotic cells after exposure of HUVEC to Hi compared with exposure to medium alone (data not shown). When all cells, adherent as well as detached, were examined using the Vybrant Apoptosis assay, no increase in the number of apopototic cells or the number of necrotic cells was observed when Hi was added compared with medium alone (p=0.4 and p=0.5, respectively) (Fig. 6). Moreover, when Hi was combined with melphalan, still no increase in the number of apoptotic or necrotic cells was seen.

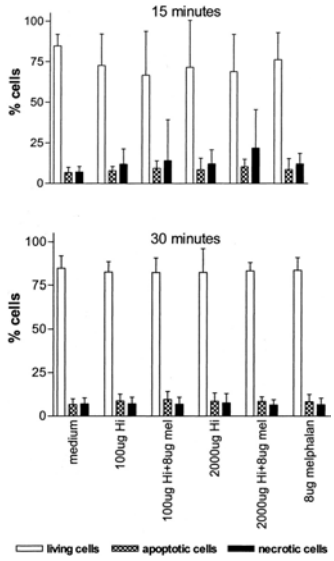


**Figure 6.** Viability of human umbilical vein endothelial cells (HUVECs) after short incubation with Hi. HUVECs were plated 24 hours before treatment at $6x10^4$ cells per well and grown to confluence. Cells were cultured at 37°C in 5% CO2 with different concentrations of Hi (0 to 200 μg/mL) and melphalan (0 to 8 μg/mL) for 15 or 20 minutes. The Vybrant Apoptosis assay kit was used to detect apoptosis and necrosis of adherent and detached cells prior to flow cytometric evaluation of the cells. Experiments were done three times in duplicate. The mean percentage , per group of cells, and upper 95% confidence intervals are shown.

***Hi and paracellular permeability in vitro***.

We observed an increase in melphalan concentration in tumors treated with both drugs, which was accompanied by strong effect of Hi on the tumor associated vasculature after ILP (Fig 2 and 3). Histopathologic examination revealed the Hi-induced formation of gaps *in vivo* in a concentration-dependent manner, requiring a minimum concentration of 200 μg/mL (data not shown). Andriopoulou *et al*. (17) reported that incubation of microvascular endothelial cells for 25 minutes with a relatively low Hi concentration (11 μg/mL) resulted in a 120% and 45% increase in permeability, for long and recently confluent cultures, respectively. We investigated the pattern of permeability using Hi concentrations 10-fold higher than in that study. In line with the findings of Andriopoulou *et al* (17), we found a concentration and time-related effect of Hi on HUVEC monolayer

permeability as well as a sharper increase in permeability in the first 15 minutes. The results presented in Fig. 7A show that exposure of HUVEC to 200 μg/mL Hi alone resulted in an increase in permeability of five-fold (5.6, 95% CI = 3.5 to 7.7) compared with the control, and 100 μg/mL Hi alone resulted in a two to three-fold (2.8, 95% CI = 1.5 to 4.1) increase compared with the control. Incubation with 50 μg/mL Hi caused only a very slight increase of about 1.5-fold (1.5, 95% CI = 1.0 to 2.0). Interestingly, when HUVEC were exposed to 50 μg/mL or 100 μg/mL Hi, no additional effect on permeability was observed after 15 and 30 minutes of incubation (curves start to parallel the control), respectively. Exposure of HUVEC to 200 μg/ml resulted in an ongoing response of HUVECs as shown by the continuing permeability increase compared with control. Even at 60 minutes, the response of HUVECs to Hi did not parallel the control curve. Incubation with melphalan had no effect on the permeability of HUVEC monolayer, neither alone nor in combination with Hi (Fig. 7). The ongoing permeability increase might be essential to the observations *in vivo*.

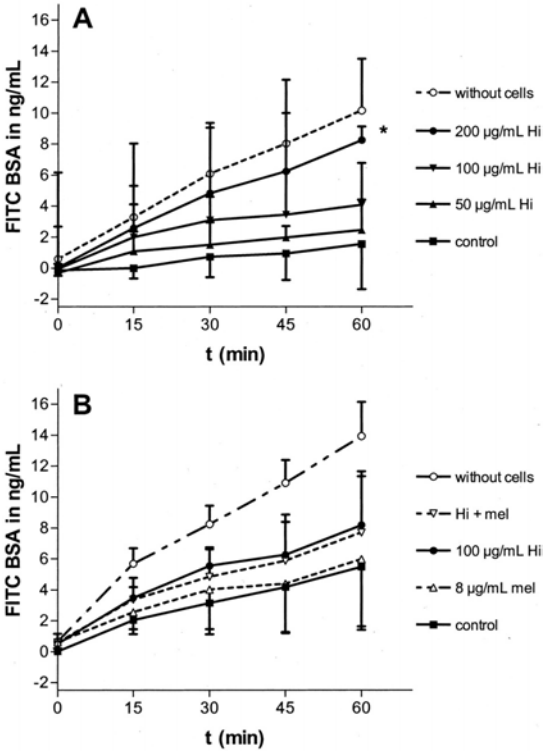


**Figure 7**. Effect of Hi on human umbilical vein endothelial cell (HUVEC) monolayer permeability. HUVECs were cultured on the filter of a transwell unit for 48 hours before the addition of fluorescein isothiocyanate and bovine serum albumin (FITC-BSA)-containing medium (control) or A) Hi in different concentrations, B) plus or minus melphalan to the upper compartment (see "Material and Methods"). The amount of FITC-BSA in the lower compartment was measured every 15 minutes for an hour. Values are from three experiments, each done in duplicate. Error bars show 95% confidence intervals of the mean. * p-values using repeated-measure analysis of variance test p=0.001 for 200 μg/mL Hi at 15, 30, 45 and 60 minutes compared with control.

## Discussion

This study shows for the first time, to our knowledge, the activity of Hi plus melphalan in ILP for the treatment of soft tissue sarcomas. The strong effect of Hi-based ILP with melphalan was explained by three mechanisms: 1) direct cytotoxicity to the tumor cells, 2) direct cytotoxicity to the tumor-associated vasculature, and 3) an indirect effect through Hi-mediated, increased melphalan concentration in the tumor.

The direct inhibitory effect of Hi on tumor cells is in accordance with previous reports on Hi receptor expression on different cell lines and human neoplasias, suggesting that it might regulate tumor cell growth (18,19). This growth-inhibitory effect on the tumor cells, combined with the observed direct effect on the endothelial cells, seen by us both *in vitro* and *in vivo*, might be an explanation for the antitumor effect of Hi (50% of the tumors stopped growing), compared with control perfusions (all tumors continued to grow). Nevertheless, chemotherapeutic drugs, such as melphalan, for example, must be added to the ILP to achieve a good antitumor response, which coincides with our observations in TNF-α-based ILP. (8)

The direct effect of Hi on endothelial cells *in vitro* is more pronounced than that of TNF-α, the current drug of choice for ILP, which we believe adds to the observed tumor response *in vivo*. Hi alone is capable of changing the morphology of endothelial cells after a short incubation period, resulting in gap formation and rounded cells, as is shown in Fig. 5. When combined with melphalan *in vivo*, the effect on the vasculature is much more evident, with diffuse gap formation and destruction of endothelial cell lining observed immediately after the ILP. In the standard treatment using TNF-α plus melphalan, destruction of the endothelial lining is a secondary effect and takes a couple of days to become evident (20). Therefore, ILP with Hi would likely enhance drug uptake more quickly and effectively than ILP with TNF-α.

The *in vitro* permeability results were in accordance with the *in vivo* findings of an augmented uptake of melphalan in the tumor as well as a decrease in the muscular concentration, reducing regional toxicity. It is remarkable that the Hi concentration used in the ILP (200 μg/ml) led to a continuous increase in the permeability of endothelial cells, which is different from the standard described short-term effect of Hi that occurs only for the first 15 minutes of exposure (18). We speculate that with the Hi concentration used in

the ILP, a threshold is reached that triggers a prolonged cellular response, a supposition that is currently under investigation.

Another potential advantage of Hi over TNF-α is its pharmacokinetics. Hi has a very short half-life in - 0.35 minutes versus 20 minutes for TNF-α (21). Hi is metabolized through two major pathways in humans; the main pathway involves ring methylation and is catalyzed by the enzyme histamine-N-methyltransferase, which is widely distributed in the tissues. Most of the product, N-methylhistamine is converted by monoamine oxidase (MAO) to N-methyl imidazole acetic acid. Alternatively, Hi can undergo oxidative deamination catalyzed mainly by the nonspecific enzyme diamine oxidase (DAO). The products are imidazole acetic acid and its riboside, which have little or no activity and are excreted in the urine (13). Although these data come from studies with lower dosages or endogenous Hi, the wide distribution of and fast action of the enzymes that metabolize Hi means that Hi is a potentially safer drug than TNF-α in case of leakage into the systemic circulation during ILP. Furthermore, these properties of Hi pharmacokinetics open new possibilities of application in, for example, isolated liver perfusion. More studies on the pharmacokinetics of higher doses and evaluation in the clinical setting are, however, essential for the clinical translation of Hi.

Our findings support a tumor endothelial cell-specific targeting effect of Hi resulting in dramatic haemorrhage and destruction of the endothelial cell lining of tumor vessels (confirmed with the CD-31 staining [data not shown]) in vivo. We hypothesize that the pronounced direct effect of Hi on the endothelial cell lining is fundamental for the better response than that achieved by melphalan alone in the ILP model discussed here.

$H_1$ and $H_2$ receptors were involved in Hi-induced tumor regression in our model. Each receptor inhibitor blocked the Hi effect *in vivo*. The two receptors are located in different cell types and have independent mechanisms of action: $H_{1\text{-}R}$ has a higher affinity, a rapid but short-lived effect, and is located in the endothelial cells; $H_{2\text{-}R}$ has a lower affinity, a slower but more sustained effect, and is located in the vascular smooth muscle cells.

Toxicity would be unlikely to be a limiting factor for the use of Hi in the ILP in humans because no systemic toxicity was observed, and the regional toxicity, affecting 33% of the rats receiving Hi either alone or combined with melphalan, was very mild and completely reversible after two days of recovery. Accordingly, ILP with TNF-α and melphalan in the clinical setting, as Hi plus melpahaln did in the animal model, also results in erythema and

edema, which sometimes slightly impair motility (respectively Grades II and III of Wieberdink, respectively) in most of the patients (6,22)

In conclusion, Hi combined with melphalan had a striking effect in the ILP for the treatment of soft tissue sarcomas in rats. The mechanism of action involved both direct and indirect effects - cytotoxicity on the tumor and endothelial cells and tumor-associated vasculature with a twofold increase in the tumoral uptake of melphalan combined with a reduction in the uptake in the adjacent muscle. Therefore, Hi plus melphalan in ILP seems to be a promising alternative to TNF-α , to be evaluated in the clinical setting.

## Acknowledgments

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# Chapter 3

## *Synergistic antitumor activity of IL-2 with melphalan in isolated limb perfusion in soft tissue sarcoma bearing rats*

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## Abstract

The cytokine interleukin-2 (IL-2) is a mediator of immune cell activation with some antitumor activity, mainly in renal cell cancer and melanoma. We have previously demonstrated that Tumor Necrosis Factor-alpha (TNF-α) has strong synergistic antitumor activity in combination with chemotherapeutics in the isolated limb perfusion (ILP) setting based on a TNF-mediated enhanced tumor-selective uptake of the chemotherapeutic drug followed by a selective destruction of the tumor vasculature. IL-2 can cause vascular leakage and edema and for this reason we examined the antitumor activity of a combined treatment with IL-2 and melphalan in our well-established ILP in soft tissue sarcoma-bearing rats (BN175). ILP with either IL-2 or melphalan alone has no antitumor effect, but the combination of IL-2 and melphalan resulted in a strong synergistic tumor response, without any local or systemic toxicity. IL-2 significantly enhanced melphalan uptake in tumor tissue. No signs of significant vascular damage were detected to account for this observation, although the tumor sections of the IL-2 and IL-2 plus melphalan treated animals revealed scattered extravasation of erythrocytes compared to the untreated animals. Clear differences were seen in the localization of ED-1 cells, with an even distribution in the sham, IL-2 and melphalan treatments, while in the IL-2 plus melphalan treated tumors clustered ED-1 cells were found. Additionally, increased levels of TNF mRNA were found in tumors treated with IL-2 and IL-2 plus melphalan. These observations indicate a potentially important role for macrophages in the IL-2-based perfusion.The results in our study indicate that the novel combination of IL-2 and melphalan in ILP has synergistic antitumor activity and may be an alternative for ILP with TNF and melphalan.

## Introduction

We have demonstrated that isolated limb perfusion with TNF-α and melphalan is associated with excellent antitumor effects against melanoma (1), large soft tissue sarcomas (2,3) and various other tumors in the clinical setting (4-6). We have previously shown that the basis for the synergy is, on one hand a significant enhancement of tumor selective melphalan uptake and on the other hand the subsequent complete destruction of tumor vasculature (2,7). The enhanced uptake of different cytotoxic agents shown in various limb

and liver tumor models in our laboratory prompted us to investigate a number of vasoactive substances for similar potential effects (7-12).

One of these agents is the cytokine IL-2 that is known to cause significant changes in vascular permeability and to cause a vascular leakage syndrome when administered at high concentrations (13-15). IL-2 is a pleiotropic cytokine that is mainly known as a molecule of central importance in the long-term culture of T lymphocytes and as a mediator of immune cells (16). IL-2, as a single agent, has been shown to have antitumor activity in both animal models (17,18) and some antitumor activity in mainly renal cell cancer or melanoma patients (19-21).

Here we report on the evaluation of the effects of high concentrations of IL-2 in combination with melphalan in the ILP setting.

## Materials and methods

### *Chemicals*

Human recombinant interleukin 2 (IL-2) was kindly provided by Chiron (Amsterdam, the Netherlands). The content of one vial of lyophilized IL-2 (1 mg per vial, specific activity $18x10^6$ IU/mg) was diluted in 1 ml sterile water for injections according to the manufacturer's instructions. Melphalan (Alkeran, 50 mg per vial, Wellcom, Beckenham, UK) was dissolved in 10 ml diluent solvent. Further dilutions were made in phosphate buffered saline to a concentration of 2 mg/mL. Fluorescein and fluorescein isothiocyanate conjugated to bovine serum albumin (FITC-BSA) were purchased from Sigma (Zwijndrecht, the Netherlands) and dissolved in phosphate buffered saline to a concentration of 10 mg/mL.

### *Animals and tumor model*

Male inbred Brown Norway rats were used for the soft tissue sarcoma model (BN175). Rats were obtained from Harlan-CPB, (Austerlitz, the Netherlands), weighing 250-300 grams, and were fed a standard laboratory diet *ad libitum* (Hope Farms Woerden, the Netherlands). Small fragments (3 mm) of the syngeneic BN175 sarcoma were implanted subcutaneously in the right hind leg just above the ankle as previously described (8,10). Tumor growth was recorded by caliper measurement and tumor volume calculated using the formula $0.4(A^2xB)$ (where B represents the largest diameter and A the diameter perpendicular to B).

Rats were sacrificed if tumor diameter exceeded 25 mm or at the end of the experiment. All animal studies were done in accordance with protocols approved by the committee on Animal Research of the Erasmus MC, Rotterdam, the Netherlands.

***Isolated limb perfusion***

The experimental isolated limb perfusion (ILP) technique was previously published (8,10). ILPs were performed at a tumor diameter of 12-15 mm at least 7 days after tumor implantation. During perfusion animals were anaesthetized with Hypnorm and Ketamine (Janssen Pharmaceutica, Tilburg, the Netherlands). The femoral vessels were approached through an incision parallel to the inguinal ligament after systemic heparin administration of 50 IU (Leo Pharmaceutical Products, Weesp, the Netherlands) to prevent coagulation in the collateral circulation and in the perfusion circuit. The femoral artery and vein were cannulated with silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter; 0.64 mm inner diameter, 1.19 mm outer diameter, respectively; Dow Corning, Ann Arbor, MI). Collaterals were temporarily occluded by applying a tourniquet around the groin. An oxygenation reservoir filled with 5 mL Haemaccel (Behring Pharma, Amsterdam, the Netherlands) and a low-flow roller pump (Watson Marlow type 505 U, Falmouth, UK) were included into the circuit. Drugs, 50 µg IL-2 and/or 40 µg melphalan, were added to the Haemaccel reservoir. The roller pump circulated the perfusate at a flow of 2.4 mL/min for 30 minutes. A washout with 5 mL oxygenated Haemaccel was performed at the end of the perfusion. During ILP and washout, the hind leg was kept at a constant temperature of 38-39°C by a warm-water mattress applied around the leg. The pH of the perfusate was monitored during ILP with a pH probe in the perfusion reservoir (pH meter HI 8424, Hanna Instruments, Inc, Ann Arbor, USA).

***Assessment of tumor response***

Tumor size was recorded daily caliper measurements. The classification of tumor response was: progressive disease (PD) = increase of tumor volume of more than 25%; no change (NC) = tumor volume equal to volume during perfusion (in a range of -25% to + 25%); partial response (PR) = decrease of tumor volume (-25% and -90%); complete remission (CR) = no palpable tumor (10). To test synergy of IL-2 and melphalan, the tumor response ratio is calculated by dividing the tumor volume at day 0 by the tumor volume at day 8. The

ratio of IL-2 alone treated rats plus the ratio of melphalan alone treated rats was compared with the ratio of IL-2 plus melphalan treated rats (Mann Whitney U test).

***In vitro response of endothelial and tumor cells to IL-2***

Cells isolated from the BN175 soft tissue sarcoma were maintained in cell culture in RPMI 1640 supplemented with 10% fetal calf serum and 0.1% penicilline-streptomycine. Media and supplements were obtained from Life Technologies (Breda, the Netherlands).

Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from normal human umbilical cords by the method of Jaffe *et. al*. (22). Cells were cultured in fibronectin coated tissue culture flasks in culture medium (Human Endothelial-SFM (GIBCO-BRL, Life Technologies, Breda, the Netherlands), with 20% Newborn Calf Serum, 10% Human Serum (Cambrex, Verviers, Belgium), 20 ng/mL bFGF and 100 ng/mL EGF (Peprotech, London, UK). Passages 5-7 were used for the experiments.

BN175 cells were added in 100 µL aliquots to 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA, USA) at a final concentration of $1x10^4$ cells per well and allowed to grow as a monolayer. HUVECs were plated in fibronectine coated 96-well plates at a final concentration of $6x10^3$ cells per well. Cells were incubated at 37ºC in 5% $CO_2$ for 72 hours in the presence of various concentrations of IL-2 and melphalan with or without leukocytes in a total volume of 150 µL.

Growth of tumor cells was measured using the Sulphorhodamine-B (SRB) assay according to the method of Skehan (23). In brief, cells were washed twice with phosphate buffered saline, incubated with 10% trichloric acetic acid (1 hour, 4ºC) and washed again. Cells were stained with 0.4% SRB (Sigma, Zwijndrecht, the Netherlands) for 15-30 min, washed with 1% acetic acid and were allowed to dry. Protein bound SRB was dissolved in TRIS (10 mM, pH 9.4). The optical density was read at 540 nm. Tumor growth was calculated using the formula: percentage of tumor cell growth = (absorbance of test well/absorbance of control well) x 100%. The concentration of IL-2 leading to 50% reduction in absorbance compared to control (i.e. 50% inhibitory concentration [$IC_{50}$]) was determined from the growth curve ($IC_{50}$). The experiments were repeated at least 5 times.

***Preparation of leukocytes***

Venous blood from healthy adult volunteers was collected in Na-heparin tubes (Becton Dickinson, Alphen aan den Rijn, the Netherlands). After centrifuging for 20 min (1500 g, room temperature), total white blood cell fraction was collected and remaining red blood cells were lysed with lysis buffer (0.83% $NH_4Cl$, 10 mM Hepes, pH = 7.0) for 30 min at room temperature. After centrifuging for 30 min (1500 rpm, room temperature), the leukocytes were dissolved in HUVEC medium at a concentration of $120.10^4$ cells/mL.

***Measurement of melphalan in tissue***

At the end of the perfusion directly after the washout, the tumor and part of the adjancent muscle were excised. The tissues were immediately frozen in liquid nitrogen to stop metabolism of melphalan, and stored at -80°C. Tumor and muscle tissues were homogenized in 2 ml acetonitrile (Pro 200 homogenizer, Pro Scientific, CT, USA) and centrifuged at 2500 g. Melphalan was measured in the supernatant by gas chromotography-mass spectrometry (GC-MS). p-[Bis(2-chloroethyl)amino]-phenylacetic methyl ester was used as an internal standard. Samples were extracted over trifunctional C18 silica columns. After elution with methanol and evaporation, the compounds were derived with trifluoroacetic anhydride and diazomethane in ether. The stable derivates were separated on a methyl phenyl siloxane GC capillary column and measured selectively by single-ion monitoring GC-MS in the positive EI mode described earlier by Tjaden and de Bruijn (24).

***Vascular permeability***

During ILP 400 µL FITC BSA was added to the perfusate. After perfusion the tumor was excised and frozen in liquid nitrogen. Acetone-fixed frozen sections were fixed for 30 min with 4% formaldehyde. After washing with PBS, the slides were incubated for 1 hour with mouse-anti-rat-CD31PE (Becton Dickinson, Alphen aan den Rijn, the Netherlands) diluted 1:50 in PBS with 5% rat serum. Thereafter, the sections were rinsed with PBS and counterstained with 300 µg/mL Hoechst (Molecular Probes, Leiden, the Netherlands) and mounted with mounting medium containing polyvinyl alcohol (Mowiol 4-88, Fluka, Zwijndrecht, the Netherlands) and 2.5% (w/v) DABCO (Sigma). The sections were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera.

***Human umbilical vein endothelial cell (HUVEC) permeability assays***

To study the effect of IL-2 on transendothelial monolayer permeability a transwell device (Costar, Cambridge, MA, USA) consisting of an upper chamber with a polycarbonate membrane (6.5 mm diameter, 0.4 µm pore size), placed inside a 24-well plate (lower chamber) was used. Confluent HUVECs were trypsinized and $1.2x10^4$ cells were seeded on the fibronectin coated upper chamber. In the lower compartment 1 mL of HUVEC medium was added. Two days after seeding, non-adhering cells were removed and the medium was replaced with 250 µL of 10 µg/mL IL-2 together with 50 µL FITC-BSA or fluorescein (1 mg/mL). The medium in the lower chamber was replaced with 700 µL of HUVEC medium. At 0.25, 0.5, 1, 2, 4, 8 and 24 hours, 50 µL medium of the lower chamber was taken and fluorescence activity was measured under excitation at 490 nm and emission at 530 nm. A standard curve was prepared with known concentrations of FITC-BSA or fluorescein. Induction of permeability was indicated by a higher concentration of FITC-BSA or fluorescein in the lower chamber of the transwell, relative to untreated controls.

***HE staining***

Directly after ILP tumors were excised, stored in formalin and embedded in paraffin. The 4 µm-sections were stained with hematoxylin and eosin using standard procedures. Three or four different tumors in each experimental group were subjected to blind evaluation. At least 6 slides were examined from each tumor. The sections were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera.

***Apoptosis Assays: TUNEL/CD31PE double staining***

Apoptotic cell death was detected using the technique of 3'hydroxy end labeling. A commercially available end-labeling kit (*In Situ* Cell Death detection Kit, Fluorescein labeled, Roche, Almere, the Netherlands) was used. Tumor tissues were also stained for endothelial cells to differentiate between apoptosis of the endothelium and apoptosis of tumor cells. Aceton-fixed frozen sections were fixed in 4% paraformaldehyde for 30 minutes and incubated for 1 hour with mouse-anti-rat CD31PE (Becton Dickinson) diluted 1:50 in PBS with 5% rat serum. After washing with PBS the sections were again fixed in 4% paraformaldehyde for 10 minutes and incubated in 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice to allow permeabilization. The slides were incubated with the

TUNEL mixture for 60 min at 37°C. After incubation, the slides were rinsed three times in PBS and counterstained with 300 µg/mL Hoechst (Molecular Probes) for 10 min. After washing with PBS the slides were mounted with mounting medium containing polyvinyl alcohol (Mowiol 4-88, Fluka). The slides were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera.

