

***In vivo* upregulation of CD95 and CD95L causes synergistic inhibition of angiogenesis by TSP1 peptide and metronomic doxorubicin treatment**

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Abstract

Antiangiogenic thrombospondin-1 (TSP1) induces endothelial cell death via a CD95-mediated cascade. We used this signaling pathway, where CD95/Fas is a rate-limiting intermediate, as a target to optimize the efficacy of TSP1 active peptide, DI-TSP. Like TSP1, DI-TSP upregulated endothelial CD95L *in vivo*. To modulate CD95 levels, we chose chemotherapy agent doxorubicin (DXR). DXR caused sustained upregulation of CD95 in the activated endothelium at 1/100 of the maximal tolerated dose. DI-TSP and DXR synergistically induced endothelial apoptosis *in vitro*, and *in vivo*, in developing murine vessels. Fas decoy, TSP1 receptor antibody and Pifithrin, a p53 inhibitor, severely decreased apoptosis and restored angiogenesis by DXR–DI-TSP combination, evidencing critical roles of CD95 and TSP1. Combined therapy synergistically blocked neovascularization and progression of the bladder and prostate carcinoma. Such informed design of a complex antiangiogenic therapy based on the rate-limiting molecular targets is a novel concept, which may yield new approaches to cancer treatment.

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Abbreviations: TSP1, thrombospondin-1; DXR, doxorubicin

Introduction

During oncogenic conversion, the cells undergo angiogenic switch and gain the ability to promote angiogenesis.¹ Growing

tumors rely on the oxygen and nutrients supplied via autonomous capillary network, and disrupting tumor-associated angiogenesis is becoming a promising new strategy to control tumor growth. Among angiogenesis inhibitors, an important place belongs to the naturally occurring peptides, frequently proteolytic fragments of the extracellular matrix proteins, which trigger apoptosis in proliferating but not in quiescent endothelium.² More than 30 natural and synthetic inhibitors are in clinical trials: the lack of toxic effects makes antiangiogenic agents an attractive long-term treatment alternative for traditional chemotherapy; however, their high cost and relatively low specific activity present a challenge (Kerbel and Folkman² and <http://clinicaltrials.gov>).

Thrombospondin-1 (TSP1) is an angiogenesis inhibitor,³ a multidomain protein that binds a variety of receptors, including integrins, CD36, integrin-associated protein, heparan sulfate proteoglycans and more.⁴ TSP1 blocks angiogenesis via CD36, a receptor constitutively expressed in a subset of endothelial cells.^{5–7} The ability to bind multiple receptors complicates the therapeutic use of nascent TSP1. This obstacle has been circumvented by introducing minimal antiangiogenic fragments of TSP1.⁸ One of them, DI-TSP is active at nanomolar concentrations that interacts with CD36 and causes endothelial cell apoptosis via the same signaling cascade⁹ (Zaichuk and Volpert, unpublished observations) while lacking undesirable effects due to binding receptors other than CD36.^{8,9} This is a C-terminus capped nonapeptide based on the GVITRIR sequence from the second properdin repeat, containing an L- to D-isoleucyl substitution.⁹ TSP1, or DI-TSP binding to CD36 triggers signaling cascade that begins with recruitment of Src-related kinase p59^{l^yn} and ultimately causes endothelial cells apoptosis.¹⁰ TSP1 selectively destroys proliferating, but not quiescent endothelium: our recent studies attribute this selectivity to the CD36-dependent upregulation of the cell surface CD95L, a ligand for the death receptor CD95/Fas.¹¹ Binding of CD95, a member of the TNF receptor superfamily to its ligand^{12–16} triggers apoptotic response via an exhaustively studied pathway that involves formation of the intracellular death-inducing signaling complexes (DISCs).¹⁷ CD95 itself is constitutively low on the resting endothelial cells and its surface expression is induced by proangiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or interleukin-8 (IL-8). Thus, CD95/CD95L-mediated apoptosis can only occur in the endothelial cells exposed to both angiogenic inhibitors and stimuli.

Proapoptotic and antiangiogenic effect of TSP1 and peptide mimetics is restricted by the availability of endothelial CD95. CD95 presentation could be improved with increased stimulation by inducers of angiogenesis, however it would cause concomitant increase in survival signals. Seeking other ways to augment an efficacy of DI-TSP, we chose doxorubicin (DXR), a genotoxic agent used for the chemotherapy of

human leukemias, lymphomas, breast and prostate cancer. DXR at high doses increases surface CD95 and triggers apoptosis in proliferating endothelium.¹⁸ Interestingly, although at these doses, DXR induced apoptosis independent of CD95/CD95L interactions, the upregulated CD95 remains functional and CD95-dependent apoptotic cascade can be triggered with the activating monoclonal antibodies in DXR-treated cells but not in untreated control population.¹⁸

Here we show that at low concentrations, DXR resulted in sustained upregulation of the endothelial CD95 without noticeable cytotoxic effects towards endothelial or cancer cells. The increase in the endothelial CD95 by low DXR doses significantly improved the antiangiogenic and antitumor efficacy of DI-TSP. The use of the low DXR doses administered continuously at regular short intervals (metronomic chemotherapy) as opposed to administration of several bolus doses may lower cardiac and liver toxicity commonly associated with genotoxic DXR treatment, as well as the emergence of multidrug resistance.^{19–21} Indeed, we showed that DXR at doses 100 times lower than those effectively used as monotherapy^{19,22,23} significantly upregulated endothelial CD95 *in vitro* and *in vivo*: this DXR-generated CD95 increase contributed to the augmented endothelial cell apoptosis by DI-TSP leading to the synergistic reduction of angiogenesis and tumor growth while avoiding toxic side effects of the high therapeutic doses.