***Immunohistochemistry***

After ILP the tumor was excised and immediately frozen in liquid nitrogen. Immunohistochemical studies were performed on acetone-fixed 7 µm-cryostat sections. The tumor sections were fixed for 30 min with 4% formaldehyde and, after rinsing with PBS the endogenous peroxidase activity was blocked by incubation for 5 minutes in methanol/3% $H_2O_2$. The slides were incubated for 1 hour with 1:50 mouse-anti-rat-CD31, CD4, CD8, antibodies to granulocytes (clone HIS48) (Becton Dickinson, Alphen aan den Rijn, the Netherlands) or macrophages (ED-1) (Serotec, Breda, the Netherlands) diluted in 5% rat serum/PBS. Thereafter, sections were washed with PBS and incubated for 1 h with goat-anti-mouse peroxidase-labeled antibody (DAKO, ITK Diagnostics BV, Uithoorn, the Netherlands) diluted 1:100 in PBS with 5% rat serum. After rinsing with PBS, positive cells were revealed by immunoperoxidase reaction with DAB solution (DAB-kit, DAKO) and counterstained lightly with haematoxylin (Sigma).

For quantification of macrophage, CD4+ cell, CD8+ cell, and granulocyte infiltration, and microvessel density two independent persons performed blinded analysis. Six representative fields (magnification 16x) in each slide and three tumors per treatment were evaluated. The sections were examined on a Leica DM-RXA and photographed using a Sony 3CCD DXC 950 camera. For macrophage, T cell and granulocyte infiltration the total number of positive cells per field of interest were counted. For the microvessel quantification, the area of vessels per field of interest was measured in calibrated digital images (Research Assistant 3.0, RVC, Hilversum, the Netherlands) and number of vessels counted.

**Table 1.** RT-PCR primers for the immune related genes and for β-actin, which was used as a housekeeping gene.

| Gene | Primers | Annealings Temp. | Product size (bp) |
|---|---|---|---|
| **B-actin** | **f: 5'-ATGGATGACGATATCGCTG-3'**<br>**r: 5'-ATGAGGTAGTCTGTCAGGT-3'** | **60ºC** | **569** |
| **IL-6** | **f: 5'-GACTTCACAGAGGATACC-3'**<br>**r: 5'-AAGTTGTTCTTCACAAACTCC-3'** | **55ºC** | **294** |
| **GRO/CINC-A** | **f: 5'-GAAGATAGATTGCACCGATG-3'**<br>**r: 5'-CATAGCCTCTCACACATTTC-3'** | **57ºC** | **367** |
| **IL-10** | **f: 5'-TGACAATAACTGCACCCACTT-3'**<br>**r: 5'-TCATTCATGGCCTTGTAGACA-3'** | **60ºC** | **402** |
| **IL-12** | **f:5'-TCATCAGGGACATCATCAAACC-3'**<br>**r: 5'-CGAGGAACGCACCTTTCTG-3'** | **65ºC** | **210** |
| **TNF-α** | **f: 5'-CTGAACTTCGGGGTGATCGGTCC-3'**<br>**r: 5'-CAGCCTTGTCCCTTGAAGAGAACC-3'** | **60ºC** | **295** |
| **IFN-γ** | **f: 5'-GCCTCCTCTTGGATATCTGG-3'**<br>**r: 5'-GTGCTGGATCTGTGGGTTG-3'** | **60ºC** | **239** |
| **MCP-1** | **f: 5'-ATGCAGGTCTCTCTGTCACG-3'**<br>**r: 5'-CTAGTTCTCTGTCATACT-3'** | **57ºC** | **446** |
| **MIP-2** | **f: 5'-GGCACAATCGGTACGATCCAG-3'**<br>**r: 5'-ACCCTGCCAAGGGTTGACTTC-3'** | **55ºC** | **287** |
| **TGF-β1** | **f: 5'-TGGAAGTGGATCCACGAGCCCAAG-3'**<br>**r: 5'-CAGGAGCGCACGATCATGTTGGAC-3'** | **55ºC** | **240** |

**f: forward primer, r: reverse primer**

***RT-PCR***

Total RNA was extracted from frozen tumor tissue using the guanidine isothiocyanate based TRIzol reagent (Life Technologies, Breda, the Netherlands) according to the manufacturer's specifications. BN175 cells *in vitro* were treated with medium, 10 μg/mL IL-2, 8 μg/mL melphalan or IL-2 plus melphalan and after 30 min of incubation total RNA was extracted. All procedures were carried out with sterile, RNase-free solutions, reagents and disposables. Total RNA was quantified by spectrophotometric analysis at wavelengths of 260 and 280 nm. To assure the quality of the RNA isolates, samples were analyzed by electrophoresis in agarose gel.

A volume of 20 µL containing 1.0 µg of total RNA of each sample was used for generation of cDNA with Omniscript Reverse Transcriptase (Qiagen, Leusden, the Netherlands) and oligo $d(T)_{16}$ (Life Technologies, Breda, the Netherlands). After incubation at 42˚C for 1 hour, the samples were heated for 5 min at 93˚C to terminate the reaction. Titanium Taq DNA polymerase (Becton Dickinson, Alphen aan den Rijn, the Netherlands) was used for the PCRs and 1.5 µL of cDNA per 37.5 µL of reaction mixture was used. The primers were purchased from Life Technologies (Breda, the Netherlands) and primer sequences are shown in table 1. β-actin was used as an internal standard. PCRs were performed on a Biometra T-gradient PCR machine using the following parameters: initial denaturation at 94˚C for 5 min followed by a maximum of 40 cycles of 94˚C for 45 sec, annealing for 45 sec (temperatures see table 1) and extension 72˚C for 1 min and a final extension step at 72˚C for 7 min. The resulting DNA fragments were electrophoretically separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. A 100-bp ladder was used as the standard.

***Semi-quantitative RT-PCR***

Total RNA isolation, cDNA preparation and RT-PCR were performed as described above (see RT-PCR). Semi-quantification of cytokine expression was carried out as followed, every 2 cycles, 5 µL of PCR product was collected and the samples were electrophoretically separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. The threshold cycle was determined as the cycle where the visible band of a specific PCR product first appeared on the gel. Intensities of the PCR product bands were determined by ImageJ v1.34 software (W. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA) and normalized for b-actin.

***Statistical analysis***

Results were evaluated for statistical significance with the Mann Whitney U test. P-values below 0.05 were considered statistically significant. Calculations were performed on a personal computer using GraphPad Prism v3.0 and SPSS v11.0 for Windows 2000.

## Results

### *Tumor response in IL-2 based ILP*

To evaluate the antitumor activity of melphalan when combined with IL-2 in an isolated limb perfusion, soft tissue sarcoma-bearing rats were perfused with the agents alone or combined. Sham perfusion with haemaccel alone resulted in progressive disease in all animals (figure 1 and table 2), whereas application of melphalan resulted in a slight inhibition of the tumor growth, with a tumor response rate of 17% (PR and CR combined). Progressive disease was also seen in all animals perfused with 50 µg IL-2. Perfusion with IL-2 plus melphalan resulted in a strong synergistic anti-tumor response and tumor response reaching 67% ($p<0.05$ compared with melphalan alone). We statistically proved the IL-2 plus melphalan synergy ($p<0.02$). No obvious regional or systemic toxicity was observed in any of the treatments.

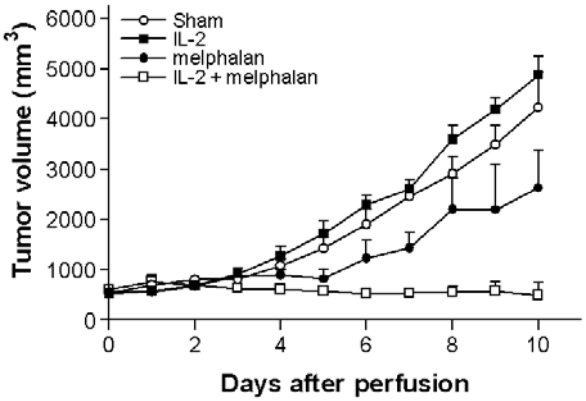


**Figure 1**. Tumor volumes of the subcutaneously implanted soft-tissue sarcoma BN175 after isolated limb perfusion with perfusate alone (sham), 50 µg IL-2, 40 µg melphalan or the combination of 50 µg IL-2 and 40 µg melphalan. Mean tumor volumes are shown ± SE. Number of rats per group is shown in Table 2.

### *Direct effect of IL-2 and melphalan on BN175 and endothelial cells*

*In vitro* experiments were performed to define whether direct cytotoxicity contributed to the improved tumor response. Because the target can be tumor vascular endothelial cells as well as tumor cells, both HUVECs and BN175 cells were tested. No direct cytotoxicity could be observed when BN175 cells or HUVECs were exposed to concentrations of IL-2 up to 10 µg/mL. Exposure of BN175 cells or HUVECs to melphalan resulted in a response curve with an $IC_{50}$ of 0.25 and 11.4 µg/mL respectively. Addition of IL-2 to melphalan did

not alter the $IC_{50}$ of melphalan in both cell types. Incubation of HUVEC with IL-2 did not change the typical cobblestone-shape of these cells. IL-2 had also no additive effect on HUVECs when co-incubated with leukocytes (data not shown).

**Table 2.** Tumor response in soft tissue sarcoma-bearing rats after isolated limb perfusion with IL-2 and melphalan over a total period of 10 days.

| Treatment | PD (%) | NC (%) | PR (%) | CR (%) |
|---|---|---|---|---|
| Sham (n=8) | 100 | - | - | - |
| IL-2 (n=9) | 100 | - | - | - |
| Melphalan (n=6) | 66 | 17 | 17 | - |
| IL-2 + melphalan (n=8) | 22 | 11 | 56 | 11 |

PD = progressive disease, increase of more than 25%; NC = no change, volume between – 25% and + 25%; PR = partial response, decrease between –25% and –99%; CR = complete response, no palpable tumor. Values are expressed in percentage of animals per response per group

***Tissue concentrations of melphalan***

Accumulation of melphalan in tumor and normal muscle tissue was determined. A highly significant tumor-selective increase of melphalan uptake was observed in the rats treated by

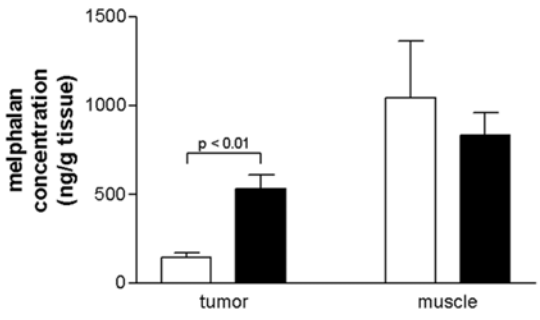


**Figure 2**. Accumulation of melphalan in soft tissue sarcoma BN175 and adjacent muscle after isolated limb perfusion (ILP). Rats were perfused with 40 μg melphalan alone or 40 μg melphalan plus 50 μg IL-2. Tumor and adjacent muscle were excised immediately after ILP and snap-frozen in liquid nitrogen. Melphalan was measured by gas chromatography-mass spectrometry on at least three different pieces per sample as described in "Materials and Mehtods". Mean values with upper SD are shown

an ILP with IL-2 and melphalan in comparison to rats treated with an ILP with melphalan alone. Figure 2 demonstrates a 3.7-fold increase in melphalan concentration in tumor tissue after perfusion with IL-2 plus melphalan (n=4) in comparison with perfusion with melphalan alone (n=4) ($p<0.01$). Importantly, IL-2 had no effect on the uptake of melphalan by muscle tissue.

***Vascular permeability and damage by IL-2***

Since we observed an increased accumulation of melphalan especially in tumor tissue after ILP with IL-2 and melphalan, we investigated the effect of IL-2 on the tumor vascular lining in more detail. First, we looked at extravasation of a larger tracer molecule (FITC-BSA) into the tumor tissue. During the perfusion, FITC-BSA was added to the perfusate and after ILP tumors were excised and frozen sections were stained with CD31PE to visualize blood vessels. We saw no increased vascular permeability for this molecule in the IL-2 plus melphalan group compared to the sham group (data not shown). Most of the FITC-BSA was still present in the blood vessels and hardly any extravasation of the albumin had taken place. For this we hypothesized that permeability of the relatively small melphalan (0.3 kD) molecule is differently affected as compared to FITC-BSA (66.4 kD).

These results were confirmed *in vitro* in which we assessed the capacity of IL-2 to induce permeability in endothelial cell monolayers. A transwell insert with only fibronectin coating and no cells was used to determine the maximum passage of FITC-BSA or fluorescein across the membrane. Incubation of HUVECs with 10 μg/mL IL-2 did not cause an increase in monolayer permeability for FITC-BSA in a period of 24 hours (data not shown). These experiments were repeated with fluorescein, a smaller molecule. Incubation with IL-2 resulted in a 1.6-fold increased permeability compared to untreated cells after 15 min of exposure and incubation times of 1 hour or longer showed no increased permeability anymore (figure 3). In conclusion, there was a transient effect of IL-2 on the permeability of endothelial cells *in vitro* for small molecules like fluorescein and not for proteins like BSA. This could explain why an increased melphalan uptake *in vivo* directly after ILP was seen and no increased permeability of FITC-BSA *in vivo*.

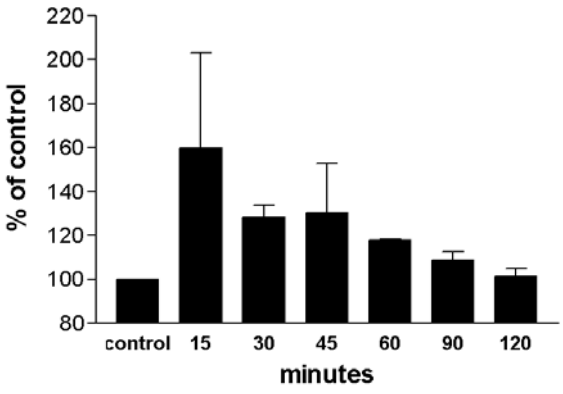


**Figure 3.** Effect of IL-2 on human umbilical vein endothelial cell (HUVEC) monolayer permeability. HUVECs were cultured on the filter of a transwell unit for 48 hours before the addtion of fluorescein containing medium (control) or fluorescein containing medium plus various concentrations of IL-2 as describe in "Material and Methods". The amount of fluorescein in the lower compartment was measured for various time points for 2 hours period. Values are from two experiments, each done in duplicate. The mean is shown with upper SD.

***pH measurement in perfusate***

Before and during perfusion the pH of the perfusate was measured. The pH of Haemaccel is 6.9 and oxygenation lowered the pH to 6.2. Directly after start of the perfusion the pH increased up to 6.5 ± 0.3. Different treatments did not have an effect on the pH of the perfusate and the pH at the end of the perfusion was 6.9 ± 0.1 for all four treatments.

***HE staining and apoptosis in vivo***

Histopathological examination was performed on the tumors from animals treated with sham, IL-2, melphalan or IL-2 plus melphalan to evaluate damage to the endothelial lining of tumor vessels. The animals were autopsied directly after ILP and the tumor slides were stained with hematoxylin. The tumor sections of the IL-2 and IL-2 plus melphalan treated animals revealed scattered extravasation of erythrocytes compared to the sham and melphalan treated animals, although the endothelial lining seemed mostly to be intact (figure 4). In the IL-2 and the IL-2 plus melphalan treatment there was a small increase in edema in the tumor in comparison with the other two groups. Because the BN175 tumor is a fast growing tumor, necrotic areas were seen in all four treatments (data not shown). At this immediate post-ILP time-point there seemed to be no difference in the number and size of the necrotic areas between the treatments.

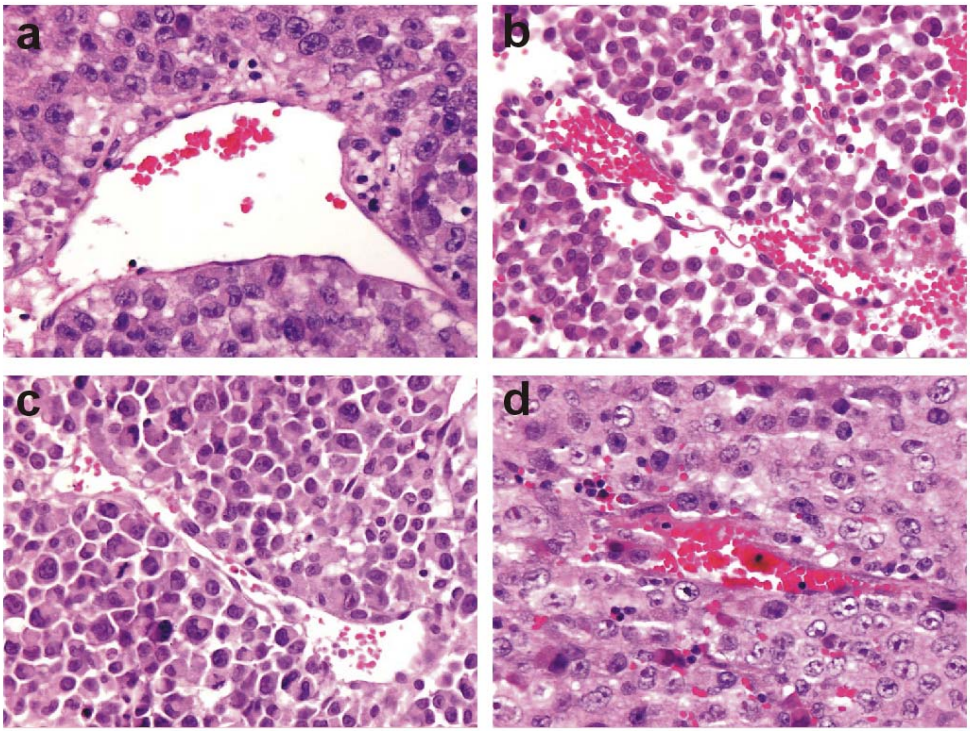


**Figure 4.** Histology of BN175 tumor after isolated limb perfusion (ILP). Tumors were excised immediately after ILP for each treatment, fixed in 4% formaldehyde solution and embedded in paraffin for hematoxylin-eosin staining. (a) sham (perfusate alone) ILP; (b) 50 μg IL-2 ILP; (c) 40 μg melphalan ILP; and (d) 50 μg IL-2 plus 40 μg melphalan ILP. . Pictures illustrate representative examples of each treatment. Original magnification of 40x

The subtle changes in vascular leakage and damage to the tumor vessels evoked by IL-2 were further confirmed by double staining of tumor sections for apoptosis and for CD31 expression. Only a few apoptotic tumor and endothelial cells were detected in both the sham and the IL-2 treated rats, without differences between the two groups (data not shown). These results indicate that IL-2 has a much less pronounced effect on the tumor vasculature when compared to TNF, which inflicts massive hemorrhagic necrosis when used in ILP (25).

**Table 3.** Microvessel density, area of the vessels and tumor infiltration after IL-2 based - isolated limb perfusion (ILP)

| | sham | IL-2 | Melphalan | IL-2 + melphalan |
|---|---|---|---|---|
| Number of vessels[a] | 33 ± 9 | 64 ± 24 | 22 ± 2 | 29 ± 5 |
| Area endothelium[b] | 6.8 ± 0.6 | 9.2 ± 1.3 | 5.9 ± 0.9 | 6.4 ± 0.8 |
| Area endothelium/vessel[c] | 0.30 ± 0.09 | 0.16 ± 0.04 | 0.28 ± 0.03 | 0.24 ± 0.02 |
| CD4[d] | 0.4 ± 0.2 | 2.4 ± 0.2 | 0.2 ± 0.2 | 0.1 ± 0.1 |
| CD8[e] | 51 ± 7 | 36 ± 10 | 67 ± 47 | 37 ± 10 |
| Granulocytes[d] | 60 ± 4 | 79 ± 4* | 48 ± 3* | 63 ± 4 # |
| Macrophages[d] | 210 ± 7 | 222 ± 52 | 201 ± 20 | 189 ± 20 |

Immediately after ILP tumors were excised and snap frozen. 7 µm-cryostat sections were stained for granulocytes, macrophages and for CD31, CD4 and CD8 positive vessels. At least 2 animals per group and 6 fields of interest per tumor were evaluated. Average ± SEM is shown. [a] number of vessels per field of interest; [b] % of total vessel area per field of interest; [c] area per vessel; [d] amount of positive cells per field of interest. * p=0.05 compared with sham ILP; # p=0.05 compared to melphalan treatment.

***Assessment of tumor vascular functionality***

The increased uptake of melphalan might correlate with the functionality of the tumor-associated vasculature. Quantification of the microvessel density and functionality was performed by immunohistochemical staining of endothelial cells. The number of vessels as well as vessel area was measured. The area per vessel is computed by dividing the total area of vessels by the number of vessels. There was no significant difference between the treatments in the number of vessels, area endothelium or the area per vessel (table 3).

***Tumor infiltration of leukocytes and macrophages***

*In vitro* IL-2 seems to have a small and transient effect on endothelial cells and no effect on tumor cells, which is also seen with TNF. We hypothesized that IL-2 stimulates immune-cells to contribute to the increased vascular leakage. To see if there is an increased infiltration of leukocytes into the tumor tissue, tumors were excised directly after isolated perfusion with sham, IL-2, melphalan or IL-2 plus melphalan. Immunohistochemical staining for CD4 and CD8 was performed on frozen sections. There were hardly any CD4 positive cells present in all four treatments (table 3). The number of infiltrating CD8 cells

was much higher compared to the number of CD4 cells. However, no clear differences in the amount of infiltrating CD8 cells were seen between the four groups.

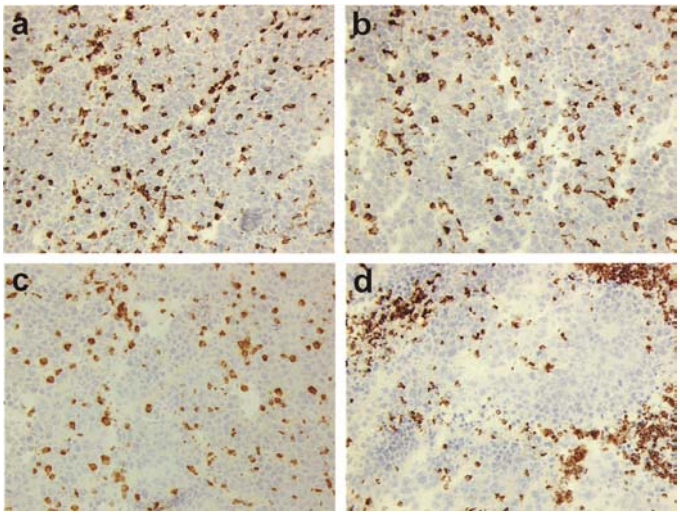


**Figure 5**. Macrophage infiltration and distribution after IL-2 based isolated limb perfusion (ILP). Tumors were excised immediately after ILP for each treatment and snap frozen. 7 μm-cryostat sections were stained for ED-1 positive cells (macrophages). (a) sham (perfusate alone) ILP; (b) 40 μg melphalan ILP; (c) 50 μg IL-2 ILP; and (d) 50 μg IL-2 plus 40 μg melphalan ILP showing a redistribution of macrophages and ED-1 cells. Pictures illustrate representative examples of each treatment. Original magnification of 16x

Infiltrated granulocytes were detected in tumor tissue in slightly larger numbers than CD8 cells. ILP with melphalan resulted in a decreased number of granulocytes compared to sham perfusions ($p=0.05$). Addition of IL-2 to the sham perfusion increased the number of infiltrating cells and addition of IL-2 to melphalan ILP also resulted in an increase in infiltrating granulocytes ($p=0.05$). However, no increased infiltration of granulocytes in the IL-2 plus melphalan group compared to sham perfusions was found. In none of the treatments a difference in distribution pattern of granulocytes was found.

Macrophages were present is all treated and untreated tumors in larger proportions than T cells and granulocytes, without differences between the treatments. But, clear differences were seen in the localization of ED-1 cells, with an even distribution in the treatments sham, IL-2 and melphalan, while in the IL-2 plus melphalan group clustered ED-1 cells were found (figure 5).

***Cytokine expression in tumor tissue and tumor cells in vitro***

The different macrophage distribution after perfusion with IL-2 and melphalan indicates possible macrophage activation. These activated macrophages could produce non-specific effector molecules like cytokines and reactive oxygen and nitrogen intermediates, all of which exhibit potent antitumor properties. Tumor biopsies were obtained directly after ILP and RNA extracted from these samples was amplified in order to create an overview of cytokine-profile in the tumor microenvironment and the effect of treatment on this profile.

All cytokines tested were expressed in sham treated tumors (data not shown). In these tumors, TGF-β1 had the highest expression level followed by MCP-1 and expression of TNF was the lowest compared to the other cytokines tested. Strikingly, only for TNF expression a response to IL-2 treatment was observed. The threshold cycle of TNF expression in IL-2 and IL-2 plus melphalan treated tumors was 5 cycles lower than that of sham treated tumors ($p<0.05$). Integrated density was measured and TNF mRNA levels were expressed as a ratio of TNF to b-actin (figure 6). A 5.7-fold increase in TNF mRNA expression was found in tumor tissue treated with IL-2 plus melphalan compared to sham treatment ($p<0.05$). IL-2 ILP led to a 3.2-fold increase in TNF mRNA expression compared to sham ILP ($p<0.05$), whereas melphalan ILP had no effect on TNF mRNA expression.

To investigate which cytokines BN175 tumor cells produced, RNA was isolated from BN175 tumor cells *in vitro*. Cells were also incubated with 10 µg/mL IL-2 and 8 µg/mL melphalan or the combination of 10 µg/mL IL-2 and 8 µg/mL melphalan for 30 minutes. Clear differences were seen between cytokine expression levels *in vivo* and *in vitro*. mRNA expression in tumors showed higher levels of IL-12, MCP-1 and TGF-β1 than tumor cells *in vitro*. Levels of the other cytokines tested were comparable. Treatment with IL-2 and/or melphalan had no effect on the cytokine expression of tumor cells *in vitro* (data not shown).

## Discussion

In this study we have examined the antitumor activity of a combined treatment with IL-2 and melphalan in an isolated limb perfusion in soft tissue sarcoma-bearing rats. We demonstrate for the first time that ILP with the combination of IL-2 and melphalan in rats bearing limb soft tissue sarcoma BN175 resulted in a strong synergistic antitumor response. The tumor response (PR and CR combined) of 67% after IL-2 plus melphalan ILP was

much higher than the tumor response after melphalan ILP alone (17%). Progressive disease was seen in all animals treated with IL-2 alone ILP. These results are comparable with those of our previous study with TNF and melphalan where we found similar synergy for the combination of TNF and melphalan (8,10). Importantly, the application of high dose IL-2 in ILP was without any local or systemic toxicity indicating possible translation of this cytokine to loco-regional settings in the clinic.

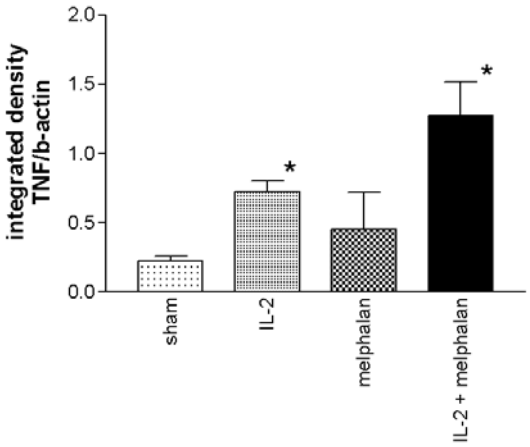


**Figure 6.** Cytokine expression in tumor tissue after IL-2 –based isolated limb perfusion (ILP). Immediately after ILP tumors were excised. RNA was isolated and semi-quantitative RT-PCR with TNF primers was carried out. Integrated density of the PCR bands was measured and TNF mRNA levels are presented as a ratio of TNF to b-actin. The mean of 3 animals per group is shown with upper SEM. * $p < 0.05$ compared to sham ILP

In different animal models IL-2, as a single agent, has been shown to have antitumor activity (17,18). In the systemic setting this requires high doses and multiple dosing, which is associated with serious systemic toxicity with hypotension, massive vascular leakage syndrome and multiple organ failure leading to death (13-15,26). The advantage of a loco-regional application, i.e. an ILP, is the minimal systemic exposure while maintaining a high therapeutic dose locally. For this reason we hypothesized that IL-2 could be a good candidate to be used in a melphalan-based ILP. IL-2 is known not to have direct antitumor effects and thus seems to be an unlikely candidate to be used in the isolated limb perfusion setting. However, we speculated that IL-2 with its multiple effects, could well impact on the pathophysiology of large tumors in a similar way as TNF and might thus significantly potentiate the distribution and uptake of melphalan throughout the tumor. A critical step for TNF-based ILP is the accurate and real-time monitoring of systemic leakage with the aim of avoiding severe systemic TNF mediated toxicity. Because IL-2 is used in a systemic

clinical setting, we hypothesized that IL-2 is a potentially safer drug than TNF and therefore a useful alternative for ILP with TNF. Moreover, the lack of toxicity warrants exploration of IL-2 in loco-regional treatment of liver cancer, in which TNF cannot be used effectively due to its inherent dose limiting liver toxicity.

Here we demonstrate that IL-2 alone in the ILP-setting had no effect on tumor growth, in spite of the high dosage we were using. A strong synergistic tumor response was observed when IL-2 was combined with melphalan. A set of experiments to investigate potential mechanisms behind the observed synergy between IL-2 and melphalan was performed both *in vitro* and *in vivo*. We showed that IL-2 did not have an effect on the proliferation or morphology of HUVECs or BN175 tumor cells *in vitro*. We speculate that the improved anti-tumor effect shown *in vivo* is probably not caused by a direct cytotoxic effect on tumor or endothelial cells.

IL-2 is a 15 kD glycoprotein produced by antigen-activated T lymphocytes that plays a varied and critical role in immunoregulation. IL-2 binds to the IL-2 receptor and the IL-2R is expressed not only on hematopoietic cells, but also on non-hematopoietic cells. Different reports have indicated the presence of IL-2 receptors on cells in head and neck squamous cell carcinoma (27) and different human melanomas (28). We evaluated the direct effect of IL-2 on endothelial cells. We did not see an effect on the proliferation of HUVECs, nor morphologic changes. Holzinger et al. showed that HUVECs possess low numbers of IL-2 receptor, although IL-2 had no effect on the proliferation of the endothelial cells neither on the typical cobblestone-shape morphology of the cells (29). This in contrast with the study of Hicks et al. where they showed that HUVECs do proliferate in response to IL-2 (30). To test whether IL-2 had an indirect effect on HUVECs we co-cultured leukocytes with HUVECs and treated them with different concentrations of IL-2. We showed that there was no effect on proliferation of endothelial cells nor did we observe morphologic changes.

Systemic IL-2 administration is often complicated by significant capillary leakage, with consequent extravasation of interstitial fluid and plasma proteins (14,15,26). In this study we evaluated whether IL-2 could cause capillary leakage in the tumor and therefore enhance the delivery of melphalan at the tumor site. We showed that IL-2 caused a 3.7-fold augmented accumulation of melphalan specifically in tumor tissue, which correlated closely with the enhanced tumor responses. This increase could very well explain the improved efficacy, as ILP with IL-2 alone did not induce any tumor response. The 3.7-fold increase

in local melphalan results in a shift from a hardly effective dose of 0.14 µg/ml to an effective dose of 0.53 µg/ml, when translated to the in vitro cytotoxicity profile of melphalan on BN175 tumor cells. Taken into account that we expect a heterogeneous distribution of melphalan especially around the tumor vessels in the well-perfused region of the tumor, actual local drug levels are likely to be even higher. Further more, IL-2 did not have an effect on the accumulation of melphalan in muscle tissue, indicating that IL-2 works specifically on the tumor-associated vessels.

The mildly acidic condition of the perfusate (pH 6.2) might enhance the antitumor effect of melphalan as suggested by a study of Kelley *et al*. (31). Others showed that hypoxia and acidosis both in vitro and in vivo are able to augment the cytotoxicity of melphalan (32,33). Addition of IL-2 did not have an effect on the pH of the perfusate. The final pH for all treatments was 6.9.

We showed in a previous study that TNF comparably augmented the accumulation of chemotherapeutic drugs specifically in tumor tissue 4- to 6-fold (7,11). As the augmented melphalan accumulation in tumor tissue induced by IL-2 correspond with our observations in TNF-based ILP we expected comparable histological changes. However, the HE slides of tumor tissue after IL-2-based ILP showed a much less pronounced extravasation of erythrocytes when compared to TNF-based ILP (25). However, in IL-2 and IL-2 plus melphalan treated tumors, although the endothelial lining seemed to be intact, scattered extravasation of erythrocytes was observed next to locally increased edema. These findings indicate that IL-2 has a much more subtle effect on the endothelial lining, compared to TNF. Moreover, IL-2 did not increase the permeability to FITC-BSA *in vivo*. Also *in vitro* endothelial permeability for FITC-BSA was not increased when HUVECs were treated with IL-2, while an enhanced permeability of 1.6-fold for fluorescein was seen after 15 minutes of incubation with IL-2. An explanation can be that FITC-BSA is a much larger molecule than fluorescein (molecular weight 66.400 D and 332 D, respectively) and the molecular weight of melphalan (305 D) is comparable with fluorescein. The lack of a strong tumor-vascular effect was confirmed by staining of tumor section for apoptosis. We could only detect few apoptotic tumor and endothelial cells and no differences between sham and IL-2-based ILP were noted. The increased tumor accumulation of melphalan might be correlated with the vessel functionality of the tumors. However, we did not see any differences between the treatments in the vessel area.

As IL-2 did not seem to have an effect on endothelial or tumor cells directly we hypothesized that immune cells are involved. We performed immunohistochemical stainings on tumor tissue collected directly after ILP. We did not see an effect on the number of infiltrating CD4 or CD8 cells. A reason why we do not see more pronounced differences in the amount of infiltrating cells between the treatments could be explained by the time-point at which tissues were collected, after only 30 minutes of treatment. None of the perfusions had an effect on the number of infiltrating macrophages, but clear differences were seen in the localization of macrophages. After ILP with IL-2 and melphalan clustered macrophages were present, while in the other treatments macrophages were evenly distributed. LPS, IFNγ and IL-2 are established as activating agents for monocytes/macrophages. Activated monocytes/macrophages produce cytokines (such as TNF) and free radicals (superoxide and nitric oxide) that have cytotoxic effects on tumor cells (34-37). We showed in tumor tissue treated with IL-2 alone or IL-2 plus melphalan a 5.7-fold increased expression of TNF, while *in vitro* stimulation of tumor cells did not result in an increased TNF expression. These results indicate that activated macrophages could play a role in the anti-tumor response of IL-2-based ILP. Additional studies are ongoing to further elucidate the mechanism.

In conclusion, IL-2 in a melphalan-based ILP is causing a strong synergistic anti-tumor response in soft-tissue sarcoma BN175. Importantly, the addition of IL-2 inflicted no toxicity locally or systemically. The results in our study indicate that the novel combination of IL-2 and melphalan in an ILP can be of value and therefore possibly a useful alternative for ILP with TNF and melphalan, or as a novel candidate for isolated hepatic perfusion.

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# Chapter 4

## *Decreased response rates by the combination of Histamine and IL-2 in melphalan-based isolated limb perfusion*

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## Abstract

Histamine (Hi) combined to melphalan in a rat isolated limb perfusion (ILP) model for lower limb soft tissue sarcoma, resulted in overall response rates (OR) of 66%. Likewise, ILP with interleukin-2 (IL-2) resulted in OR of 67%, when combined to melphalan, in the same experimental model. In systemic immunotherapy, the combination of IL-2 and Hi has been used for solid tumor treatment based on immunomodulatory effects. In this study we used our well-established ILP experimental model to evaluate whether the synergistic effect between the two drugs seen in the systemic setting, could further improve response rates in a loco-regional setting. Histological evaluation was done directly and 24 hours after ILP. Melphalan uptake by tumor and muscle were measured. Hi and IL-2 together, combined to melphalan in the ILP led to OR of only 28%. Histology of tumors demonstrated partial loss of Hi-induced haemorrhagic effect when IL-2 was present. Melphalan accumulation in the tumor when both Hi and IL-2 were added (3.1 fold) was very similar to accumulation with Hi only (2.8 fold), or IL-2 only (3.5 fold) combined to melphalan. In vitro there was no synergy between the drugs. In conclusion there was a negative synergistic effect between IL-2 and Hi in the regional setting.

## Introduction

Isolated limb perfusion with TNF and melphalan is a well-established limb salvage procedure for the treatment of melanoma [1], large soft tissue sarcomas [2,3] and various other tumors in the clinical setting [4,5]. The mechanism of action being primarily a significant enhancement on tumor-specific melphalan accumulation and secondarily the complete destruction of tumor associated vasculature [5,6]. Based on our previous studies documenting an enhanced tumor uptake of different cytotoxic agents in TNF-based perfusion of limb and liver, other vasoactive drugs were considered as potential candidates to be evaluated for similar effects [6-10]. The inflammatory mediator histamine (Hi), due to its effects on fine vessels with the formation of edema and also the formation of gaps between endothelial cells [11] appeared as an almost obvious first candidate. Indeed, it strongly augmented tumor response in melphalan-based ILP with OR of 66% [12]. Next, we found that the cytokine IL-2, known to cause significant changes in vascular

permeability and responsible for the vascular leakage syndrome when administered at high concentrations [13,14], also dramatically improved tumor response in melphalan-based ILP with OR of 67% [15].

Interleukin-2 (IL-2) has been widely used in the systemic treatment of solid tumors and leukemias [16] based on immunomodulatory effects, which can be further enhanced when histamine is added [17,18]. These observations granted evaluating whether this synergy could be also present in the loco-regional setting to further improve response rates observed with Hi or IL-2 alone in melphalan-based ILP.

## Material and Methods

### *Animals and Tumor cell line*

Male inbred Brown Norway (BN) rats were obtained from Harlan-CPB (Austerlitz, the Netherlands), weighing 250-300g. Animals were housed at the Central Animal Facility of the Erasmus MC Rotterdam and fed a standard laboratory diet ad libitum (Hope Farms Woerden, the Netherlands).

The syngeneic spontaneous rapidly growing and metastasizing BN-175 soft tissue sarcoma [19] was kept in liquid nitrogen and implanted on the dorsum of a BN rat for further growth before being transplanted to the right hind limb of rats.

All animal studies were done in accordance with protocols approved by the Animal Care Committee of the Erasmus University Rotterdam, the Netherlands.

### *Chemicals*

Human recombinant interleukin 2 (IL-2) was kindly provided by Chiron (Amsterdam, the Netherlands). The content of one vial of lyophilized IL-2 (1 mg per vial, specific activity 18 x $10^6$ IU/mg) was diluted in 1 mL sterile water for injection according to the manufacturer's instructions. Melphalan (Alkeran, 50 mg per vial, Wellcom, Beckenham, United Kingdom) was dissolved in 10 ml of diluent solvent. Further dilutions were made in PBS to a concentration of 2 mg/mL. Histamine, kindly provided my Maxim Pharmaceuticals Inc. (San Diego, CA, USA) was already diluted at a concentration of 1mg/mL.

***Isolated limb perfusion protocol.***

Small fragments (3 mm) of the syngeneic BN-175 sarcoma were inserted subcutaneously in the right hind leg of rats as previously described [6,8]. Tumor growth was measured daily with a caliper and volume was calculated using the formula $0.4(A^2xB)$ (where B represents the largest tumor diameter and A the diameter perpendicular to B). When tumor diameter exceeded 25 mm or at end of the experiment rats were killed by cervical dislocation, under anesthesia.

The treatment consisted of the experimental ILP as described previously [6,8]. Briefly, 7-10 days after transplantation of tumor fragments a diameter between 12-15 mm was reached, and rats were randomly assigned to the treatment groups. Under anesthesia (intraperitoneal ketamine and intramuscular hypnomidate), the animals were laid in dorsal decubitus and fixed to the operating table. Inguinal vessels were reached through an incision parallel to the inguinal ligament, heparin at 50 IU (Leo Pharmaceutical Products, Weesp, the Netherlands) was systemically administered via penile vein before cannulation of the artery and vein with silastic tubing (0.30 mm inner diameter, 0.64 mm outer diameter and 0.64 mm, 1.19 mm outer diameter, respectively; Dow Corning, Ann Arbor, MI). Cannulas were connected via a roller pump to an oxygenated reservoir in which drugs were added as boluses. A groin tourniquet occluded collateral vessels, warranting a proper isolation of the limbs. Perfusion was maintained for 30 minutes at a mean flow rate of 2.5 mL/min. During the whole procedure the hindleg of the rat was kept at a temperature of $38^o$ to $39^oC$ by means of a warm mattress involving the limb, and constantly controlled by a thermometer placed on the skin. At the end of the 30 minutes perfusion a washout with 2 mL of oxygenated haemaccel was performed and the content of the perfusate was discarded. Cannulas were taken out, vessels were ligated and the skin closed. Rats were then placed under a warm light source for maintenance of body temperature until completely recovered from anesthetic effect.

The 5 mL total volume perfusate consisted of: 1) hemaccel alone (Boehring Pharma, Amsterdam, the Netherlands); 2) hemaccel plus 40 μg melphalan (Alkeran® Wellcome, Beckenham, United Kingdom); 3) hemaccel plus 1000 μg Hi; 4) haemaccel plus 50 μg IL-2; 5) hemaccel, 40 μg melphalan and 50 μg IL-2; 6) hemaccel, 40 μg melphalan and 1000

μg Hi; or 7) hemaccel, 40 μg melphalan, 1000 μg Hi and 50 μg IL-2. Between five and nine rats were included in each group.

Tumor dimensions were measured every day and used to monitor tumor volume. Tumor volume on day 10 was compared to that on day 0 and response was classified as follows: progressive disease (PD), volume increase of more than 25%; no change (NC) volume change in the range of –25% to +25%; partial remission (PR) decrease between –25% and –99% or complete response (CR), no palpable tumor. Overall response (OR) is defined as the percentage of partial plus complete response.

Limb function was a clinical observation in which the rat's ability to walk and stand on the perfused limb was scored after ILP. On this scale grade 0 means a severely impaired function where the rat drags its hindlimb without any function; grade 1 indicates a slightly impaired function, meaning the hindlimb is not used in a normal way but the rat stands on it when rising; finally grade 2 indicates an intact hindlimb function and normal walking and standing pattern is observed.

***HE staining after ILP***

Two animals for each group were killed right after ILP, tumors were excised, fixed in 4% formaldehyde solution and embedded in paraffin before staining with hematoxylin and eosin. Whole tumors were cut in 5 μm thickness slides, so as to cover inner and outer areas. Next, different spots in the slides were carefully examined under the microscope to obtain a broader view of the effect of each treatment. Images were taken on a Leica DM-RXA microscope equipped with a Sony 3CCD DXC camera.

***Immunohistochemistry***

Two rats bearing untreated BN-175 tumors and two submitted to each of the following ILP: (1) Hi+melphalan; (2) IL-2 plus melphalan or (3) Hi+IL-2+melphalan were included in this study. Tumors were excised and immediately frozen in liquid nitrogen (right after the ILP for those treated). Immunohistochemical studies were performed on acetone-fixed 7 μm cryostat sections. Tumor slides were fixed in 4% paraformaldehyde for 30 min, washed in PBS three times and incubated for 5 minutes in methanol/3% $H_2O_2$ to block endogenous peroxidase activity. Next, slides were incubated for 1h with 1:50 mouse-anti ED-1

(macrophages) (Serotec, Breda, the Netherlands) diluted in 5% rat serum/PBS. Then slides were washed with PBS three times before incubation for 1h with 1:100 goat-anti-mouse peroxidase-labeled antibody (DAKO, ITK Diagnostics BV, Uithoorn, the Netherlands) in 5% rat serum PBS. After washing with PBS for three times, positive cells were revealed by immunoperoxidase reaction with DAB solution (DAB-kit, DAKO) and counterstained with haematoxylin according to Meyer (Sigma). Finally, slides were mounted with Mowiol. Images were taken on a Leica DM-RXA microscope equipped with a Sony 3CCD DXC camera.

***In vivo Melphalan uptake***

Right after ILP with either melphalan alone; melphalan plus Hi; melphalan plus IL-2 or melphalan plus Hi and IL-2, rats were killed by cervical dislocation. Tumors and muscle were removed and quickly frozen in liquid nitrogen and stored at –80$^{o}$C. Tissues were thawed and homogenized in 2 ml acetonitrile (PRO 200 homogenizer, Pro Scientific, CT, USA) and centrifuged before melphalan concentration was measured by gas chromatography-mass spectrometry (GC-MS), as previously described [20]. Between five and six rats were included in each group.

***Cytotoxicity assay***.

BN-175 tumor cells (isolated from the spontaneous, rapidly growing and metastasizing soft tissue sarcoma) [19] were first grown in RPMI-1640 medium (Life Technologies, the Netherlands) supplemented with 10% fetal calf serum and 0.1% penicillin-streptomycin (Life Technologies, the Netherlands).

Cells were plated 24 hours before treatment in 96-wells, flat-bottomed, microtiter plates (Costar, Cambridge, MA, USA) at $10^4$ cells per well (100μL) and allowed to grow as a monolayer. Cells were incubated at 37$^{o}$C in 5% $CO_2$ for 72 hours in the presence of medium alone or medium plus different concentrations of IL-2 (0 to 20 μg/mL) and Hi (0 to 200 μg/mL).

Growth of tumor cells was measured using the Sulphorhodamine-B (SRB) assay [21]. In brief, cells were washed with phosphate buffered saline, incubated with 10% trichloric acetic acid for one hour at a temperature of –4$^{o}$C and washed again. Cells were then stained with SRB for about 15 to 30 minutes, washed with 1% acetic acid and allowed to dry.

Protein-bound SRB was dissolved in TRIS (10mM, pH 9.4). Extinction was measured at 540 nm and the percentage of growth inhibition was calculated according to the formula: percentage of tumor cell growth = (test well/control well) x 100%. The drug concentration leading to 50% reduction in the absorbance, as compared to control ($IC_{50}$), was determined from the growth curve. The experiments were repeated four times in duplicate.

Human umbilical vein endothelial cells (HUVEC) were isolated as described by Jaffe et al. [23] and cultured in Human endothelial – SFM/RPMI medium (Biotechnologies, the Netherlands) supplemented with heat inactivated human serum (Biowhitaker, the Netherlands), new born calf serum, human EGF, human vFGF and 0.1% penicillin-streptomycin (Life Technologies, the Netherlands).

HUVEC were plated 24 hours before treatment at $6x10^3$ cells per well (100 μl) and cultured as described above. Growth inhibition and $IC_{50}$ were determined as for the BN-175.

***Statistical analysis***

Kruskal-Wallis and Mann-Whitney U tests were used to evaluate statistical significance of the results. All statistical tests were two-sided and P values less than 0.05 were considered as statistically significant. Calculations were performed with Prism v3.0 software (GraphPad Software Inc.) and SPSS v10.0 for Windows 2000.