Results

Low DXR doses augmented CD95/Fas surface expression by endothelial cells

In vitro DXR at concentrations equivalent to the maximum tolerated therapeutic doses (MTD, 250 ng/ml)^{23,24} induces upregulation of a functional cell surface CD95 by the large vessel endothelium (HUVEC).¹⁸ Here, we tested whether DXR doses below MTD (50–200 ng/ml) were sufficient to upregulate endothelial CD95. We analyzed the effect of DXR between 50 and 500 ng/ml on apoptosis and CD95 presentation of HUVECs and microvascular endothelial cells (HMVECs) cultured in full serum. DXR induced significant endothelial cell apoptosis only at 500 ng/ml. Although lower DXR concentrations failed to trigger apoptosis in HUVECs and HMVECs, or to delay the cell cycle progression (Figure 1a, b and supplementary Figure 1a), quantitative flow cytometry analysis showed a substantial increase of the surface CD95 (Figure 1c, d and data not shown). Baseline fluorescence of the cells stained with the secondary antibody alone was not affected by the isotype control IgG. Thus, DXR significantly increased surface CD95 at doses at least 10 times lower than those required to induce endothelial cell apoptosis *in vitro*.¹⁸

DI-TSP upregulated CD95L expression and induced HMVECs apoptosis in synergy with DXR

TSP1 increases mRNA and surface CD95L in the endothelial cells via signaling cascade mediated by its antiangiogenic receptor, CD36.^{10,11,25} Since HUVECs are CD36 negative,⁶ we examined the ability of DI-TSP, a short peptide derivative

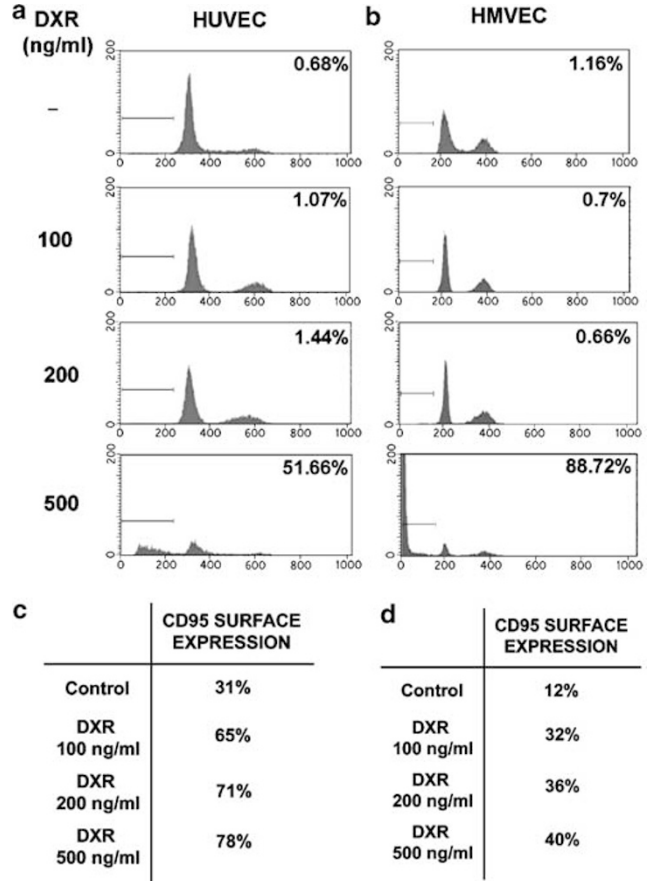


Figure 1 Cell cycle and CD95 surface levels in DXR-treated endothelial cells. HUVECs (a) and HMVECs (b) were treated 48 h with the indicated DXR concentrations, stained with propidium iodide and DNA content analyzed by flow cytometry. Note the significant percentage of the apoptotic cells in the presence of 500 ng/ml DXR. (c, d) Quantification of flow cytometry analysis of surface CD95 expression in DXR-treated HUVECs (c) or HMVECs (d). Note the considerable increase in CD95-positive cell population at lower DXR concentrations. Control values reflect the percentage of FITC-positive cells after incubation with the FITC-conjugated secondary antibody alone

of TSP1, to induce similar events only in the CD36-positive HMVECs.^{5,6} Treatment (24 h) with DI-TSP at antiangiogenic, proapoptotic concentration (10 nM) resulted in pronounced upregulation of CD95L that was preserved in the presence of DXR (Figure 2a).

We then combined DXR doses, which in a single treatment increased endothelial CD95 surface expression but not apoptosis, with DI-TSP and tested their ability to cooperatively induce apoptosis in proliferating HMVECs. Importantly, up to 70% cells displayed signs of apoptosis in HMVEC population treated with DI-TSP/DXR combination. Single agents produced little increase in cell death compared to the baseline control levels as was determined by TUNEL (Figure 2b) and by flow cytometry analysis of DNA content (data not shown). Thus, DXR and DI-TSP acted in synergy (see below) to induce HMVEC apoptosis *in vitro*. As we anticipated, this synergistic induction of apoptosis was CD95 and CD95L dependent as CD95 decoy receptor diminished apoptosis rate to ~9.5% (Figure 2b). Basal levels of CD95L combined with the modest

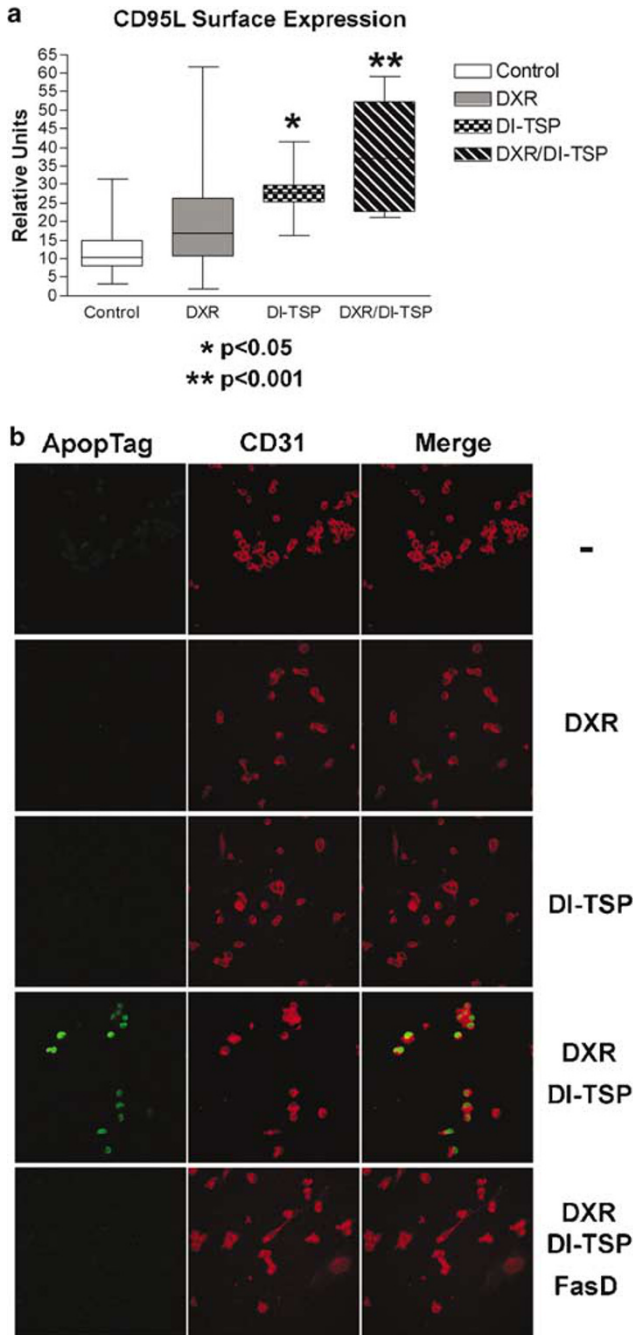


Figure 2 Combination of low doses of DXR and DI-TSP induce CD95, CD95L and apoptosis in cultured HMVECs. (a) HMVECs were treated 24 h with 10 nM DI-TSP and/or 100 ng/ml DXR as indicated and stained *in situ* for CD95L and CD31 endothelial cell marker. CD95L expression was measured on digital images using LaserPix software and normalized against CD31 levels. Note the upregulation of CD95L expression in DI-TSP-treated cells. (b) Apoptosis was analyzed by *in situ* TUNEL assay. DI-TSP/DXR treatment was repeated in the presence of soluble CD95 decoy receptor (FasD). Cell population was counterstained with CD31 (red). Each field includes roughly equal number of cells. Note high apoptosis levels in the presence of DI-TSP/DXR combination and reversal by CD95 decoy

CD95 increase due to the low dose of DI-TSP were insufficient to induce apoptosis at significant levels in the cells cultured in serum-rich medium (Figure 2a,b).

DXR and DI-TSP treatment upregulated CD95, CD95L on remodeling capillaries and increased apoptosis *in vivo*

We next sought to determine whether DI-TSP and low DXR doses cooperatively block angiogenesis *in vivo*. We chose 0.2 mg/kg/5 days DXR regimen for the *in vivo* treatment: it is 25 times lower than the bolus therapeutic dose of DXR conventionally used to block tumor growth in mouse models and 100 times lower than maximum tolerated dose (MTD).^{19,22,23} We used Matrigel plug model where murine vessels develop subcutaneously towards and into the extracellular matrix plug. This model allows the study of developing blood vessels over time, while excluding interference by the tumor cells. Detection of DXR-dependent increase in CD95/Fas proved difficult, as the plugs were supplemented with the excess of exogenous proangiogenic factors (VEGF or bFGF), which are known to induce endothelial CD95.¹¹ Seeking conditions that would allow unequivocal demonstration of *in vivo* CD95 upregulation by the chosen DXR dose, we performed the assay using Matrigel plugs lacking exogenous VEGF and heparin. We were able to detect at least a 10-fold increase in CD95 staining in the presence of DXR (0.2 mg/kg) (Figure 3a,b). In standard Matrigel supplemented with proangiogenic factors, we were able to detect CD95L in the new capillaries treated with either DI-TSP or DI-TSP/DXR combination (Figure 3c). Thus, endothelial cells of the remodeling capillaries *in vivo* express higher levels of CD95 in response to DXR and increase CD95L in response to DI-TSP.

We were able to detect an apparent increase in the apoptotic endothelial cell population in response to DI-TSP/DXR combination, where both CD95 and CD95L were upregulated (Figure 3d). Interestingly, the expression of CD31 (PECAM-1) by the endothelial cells undergoing apoptosis was markedly lower (see Discussion); however, localization of CD31 to the apoptotic cells was evident. (Figure 3d, inset). Finally, DXR alone caused no significant change in MVD in the Matrigel plugs compared to the untreated control. DI-TSP at 1 mg/kg caused a 1.4 decrease in the median vascular density ($P < 0.06$), and the combination treatment produced a striking 2.9-fold decrease ($P < 0.002$).

DI-TSP and DXR at low doses delayed tumor progression and diminished microvessel density

In order to investigate whether DXR and DI-TSP combination would affect tumor progression and tumor-associated angiogenesis, we used xenograft model of the human carcinoma of the bladder 253J B-V, a highly aggressive cell line completely lacking secreted TSP1.²⁶ Animals that received DI-TSP/DXR combination treatment showed a significant delay in tumor onset compared to the single treatments and to the control vehicle. This delay was followed by tumor stabilization (Figure 4a). When combined DI-TSP/DXR treatment was applied to a less aggressive prostate carcinoma cell line LNCaP, the results were even more striking (Figure 4b). While low dose of DXR alone had no significant effect on the tumor progression and DI-TSP caused only modest several days' delay, DI-TSP/DXR combination treatment resulted in the