## Results

***Tumor response study***

As shown in Figure 1 and Table 1, Sham perfusion and IL-2 alone perfusions did not affect tumor growth leading to PD in all treated animals (100%). Hi alone ILP resulted in tumor growth stabilization in 3 out of 6 rats (50% NC). Melphalan ILP arrested tumor growth in 2 out of 6 rats (34%), including one (17%) PR and 1 (17%) NC but no complete response was observed. As previously reported, Hi plus melphalan and IL-2 plus melphalan had 66% and 67% OR, including 33% CR (2 rats) and 11% CR (1 rat), respectively ($P<0.05$ for both IL-2+melphalan versus melphalan and for Hi+melphalan versus melphalan).

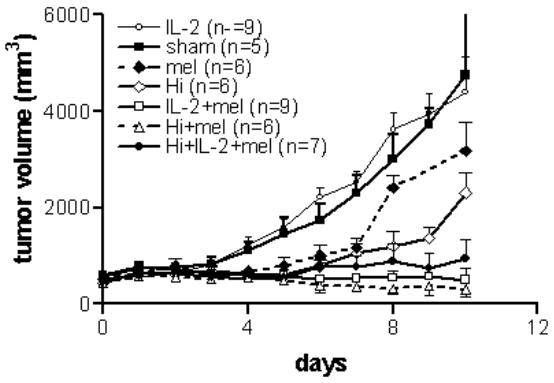


**Figure 1**. Tumor response after isolated limb perfusions. Small fragments of BN-175 soft tissue sarcomas were inserted in the right hind limb of Brown Norway rats (see Material and methods). When tumors reached 12-15 mm (7-10 days), rats were randomly submitted to ILP with perfusate alone (sham); 8 µg/mL melphalan (mel); 200 µg/mL Histamine (Hi); 10 µg/mL IL-2; 10 µg/mL IL-2 plus 8 µg/mL melphalan; 200 µg/mL Hi plus 8 µg/mL melphalan or 200 µg/mL Hi, plus 10 µg/mL IL-2 and 8 µg/mL melphalan. Tumors were measured daily with a caliper and volumes were calculated. Mean tumor volumes +/- SEM are depicted in the graph. The number of independent experiments (rats) for each treatment is shown in parentheses

ILP with the triple combination of IL-2 and Hi plus melphalan resulted in reduced tumor growth inhibition as compared to ILP with IL-2 or Hi combined with melphalan. The best response achieved for this group was a PR in 2 out of seven treated rats (29%). It is also of note that 4 of the rats (42%) from the triple combination group presented with PD, against none out of 6 for the Hi plus melphalan group ($P=0.014$ for Hi+melphalan versus Hi+IL-2+melphalan; $P<0.05$ for IL-2+melphalan versus Hi+IL-2+melphalan).

No systemic side effects or toxicity was observed in any of the treatments administered. As for regional toxicity only some mild temporary edema after Hi-ILP, either alone or combined to melphalan with and without IL-2, was observed leading to a temporary grade 1 toxicity in two rats for each group. After edema resolution, within 3 to 4 days, all animals recovered a normal grade 2 limb function.

***In vitro synergy between Hi and IL-2***

Searching for an explanation for the decreased response rates observed when IL-2 was combined to Hi, we first used *in vitro* cytotoxicity assays.

We knew from previous studies that IL-2 had no direct cytotoxic effect on BN-175 nor on HUVEC [16] whereas with Hi we observed an $IC_{50}$ of respectively 30 µg/mL (BN-175) and 100 µg/mL (HUVEC) [12]. We tested the hypothesis whether IL-2 could decrease the

direct effect of Hi on the cells. The addition of IL-2 was however ineffective with all curves overlapping each other (figure 2).

**Table 1**: Response in BN-175 soft tissue sarcoma-bearing rats after melphalan-based ILP in combination with Histamine and IL-2 over a total period of 10 days.

| Treatment[b] | CR[a] | PR | NC | PD |
|---|---|---|---|---|
| Sham (n=5) | - | - | - | 100% |
| Melphalan (n=6) | - | 17% | 17% | 66% |
| Hi+IL-2+Melphalan (n=7) | - | 29% | 29% | 42% |
| IL-2+Melphalan (n=9) | 11% | 56% | 11% | 22% |
| Hi+melphalan (n=6) | 33% | 33% | 33% | - |

a) responses were scored as described in materials and methods. CR complete response, PR Partial response, NC no change, and PD progressive disease.

b) Melphalan (40 µg), Histamine (Hi, 1000 µg) and IL-2 (50 µg) were added as boluses to the perfusate (5 ml).

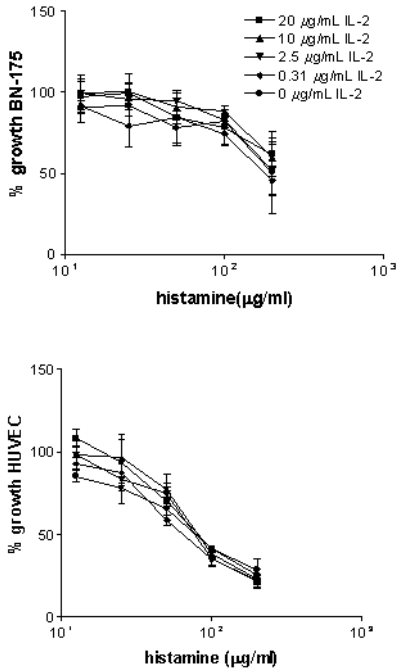


**Figure 2**. In vitro cytotoxicity of Hi and IL-2 according to percentage of tumor growth inhibition. Cells were incubated for 72h with different concentrations of Hi plus IL-2 and cell growth was evaluated by the sulphorhodamine assay as described in materials and methods. (A) BN-175 soft tissue sarcoma cells and (B) Human umbilical vein endothelial cells. Each point represents an average of four independent experiments in duplicate +/- SEM.

***Direct effects on tumor cells and tumor-associated vasculature (TAV)***

Next we evaluated tumor histology right after ILP. Observations right after and 24 hours after ILP were quite similar with a striking loss in the haemorrhagic effect related to Hi. Although some extravasation of red blood cells was observed the diffuse pattern seen after Hi plus melphalan was gone.

Moreover, the massive destruction of the endothelial cell-lining characteristic of Hi plus melphalan ILP was much less pronounced. Also edema between tumor cells was reduced and vessels appeared less dilated when compared to ILP with Hi (Figure 3).

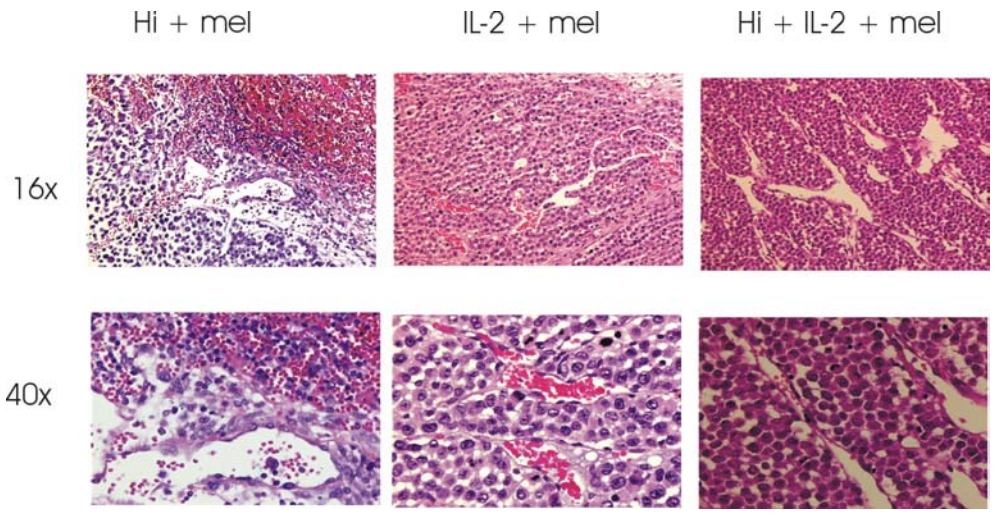


**Figure 3**. Histology of tumors after isolated limb perfusion (ILP). Tumors were excised immediately after ILP for each treatment, fixed in 4% formaldehyde solution and embedded in paraffin for hematoxylin-eosin staining. Hi + melphalan ILP resulted in severe damage to tumor vessels and massive hemorrhage. IL-2 + melphalan inflicted a slight edema while after ILP with Hi + IL-2 + melphalan a striking loss in the Hi-induced hemorrhagic effect was observed. Also less destruction of the endothelial cell lining and reduced edema between tumor cells was seen.

***Indirect effect on TAV***

Finally we evaluated melphalan accumulation in tumor and muscle and compared these values to those previously reported for Hi and IL-2. Accumulation of melphalan after ILP with the triple combination of Hi, IL-2 and melphalan is very similar to the levels obtained after ILP with Hi or IL-2. An increase in melphalan accumulation in tumor of 3.1 fold for Hi plus IL-2 was observed, against a 2.8 fold and 3.5 fold increased melphalan concentration after ILP with respectively Hi or IL-2.

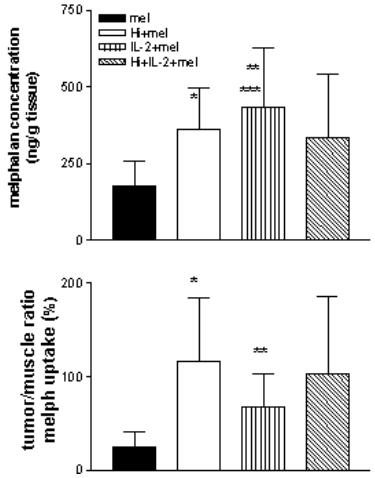


**Figure 4.** Melphalan accumulation in tumor tissue during melphalan-based ILP. Tumors and muscle were excised right after ILP and quickly frozen in liquid nitrogen. Melphalan was measured by gas chromatography-mass spectrometry (GC-MS) as described in material and methods. Values are depicted in bars graph +/- SD. A) Values obtained in the tumor. * P=0.03 (Hi+ melphalan as compared to melphalan); ** P=0.006 (IL-2+ melphalan as compared to melphalan); *** P=0.05 (IL-2+melphalan as compared to Hi+IL-2+melphalan). B) Ratio between tumor and muscle melphalan uptake. * P=0.02 (Hi+melphalan as compared to melphalan); ** P=0.01(IL-2+melphalan as compared to melphalan)

### *Tumor infiltration of macrophages*

Figure 5 shows that macrophages were clearly present both in treated and untreated tumors. As previously published IL-2+melphalan ILP led to a redistribution of macrophages with clustered ED-1 cells [15] . The addition of Hi, in the triple combination treatment, reverted this effect restoring the even distribution observed for all the other groups. This even distribution is also found after Hi+melphalan ILP.

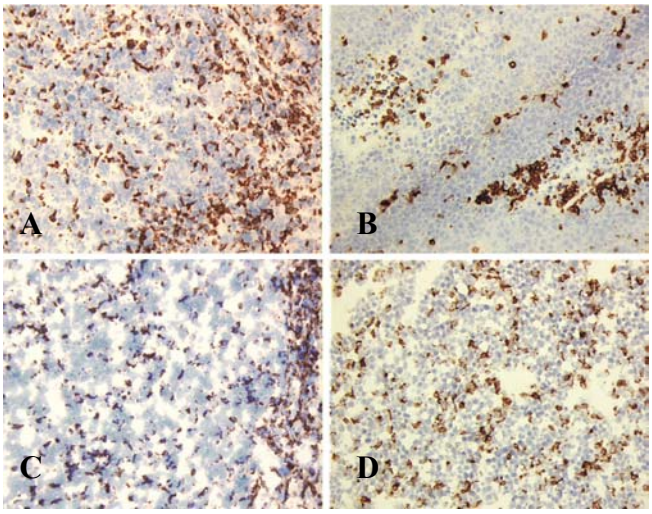


**Figure 5**: Immunohistochemical staining for ED-1 positive cells (macrophages) on frozen tumor sections of BN-175 soft tissue sarcoma. Tumors were excised and immediately frozen in liquid nitrogen. Representative pictures are shown for (a) not treated BN-175 tumor; (b) perfused with IL-2+mel; (c) perfused with Hi+mel and (d) perfused with Hi+IL-2+mel. ILP with IL-2 and melphalan resulted in redistribution of macrophages with clustered ED-1 cells. This effect was lost with the combination of Hi to the IL-2+mel ILP in the triple drug treatment. Original magnification 16X.

## Discussion

We have shown in this study that synergy between Hi and IL-2 seen in systemic treatment is lost in the regional setting, with a 38% decrease in OR rate, as compared to each drug alone combined to melphalan.

The mechanism of action in the regional setting is quite different from the one seen in the systemic setting. Instead of the immunological effect involved in the latter one, there is a triplet action of Hi in the ILP: 1) a direct cytotoxic effect on TAV, 2) a direct cytotoxic effect on tumor cells and 3) an indirect effect on tumor vasculature, increasing drug accumulation in the tumor [12].

A possible explanation might be the dose used in the regional setting, which is in the order of 6 to 8 times higher than those reported for the systemic treatment [12,22], or the much shorter exposure time in the ILP. In this different pharmacokinetic scenario the combination of two inflammatory agents could lead to a down regulation of their action and consequently result in a loss in efficacy. A similar effect was previously reported with the combination of histamine and TNF-α [24,25,26]. Supporting this theory is the finding of reversed clustering effect of IL-2 on ED-1 cells by the addition of Hi, as shown in Figure 5.

Macrophages are involved in many different processes such as tissue remodeling during embryogenesis, wound repair, removal of damaged or senescent cells subsequent to injury or infection, hemopoiesis and homeostasis. Tumor infiltrating macrophages have a complex role in tumor development being capable of either supporting or blocking its growth, according to its activation status [27]. Activated macrophages are important actors in the defense against tumor cells, recognizing and lysing them by the production of cytotoxic products such as oxygen radicals and Tumor Necrosis Factor (TNF) [28]. We previously showed, in tumor tissue treated by IL-2-based ILP, either alone or in combination with melphalan, a 5.7 fold increase in TNF expression [15]. Contrarily to this, Histamine an immunomodulatory agent has been shown to indirectly block the release of secondary cytokines such as IL-1 and TNF-α [29].

The reduction on hemorrhagic effect seen when IL-2 is combined to ILP with Hi and melphalan is surprising, as IL-2 apparently had no direct effect on endothelial cells nor on the activity of Hi towards endothelial cells in vitro. On the other hand, it is known that IL-2 induces vasopermeability when administered systemically by causing the efflux of

intravascular fluids to extravascular spaces, a phenomenon described as capillary leak syndrome [30]. Accordingly, IL-2 increased melphalan accumulation in the tumor after ILP (Figure 4). Some authors suggest an indirect role of IL-2 in induction of vasopermeability by the generation of lymphokine activated killer cells and/or secondary cytokines which in turn damage the endothelial cells. Secondary cytokines implicated in this indirect effect include TNF-α among others, which may increase the expression of the inducible form of nitric oxide synthase (iNOS) and secondarily an increase in NO, toxic to endothelial cells [30].

According to our findings, there were very similar tumoral melphalan values, whether or not IL-2 and Hi were combined. Based on these observations we speculate that the loss in Hi-induced hemorrhage and vascular destruction is most likely responsible for the reduced OR (from 66% to only 28%), when IL-2 was combined to the treatment.

In conclusion the association of Hi and IL-2 in the Melphalan-based ILP setting does not improve response, but more so a strongly diminished response rate was observed compared to ILP with either drug alone combined with melphalan.

## Acknowledgments

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# Chapter 5

## *Histamine, a vasoactive agent with vascular disrupting potential, improves tumor response by enhancing local drug delivery*

Flavia Brunstein, Joost A. Rens, Sandra T. van Tiel, Alexander M.M. Eggermont, Timo L.M. ten Hagen



Submitted

## Abstract

TNF-based Isolated Limb Perfusion is an approved and registered treatment for sarcomas confined to the limbs in Europe since 1998, with limb salvage indexes of 76%. TNF improves drug distribution in solid tumors and secondarily destroys the tumor-associated vasculature (TAV). Here we explore the synergistic antitumor effect of another vasoactive agent, Histamine (Hi) in doxorubicin-based ILP and evaluate its antivascular effects on TAV.

We used our well-established rat ILP model for in vivo studies looking at tumor response, drug distribution and effects on tumor vessels. *In vitro* studies explored drug interactions at cellular level on tumor cells (BN-175) and endothelial cells (HUVEC). There was a 17% partial response and a 50% arrest in tumor growth when Hi was combined to DXR, without important side effects, against 100% progressive disease with DXR alone and 29% arrest in tumor growth for Hi alone. Histology documented an increased DXR leakage in tumor tissue combined to a destruction of the TAV, when Hi was added to the ILP. *In vitro* no synergy between the drugs was observed. In conclusion, Hi a vasoactive drug, targeted primarily the TAV and synergized with different chemotherapeutic agents.

## Introduction

TNF-based Isolated Limb Perfusion (ILP) is an approved and registered treatment for sarcomas confined to the limb in Europe since 1998 and is currently carried out in approximately 30 cancer centers with referral programs for limb salvage around the continent (Eggermont *et al*, 1996a). ILP with TNF and melphalan also yields excellent antitumor effects against melanoma (Lienard *et al*, 1992) and various other tumors in the clinical setting (Olieman *et al*, 1999; Bickels *et al*, 1999; Eggermont *et al*, 2003). The mechanism of action is based on the vasoactive effects of TNF, leading to a significant enhancement of tumor selective melphalan uptake (de Wilt *et al*, 2000) and, secondarily to a complete destruction of tumor vasculature (Eggermont *et al*, 1996b).

An important drawback of the use of TNF is its highly toxic nature mandating strict monitoring of leakage to the systemic compartment during ILP. Moreover, this toxic profile of TNF limits expansion of its use to less controllable sites. Therefore, other possible vaso-active drugs were sought and tested in our preclinical rat ILP model as potential candidates

(Brunstein *et al*, 2004; Hoving *et al*, 2005). In this perspective we showed strong synergy of Histamine (Hi), an inflammatory mediator, when combined to melphalan in ILP, including a 66% overall response rate (OR) with 33% complete responses (CR) (Brunstein *et al*, 2004).

The aim of this study is to evaluate the effects of Hi on TAV by means of histological studies and also explore whether the synergistic effect of Hi would also apply to the combination with doxorubicin (DXR), an important chemotherapeutic drug in solid tumor treatment (Santoro *et al*, 1995; O'Byrne and Steward, 1999). Based on the assumption that DXR is the best single agent for systemic therapy, with activity in more than 20% of the treated patients, some Italian centers use it in the TNF based ILP instead of melphalan, with circa 26% complete histologic necrosis (Rossi *et al*, 1999; Rossi *et al*, 2005). Using the experimental ILP model in rats bearing syngenic soft tissue sarcomas, the ability of the combined treatment to improve tumor response is evaluated. The effects of Hi on endothelial cells and TAV as well as on drug distribution are evaluated *in vivo*, taking advantage of the natural fluorescence of DXR and combining different histological stainings.

## Materials and Methods

***Isolated limb perfusion protocol.***

Male inbred Brown Norway rats were obtained from Harlan-CPB (Austerlitz, the Netherlands), weighing 250-300g and were fed a standard laboratory diet *ad libitum* (Hope Farms Woerden, the Netherlands).

Small fragments (3 mm) of the syngeneic BN-175 sarcoma were inserted subcutaneously in the right hind leg of the animals as previously described (de Wilt *et al*, 1999). Tumor growth was measured daily with a caliper and the volume was calculated using the formula $0.4(A^2xB)$ (where B represents the largest tumor diameter and A is the diameter perpendicular to it). When tumor diameter exceeded 25 mm or at the end of the experiment rats were killed by cervical dislocation, under anesthesia.

The treatment consisted of the experimental ILP, previously described (de Wilt *et al*, 1999). Briefly, 7-10 days after implantation tumors reached a diameter between 12-15 mm and were amenable to the procedure. Under anesthesia (intraperitoneal ketamine and intramuscular hypnomidate), the inguinal vessels were reached through an incision parallel

to the inguinal ligament, canulated and connected via a roller pump to an oxygenated reservoir where drugs were added in boluses. A groin tourniquet occluded collateral vessels, warranting a proper isolation of the limb.

The 5 mL total volume perfusate consisted of: haemaccel alone (Boehring Pharma, Amsterdam, the Netherlands); haemaccel plus 400 μg DXR (80 μg/mL) (Adriablastina®, Farmitalia Carlo Erba, Brussels, Belgium); haemaccel plus 1000 μg Hi (200 μg/mL) (kindly provided by Maxim Pharmaceuticals Inc., San Diego, CA) or haemaccel with 400 μg DXR and 1000 μg of Hi.

Tumor dimensions were measured every day for volume calculation. Response was classified as: progressive disease (PD) increase of more than 25%; no change (NC) volume kept in the range of –25% to +25%; partial remission (PR) decrease between –25% and –99% or complete response (CR), no palpable tumor, initial volume as compared to volume on day nine.

Limb function was clinically observed as the ability to walk and stand on the perfused limb after ILP. On a scale from 0 to 2, grade 0 is a severely impaired function where the rat drags its hind limb; grade 1, a slightly impaired function (can not use it in a normal way, but stand on it); finally grade 2 is an intact function (normal walking and standing pattern).

The studies were done in accordance with protocols approved by the Animal Care Committee of the Erasmus University Rotterdam, the Netherlands.

***Histologic evaluation after Hi-based ILP.***

Two animals for each group were killed by cervical dislocation directly and 24 hours after ILP, tumors and a piece of underlying muscle were excised, fixed in 4% formaldehyde solution and embedded in paraffin. The slides were stained with hematoxylin and eosin and CD-31 by the Pathology department of the Erasmus MC. Images were taken on a Leica DM-RXA microscope supplied with a Sony 3CCD DXC camera.

***Perls Iron staining - histologic evaluation after Hi-based ILP.***

Two animals for each group were killed by cervical dislocation seven days after ILP, tumors and part of the adjacent limb muscle were excised, fixed in 4% formaldehyde solution and embedded in paraffin. The slides were stained by Perl's method, a qualitative

technique based on the release of ferric iron from hemosiderin by acid treatment, forming ferric chloride. The ferric iron reacts with potassium ferrocyanide to form ferric ferrocyanide, an insoluble blue compound known as Prussian blue (Bancroft and Cook**,** 1984). Images were taken on a Leica DM-RXA microscope supplied with a Sony 3CCD DXC camera.

***Doxorubicin distribution and evaluation of vascular function.***

To gain insight in *in vivo* intratumoral drug distribution, three animals for DXR alone and three for Hi plus DXR were submitted to standard ILP plus the addition of 20 μL of the vessel staining FITC-lectin (*Bandeireae simplicifolia*, BS-I Isolcetibe B4, Sigma) to the perfusate. Directly after the procedure the animals were killed by cervical dislocation, tumors were excised, snap frozen in liquid nitrogen and stored at –80$^{o}$C. Thick sections of 25 μm were mounted with Mowiol and evaluated by confocal microscopy with a Zeiss LSM 510 Meta (488 nm laser with 505-505 band pass filter (FITC) and 543 nm laser with 560 long pass filter (DXR). Nine different fields per animal were selected and photographed (three fields per slide, in a total of three slides per animal). Images were further processed, using Image Tool ® for Windows 2000. First, the colors of the images were separated for quantification of vessel density (green) and drug distribution (red). Next, images were binarized, with a lower threshold setting based on the negative control, and the percentage of positive pixels was determined. Data was plotted with GraphPad Prism for Windows 2000.

***Cytotoxicity assay***.

Direct interaction between DXR and Hi was evaluated in vitro on BN175 tumor cells and endothelial cells.