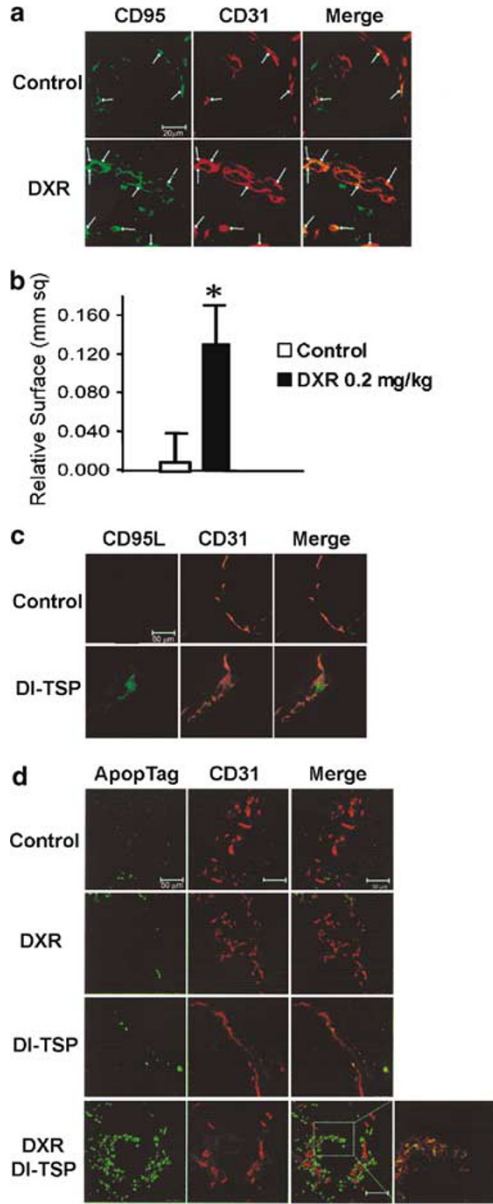


Figure 3 The effect of DI-TSP and DXR on CD95 and CD95L expression in newly formed capillaries in the Matrigel plugs *in vivo*. (a) CD95 levels (green) evaluated in the DXR-treated neovessels (red) in the absence of stimulatory VEGF in the Matrigel plugs. Arrows indicate CD95-positive endothelial structures. (b) Quantitative analysis of the experiment in (a). * $P \leq 0.002$. (c) Mice were given Matrigel plugs containing VEGF, treated as indicated and CD95L (green) assessed by immunofluorescence. Endothelial structures are visualized by CD31 staining (red). (d) Similarly treated Matrigel plugs were stained for apoptosis (TUNEL, green). Note the significant increase in the endothelial cell death by DI-TSP/DXR combination. CD31 (red) was used as an endothelial cell marker. Also, note weaker CD31 staining of the apoptotic endothelial structures (inset)

eight-fold reduction of the tumor volume and a considerable progression delay. Interactive coefficient I for DI-TSP calculated using modified isobologram analysis²⁷ was > 1 (~70 and 50 for the bladder and prostate cancer models, respectively), indicating strong synergy. Microvessel density (MVD) of these tumors followed the same pattern in that it was

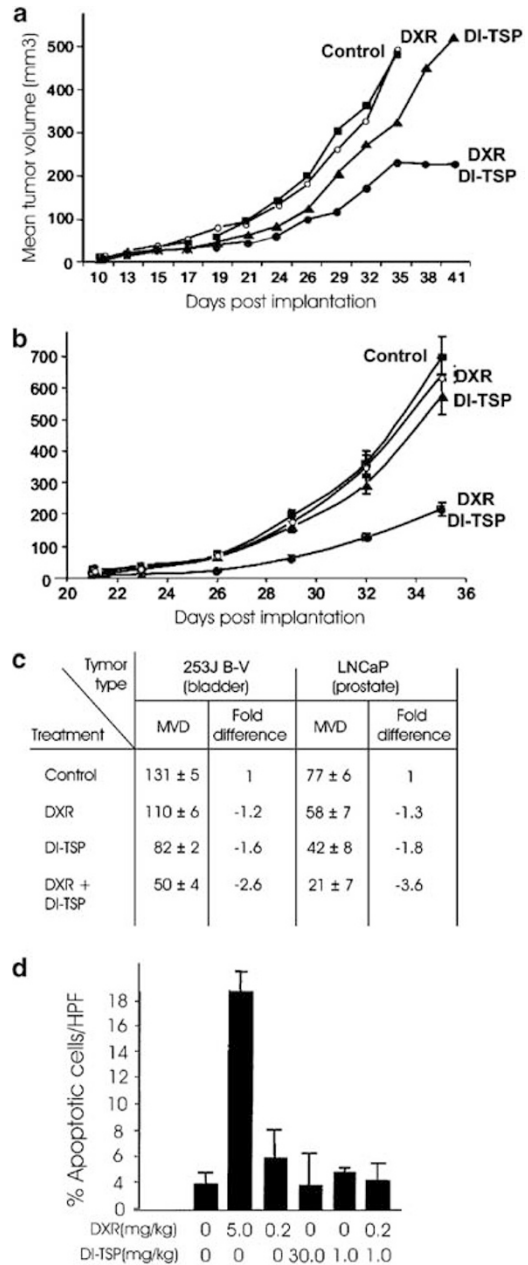


Figure 4 Combination of low DXR doses with DI-TSP treatment augments the delays in tumor progression and further reduces tumor vascularity. Nude mice were given subcutaneous bladder carcinoma tumors and treated with control saline or DI-TSP ± DXR. (a) The effect of DI-TSP/DXR combination on progression of bladder carcinoma xenografts. (b) The effect of the same treatment on progression of prostate carcinoma xenografts. (c) Tumor vascularity in 253J B-v and LNCaP tumors was assessed by CD31 staining of tissue sections and quantified using ImageQuant software. Microvessel counts and fold reduction are shown. All the differences are significant ($P < 0.05$). (d) Apoptosis in the cardiac muscle measured by *in situ* TUNEL assay

significantly lower in the tumors extracted from mice treated with the combination therapy, DI-TSP/DXR (Figure 4c and supplementary Figure 1b). DI-TSP alone produced a mild decrease in the tumor MVD and size while DXR alone had no significant effect (Figure 4c and supplementary Figure 1b). Interestingly, higher DXR doses resulted in a different pattern

of tumor progression: after the initial delay, tumors in the groups treated with DXR alone or in combination with DI-TSP grew at a faster rate (see Discussion).

Bolus DXR administration at MTD causes apoptosis in multiple organs, often resulting in heart and liver failure. We measured apoptosis in the hearts of mice after 2 weeks of DXR boli, metronomic DXR and DI-TSP/metronomic DXR combination. MTD resulted in >four-fold higher apoptosis in the cardiac muscle compared to the control vehicle, no measurable increase was observed with DI-TSP/DXR combination (Figure 4d). Animals treated with MTD lost ~15% of their body weight while combined treatment caused no significant weight loss (data not shown).

DXR/DI-TSP combination induced CD95, CD95L and apoptosis in tumor vasculature

Compared to the subcutaneous Matrigel plugs, the environment of the tumor vasculature is extremely complex. Thus, it was nonevident that the effect of DI-TSP and DXR on CD95/CD95L expression would remain the same. Nevertheless, in mice treated with DXR/DI-TSP combination, we detected profound upregulation of CD95L in tumor vessels in the presence of DI-TSP alone or in combination with DXR (Figure 5a, right). An increase in CD95 was less evident due to the high background levels caused by tumor-derived proangiogenic factor(s); however, quantitative evaluation of immunostained tumor sections showed increased number of the CD95-positive endothelial structures (Figure 5a, left).

At-a-glance, evaluation of the apoptosis levels in tumor xenografts showed a dramatic increase in total numbers of TUNEL-positive cells in the tumors treated with DI-TSP/DXR

combination compared to the single treatments and to the sham-treated control (Figure 5b). To distinguish between the antiangiogenic and tumoricidal effects of the treatments, we performed quantitative analysis of the apoptotic cell populations separately in the endothelial (CD31-positive) and non-endothelial (CD31-negative, tumor and inflammatory cells) compartments of the tumors. The combined therapy caused striking increase in the apoptosis in both endothelial and nonendothelial compartments of the tumor, while the same dose of DXR alone failed to affect endothelial cells and had very modest effect on the tumor cells, suggesting that tumor cell apoptosis was largely due to the restrictions posed by the lack of nourishing vasculature.

Angiogenesis blockade by DI-TSP/DXR combination was mediated by TSP1 receptor and required CD95/CD95L death cascade

The synergy between DXR and DI-TSP *in vivo* was dependent on the signal generated by CD95L binding to CD95/Fas on the microvascular endothelium. Soluble CD95/Fas-Fc receptor (Fas decoy) significantly attenuated MVD reduction by the combined treatment and inhibited endothelial cell apoptosis in Matrigel plug assay (Figure 6a–c). The inhibitory activity of the combination therapy was clearly associated with TSP1 signaling cascade. Neutralizing antibody against TSP1 receptor CD36 were previously shown to inhibit TSP-mediated induction of CD95L and apoptosis.^{10,11} The same antibody completely abrogated endothelial cell apoptosis and antiangiogenesis by DI-TSP/DXR combination (Figure 6a–c). Since CD95 upregulation by DXR is p53-dependent¹⁸ while apoptosis by DI-TSP is not,¹⁰ we used Pifithrin- α (PFT- α), an inhibitor of p53-dependent transcription,²⁸ in order to eliminate DXR induction of CD95 mRNA and protein. Predictably, PFT- α relieved the effect of DI-TSP/DXR on both microvessel counts and endothelial cell.

Discussion

Angiogenesis inhibitors are gaining their due place among cancer treatments. Since their primary target is normal, genetically stable endothelium, and not the labile tumor cells, the selection pressure is less likely to evoke resistance. Antiangiogenics target no particular tumor lineage and thus can be applied to a variety of tumors. Such therapies delay progression of highly and of poorly vascularized tumors²⁹ and the reports from the preclinical studies hold promise.³⁰ However, some caveats already emerged, for example, persistent hypoxia due to angiogenesis blockade causes selection of the tumor cells with higher resistance to the stress-induced apoptosis.² These findings imply the importance of maximizing the efficacy of inhibitors in order to limit the time of exposure to acute hypoxia. Here, we developed a rational approach to maximize the efficacy of angiogenesis inhibitor, TSP1 based on its molecular mechanism of action.

The first steps to make TSP1 suitable for clinical use have already been taken. Although nascent protein strongly inhibits angiogenesis,³¹ its ability to bind multiple receptors is disconcerting, since some of these receptors, including

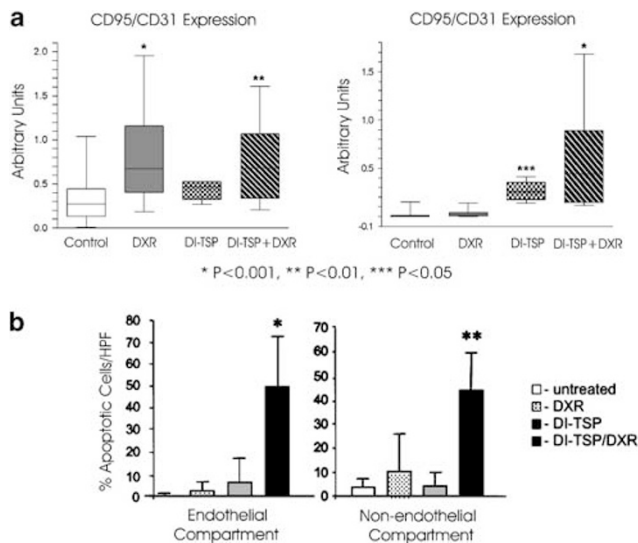


Figure 5 Combination of low DXR doses with DI-TSP treatment augments CD95 and CD95L expression in tumor endothelial cells and increase apoptosis in the tumors. (a) CD31-positive structures (blood vessels) within tumors subjected to the indicated treatments were evaluated for the expression of CD95 (left) and CD95L (right). Note the significant upregulation of CD95 by DXR and of CD95L by DI-TSP. (b) Apoptosis in the endothelial (left panel) and in the tumor cell compartments (right) are shown separately. * $P \leq 0.002$, ** $P \leq 0.003$