BN-175 tumor cells (isolated from the spontaneous, rapidly growing and metastasizing soft tissue sarcoma) (Kort *et al*, 1984) were grown in RPMI-1640 essential medium (Life Technologies, the Netherlands) supplemented with 10% fetal calf serum and 0.1% penicillin-streptomycin (Life Technologies, the Netherlands).

Cells were plated 24 hours before treatment in 96-wells, flat-bottomed, microtiter plates (Costar, Cambridge, MA, USA) at a concentration of $10^5$ cells per mL, final volume of 100 μL and allowed to grow as a monolayer. Next, they were incubated at 37$^{o}$C in 5% $CO_2$ for

48 hours in the presence of medium alone or medium plus different concentrations of DXR and Hi. Hi ranged from 0 to 200 μg/mL and DXR from 0 to 5 μg/mL.

Growth of tumor cells was measured using the Sulphorhodamine-B (SRB) assay (Skehan *et al*, 1990). In brief, cells were washed with phosphate buffered saline, incubated with 10% trichloric acetic acid for one hour at a temperature of –4°C and washed again. Cells were stained with SRB for about 15 to 30 minutes, washed with 1% acetic acid and allowed to dry. Protein-bound SRB was dissolved in TRIS (10mM, pH 9.4). Extinction was measured at 540 nm and the percentage of growth inhibition was calculated according to the formula: percentage of tumor cell growth = (test well/control well) x 100%. The drug concentration leading to 50% reduction in absorbance, as compared to control ($IC_{50}$), was determined from the growth curve. The experiments were repeated four times.

Human umbilical vein endothelial cells (HUVEC) were prepared by collagenase treatment of freshly obtained human umbilical veins and cultured in Human endothelial – SFM/RPMI medium (Biotechnologies, the Netherlands) supplemented with 10% heat inactivated human serum (Biowhitaker, the Netherlands), 20% new born calf serum, human EGF, human bFGF and 0.1% penicillin-streptomycin (Life Technologies, the Netherlands).

HUVEC were plated 24 hours before treatment in 96 well plates at $6x10^4$ cells per mL, total volume of 100 μL and allowed to grow as a monolayer. Next, they were cultured for 48 hours with Hi, in concentrations ranging from 0 to 200 μg/mL and DXR from 0 to 0.5 μg/mL. The growth and $IC_{50}$ were determined in the same way as for the tumor cells.

***Statistical analysis.***

Kruskal-Wallis and Mann-Whitney U tests were used to evaluate statistical significance of the results. All statistical tests were two-sided and P values less than 0.05 were considered as statistical significant. Calculations were performed on a personal computer using Prism v3.0 software (GraphPad Software Inc.) and SPSS v10.0 for Windows 2000.

## Results

***Tumor response after ILP***.

While tumors grew exponentially in all rats submitted to either control or DXR alone ILP, Hi alone could arrest tumor growth for four days in 2 out of 7 animals (29%). As expected,

the best response was seen with the combination of Hi and DXR showing a partial regression in 2 animals (33%) and arrest of tumor growth for approximately six days in 3 animals (50%) (P<0.01 on day 8 for Hi plus DXR as compared to Sham; P = 0.027 on day 8 for Hi plus DXR as compared to DXR alone). (Figure1) (Table 1).

As previously seen in Hi plus melphalan ILP, Hi either alone or combined with DXR did not inflict systemic side effects. As for regional toxicity only some edema after Hi ILP, both with and without DXR, was observed leading to a temporary grade 1 toxicity in two rats for each group. ILP with DXR alone also caused a temporary regional toxicity in two rats scoring a grade 1 function with limb edema lasting for 3 to 4 days.

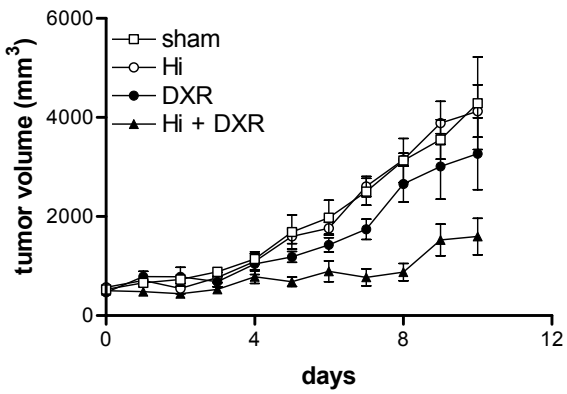


**Figure 1**: Tumor response in soft tissue sarcoma bearing rats after Histamine-based Isolated Limb Perfusion (ILP). Tumor-bearing rats were submitted to ILP with sham, doxorubicin (DXR), Histamine (Hi) or DXR plus Hi as described in Materials and Methods. Mean tumor volumes +/- SEM are depicted. * P<0.01 on day 8 for Hi+DXR as compared to Sham; P = 0.027 on day 8 for Hi+DXR as compared to DXR alone

***Histology***.

Immediately after ILP with Hi plus DXR vasodilation was observed, accompanied by tumoral endothelial cell damage and hemorrhage. Next to that some edema in the tumor was observed. Sham or DXR alone ILP had no effect on vasodilation or hemorrhage and predominantly intact tumor cells and few necrotic spots were seen. CD-31 staining corroborated the observation of Hi-mediated destruction of the TAV-associated endothelial cell lining in treated tumors. (Figure 2).

**Table 1**: Response in BN-175 soft tissue sarcoma-bearing rats after doxorubicin-based ILP in combination with Histamine over a total period of 08 days.

| Treatment[b] | CR[a] | PR | NC | PD |
|---|---|---|---|---|
| Sham (n=5) | - | - | - | 100% |
| DXR (n=6) | - | - | - | 100% |
| Histamine (n=7) | - | - | 29% | 71% |
| Hi + DXR (n=6) | - | 17% | 50% | 33% |

[a]) responses were scored as described in materials and methods. CR complete response, PR Partial response, NC no change, and PD progressive disease.

[b]) Doxorubicin (400 µg) and Histamine (Hi, 1000 µg) were added as boluses to the perfusate (5 ml).

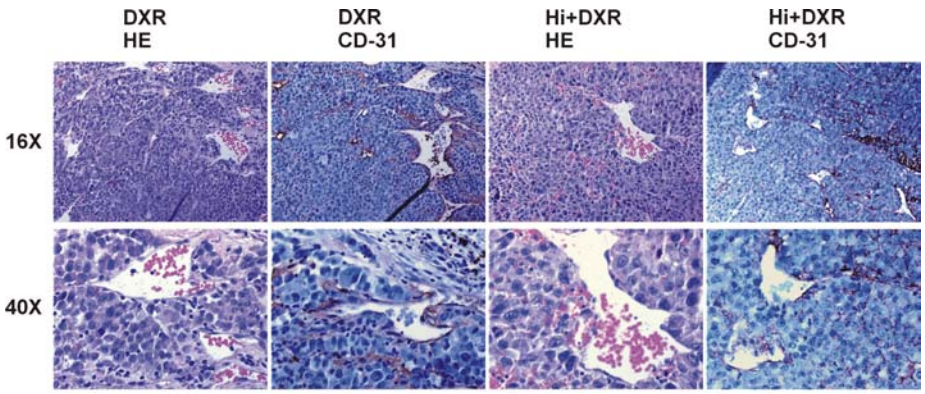


**Figure 2**: Tumor vascular destructive effect and hemorrhagic necrosis upon Histamine-based Isolated Limb Perfusion (ILP) (A). Pictures of representative tumor histology (HE) and vascular destruction (CD31) right after ILP with doxorubicin (DXR) or DXR plus Histamine (Hi) are shown. Orange bar on 16x magnification pictures corresponds to 100 µm and red bar on 40x magnification to 50 µm.

***Perls Iron staining - histologic evaluation after Hi-based ILP***

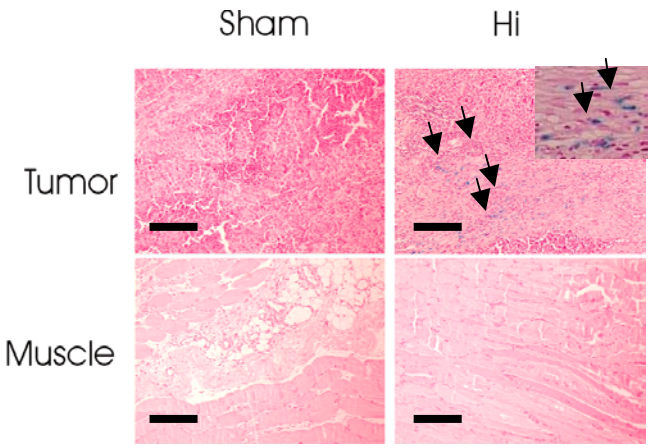


**Figure 3**: Staining by Perl's method shows Histamine-related induction of hemorrhagic necrosis at 7 days after ILP, which is absent in muscle. Original magnification 10X. Black bar corresponds to 100 μm.

In agreement with HE and CD-31 findings, Perl's method documented a hemorrhagic effect linked to Hi administration. Hi treated tumors had iron deposits seven days after ILP, mainly in the tumor tissue. Muscle tissue showed few and much smaller foci. Sham had no iron deposits neither in muscle, nor in tumor. These findings further support the specific TAV-targeting action of Hi. (Fig. 3)

***Doxorubicin distribution and TAV evaluation by FITC- lectin ILP.***

Taking advantage of the natural red fluoresce of DXR we evaluated drug distribution within tumor and muscle by confocal microscopy of thick slides. When ILP was performed with DXR alone some extravasation was observed around perfused (lectin-positive) vessels. Increased extravasation of DXR was seen around tumor vessels, when Hi was co-administered, while in muscle no major effects were noted. Moreover, some areas of DXR leakage could be observed in the tumor, with diffuse or even absent lectin staining, indicating severe damage to the endothelial lining of the tumor vasculature. These observations indicate an increased leakage specifically from the tumor vascular bed when

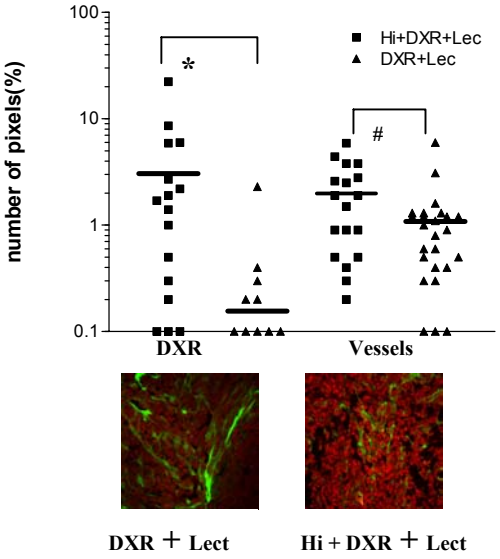


**Figure 4**: Effect of Histamine-based Isolated Limb Perfusion (ILP) on intratumoral doxorubicin (DXR) distribution in relation to tumor vessel presence. Directly after ILP with DXR and Lectin-FITC (DXR+Lec), or Histamine plus DXR and Lectin-FITC (Hi+DXR+Lec) tumors were excised and snap-frozen. 25 µm-thick slides were examined by confocal microscopy. DXR distribution and vessel density in digital images were measured as described in Materials and Methods. Representative pictures of intratumor DXR distribution right after ILP with DXR and Lectin-FITC (DXR+Lec), or Histamine plus DXR and Lec (Hi+DXR+Lec) are shown. * $p<0.001$; # $p=0.06$

Hi was added to the ILP (Figure 4). This finding was confirmed by means of intratumoral drug distribution quantification done through pixels intensity measurement ($P < 0.001$, DXR alone compared to DXR combined to Hi). The lectin staining also revealed large areas devoid of functional vessels, mainly in the tumor center, in which no DXR could be delivered during the ILP.

***Direct cytotoxicity of histamine***.

To evaluate the potential synergistic action between DXR and Hi, in vitro cytotoxicity assays were done on BN-175 tumor cells and on HUVEC. As shown in Figure 5, both agents were capable of killing endothelial cells with an $IC_{50}$ of 200 µg/mL for Hi and an $IC_{50}$ of 0.1 µg/mL for DXR. While BN-175 tumor cells, were effectively killed by DXR with an $IC_{50}$ of 0.08 µg/mL, hardly any effect of Hi was noticed with an $IC_{50}$ as high as 500 µg/mL. Combining DXR and Hi in vitro had only an additive effect.

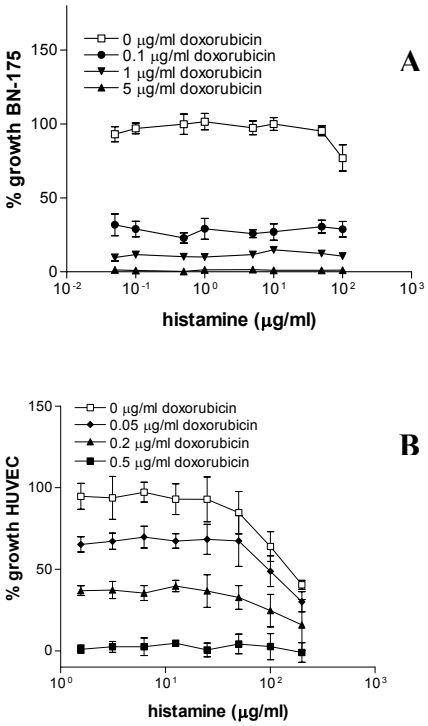


**Figure 5**: Evaluation of possible direct effects of Histamine (Hi) and doxorubicin (DXR) on BN-175 soft tissue sarcoma cells and HUVEC. BN175 tumor cells (A) or HUVEC (B) were exposed to 0 – 5 μg/mL DXR with Hi 0 – 200 μg/mL for 72 hours. Each point represents an average of four readings. Error bars show standard deviation values

## Discussion

In this study we show, by combining the natural red fluorescence of DXR with lectin-FITC staining of functional vessels during ILP, that the vascular disrupting effect of Histamine augments intratumoral delivery of DXR. Based on these observations we hypothesize that both destruction of the tumor-associated vasculature and better tumor drug distribution, when Hi was administered, added to the observed augmented tumor response. Additional histological stainings, such as CD-31 and Perl's method, further demonstrate Hi related endothelial lining disruption and tumor hemorrhage, respectively. It's of note that these effects were more intense in tumor than muscle tissue, which is in agreement with previous findings showing a four-fold increase in melphalan uptake by tumor as compared to muscle (Brunstein et al, 2004). The direct hemorrhagic effect of Histamine, mainly on tumor vasculature, is an advantage compared to the standard drug TNF, for which this is a secondary effect seen only around 6 hours after ILP (Nooijen et al, 1996).

No major effects on normal vasculature (e.g. in muscle) were seen, indicating a potentially safe profile of Hi in terms of side effects and damage to normal tissues. Indeed no serious side effects linked to Hi were observed, only some temporary limb edema, which were completely reversible after 3 to 4 days. It is also noteworthy that no systemic side effects were seen.

Furthermore, in this study we show that the anti-tumor effect of Histamine in a regional therapy model, was not restricted to melphalan but was also present in combination with DXR, resulting in tumor regressions or tumor growth arrest in 67% of the rats. Hi alone arrested tumor growth in 29% of the animals, whilst tumor progression was observed in virtually all rats in sham or DXR alone groups.

Tumor response rates in this study were not as good as those previously reported for the combination of Hi plus melphalan. Yet, DXR alone was also less active than melphalan alone in our ILP model, with no antitumor effect (100% progressive disease) for DXR, against 17% partial response and 17% tumor growth arrest for melphalan (Brunstein et al, 2004). These findings are in accordance with the literature where melphalan is described as the drug of choice for ILP in most centers worldwide with the best response rates and lower complication indexes (de Wilt et al, 1999; Thompson et al, 1998). A possible explanation for the reduced efficacy of DXR could be its cycle dependency, while melphalan does not have this restriction. Taking into account that during an ILP drugs are delivered in a higher dosage but only once, apposed systemic chemotherapy where drugs are given repeatedly, this difference in activity might play an important role.

Besides the above mentioned, the Hi batch used in this study appeared less active in vitro on both tumor and endothelial cells compared to previous results (Brunstein et al, 2004), possibly resulting in a partial lost of the direct effect of Hi towards tumor and endothelial cells in vivo. Strikingly, tumor endothelial lining destruction and hemorrhage remained similar in vivo after Hi-based ILP compared to previous results, an observation well documented by the different histologic stainings used.

TNF-based ILP, an approved and registered treatment for sarcomas in Europe is currently carried out in approximately 30 cancer centers with referral programs for limb salvage around the continent (Eggermont et al, 1996b). This success demands further extrapolation to organ perfusions and systemic approach; however, the strong dose limiting toxicity seen with the use of TNF impairs its broader use. We show here that Hi was capable of

augmenting tumor response when combined to a different chemotherapeutic drug. The target of both vasoactive drugs, TNF and Hi, is the TAV, which explains its beneficial effects on different tumor types and in combination with different chemotherapeutic drugs. Taking the very short circulation time of Hi and the limited toxicity profile into account Hi has potentially a broader application for the treatment of different tumor types and organ perfusion making it a potentially good alternative for TNF.

In conclusion, the inflammatory mediator Hi acts as a vasoactive drug, targeting the tumor-associated vasculature and is capable of synergizing with different chemotherapeutic agents. These findings support a potential role of Hi in regional treatment and organ perfusions opening new frontiers for further development of these treatment modalities in the clinic, as an important tool in the surgical oncology field.

## Acknowledgements

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# Chapter 6

## *Synergistic antitumor effects of histamine plus melphalan in isolated hepatic perfusion for liver metastases*

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## Abstract

Non-resectable primary and metastatic liver tumors remain an important clinical problem. Melphalan based Isolated Hepatic Perfusion (M-IHP) leads to more than 70% objective responses in selective groups of patients with non-resectable metastases confined to the liver. Complete responses are rare and progression free survival limited. TNF, a very active agent in isolated limb perfusion, is linked to serious hepatotoxicity restricting its use in IHP. Due to its vaso-active properties Hi stands as an alternative to TNF. Here we evaluate its potential synergistic effect in M-IHP, improving response rates. Our experimental rat IHP model is used for the treatment of soft tissue sarcoma liver metastases. Blood samples are collected for monitoring liver enzymes. Livers are excised 72 hours and 7 days post treatment for histological evaluation. After Sham-IHP and Hi-IHP tumor progression was observed in 100% of treated animals while after M-IHP this number felt to 62% and, after Hi+M-IHP to only 22% ($p=0.006$). Overall response rates were of 55% for Hi+M-IHP against 25% for M-IHP and more importantly, CR were only observed after Hi+M-IHP (22%) ($p= 0.009$). Hepatotoxicity peaked within 24 hours post IHP, independently of the treatment administered; recovered in 48 hours and was mainly related to transaminases elevation (grade 3 ASAT and 1 ALAT for control group and grade 3 and 4, respectively, for all other treatments). No serious systemic toxicity was observed. Histology of liver showed no serious damage. Hi+M IHP has synergistic antitumor effects without any increase in regional or systemic toxicity.


## Introduction

As many as 50% of the patients with a primary malignancy will eventually develop metastases in the liver, more than in any other organ. Primary tumors draining into the portal circulation more likely develop liver metastasis but those arising in other sites such as breast and lungs might also develop them [1]. Surgical approach, when possible, remains the best option prolonging survival rates and even granting some patients cure, while unresectable primary or metastatic cancers confined to the liver still pose an important clinical challenge. Mean survival of patients with unresectable colorectal liver metastases is 12 to 18 months while for those with ocular melanoma metastases, reported survival expectancy is less than one year [2]. The use of neoadjuvant therapies might render an unresectable tumor resectable and these patients will still benefit from the advantages of

surgical treatment. Systemic therapy has a limited role, depending on the histological type of the primary lesion.

Regional approaches such as Hepatic arterial chemotherapy infusion and perfusion allow high doses of chemotherapeutic drugs, typically delivering higher drug concentrations to tumor as compared to the hepatic parenchyma and the body as a whole. The advantages are a direct tumor administration, with limited systemic toxicity and the treatment of the whole liver including the micrometastases. Response rates are increased to the range of 42%-62% using different chemotherapeutic agents such as 5-FU and melphalan. In spite of promising results in a selective group of patients, IHP remains an experimental procedure and further development is needed to improve its efficacy and broaden its applicability, including the enhancement of melphalan effect [2,3]. In this scenario, despite of its striking effect in Isolated Limb Perfusion (ILP), the use of TNF in IHP was disappointing. Preclinical studies suggested a synergistic effect, increasing drug uptake mainly for highly vascularized tumors [4,5] but, unfortunately its use in the clinical setting was hampered by serious hepatotoxicity limiting the use of higher doses [6,7].

We showed previously that Histamine (Hi), an inflammatory mediator, stood as a potential alternative to TNF-α, strongly augmenting tumor response rates in melphalan-based ILP (overall response rates of 66%). The mechanism of action being based on (1) a direct cytotoxic effect on tumor-associated vasculature (TAV), (2) a direct cytotoxic effect on tumor cells and (3) an indirect effect on TAV, increasing tumoral drug accumulation [8].

Based on reports of systemic use of Hi combined to IL-2 in the treatment of stage IV melanoma patients, including those with liver metastases, no serious treatment related hepatotoxicity was expected.

Here we explore the synergistic effect of Histamine in melphalan-based IHP (M-IHP) potentially improving the efficacy of the method. The previously described leakage free IHP experimental model in rats [5,9] is used for the treatment of soft tissue sarcoma liver metastases.

## Materials and Methods

### *Animals and Tumor cell line*

Male inbred Brown Norway (BN) rats were obtained from Harlan-CPB (Austerlitz, the Netherlands), weighing 253-308 g (mean 276 g). Animals were housed at the Central

Animal Facility of the Erasmus MC Rotterdam and fed a standard laboratory diet ad libitum (Hope Farms Woerden, the Netherlands).

The syngeneic, spontaneous, rapidly growing and metastasizing BN-175 soft tissue sarcoma [10] was kept in liquid nitrogen and implanted on the dorsum of a BN rat for further growth before being inserted in the liver of the experimental animals.

All animal studies were done in accordance with protocols approved by the Animal Care Committee of the Erasmus University Rotterdam, the Netherlands.

***Chemicals***

Melphalan (Alkeran, 50 mg per vial, Wellcom, Beckenham, United Kingdom) was dissolved in 10 mL of diluent solvent. Further dilutions were made in PBS to a concentration of 2 mg/mL. Histamine, kindly provided by Maxim Pharmaceuticals Inc. (San Diego, CA, USA), came in vials already diluted in the concentration of 1 mg/mL

***Isolated liver perfusion protocol***.

Small viable fragments (1-2 mm) of the syngeneic BN-175 sarcoma were implanted under the liver capsule in the left and right liver lobe of each rat, with a 19 G Luer lock needle in a standardized manner [5]. IHP was performed six days after implantation when tumors reached a diameter of approximately 6 mm. During follow-up tumor diameters were assessed through a small midline incision by caliper measurement. Tumor volume was calculated by the formula $0.4(A^2xB)$ (where B represents the largest tumor diameter and A is the diameter perpendicular to B). When tumor diameter exceeded 20 mm, or abdominal adhesions made further assessment of tumor size impossible or at the end of the experiment rats were killed by cervical dislocation, under anesthesia [4,5].

The treatment consisted of a modification of the experimental IHP technique described by de Brauw et al [11] which was previously described [5,9]. Briefly, 6 days after implanting tumor fragments they reached a diameter around 6 mm and were amenable to the procedure. Under anesthesia (maintained and induced with ether) a mid-line laparotomy was performed and the hepatic ligament exposed. The pyloric side branch of the portal vein and the gastroduodenal side branch of the common hepatic artery were cannulated using silastic cannulas (0.025 inch outer diameter (OD) and 0.012 inch inner diameter (ID) – Dow Corning, Michigan, USA). Through an inguinal incision the femoral vein was exposed and cannulated retrogradely so as to reach the caval vein with the tip positioned at the level of

the hepatic veins. Isolation of the hepatic vascular bed was obtained by temporary ligating the common hepatic artery and the portal vein. The venous outflow limb was isolated by temporarily clamping the supra-hepatic caval vein and by applying a temporary ligature around the infra-hepatic caval vein containing the cannula, cranial to the right adrenal vein. During isolation the mesenteric artery was clamped to reduce splanchnic blood pressure and the risk for translocation of intestinal bacteria. After the IHP procedure clamps on caval vein, portal vein, hepatic artery and mesenteric artery were released. Gastroduodenal artery, pyloric vein and femoral vein were ligated and gastroduodenal and pyloric cannulas removed.