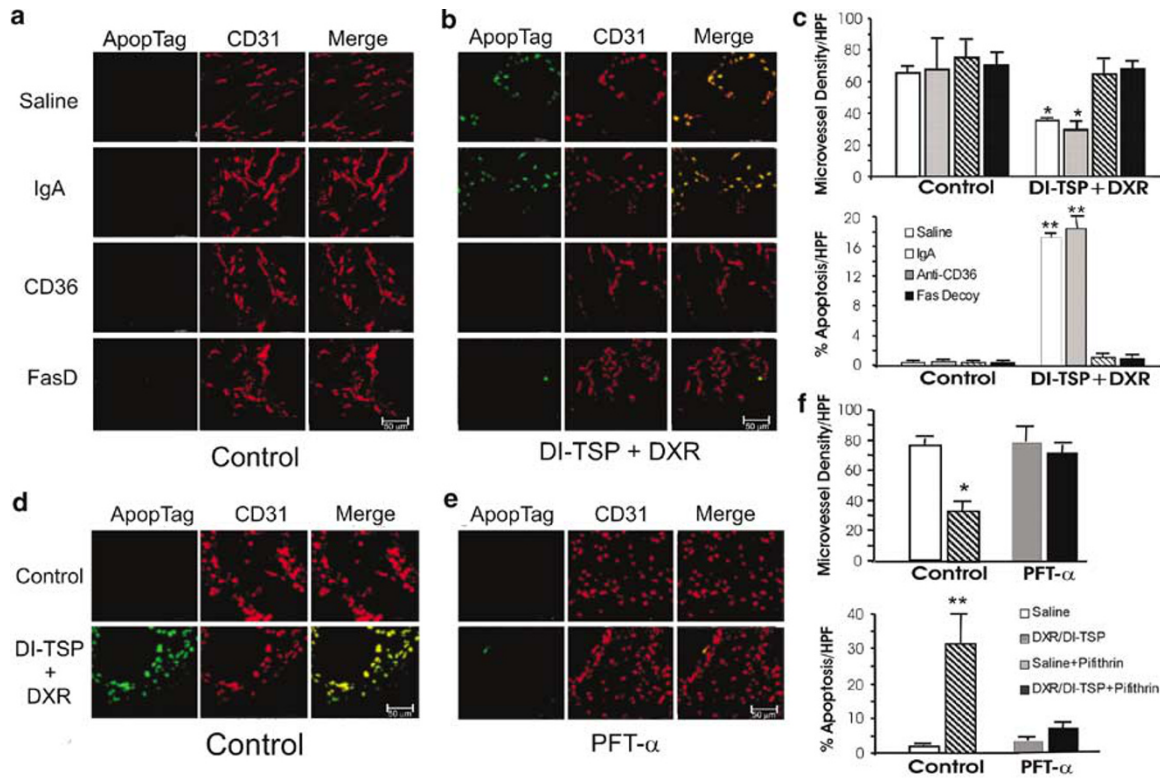


Figure 6 The role of CD35, CD95 and p53 in the anti-angiogenesis and apoptosis by DI-TSP/DXR combination. (a–c) Mice given subcutaneous Matrigel plugs containing VEGF were treated with vehicle PBS (a) or DI-TSP/DXR combination (b). CD36-neutralizing antibodies, anti-mouse IgA isotype control antibody and CD95-FC decoy receptor were added where indicated. Note that high apoptosis levels in treated plugs *versus* control were decreased visibly by either CD36 antibody or by CD95 decoy, but not by isotype control antibody. (c) Quantitative analysis of the experiment shown in (a, b). □ PBS, ▨ anti-CD36, ▩ CD95 decoy. * $P \leq 0.004$, ** $P \leq 0.002$ (d–f) Mice with subcutaneous Matrigel plugs were treated with control PBS (d and e, upper panel) or DI-TSP/DXR combination (d and e, lower panels), alone (d) or with PFT- α (e), an inhibitor of p53-dependent transcription. (f) Quantification of the experiment in (d, e). □ PBS, ▨ DI-TSP/DXR, ▩ PFT- α , ▩ DI-TSP/DXR + PFT- α . * $P \leq 0.001$, ** $P \leq 0.0003$

integrin-associated protein, mediate functions incompatible with antiangiogenic therapy.³² Minimal antiangiogenic fragments of TSP1 mapping to the type I repeats and preprocollagen homology domain share GVTRIR consensus sequence^{8,33} and block angiogenesis *in vitro* and *in vivo* by inducing endothelial cell apoptosis that occurs upon binding to CD36, a cell surface receptor expressed predominantly on the microvascular endothelium.^{5,6,10} DI-TSP, a modified GVTRIR-based peptide, blocks endothelial cell functions, angiogenesis and tumor growth with relatively high specific activity.³⁴ In this study,³⁴ the dose of DI-TSP that inhibited tumor growth by 70% and diminished MVD by 50% was 30 mg/kg twice a day, 60 times higher than the dose used in this report. A total of 1 mg/kg/twice daily (2 mg/kg) caused a modest 30% decrease in tumor volume, similar to what we observe in a subcutaneous model (Figure 4). These peptides are now in Phase 1 and 2 clinical trials with the evidence of favorable outcomes mounting (see June 2003 press release from Abbott Labs). Another option is modulation of the primary receptor or TSP1, CD36 that can be achieved by treatment with the ligands of the nuclear receptor PPAR- γ .³⁵

The ability of TSP1/DI-TSP to selectively eliminate remodeling endothelium is based on the secondary receptor-mediated signaling cascade where TSP-dependent expression of CD95L triggers apoptosis of the CD95/Fas-positive

endothelial cells.¹¹ CD95 expression is a function of remodeling induced by angiogenic stimuli (VEGF, bFGF, etc.), it is independent of TSP1, and limits its efficacy.^{11,36}

Seeking factors to improve antiangiogenic effect of DI-TSP, we examined DXR, a common chemotherapy agent that increases CD95/Fas presentation by the activated endothelium. We recently showed that DXR at 500 ng/ml induces apoptosis in proliferating endothelial cells, and induces p53-dependent increase in the mRNA and cell surface expression levels of CD95. Although this increase in the expression of CD95 was not involved in the signaling cascade that led to caspase activation and endothelial cell apoptosis by DXR, CD95 itself was functionally active and capable of inducing cell death upon stimulation with agonistic CD95 antibodies, showing that the required molecular machinery remained functional.¹⁸ Here, we showed that DXR concentrations as low as 50 ng/ml still increased CD95, but caused no significant apoptosis in EC nor in cancer cells. These noncytotoxic concentrations augmented *in vitro* EC sensitivity to apoptosis by DI-TSP via CD95 cascade. Modified isobologram analysis²⁷ yielded interaction coefficient of > 50, clearly pointing to synergy.

When DISC formation is followed by caspase activation, CD95/Fas is internalized to be recycled via clathrin-coated pits and caveolae (reviewed in Peter and Krammer³⁷), similar

events are involved in the CD95 trafficking and surface upregulation by the endothelial cells in response to angiogenic stimuli.¹¹ Thus, the intracellular pool of CD95/Fas protein clearly limits its availability for the surface exposure by inducer-stimulated endothelium. Conversely, DXR treatment triggers a sustained, p53-dependent upregulation of CD95 mRNA and consequently increases the total CD95 available for the surface presentation and ligand interaction.

On the other hand, strong and sustained p53 expression in the DXR-treated endothelial cells may render these cells more susceptible to apoptosis by TSP1/DI-TSP.^{38,39} We found that DXR-induced p53 increased APAF-1, a component of the mitochondrial apoptotic pathway⁴⁰ and PUMA expression levels (see supplementary Figure 2),⁴¹ which, in turn could sensitize endothelial cells to the death by CD95 ligation. Indeed, dramatic reduction of antiangiogenesis by the DXR/DI-TSP combination by p53 inhibitor, PFT- α pointed to a major contribution of the p53 to their synergy.

On the other hand, hypoxic conditions due to the antiangiogenic arm of the treatment altered the tumor cells responsiveness to the low doses of DXR. Hypoxia severely diminished the increase of p21 in response to DXR while significantly increasing the proportion of cells arrested in G2/M phase and somewhat increasing apoptosis (see supplementary Figure 2). Although p21 predominantly serves as an inhibitor of cell proliferation, it also plays an important role in drug-induced tumor suppression. Nevertheless, a number of recent studies have shown that p21 (WAF1/Cip1) can assume both pro- or antiapoptotic functions in response to antitumor agents depending on cell type and cellular context.⁴²

Continuous application of the low doses of chemotherapy agents (metronomic dosing) was proposed as a replacement for the discontinuous use of the same agents at MTD in a 'front-loading' manner. Such treatments are thought to minimize the long-term tumor burden by acting as antiangiogenic agents as well as by reducing the selection for treatment-resistant population of the tumor cells.⁴³ That is one possible explanation of how higher DXR doses, although upregulating CD95 expression on the endothelial cells, trigger different tumor progression pattern, in which tumor development, albeit initially slower, later gained momentum. On the other hand, rapid regrowth of the tumor in the periods between bolus doses of chemotherapy causes higher levels of secreted growth factors and thus more vigorous angiogenesis.⁴³ In addition, angiogenesis inhibitors, such as TNP470 minocyclin and DC101, have been shown to improve the efficacy of chemotherapy agents, like cisplatin, cytophosphamide and paclitaxel by improving small molecule delivery to the tumor mass due to reduced interstitial pressure.^{44,45}

Here, we investigate the combination of DI-TSP, an antiangiogenic agent, and of metronomic dosage of a chemotherapy agent DXR that have cooperative effect in reducing tumor angiogenesis. DXR greatly increased the ability of DI-TSP to block angiogenesis and tumor growth *in vivo*. The dose of DXR used in our treatment regimens was 25–100 lower than MTD commonly used in mouse tumor models.^{22,23,46} Interaction index was far greater than 1, clearly indicating synergy, which was critically dependent on DI-TSP signaling intermediates CD36 and CD95L. Combined DI-TSP/DXR inhibition of angiogenesis was relieved in the presence of

anti-CD36-blocking antibodies. Moreover, this synergy was based on CD95-dependent signaling events because the combined antiangiogenic effect and enhanced endothelial cell apoptosis *in vivo* were eliminated by soluble CD95 decoy. Apparently low doses of DXR rendered tumor cells more sensitive to the stress-induced apoptosis due to the vascular reduction by DI-TSP by upregulating proapoptotic genes and increasing G2/M arrest. Interestingly, higher CD95/CD95L expression by remodeling capillaries in the presence of DI-TSP and DXR associated with apoptosis and antiangiogenesis invariably correlated with a dramatic decrease in CD31 levels. CD31 ligation has been implicated in survival signals such as Akt-dependent increase of Bcl-2 A1 and Bcl-X.^{47,48} It is also possible that CD31 was decreased due to dissociation of the cells within affected vessels: strong lateral expression of CD31 is typical for the endothelial cells forming contacts and is thought to play a role in vascular permeability.⁴⁹ In this case, decrease in CD31 is likely treatment-related and further facilitates apoptosis by DI-TSP/DXR combination.

One obvious advantage provided by the metronomic dosing of chemotherapy agents is their decreased toxic effect towards noncancerous cells.⁵⁰ Indeed apoptosis levels induced by metronomic DXR treatment in experimental animals were considerably lower compared to MTD treatment and had no significant effect on background apoptosis levels in the heart and liver.

The increase in CD95/Fas expression by the resting capillaries and large vessels was an important concern as such upregulation could compromise the integrity of pre-existing CD36-positive vasculature. We detected slight upregulation of CD95 by DXR on the resting vessels (unpublished observation). However, no vascular complications or increased endothelial cell apoptosis were observed in these vessels in the course of 35-day treatment, consistent with the observation that quiescent endothelium is more resistant to CD95-mediated apoptosis,^{32,51–54} possibly due to the higher levels of antiapoptotic protein FLIP,⁵⁵ increased stability of Ang1 provided by accessory cells⁵⁶ and other survival signals generated by cell–cell contacts and paracrine factors secreted by surrounding stroma.

Although the effect of DXR/DI-TSP combination *in vitro* was specific for the endothelial cells, combined treatment caused apoptosis in both endothelial and nonendothelial compartments of the tumor. Such dual *in vivo* effect is typical for the antiangiogenic therapies and is largely explained by hypoxia and hypoglycemia caused by diminished vascularization.⁵⁷ Recently, a model was proposed where hypoxia-insensitive p53-negative cells are selected during continuous antiangiogenic treatment.⁵⁸ Such p53-negative cells have diminished ability to enter apoptotic program or to undergo cell cycle arrest in response to HIF-1 α activation.^{59–61} Interestingly, when MTD of DXR were used in conjunction with DI-TSP, the resulting tumors were considerably larger than control-treated ones despite similar decrease in MVD, with the majority of the cells staining negative for p53 (Nelius and Volpert, unpublished observations). Such rapid emergence of the p53-negative, hypoxia-resistant cells could be attributed to the extensive DNA damage/repair due to the high genotoxic dose of DXR. Therefore, minimal effective dose of DXR should be considered for the future treatment regimens.

Recent studies provide another link between TSP1 and chemotherapeutic agents: low-dose metronomic therapy was found to increase TSP1 production and angiogenesis,⁶² while metronomic chemotherapy with cytoxin was shown to decrease circulating endothelial cell progenitors⁶³ possibly due to increased TSP1 production.⁶⁴ Here, we demonstrate high antiangiogenic potential of the combination therapy that includes TSP1 antiangiogenic peptide with high specific activity and a metronomic dose of another chemotherapy agent DXR. We show that these agents act synergistically to induce endothelial cell apoptosis and to block angiogenesis and elucidate a mechanism that underlies this synergy. Furthermore, we demonstrate convincingly that the toxic effect of this combination therapy *in vivo* is extremely low, thus making it an attractive option for a long-term preventative treatment aimed to minimize long-term tumor burden in cancer patients due to residual tumors after primary tumor resection or to the dormant micrometastases.

Experimental Procedures

Cell culture and reagents

HUVECs from umbilical cords isolated as described⁶⁵ or purchased from Clonetics (Cambrex BioSciences, Rockland, MD) were maintained as in Lorenzo *et al.*¹⁸ HMVECs isolated from neonatal foreskins^{66–68} or purchased from Clonetics were maintained in MCDB131 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10 mM HEPES, 15% FCS, 10 ng/ml EGF (Promega, Madison, WI, USA), 2.5 μ g/ml Fungizone, penicillin, streptomycin, 1 μ g/ml hydrocortisone and 2 mM L-glutamine. Bladder cancer cell line 253J, B-V was maintained in DMEM, 10% FCS, 2 mM L-glutamine, penicillin and streptomycin. Prostate cancer cells LNCaP were maintained in RPMI medium with 10% FCS. DXR was from Ferrer Farma (Barcelona, Spain). Recombinant human VEGF was from PeproTech (London, UK). DI-TSP generated as described in Reihel *et al.*³⁴ was generously provided by Abbott Laboratories.