The perfusion circuit consisted of an arterial inflow limb in the hepatic artery, a venous outflow limb in the caval vein and a collection reservoir/oxygenator. The circuit was primed with 10 mL haemaccell (Behring Pharma, Amsterdam, the Netherlands) containing 50 IU of heparin. The perfusate was oxygenated in the reservoir with a mixture of O2:CO2 (95%: 5%) and kept at 38-39$^{o}$ C through a heat exchanger connected to a warm water bath. A temperature probe was positioned in the lumen of the portal catheter 5 cm from the catheter tip. Arterial flow of 5 mL/min was maintained with a low-flow roller pump (Watson Marlow type 505 U, Falmouth, UK). Rats were perfused for 10 minutes with Haemacell and dissolved agents followed by a washout with oxygenated haemaccell for two minutes.

During the whole surgical procedure, which took in average 60-80 minutes, rats were kept at constant temperature with a warmed mattress.

Rats were randomly perfused with: 1) haemaccel alone (Boehring Pharma, Amsterdam, the Netherlands); 2) haemaccel plus 50 μg melphalan (Alkeran® Wellcome, Beckenham, United Kingdom); 3) haemaccel and 1000 μg of Hi; or 4) haemaccel, 50 μg melphalan and 1000 μg Hi. Between four and six rats were included in each group, with a total of evaluable tumors ranging from seven to twelve.

Tumor dimensions were measured every four days. Volume on day 08 was compared to day 0 and response was classified as: progressive disease (PD) when there was a volume increase of more than 25%; no change (NC) volume kept in the range of –25% to +25%; partial remission (PR) decrease between –25% and –99% or complete response (CR), no palpable tumor on day 8.

Weight, food and water intake, general aspect of the animal (hair and behavior) were evaluated daily for grading toxicity of the different treatments administered.

***Hepatotoxicity***

Blood samples were withdrawn when canulating the vessels before starting the IHP (t=0 min); right after the end of the procedure, before taking the cannulas out (t=10 minutes); 24 hours and 72 hours after IHP. Alanine aminotrasferase (ALAT), Aspartate aminotransferase (ASAT), gamma-glutamyltranspeptidase (gGT) and Alkaline phosphatase (AP) were measured at all above-mentioned time points. Toxicity was graded according to WHO common toxicity criteria (WHO Handbook for Reporting Results of cancer Treatment. CTC Version 2.0 Published on 30 April 1999).

***Histologic evaluation after ILP***

Two animals for each group were killed 72 h and one week after IHP, tumor and liver were excised, fixed in 4% formaldehyde solution and embedded in paraffin. The liver of a untreated BN rat was used as control. Slides were stained with HE; CD-31 and Periodic Acid Schiff (PAS) method. Shortly, for the PAS method, slides were deparaffinized and hydrated to water, oxidized in 0.5% periodic acid solution, rinsed in distilled water, placed in Schiff reagent and washed in tap water. Next, they were counterstained with Hematoxylin and mounted with mowiol. HE and CD-31 staining were performed by the Pathology Department of the Erasmus University. Images were taken on a Leica DM-RXA microscope supplied with a Sony 3CCD DXC camera.

***Statistical analysis.***

Repeated-measures analysis of variance on days 4, 8 and 12 was performed with SPSS software release 11.0 for Windows 2000. Main effects of day and treatment were included in the model as well as their interaction. Response rates were subjected to analysis of variance and post hoc to the least significant difference (LSD) multiple comparison test. All statistical tests were two-sided and P values less than 0.05 were considered as statistically significant. Whether synergy was obtained with the combined treatment was calculated as described previously [8]. Calculations were performed on a personal computer using Prism v3.0 software (GraphPad Software Inc.) and SPSS v11 for Windows 2000.

## Results

***Tumor response after Isolated Hepatic Perfusion***

**Table 1**. Response in Brown Norway rats bearing liver metastasis of BN -175 soft tissue sarcoma after melphalan-based IHP in combination with Histamine over a total period of 12 days.

| Treatment[a] | CR[b] | PR | NC | PD |
|---|---|---|---|---|
| Sham (n=12) | - | - | - | 100% |
| Melphalan (n=8) | - | 25% | 13% | 62%* |
| Hi (n=12) | - | - | - | 100% |
| Hi + melphalan (n=9) | 22%+ | 33% | 22% | 22% |

[a]) Melphalan (40 µg) and Histamine (Hi, 1000 µg) were added as boluses to the perfusate (10 mL).

[b]) responses were scored as described in materials and methods. CR complete response, PR Partial response, NC no change, and PD progressive disease.

* p=0.003 as compared to Hi+melphalan-IHP

+ p=0.03 as compared to melphalan-IHP. The combination of Hi to melphalan had a significant synergistic effect (p= 0.002).

All the rats submitted to sham and Hi alone IHP presented with progressive disease, 62% of rats had progressive disease after a melphalan-IHP and only 22% progressed after HI+M-IHP (p = 0.03).

After M-IHP overall response rate (OR) was 25% and consisted only of PR whereas after Hi+M-IHP the OR was of 55% including 22% CR and 33% PR (P=0.009 for Hi versus Hi plus melphalan on day 12; P=0.03 for melphalan versus Hi plus melphalan on day 12) (Figure 1) and (Table 1).

The increased response rates seen by the addition of Hi to Melphalan was clearly synergistic (p= 0.002).

All animals had a mean weight loss in the range of 7.8% to 12.4%, independently of the treatment administered; even those who underwent a sham IHP had a 10% mean weight loss.

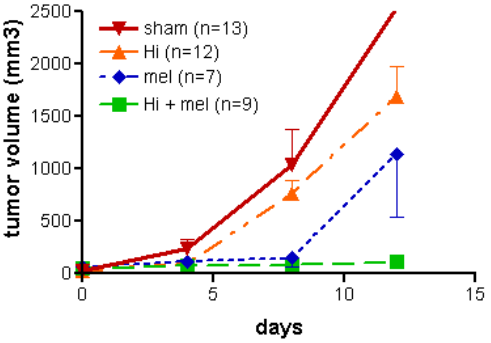


**Figure 1.** Tumor response after isolated hepatic perfusions (IHP). Brown Norway rats bearing liver implants of BN-175 soft tissue sarcoma were randomly submitted to IHP with perfusate alone (sham), 50 µg melphalan, 1000 µg Hi or 1000 µg Hi plus 50 µg melphalan. Tumors were measured every four days with a caliper and volumes were calculated. Mean tumour volumes +/- SEM are depicted in the graph. The number of independent experiments (rats) for each treatment is shown in parentheses

***Liver toxicity***

Hepatotoxicity peaked at 24 hours post IHP for all the different treatments administered (Figure 2 and Table 2). The control group (Sham IHP) presented with grade 3 increase of ASAT (median 315.6 IU/L; range 218.0 to 415.2 IU/L) and grade 1 increase of ALAT (median 109.4 IU/L; range 59.2 to 124 IU/L), which recovered to grade 0 within the following 48 hours (median 67.7 and 10.6 IU/L, respectively). All the other treatments (melphalan, Hi and combination treatment Hi plus melphalan) led to grade 4 increase of ASAT and grade 3 of ALAT yet, the recover pattern was similar to those described above for Sham perfusions (Table 2).

As for AP there was a grade 1 toxicity for all the treated groups (Hi 159.9 IU/L; Melphalan 145.0 IU/L and combination Hi plus melphalan 158.4 IU/L – all values are medians). Control IHP (Sham) led to grade 0 (95.3 IU/L).

Finally gGT presented with grade 0 toxicity for all the different groups (medians ranged from 2.2 IU/L for melphalan alone to 4.6 IU/L in the Sham perfused group). Interestingly enough, Sham group still showed a trend towards increasing values 72 hours after the procedure while the three other treated groups presented a recovery profile.

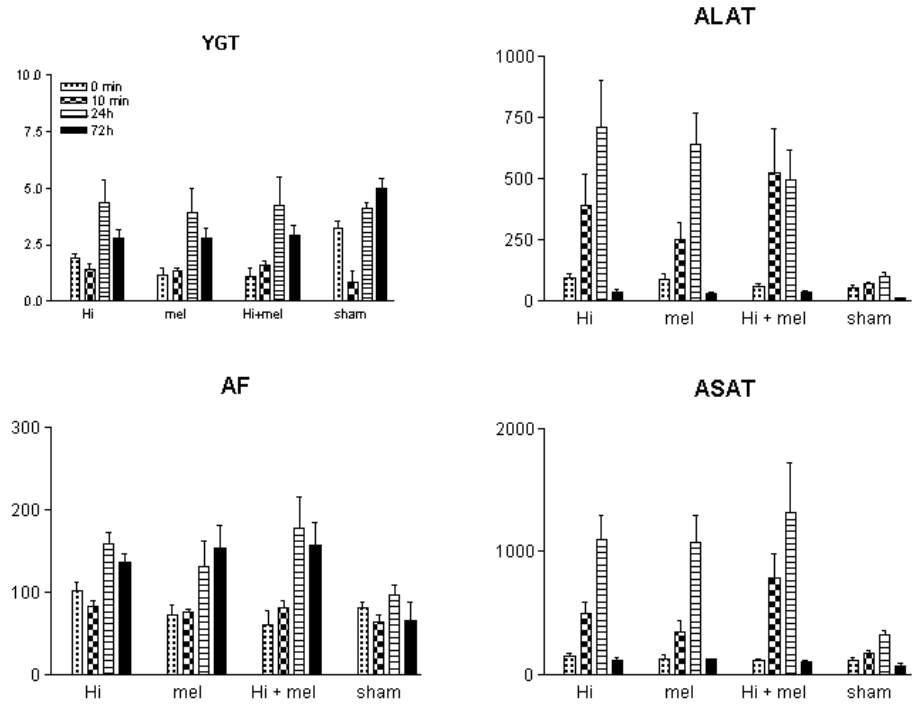


**Figure 2.** Hepatotoxicity post IHP. Blood samples were withdrawn when canulating the vessels before starting the IHP (t=0 min); right after the end of the procedure, before taking the cannulaes out (t= 10 min); 24 hours and 72 hours after IHP. Alanine aminotrasferase (ALAT), Aspartate aminotransferase (ASAT), gamma-glutamyltranspeptidase (GGT) and Alkaline phosphatase (ALP) were measured in all the above-mentioned time points Toxicity was graded according to WHO common toxicity criteria. Median values are depicted +/- SEM. N=6 per group.

**Table 2**: Liver enzymes range after different treatment IHP, showing the highest and lowest value observed for each group (All values are in IU/L).

| Treatment | ALAT | ASAT | GGT | ALP |
|---|---|---|---|---|
| **Sham** | **10.6 – 109.4** | **67.7 – 315.6** | **0.6 – 4.6** | **66.8 – 95.3** |
| **Mel** | **26.2 – 647.2** | **108.5 – 1130.1** | **1.2 - 3.7** | **78.2 – 145.0** |
| **Hi** | **28.0 – 604.9** | **94.3 – 1142.7** | **1.5 – 3.5** | **85.6 – 159.9** |
| **Hi + mel** | **23.4 – 478.5** | **98.5 – 1034-9** | **0.9 – 3.0** | **52.4 – 158.4** |

***Histology***

HE slides showed normal liver anatomy with preserved structures similar to the ones seen in normal liver. We can clearly see the lobes, the portal vein, central vein and biliary ducts. There are some mild eosinophilic deposits around the biliary ducts and some necrotic areas for melphalan treated specimens (both alone and in combination with Hi). (Figure 3)
PAS staining on 72 hours and seven days post IHP showed that the same pattern independently of the treatment administered was maintained, disclosing negative areas around the central vein, as compared to normal liver with extensive glycogen positive stained areas.

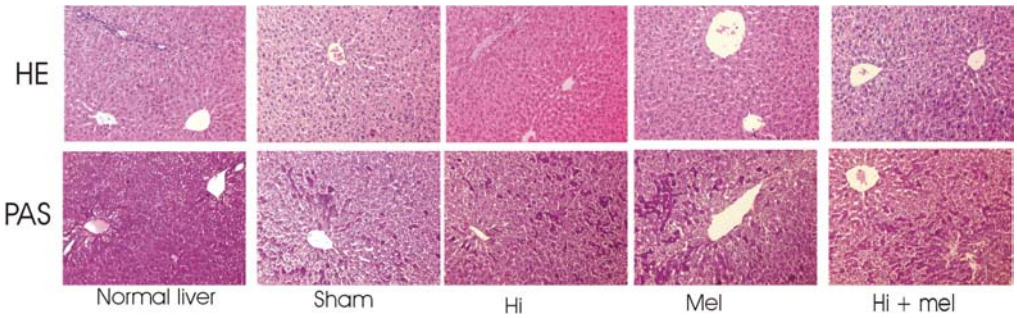


**Figure 3**. Histological evaluation of livers extracted from an untreated BN rat and seven days after sham, melphalan, Hi or Hi plus melphalan IHP. Livers were fixed in 4% formaldehyde solution and embedded in paraffin before staining with HE and Periodic Acid Schiff (PAS) method. HE staining show preserved anatomical structures as compared to normal liver. An even reduction on glycogen for all the treatments, even sham, as compared to normal untreated liver is illustrated by the PAS method. Images were taken on a Leica DM-RXA microscope supplied with a Sony 3CCD DXC camera.

## Discussion

We show here for the first time, a very promising synergistic effect of the combination of Histamine with melphalan in Isolated Hepatic Perfusion for the treatment of soft tissue sarcoma metastasis. More importantly with no systemic toxicity and an acceptable regional toxicity, which was not greater than the one seen after IHP with melphalan alone.
IHP was first applied more than 40 years ago [12] but there was limited clinical experience then. Most of the early reports lacked documented efficacy and unacceptable mortality rates of 10% to 25% were reported. More recently, thanks to technical advances made and

standardization of the techniques for vascular isolation of the liver, it returned as a treatment option for patients with unresectable cancers of the liver. Also, the results obtained in isolated limb perfusion (ILP) had a strong impact on the further exploration of organ perfusion methodologies as a neoadjuvant approach for those patients with unresectable hepatic lesions.[6,13-15]

The number of drugs considered for IHP is still very limited as these agents must be effective after a single short exposure of no longer than 60 minutes, without serious hepatotoxicity. Melphalan is described as effective against both colorectal cancer and melanoma after relatively short exposure times and has a steep dose-response curve, being widely accepted as a good option for locoregional treatment of liver metastasis. [3].

Although in the clinical setting melphalan is used at a concentration of 1.0 mg/kg [16], here we used it at 0.2 mg/kg so as to better evaluate the effect of the combination treatment with Hi. Still, melphalan alone in this lower dosage led to 25% PR associated with grade 3 and 4 hepatotoxicity measured by transaminases. The combination treatment, with melphalan plus Hi, did not add further to the toxicity as observed with each drug alone, yet significantly improved response rates with 55% OR, including 22% complete responses. (Table 2).

TNF-α related hepatotoxicity in IHP [4,17] has been clearly shown and precludes its use in IHP. As previously shown it not only preferentially accumulates in the liver instead of the tumor tissue but also leads to endogenous TNF-α production by the Kupffer cells abundantly present in the normal liver tissue [18]. In fact it does not come as a total surprise, since the production of this cytokine is known as one of the initial events in liver injury. TNF-α recruits inflammatory cells that cause hepatocyte injury and promote production of type I collagen fibers by hepatic stellate cells as a healing response. Additionally it acts on biliary ducts to interfere with the flow of bile causing cholestasis.

The previous reported systemic combination of Histamine and IL-2 for the treatment of stage IV melanoma patients with liver metastasis [19], suggested a potentially safer profile in terms of hepatotoxicity. Indeed, in spite of grade 3 and 4 toxicity for transaminases within 24 hours, we observed a satisfactory recovery in 72 hours post treatment with no systemic repercussion.

Histological findings further back-up the observation of a mild and tolerable hepatotoxicity since no important anatomical damage was seen and accordingly glycogen distribution (PAS staining) showed the same mild decrease after 72 hours for all the different treatments administered.

In conclusion, Histamine has a synergistic antitumor effect when combined to melphalan-based isolated hepatic perfusion for the treatment of BN-175 liver metastases. Based on these results and those previously reported on the synergistic effect of Histamine in melphalan-based isolated limb perfusion a Phase I-II study to explore its therapeutic efficacy in the clinics is worthwhile and currently in development.

## Acknowledgements

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# Chapter 7

## *Vasoactive drugs improving drug delivery in regional treatment - Review*

Flavia Brunstein, Alexander M.M. Eggermont, Timo L.M. ten Hagen

## Introduction

The efficacy of systemic cancer therapy largely depends on adequate delivery of the therapeutic agents to tumor cells, preventing recurrence and resistance. Cancer drugs are often administered systemically, thus drug delivery involves three processes: (1) distribution through the blood circulation (transport within a vessel); (2) transport across vasculature walls intro surrounding tissues and (3) transport through interstitial space within the tumor. These processes are influenced by both the physicochemical properties of the drug or particle (size, diffusivity, binding to macromolecules, etc) and the biologic properties of the tumor (vasculature, extracellular matrix components, interstitial fluid pressure, etc) [1] .

The adequate delivery of anticancer drugs is of crucial importance as most chemotherapeutic drugs exhibit sharp dose response curves. Regional approaches, such as Hepatic Arterial chemotherapy Infusion (HAI) and Perfusion (HAP) or Isolated Limb Perfusion (ILP), allow high doses of chemotherapeutic drugs to be administered, typically resulting in the delivery of higher drug concentrations to tumor as compared to the normal tissues, limiting systemic toxicity and, for some tumor types also potentially treating locoregional micrometastases. While IHP remains an experimental procedure, ILP is a registered treatment in Europe since 1998, for limb threatening soft tissue sarcomas [2,3].

The success of this approach demands further extrapolation, not only towards organ perfusions and systemic approach but also in the search of other vasoactive drugs that could act on the biologic properties of the tumors and thus, improve drug delivery.

Here we review the process that led to the establishment of an antivascular biochemotherapy that currently allows effective treatment of large bulky limb tumors. Included are established agents that change the pathophysiology of tumors as well as the potential "new" old drug Histamine, which, given the promising results seen in the experimental setting, may emerge as a good alternative in the clinical setting as well.

## I - Coley's Toxin – where it all started

Already in the early 1700, it was known that certain cancer patients would experience remission of their malignancies when affected by a concomitant bacterial infection, especially erysipelas. Still, it was only 100 years later that an American surgeon, William

B. Colley would transform this into a useful tool for the treatment of advanced cancer patients. He deliberately induced erysipelas in a selected group of patients, by the administration of the "Coley's toxins" made of heat killed streptococcal broth cultures and heat killed *Serratia marcescens*. The addition of endotoxins from the gram negative *Serratia* was crucial for the achievement of reproducible therapeutic effects in the clinic, as it enhanced the virulence of streptococcal cultures in rabbits [4].

An immune response to pathogens is associated with a multitude of cytokine cascades, which in turn triggers other cascades and a diversity of cellular responses. This immune cascade was readily evoked by Coley's crude bacterial vaccine, but difficult to reproduce with single cytokine therapy. Currently, the only conventional bacterial treatment in use is the bacillus Calmette-Guerin (BCG) in superficial bladder cancer. Applied directly to the tumor site and repeatedly it reduces recurrence. After intravesical application a wide range of cytokines can be identified in the urine including: interleukin (IL)-1, IL-2, IL-6, IL-8, IL-10, IL-12, IL-18, interferon gama, macrophage colony stimulating factor and TNF-α. This illustrates the point that individual immunomodulating cytokines are in fact only one small facet of the complex immunological response to infection and, correspondingly, tumor regression.

Many researches strived to identify the active component of Coley's vaccine, and which host factors were produced in response to it, that finally led to tumor regression. A couple of cytokines, such as tumor necrosis factor (TNF), interleukins and interferons were considered as possibilities, while some authors claimed that more than one cytokine is involved. Accordingly, different vaccine regimens using combinations of various cytokines have been evaluated in the treatment of advanced solid tumors and this remains an open field for further evaluation [5].

Apart from the widely accepted immunologic effect, some authors claim an additional role played by plasminogen activators, in tumor regression after Coley's toxins administration. More specifically it would be related to streptokinase produced by virulent streptococci, combined with host plasminogen and triggering a conformational change and self-cleavage resulting in the formation of enzymatically active plasmin. This potent enzyme, with relatively broad substrate specificity, is capable of initiating other protease cascades and degradation of a variety of plasma and extracellular matrix proteins, which finally compromises tumor viability [6].

## II -Tumor Necrosis Factor

In 1975 tumor necrosis factor alpha (TNF-α) was isolated from the serum of bacillus Calmette-Guérin (BCG) infected mice challenged with endotoxin[7]. It was shown that TNF-α, produced by host macrophages in response to endotoxin challenge, led to hemorrhagic necrosis of murine tumors

Despite the dispute over the ability of a single agent to reproduce the effects seen with Coley's vaccine, TNF-α was then identified as its active component.

The mouse and human TNF genes were cloned and recombinant TNF-α was produced in *E. coli* [8] in large scale, paving the way for extensive *in vivo* studies. It was then found that, besides its anti-tumour effects, TNF-α also had strong haemodynamic effects and was an important mediator of septic shock [9].

Clinical studies confirmed that the dose limiting toxicity of TNF-α was due to vasoplegia, with multiorgan failure and septic shock like syndrome. Furthermore, most tumor models showed that administration of TNF-α alone was not enough to inflict a tumor response and that definitive cure was only achieved by its combination with chemotherapeutic agents, or with interferon (INF) gamma. Also in this period, end of the 80's beginning of 90's, hyperthermia was shown to synergize with TNF, INF and chemotherapeutic agents to induce anti-tumor responses [10].

Given the toxicity and poor responses seen, in the middle 80's further clinical evaluation of systemic TNF was abandoned. Nevertheless, in 1988 a protocol for its application in the isolated limb perfusion (ILP) was developed, taking advantage of the surgical isolation of the limb which allows the regional use of doses up to 30 times higher the maximum tolerated dose (MTD)[10,11].

## III - Isolated Limb Perfusion (ILP)

ILP was first described by Creech *et al.* in 1958 and further improved in the following years[12,13]. Basically it consists of temporary isolating the circulation of the limb from the rest of the body, by cannulating the main limb vessels, connecting them to a heart-lung machine via a pumping role, with combined mild hyperthermia (39-40$^{o}$C) and a primer perfusate. The collateral vessels are ligated and a tourniquet placed in the root of the limb assuring a safe isolation so as to allow local administration of high doses of

chemotherapeutic agents, up to 15 to 25 times higher than the systemic maximum tolerated dose (MTD), without systemic side effects.

ILP with the alkylating cytotoxic agent melphalan (L-phenyl-alanine-mustard) has been used for the treatment of patients with advanced extremity melanoma achieving complete responses (CR) of about 54%. Other chemotherapeutic agents have been tried as alternatives, or combined to melphalan, such as doxorubicin, cisplatin, fotemustine, but none provided better tumor responses as compared to melphalan alone [14].

In spite of the good results seen for melanoma, mainly for small lesions, the responses of bulky tumors and sarcomas were as poor as 20% overall response rates (OR) with no CR [15,16]. The combination of TNF-α to this treatment positively changed this scenario, increasing OR to the range of 80% and a limb salvage index of 70%. Based on these data TNF-melphalan ILP (TM-ILP) was recognized as the best treatment option for limb threatening soft tissue sarcomas and licensed for this use in 1998 by the European Agency for the Evaluation of Medical Products (EMEA)[17].