Flow cytometry analysis

Endothelial cells were plated in 60 mm gelatinized tissue culture dishes (1×10^5 /plate), treated with indicated DI-TSP/DXR combinations and harvested with trypsin/EDTA. The cells were washed with cold phosphate-buffered saline (PBS), incubated 30 min on ice with anti-CD95 mouse monoclonal antibody DX-2 (1 μ g/ml, PharMingen, San Diego, CA, USA) and 30 min with FITC-conjugated rabbit anti-mouse IgG (DAKO, 1:50 dilution). Flow cytometry was performed with FACScan (Becton Dickinson) or Cyan Flow (Dako) flow cytometers and the results analyzed using CellQuest or Summit software. The baseline was determined using cells incubated with the secondary antibody alone.

Animal studies

All animal studies were handled according to the animal care and use federal guidelines approved by the National Institute of Health.

Matrigel plug assay

Matrigel plug assay was performed as described.⁶⁹ Cold growth factor depleted Matrigel (BD Biosciences) supplemented with 64U/ml Heparin (Sigma, MO, USA) and 250 ng/ml VEGF was injected into the median

abdominal area of anesthetized C57BL6 mice (400 μ l/mouse). To block CD95/CD95L interactions, Matrigel was mixed with CD95 decoy (CD95D) (Sigma, 1 μ g/ml). To block CD36, anti-CD36 IgA antibody (5 μ g/ml, Cascade Bioscience) or IgA isotype control antibody (5 μ g/ml, Southern Biotech) were added. All antibodies were dialyzed to remove NaN_3 . The plugs solidified at body temperature. DI-TSP was administered daily as intraperitoneal (i.p.) injections and DXR was given i.p. every 5 days. On days 8–14, the plugs were excised, snap-frozen at -80°C in Tissue-Tek OCT compound (Sakura, Torrance, CA, USA), and cut into 5 μ m sections.

Heterotopic implantation of tumor cells

At 70–80% confluence, bladder cancer cells, 253J B-V cells (cell line derived from aggressive carcinoma of the bladder), were transferred for 24 h in fresh medium, harvested by brief trypsinization and 1×10^6 cells were injected subcutaneously into the hindquarters of athymic mice (*nu/nu*), five animals/group, two sites/animal. LNCaP prostate cancer cells were injected into the right flanks (*nu/nu* mice, 9–10 animals/group) in 200 μ l Matrigel (BD Biosciences), at 2×10^6 cells/site. DI-TSP and DXR treatments were carried out as above. Tumors were measured with a microcaliper every 2 days. At the completion of experiment, the animals were killed, tumors removed, weighted, measured, snap-frozen in OCT compound and processed for analysis.

Immunofluorescence staining

Cryosections (5 μ m) of snap-frozen Matrigel plugs or tumor xenografts were fixed in cold acetone, 1:1 acetone/chloroform and cold acetone (5 min/treatment), rinsed in PBS and incubated with Avidin/Biotin Blocking Kit (Vector Laboratories) to block endogenous biotin/avidin. Slides were washed in PBS, blocked 30 min in 2% donkey serum, 2% goat serum in PBS and incubated 1 h with rabbit anti-CD95L (2.5 μ g/ml, Calbiochem, San Diego, CA, USA) and 4 μ g/ml rat anti-mouse CD31 (Santa Cruz, CA, USA) in blocking solution. The slides were washed in PBS and incubated 30 min with biotinylated anti-rabbit antibodies (1:200 dilution, Vector Laboratories) and donkey anti-rat RhodamineX-conjugated antibodies (1:200 dilution, Jackson ImmunoResearch, West Grove, PA, USA) in blocking solution. Slides were developed for 15 min with FITC-conjugated Fluorescein Avidin D (20 μ g/ml, Vector Laboratories). To visualize CD95/Fas, the sections were reincubated with Biotin/Avidin Kit, washed and blocked with 4% goat serum in PBS for 30 min followed by 1 h incubation with anti-CD95 antibody (2.5 μ g/ml, C-20, Santa Cruz Biotech, Santa Cruz, CA, USA) in blocking solution. Biotinylated anti-rabbit antibodies were applied in blocking solution at 1:200 dilution for 30 min, the slides washed, exposed for 15 min to 1 μ g/ml Streptavidin Cy5 (Jackson ImmunoResearch), dried and mounted using VectaShield Mounting Media (Vector Laboratories).

Microvessel counts

The tumors fixed as above were blocked in 3% donkey serum, 1% BSA in PBS. The slides were incubated 1 h (room temperature) with rat anti-mouse CD31 antibody (PharMingen, San Diego, CA, USA), washed and reblocked. RhodaminX-labeled anti-rat antibodies were added in blocking solution for 1 h, slides washed, and mounted in VectaShield Mounting Medium. Quantitative digital image analysis was performed using MetaView software. We defined MVD as the average area corresponding to the CD31-positive structures counted in at least 10 high-powered ($\times 100$) field per experimental condition. Statistical significance was evaluated with paired Student's *t*-test.

Quantification of endothelial CD95 and CD95L expression

To evaluate CD95 expression in blood vessels, the area of CD31-positive structures was measured using LSM 510 (Zeiss) and Laserpix (BioRad) software. The areas of CD95 and CD95L-positive structures, which colocalized with CD31 staining, were measured and CD95/CD31 and CD95L/CD31 ratios calculated.

Combined staining for CD95L, CD31 and apoptosis

Staining for CD95L/CD31 was performed as above, the slides were washed and cells positive for DNA fragmentation detected by TUNEL (ApopTag™ kit, Intergen, Purchase, NY, USA) (this assay allows to discriminate between apoptosis and necrosis). The slides were mounted and fluorescent images analyzed by confocal microscopy. Quantification was performed where indicated, using MetaView software.

For the *in situ* staining for CD31/apoptosis, HMVECs were plated on gelatinized coverslips, grown to 60% confluence and treated 24 h with DXR/DI-TSP combinations. The slides were fixed 10 min in cold acetone, washed with PBS and stained consecutively for CD31 and with ApopTag™ as above.

Statistical evaluation of the data was carried out using paired Student's *t*-test and a two-way ANOVA.

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Supplementary Information accompanies the paper on the Cell Death and Differentiation website (<http://www.nature.com/cdd>)