Initially it was thought that TNF had a direct cytotoxic activity on tumor cells explaining the tumor responses observed. Although this may be true for a minority of tumors, most tumor cells appear quite insensitive to TNF [18-20]. Angiograms of TM-ILP treated patients indicated the tumor vasculature as an important target for TNF [11,21]. Since TNF acts primarily on the tumor vasculature, the response to TM-ILP depends more on the tumor degree of vascularization than on the histologic type of the tumor. Moreover, normal vessels are preserved and their endothelium remains intact while the endothelium of tumor vessels is heavily disrupted, probably as a result of detachment of the endothelial cells from the underlying basement membrane [22]. Further details on TNF in cancer treatment, with an insight on molecular mechanisms can be found in a recently published review [23].

## IV - Endothelium and Endothelial Cells

The endothelium, which forms the inner lining of the blood vessels, is not an inert cell layer but rather highly metabolically active, playing a major role in many homeostatic processes such as control of vasomotor tone, trafficking of cells and nutrients, maintenance of blood fluidity, regulation of permeability and the formation of new blood vessels [24]. Furthermore, a key feature of endothelium is the variation of its properties between different sites of the vasculature and from one moment in time to the next. These characteristics reflect the

endothelium capacity to respond to unique needs of underlying tissue. This communication between the endothelium and neighbouring cell types is a critical determinant of endothelial cell phenotype. There are genetic and environmental determinants of this heterogeneity. Given this active role played by endothelium and endothelial cells they are interesting targets for better understanding of diseases and, consequently the development of new treatment strategies.

When considering its role in disease, two terms commonly appear in the literature: "activation" and "dysfunction". The concept of activation first appeared after in vitro studies demonstrating that defined stimuli (such as endotoxin, TNF and IL-1, among others) induced "activation antigens" (for example E-selectin) on the surface of endothelial cells. These in turn, correlated with the expression of pro-adhesive, antigen presenting and pro-coagulant activities. In summary, the term "activation" reflects the capacity of endothelial cells to perform new functions without evidence of cell injury or division. It's not an all-or-nothing response and not necessarily linked to disease but rather a spectrum of responses occurring both under physiological and pathophysiological conditions. The activated phenotype generally consists of some combination of increased cell adhesiveness, shift in hemostatic balance to the procoagulant side, secretion of inflammatory mediators and change in cell survival/proliferation.

The term "endothelial cell dysfunction" was first used to describe structural changes or loss of anatomical integrity, particularly in the context of arteriosclerosis. Next, there was a growing appreciation that intact endothelium may actively contribute to disease initiation and/or progression. In 1980 the term was first coined to describe hyper-adhesiveness of the endothelium to platelets [24].

Tumors, wound tissue and placenta contain fast proliferating endothelium being vulnerable to induction of hemorrhage by cytokines such as TNF. Contrarily, normal tissue endothelium has very low proliferation rates and, thus, the effect of TNF on it is more of a stimulatory nature and might be related to angiogenetic properties. Other factors playing a role in the vulnerability of tumor vasculature are those related to the tumor itself such as hypoxia, lack of nutrients and low pH [25-27].

Clearly, active proliferating endothelium, in contrast to quiescent endothelium of normal vessels, is more sensitive to certain factors, such as TNF-α. To identify potentially more active compounds or alternatives to TNF-α other vasoactive agents have been explored in

the experimental setting which primarily target the tumor associated vasculature and the fast proliferating tumor endothelium [28-30]. The inflammatory mediator, Histamine, which showed a promising activity in the experimental ILP setting, is one of the main subjects of this current review.

## V - Histamine

### *V. 1 - Historical perspective and general overview*

Histamine was first prepared in 1907 but at that time there was no knowledge of its physiological activity or potential interest. In 1910 Barger and Dale isolated it from a particular ergot extract, the *Ergotinum dialysatum*. Although Ackermann and Kutscher also succeeded in isolating it independently, it is from Dale the credit of describing its pharmacological actions almost completely.

Dale was particularly interested in the capillary dilator effect of histamine and considered the possibility that, either its release or the release of a histamine-like substance might contribute to the regulation of capillary blood flow. According to him, this liberation would be the result of metabolic activity, and could provide fine adjustment of the capillary circulation to the needs of the tissues. He also described its role in the anaphylactic shock and showed that histamine could be extracted from tissues [31].

Histamine is a primary amine synthesized and stored within secretory granules of human mast cells and basophils; but also in cells of the epidermis, gastric mucosa, neurons of the central nervous system, as well as in cells in regenerating or rapidly growing tissues. It is one of the most important mediators involved in various physiological and pathological conditions, including neurotransmission, numerous brain functions, secretion of pituitary hormones, regulation of gastrointestinal and circulatory functions and inflammatory reactions [32,33]. Histamine has also been shown to potently influence the immune response, being the principal mediator of the immediate hypersensitivity reaction that follows interaction of antigen with specific IgE molecules on the surface of mast cells and/or basophiles, and vascular endothelial cells are major targets for the biological actions of histamine. Interestingly, pending on the timing of exposure, histamine limits TNF responses through a direct effect on TNF receptor shedding, characterizing its regulatory effect [34].

***V. 2 - Vasoactive properties of Histamine***

Histamine's classical effect on fine vessels is the formation of edema by an increase in the flow of lymph and its protein content to the extracellular space and also the formation of gaps between endothelial cells, increasing transcapillary vesicular transport [35]. Inflammatory edema facilitates the transfer of immunologically active molecules and cells from the vascular space to the tissues. In spite of the beneficial effects, excessive loss of molecules and water from the vascular space can also impair organ physiology; therefore, the importance of rapidly restoring normal barrier properties. Histamine is accredited as meeting these properties, rapidly and transiently increasing endothelial permeability in post capillary venules [36]. The exact mechanism responsible for the formation of endothelial gaps and recovery in these vessels has been explored in the past decades, revealing a multiple step process.

Histamine binds to H1 receptors, activating the inositol phosphate second messenger system and consequently increasing the intracellular calcium concentration [37,38]. These events may in the end lead to endothelial contraction, the mechanism claimed responsible for gap formation [39-41].

There is a consensus that histamine-induced increased permeability is a transient event and that recovery happens, even in the presence of histamine. This permeability recovery phenomenon, mediated by increased intracellular cAMP level, also involves negative feedback inhibition, resulting in reversible modulation of the dimension of junctional gaps [42]. This pathway is shared by a number of so-called "endothelial stabilizers" preventing permeability increase by inflammatory mediators [41]. Prostaglandin (PGI2), a member of postanoid family, acts as one of these "endothelial stabilizers" to prevent the histamine induced permeability increase in venules and to decrease permeability of endothelial monolayers by activating the cAMP second messenger system. The increase in intracellular cAMP concentration in endothelial cells, linked to the gap reclosure phenomenon, is associated with an increase in the amount of F-actin in these cells. Probably the stabilization of the machinery involved in endothelial contraction occurs by increased polymerisation of actin filaments and prevention of the actin-myosin contraction [43].

PGI2 is mainly produced by endothelial cells and histamine stimulates its production after very short time incubation periods of 1 to 2 minutes [44]. In agreement with this, Wu and

Baldwin [45] used the PGI2 synthetase inhibitor tranylcypromine and showed that permeability recovery was totally abolished in some vessels, but still existed in others. Nevertheless, the extent of recovery for the latter ones was decreased.

Examining the temporal changes in the permeability coefficient over a 2-hour period Ehringer *et al* [46] showed that there are dynamic alterations in the time course of changes in HUVEC permeability, which are different for different vasoactive mediators of inflammation, namely thrombin, histamine and bradykinin. For histamine, permeability coefficients in vitro reached a maximum at 0-30 minutes and remained at this peak value for the entire 120 minutes studied. Since HUVEC cells were constantly exposed to histamine during the whole duration of the experiment, the authors speculated that it could have led to a deleterious effect on vascular integrity. Contrary to his observation we showed by a FACS apoptosis/necrosis assay that HUVEC were still viable, even when histamine remained present, while permeability continued to rise for the whole 60 minutes evaluated. Although our evaluation period ran for half the time, the concentrations of histamine used by us were 200x higher (circa $2x10^{-3}$ M against $10^{-5}$ M) [28].

The increase in microvascular permeability by histamine and other inflammatory mediators is associated with activation of the cellular contractile cytoskeleton and the formation of small gaps between adjacent endothelial cells in vitro and in vivo. In this sense, Andriopoulou *et al* [47] showed that histamine directly affected adherens junction properties, reducing intercellular cohesion and increasing vascular permeability. This effect, even in the absence of apparent intercellular gaps, was accompanied by tyrosine phosphorylation of VE-cadherin and catenins and by partial dissociation of VE-cadherin from the cytoskeleton. They also reported on different effects of histamine with respect to junction maturation, although, according to these authors the presence of frank discontinuities in the endothelial monolayers was not the absolute requirement for the histamine effect.

In line with the endothelium heterogeneity, barrier integrity differs not only between organs but also between different vascular segments of the same organ. Moreover, endothelial cells derived from conduit vessels and microvessels are phenotypically distinct and thus, present site-specific vascular responses to inflammation [48]. Discriminating between key events that regulate these responses and the signaling events activated to coordinate these process shall provide a better understanding of the microvascular biology process and open new

possibilities of treatment in sepsis, but also in oncology for a better drug delivery to tumor cells.

### *V.3 - Potential use of Histamine in Cancer treatment*

Based on the vasoactive properties of Histamine we showed its potential beneficial effect in improving drug delivery in the regional setting. Similar to the effect seen with TNF, Histamine showed a synergistic effect when combined to melphalan or doxorubicin in a rat experimental model of soft tissue sarcoma. The effect was based on a triplet: 1) direct cytotoxicity against tumor cells, 2) direct cytotoxicity against the TAV and 3) an indirect effect through Hi-mediated, increased melphalan concentration in the tumor [28,49]. There was a four times higher melphalan concentration in the tumor when histamine was combined as compared to melphalan alone. Furthermore, histamine led to tumor-associated vascular destruction with hemorrhagic necrosis while preserving normal tissue (adjacent muscle) vasculature.

Also in a different regional setting, the Isolated Hepatic Perfusion (IHP), administration of Histamine resulted in a synergistic effect when combined to melphalan. Without adding further toxicity it significantly improved response rates with a 55% OR, including 22% complete responses. Also of note, the combination treatment of Histamine and melphalan IHP decreased tumor progression rates in the perfused animals as compared to IHP with melphalan alone [50].

The synergistic results seen with the use of Histamine in ILP raised our enthusiasm to explore the possibility of combining different vaso-active and inflammatory cytokines to further improve tumor responses. It has been shown that systemic treatment of Histamine with IL-2 could benefit tumor therapy. However, it is also not unlikely that high dose local administration of these agents may as well counteract each other. We explored the combination of Histamine and IL-2 in the ILP setting and observed a 50% reduction in response rates in the ILP with Histamine and IL-2 plus melphalan compared to histamine or IL-2 alone combined with melphalan [49].

### *V - Concluding remarks and future perspectives*

Coley's toxin, described more than 100 years ago, provided a new insight in inflammatory response and its use in cancer treatment. Besides the impact it had on immunogenic

approaches, it also opened the field for antivascular biochemotherapy strategies, based on the vasoactive properties of inflammatory agents.

The registered TNF-based Isolated Limb Perfusion, currently in use in 35 centers in Europe, is a remarkable example of this accomplishment. Still the search for a better understanding of the mechanism of action of vasoactive agents, inflammatory response and endothelium properties from both normal and tumor vessels is necessary for further improvement of response rates. Currently ongoing research provides new insights and potentially new drugs for the combination treatment of solid tumors. The use of Histamine in regional treatment is a good example of the achievements obtained by these efforts, as it dramatically increased response rates in pre-clinical models of ILP and IHP.

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# Chapter 8

## *Summary & Conclusions*

## Summary

In **Chapter 1** an introduction to several experimental studies is given on the use of new vasoactive drugs as an alternative to tumor necrosis factor alpha (TNF-α), mainly the inflammatory mediator Histamine (Hi) and the cytokine Interleukin-2 (IL-2).

**Chapter 2** describes the synergistic antitumor effect of Hi combined to melphalan in a rat isolated limb perfusion (ILP) model. Hi-dependent reduction in tumor volume was blocked by $H_1$ and $H_2$ receptor inhibitors. Tumor regression was observed in 66% of the animals treated with Hi and melphalan, compared with 17% after treatment with Hi or melphalan alone. Tumor melphalan uptake increased and vascular integrity in the surrounding tissue was reduced after ILP treatment with Hi and melphalan compared with melphalan alone. *In vitro* results paralleled *in vivo* results. BN-175 tumor cells were more sensitive to the cytotoxicity of combined treatment than HUVECs, and Hi treatment increased the permeability of HUVECs. In conclusion, Hi in combination with melphalan in ILP improved response to that of melphalan alone through direct and indirect mechanisms.

Next, in **Chapter 3**, the same rat ILP model is used to evaluate the antitumor effects of the cytokine interleukin-2 (IL-2). ILP with IL-2 alone or ILP with melphalan alone has no antitumor effect, but the combination of IL-2 and melphalan in the ILP resulted in a strong synergistic tumor response (tumor regression in 67% of the rats treated by IL-2 plus melphalan ILP), without any local or systemic toxicity. IL-2 significantly enhanced melphalan uptake in tumor tissue without any major vascular damage, apart from scattered extravasation of erythrocytes seen in tumor sections of the rats treated with IL-2 and IL-2 plus melphalan, not seen in tumor sections of rats receiving control ILP. Also of note were clear differences in the localization of ED-1 cells, which presented clustered ED-1 cells on tumor slides of the rats treated by IL-2 with melphalan ILP. Tumor slides from all the other rats, receiving sham ILP, IL-2 alone ILP and melphalan alone ILP showed an even distribution of ED-1 cells. In addition, TNF mRNA levels were elevated in those tumors treated with IL-2 ILP and IL-2 plus melphalan ILP. These observations suggest an important role for macrophages in IL-2-based perfusion**.**

Based on the enthusiastic results described on Chapter 2 and Chapter 3, and given the enhancement of IL-2 effects in systemic therapy by the combination of Hi, it was tempting to combine the two of them in the ILP to further improve the response rates previously seen. Contrarily to these expectations, **Chapter 4** describes the negative synergistic effect

of the combination of Hi and IL-2 in melphalan-based ILP, with overall response rates (OR) of 28% against 66% and 67% for Hi ILP and IL-2 ILP, respectively. Histology of tumors demonstrated partial loss of Hi-induced haemorrhagic effect when IL-2 was present. The addition of Hi to IL-2 in melphalan-based ILP reverted the clustering effect on ED-1 cells (macrophages) previously described on Chapter 3. Contrarily to these, tumor melphalan uptake remained high.

**Chapter 5** addresses the combination of Hi to Doxorubicin (DXR), an important chemotherapeutic drug in solid tumor treatment showing that the synergistic effect is still present in the rat ILP model. Moreover, the effects of Hi on endothelial cells and tumor-associated vasculature (TAV) leads to a better drug distribution as shown here *in vivo* by taking advantage of the natural red fluorescence of DXR combined to green lectin-FITC staining of functional vessels during ILP. Additional histological stainings, CD-31 and Perl's method, demonstrates Hi related endothelial lining disruption and tumor hemorrhage, respectively. More importantly, these effects were more intense in tumor than in adjacent muscle tissue.

**Chapter 6** describes the synergistic antitumor effects, without any increase in toxicity, of Hi with melphalan in the isolated hepatic perfusion (IHP) where TNF-α use is restricted due to serious hepatotoxicity. A rat IHP model is used for the treatment of soft tissue sarcoma (BN175) liver metastasis with overall response rates of 55% for Hi plus melphalan IHP against 25% for melphalan alone IHP. More importantly, complete responses were only observed after Hi plus melphalan IHP (22% of the rats treated). The combination of Hi to melphalan also decreased the number of rats presenting with tumor progression, from 62% after melphalan alone IHP to 22% after Hi plus melphalan IHP. Hepatotoxicity peaked within 24 hours post IHP, independently of the treatment administered; recovered in 48 hours and was mainly related to transaminases elevation. Histology showed no serious liver damage.

Finally, in **Chapter 7** an overview of the use of vasoactive drugs, mainly focusing on TNF-α and Hi is given. Starting from the works of Coley and the use of the toxin with his name for the treatment of different solid tumors, going through the isolation of TNF and its frustrated use in systemic cancer treatment till the idea of using it in the regional setting. A short description of the ILP history is provided. Then, the endothelial cell and endothelium properties are focused. Finally the history of Hi and its vasoactive effects are reported with a view on its use as an alternative to TNF-α.

## Conclusions

Based on the studies performed in this thesis the following conclusions can be drawn:

- ILP with Hi in combination with different chemotherapeutic agents results in synergistic antitumor activities, without serious local or systemic toxicity.
- Hi has vasoactive properties, acts on tumor-associated vasculature and leads to higher tumor drug uptake and tumor vasculature destruction.
- The positive synergistic effects of Hi combined to melphalan are also observed in the isolated hepatic perfusion.
- IL-2 in combination with melphalan in the ILP results in synergistic antitumor activities. The mechanism of action is based on a higher tumor drug uptake without major damage to tumor vasculature.
- Macrophages play a major role in IL-2 based ILP.
- The combination of Hi and IL-2 in melphalan-based ILP leads to decreased response rates.

## Samenvatting en conclusie

**Hoofdstuk 1** geeft een inleiding in verscheidene experimentele studies naar het gebruik van nieuwe vasoactieve stoffen, zoals histamine en het cytokine interleukine (IL-2), als potentiële alternatieven voor tumor necrosis factor alpha (TNF).

In **hoofdstuk 2** wordt het synergistisch effect, in het geïsoleerde poot perfusie model (ILP), van histamine in combinatie met melphalan beschreven. Tumor regressie werd verkregen in 66% van de dieren behandeld met histamine in combinatie met melphalan. ILP met histamine alleen of melphalan alleen resulteerde slechts in 17% van de ratten in tumor regressie. Bij behandeling met histamine in combinatie met melphalan werd een verhoogde melphalan opname in de tumor waargenomen vergeleken met melphalan alleen. Ook was de integriteit van de vasculatuur in het omliggende tumor weefsel verminderd na histamine-melphalan ILP. Dit werd ook in vitro waargenomen. BN-175 (sarcoom) tumor cellen bleken gevoeliger voor de behandeling dan endotheelcellen. Histamine verhoogde daarentegen de permeabiliteit van de endotheelcellen. Concluderend, ILP met histamine in combinatie met melphalan zorgt voor een betere tumor respons dan ILP met melphalan alleen door zowel een direct als een indirect mechanisme.

In **hoofdstuk 3** werd gebruikt gemaakt van hetzelfde ILP model voor het testen van het antitumor effect van het cytokine IL-2. ILP met IL-2 alleen of melphalan alleen had geen antitumor effect, maar de combinatie IL-2 met melphalan resulteerde in een goede tumor respons (67% van de behandelde tumoren resulteren in een tumor regressie) zonder lokale of systemische toxiciteit. IL-2 verhoogde de melphalan opname in de tumor zonder ernstige vaatschade. Eveneens werd een uittreding van rode bloedcellen gezien in de tumor na behandeling met IL-2 alleen of IL-2 in combinatie met melphalan. Dit werd in de controle behandeling niet waargenomen. Ook waren duidelijke verschillen in lokalisatie van ED-1 cellen (macrofagen) waar te nemen. In tumor weefsel, na behandeling met IL-2 in combinatie met melphalan, gingen de ED-1 cellen groeperen. Na controle, IL-2 of melphalan ILP laggen deze cellen gelijkmatig door de tumor verspreidt. Eveneens was TNF mRNA verhoogd in tumoren behandeld met IL-2 of IL-2 in combinatie met melphalan. Deze bevindingen suggereren een belangrijke rol voor macrofagen in IL-2 perfusies.

Op basis van deze resultaten, en bevindingen dat een systemische behandeling met IL-2 in combinatie met histamine voor een beter effect zorgt, werden deze stoffen verder gecombineerd in de ILP. Echter, in tegenstelling tot de verwachtingen, beschrijft **hoofdstuk**

**4** een negatief synergistisch effect wanneer histamine, IL-2 en melphalan werden gecombineerd.

Respectievelijk werd een tumor respons verkregen van 66% en 67% van histamine-melphalan ILP en IL-2-melphalan ILP terwijl histamine-IL-2-melphalan ILP slechts een respons behaalde van 28%. Histologisch onderzoek van het tumor weefsel toonde een gedeeltelijk verlies van de histamine geïnduceerde haemorrhage aan wanneer dit werd gecombineerd met IL-2. Ook werd geen groepering van de ED-1 cellen meer waargenomen in tumoren na behandeling met histamine-IL-2-melphalan. Vreemd genoeg bleef de melphalan opname in de tumor wel hoog.

In **hoofdstuk 5** wordt de combinatie van histamine en doxorubicine (DXR), een belangrijk chemotherapeuticum in de behandeling van solide tumoren beschreven. Deze combinatie veroorzaakte een synergistisch effect in het rat ILP model. Het effect van histamine op endotheelcellen en de tumor-geassocieerde vasculatuur resulteerde in een verbeterde verdeling van het chemotherapeuticum. Dit werd aangetoond door gebruik te maken van de rode fluorescentie van het DXR gecombineerd met groen fluorescerend lectine-FITC dat gedurende de ILP de functionele bloedvaten aankleurde. Histologische kleuringen tonen aan dat histamine de endotheelcellen (CD31 positieve cellen) vernietigde en tumor haemorrhage (door perl's methode aangetoond) veroorzaakte. Deze effecten werden voornamelijk waargenomen in de tumor en niet in het omliggende spierweefsel.

**Hoofdstuk 6** beschrijft het synergistisch antitumor effect van histamine en melphalan in de geïsoleerde lever perfusie (IHP) zonder een verhoogde toxiciteit. Dit in tegenstelling tot TNF, dat wel een hoge lever toxiciteit veroorzaakt. Voor de behandeling van lever metastasen van BN-175 werd een IHP in de rat verricht. Een behandeling met histamine gecombineerd met melphalan resulteerde in een respons van 55% tegenover een respons van 25% met melphalan alleen. Slechts na IHP met histamine en melphalan (22% van de behandelde tumoren) werd een volledige response waargenomen. De combinatie van histamine en melphalan verminderde ook het aantal doorgroeiende tumoren van 62% (melphalan alleen) tot 22%. Lever toxiciteit, voornamelijk transaminase verhoging, bereikte een maximum na 24 uur, maar was echter onafhankelijk van de behandeling en herstelde na 48 uur. Histolgisch onderzoek bevestigde eveneens dat er geen ernstige leverschade na de IHP was opgetreden.

In **hoofdstuk 7** wordt een overzicht gegeven van het gebruik van vasoactieve middelen, voornamelijk TNF en histamine. Dit overzicht begint met het werk van Coley en het

gebruik van het toxine, dat zijn naam draagt, voor de behandeling van verschillende solide tumoren, de isolatie van TNF en de frustratie tijdens de systemische behandeling tot het idee om TNF in een regionale behandeling te gebruiken. Hier wordt ook een korte geschiedenis beschreven van de ILP en geconcentreerd op het endotheel. Ten laatste wordt de geschiedenis van histamine en het vasoactieve effect beschreven als alternatief voor TNF.

Gebaseerd op de uitgevoerde experimenten beschreven in deze thesis kunnen de volgende **conclusies** getrokken worden:

- Een ILP met histamine in combinatie met verschillende chemotherapeutica resulteert in een synergistisch antitumor effect zonder ernstige lokale of systemische toxiciteit.
- Histamine heeft vasoactieve eigenschappen, werkt op de tumor-geassocieerde vasculatuur, veroorzaakt verhoogde opname van de chemotherapeutica in de tumor en vernietiging van de tumor vasculatuur.
- Ook in de IHP wordt een synergistisch antitumor effect van histamine in combinatie met melphalan waargenomen
- ILP met IL-2 in combinatie met melphalan resulteert in een synergistisch antitumor effect. Het achterliggende mechanisme is gebaseerd op de verhoogde opname van melphalan in de tumor zonder duidelijke schade aan de tumor vasculatuur.
- Macrofagen spelen een grote rol in een ILP met IL-2
- De combinatie histamine-IL-2-melphalan leidt tot een verminderde tumor respons.

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## Curriculum Vitae

Flavia Brunstein, the author of this thesis was born in Sao Paulo, Brazil, where she followed her secondary school. She obtained her Medical degree at the Medical School from the Federal University of Sao Paulo (UNIFESP-EPM) followed by her training in General Surgery and Plastic Surgery at Hospital Sao Paulo, linked to the same University. After finishing this surgical training she joined the Tumors division of the Plastic Surgery Department and, under the supervision of Prof. Dr. IDAO Santos obtained her Masters degree on clinical studies on "The metabolic changes in the limb during Isolated Limb Perfusion for the treatment of irresectable melanoma limb metastases". She had visited many international Cancer centers, including the Sydney Melanoma Unit and the CPO in Lausanne. In 2000 she came for the first time to the Daniel den Hoed Cancer Center when she got to know the Experimental Surgical Oncology and the animal experimental models of regional treatment. These led to the establishment of a translational research program on the evaluation of Histamine as an alternative to TNF in loco-regional treatment, under the supervision of Prof. Dr. A.M.M. Eggermont and Dr. T.L.M. ten Hagen. The current thesis is the result of these efforts.

## Acknowledgements

Looking at this thesis finally finished, together with the published papers gives an indescribable good feeling. These years had been of hard work but also of good fun. Living in a different country and working in a completely new environment was one of the richest experiences I ever had. Although I'm aware that it required a lot of personal effort to accomplish this task I certainly wouldn't have made it without the help and support of many people, some of them not directly involved in the studies but whose friendship and support are priceless. I would like to name a few of them:

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To all my friends here and abroad who shared the ups and downs of this whole process and gave meaning to it.

And last but by no means least, to my dear family, Raquel, Oswaldo, Andreia, Marcelo and Gabriel who, in spite of the distance, were always there for me. Your belief and support gave me the strength to keep going – Thank you!

Flavia

# Appendix

## Chapter 2 (page 24)

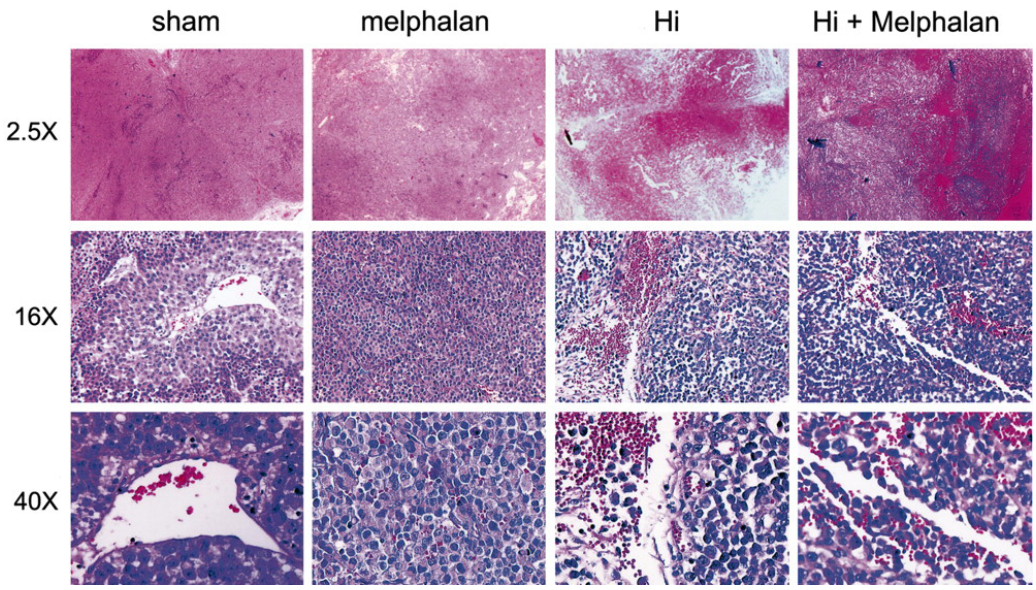


**Figure 3.** Histology of tumor after isolated limb perfusion (ILP). Tumors were excised immediately after ILP for each treatment, fixed in 4% formaldehyde solution and embedded in paraffin for hematoxylin-eosin staining. Perfusate alone (sham) ILP with intact vessels and normal tumor tissue; melphalan 8 μg/mL ILP with some spots of necrosis on tumor tissue but no vascular damage; Hi alone 200 μg/mL ILP showing vascular vasodilation, extravasation of red blood cells into the tumor and damage to the endothelial cell lining of tumor vessels; Hi plus melphalan (200 μg/mL and 8 μg/mL, respectively) ILP showing the damage to tumor vessels and massive hemorrhage. Pictures illustrate representative examples of each treatment)

## Chapter 2 (page 26)

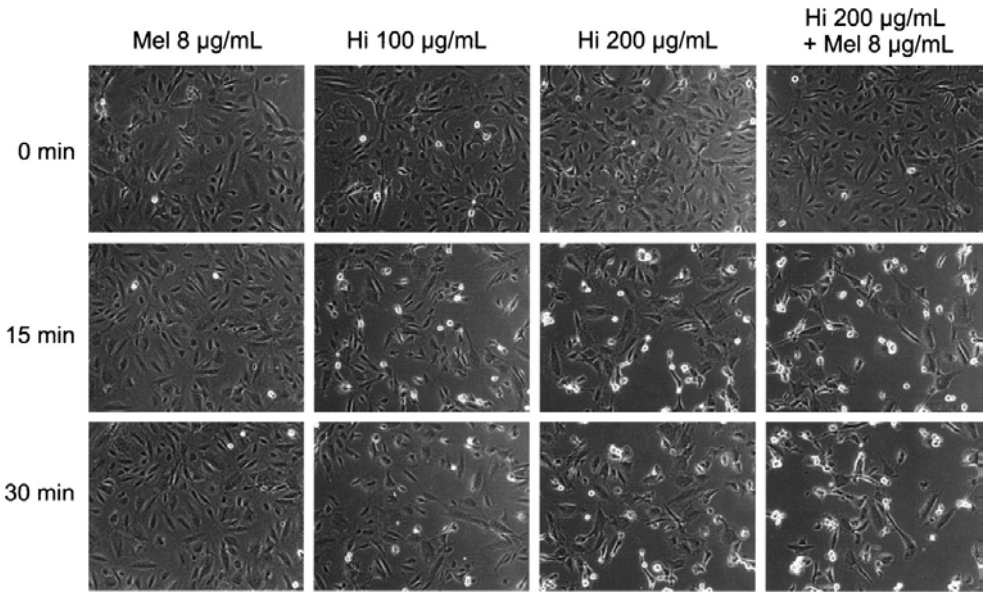


**Figure 5**. Direct effect of Hi on endothelial cells. Human umbilical vein endothelial cells (HUVECs) were incubated with medium alone; melphalan (8 µg/mL) alone, Hi (100 µg/mL or 200 µg/mL) alone or in combination for 15 and 30 minutes. Gap formation and morphologic changes can be observed already after 15 minutes incubation both with 100 and 200 µg/mL (a more pronounced effect for 200 µg/mL).

## Chapter 3 (page 47)

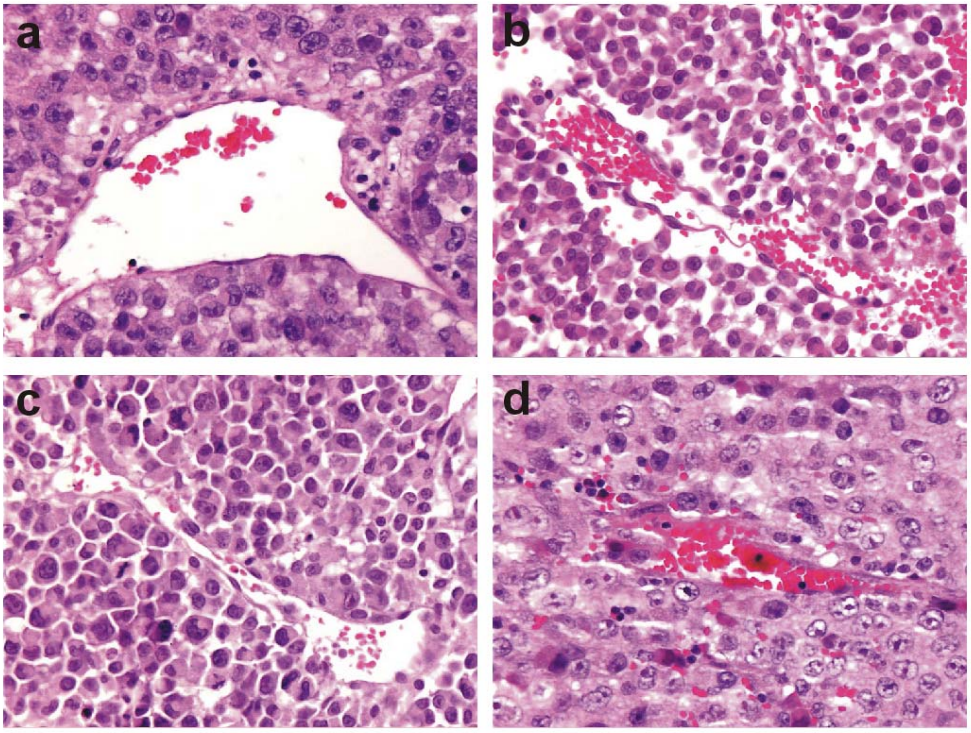


**Figure 4.** Histology of BN175 tumor after isolated limb perfusion (ILP). Tumors were excised immediately after ILP for each treatment, fixed in 4% formaldehyde solution and embedded in paraffin for hematoxylin-eosin staining. (a) sham (perfusate alone) ILP; (b) 50 μg IL-2 ILP; (c) 40 μg melphalan ILP; and (d) 50 μg IL-2 plus 40 μg melphalan ILP. . Pictures illustrate representative examples of each treatment. Original magnification of 40x

## Chapter 3 (page 49)

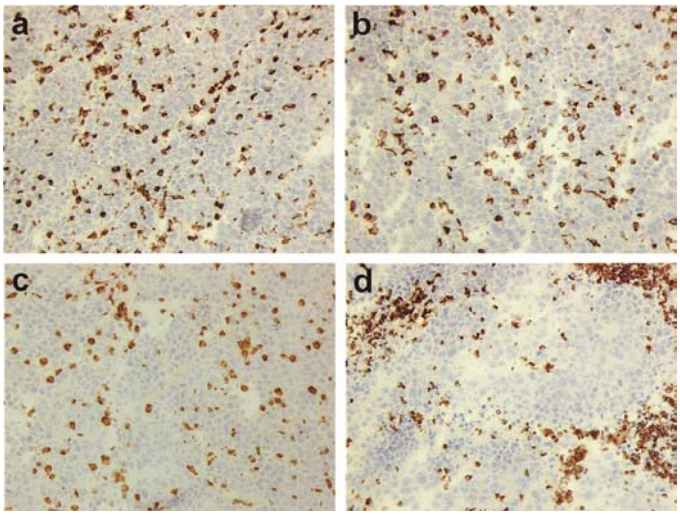


**Figure 5.** Macrophage infiltration and distribution after IL-2 based isolated limb perfusion (ILP). Tumors were excised immediately after ILP for each treatment and snap frozen. 7 μm-cryostat sections were stained for ED-1 positive cells (macrophages). (a) sham (perfusate alone) ILP; (b) 40 μg melphalan ILP; (c) 50 μg IL-2 ILP; and (d) 50 μg IL-2 plus 40 μg melphalan ILP showing a redistribution of macrophages and ED-1 cells. Pictures illustrate representative examples of each treatment. Original magnification of 16x

## Chapter 4 (page 68)

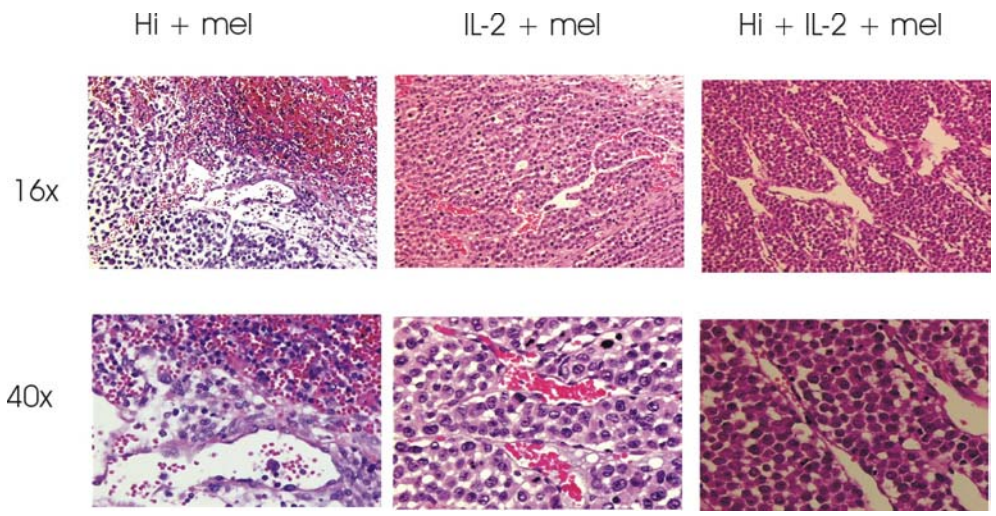


**Figure 3**. Histology of tumors after isolated limb perfusion (ILP). Tumors were excised immediately after ILP for each treatment, fixed in 4% formaldehyde solution and embedded in paraffin for hematoxylin-eosin staining. Hi + melphalan ILP resulted in severe damage to tumor vessels and massive hemorrhage. IL-2 + melphalan inflicted a slight edema while after ILP with Hi + IL-2 + melphalan a striking loss in the Hi-induced hemorrhagic effect was observed. Also less destruction of the endothelial cell lining and reduced edema between tumor cells was seen.

## Chapter 4 (page 69)

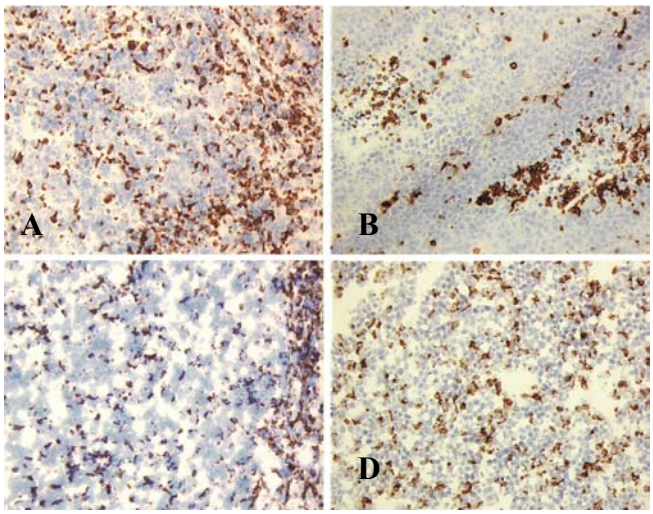


**Figure 5**: Immunohistochemical staining for ED-1 positive cells (macrophages) on frozen tumor sections of BN-175 soft tissue sarcoma. Tumors were excised and immediately frozen in liquid nitrogen. Representative pictures are shown for (a) not treated BN-175 tumor; (b) perfused with IL-2+mel; (c) perfused with Hi+mel and (d) perfused with Hi+IL-2+mel. ILP with IL-2 and melphalan resulted in redistribution of macrophages with clustered ED-1 cells. This effect was lost with the combination of Hi to the IL- 2+mel ILP in the triple drug treatment. Original magnification 16X

## Chapter 5 (page 82)

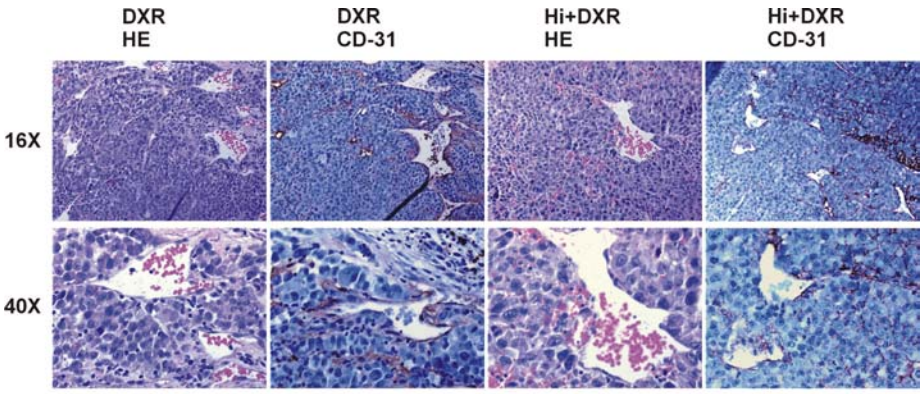


**Figure 2**: Tumor vascular destructive effect and hemorrhagic necrosis upon Histamine-based Isolated Limb Perfusion (ILP) (A). Pictures of representative tumor histology (HE) and vascular destruction (CD31) right after ILP with doxorubicin (DXR) or DXR plus Histamine (Hi) are shown. Orange bar on 16x magnification pictures corresponds to 100 μm and red bar on 40x magnification to 50 μm.

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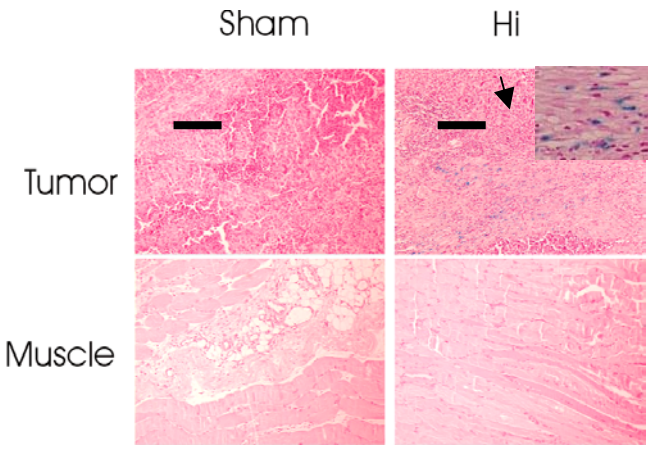
## Chapter 5 (page 83)

**Figure 3**: Staining by Perl's method shows Histamine-related induction of hemorrhagic necrosis at 7 days after ILP, which is absent in muscle. Original magnification 10X. Black bar corresponds to 100 μm.




## Chapter 5 (page 84)

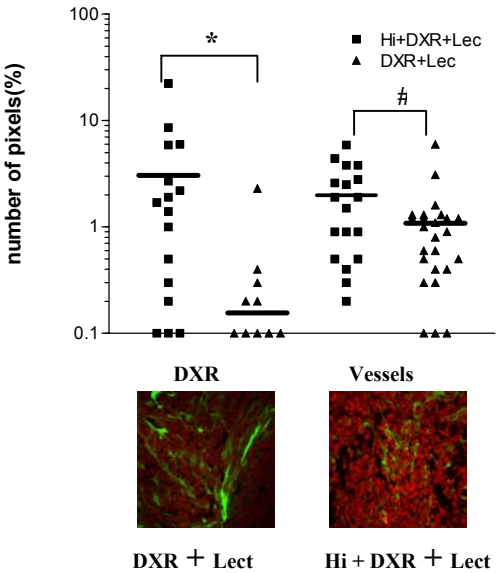



**Figure 4**: Effect of Histamine-based Isolated Limb Perfusion (ILP) on intratumoral doxorubicin (DXR) distribution in relation to tumor vessel presence. Directly after ILP with DXR and Lectin-FITC (DXR+Lec), or Histamine plus DXR and Lectin-FITC (Hi+DXR+Lec) tumors were excised and snap-frozen. 25 μm-thick slides were examined by confocal microscopy. DXR distribution and vessel density in digital images were measured as described in Materials and Methods. Representative pictures of intratumor DXR distribution right after ILP with DXR and Lectin-FITC (DXR+Lec), or Histamine plus DXR and Lec (Hi+DXR+Lec) are shown. * $p<0.001$; # $p=0.06$

## Chapter 6 (page 98)

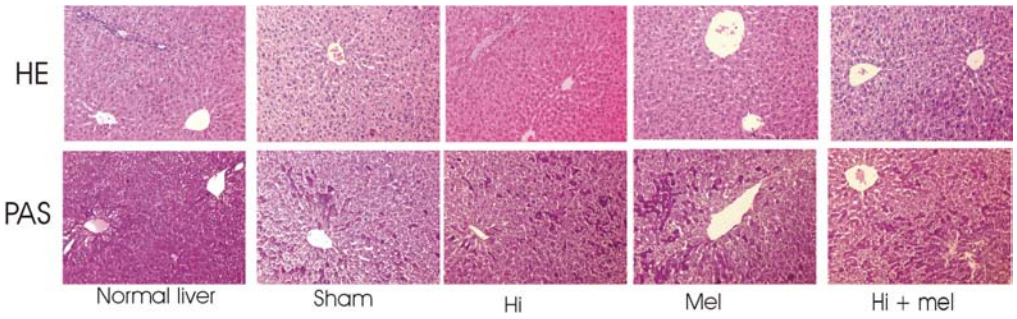



**Figure 3**. Histological evaluation of livers extracted from an untreated BN rat and seven days after sham, melphalan, Hi or Hi plus melphalan IHP. Livers were fixed in 4% formaldehyde solution and embedded in paraffin before staining with HE and Periodic Acid Schiff (PAS) method. HE staining show preserved anatomical structures as compared to normal liver. An even reduction on glycogen for all the treatments, even sham, as compared to normal untreated liver is illustrated by the PAS method. Images were taken on a Leica DM-RXA microscope supplied with a Sony 3CCD DXC camera.

# Propositions

(Belonging to the thesis Histamine's vasoactive properties improving response rates in solid tumor treatment)

1. **The immediate effect of Histamine on tumor-associated vasculature (TAV) endothelial cell lining is increased permeability, gap formation and intratumoral hemorrhage (this thesis).**

2. **Histamine improves the delivery of chemotherapeutic agents to tumor cells by changing TAV permeability and by intratumoral hemorrhage to a large degree via red cell bound drug (this thesis).**

3. **Because Histamine can block the IL-2 induced permeability enhancement mechanism, a loss of synergy with chemotherapeutic drugs is observed when the three agents are combined (this thesis).**

4. **Given the absence of Histamine-related hepatotoxicity, its use in melphalan-based isolated liver perfusion is better than the use of TNF in that setting (this thesis).**

5. **IL-2 should be evaluated in a clinical trial in ILP while Histamine is an excellent candidate for an IHP clinical trial (this thesis).**

6. **Tumor cell and stromal cell targeted delivery development will rather reset the stage for more successful use of chemotherapeutic agents, than replace chemotherapy use.**

7. **The "good" reason is usually provided instead of the "real" reason.**

8. **There are at least three different versions for how and why things happened: one from one side, one from the other side and the true one, which usually lays somewhere between the two of them.**

9. **"Primeiro estranha-se, depois entranha-se"- Fernando Pessoa ("At first, it's unfamiliar, then it strikes root")**

10. **If you don't aspire to great things you don't even achieve little ones (Folk culture)**

11. **"In order to understand a culture, you have to listen to the music they make." – Confucius**