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Advances in Brief

Taxane-Mediated Antiangiogenesis *in Vitro*: Influence of Formulation Vehicles and Binding Proteins

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Abstract

Paclitaxel (Taxol) and docetaxel (Taxotere) have been shown to inhibit angiogenesis at low concentrations that do not affect cancer cell proliferation. Here, we used rat aortic rings and human umbilical vein endothelial cells to evaluate the influence of their formulation vehicles Cremophor EL and polysorbate 80, as well as serum binding proteins on taxane-mediated antiangiogenesis. The data show that clinically relevant concentrations of the vehicles and binding proteins nullify the antiangiogenic activity of both taxanes. It is suggested that these agents may need to be used at much higher doses than anticipated for effective antiangiogenic chemotherapy.

Introduction

Inhibition of angiogenesis is being considered a promising treatment strategy for cancer. Amid the myriad of newly discovered angiogenesis inhibitors, Polverini and Novak (1) first reported the antiangiogenic property of the chemotherapeutic drugs mitoxantrone and bisantrene over a decade ago. Most cytotoxic anticancer agents have since been demonstrated to suppress angiogenesis (2). Recently, low-dose and frequent administration of some of these drugs (*e.g.*, cyclophosphamide, vinblastine, campthothecin, topotecan), known as antiangiogenic or metronomic chemotherapy, was shown to be effective against solid tumor growth in preclinical studies (3, 4). Paclitaxel (Taxol) and docetaxel (Taxotere) are microtubule stabilizing drugs with clinical activity in the treatment of breast, ovarian, endometrial, prostate, and lung cancer (5). In addition to their cytotoxicity against tumor cells, recent reports showed that taxanes also exhibit antiangiogenic properties when used at very low doses by inhibiting endothelial cell proliferation, migration, and tube formation (6–9). Cremophor EL (polyoxyethylene-glycerol triricinoleate 35) and polysorbate 80 (Tween 80; polyoxyethylene-sorbitan-20-monooleate) are nonionic surfactants used as formulation vehicles for many antineoplastic drugs including paclitaxel and docetaxel, respectively. It is well documented that these vehicles have intrinsic biological and pharmacological activities. For instance, Cremophor EL has been shown to alter bone marrow cellularity and to increase hematopoietic progenitor cells (10). Drori *et al.* (11) demonstrated that Tween 80 alters membrane fluidity and increases membrane permeability. Tumor growth inhibition by Tween 80 was reported in mice (12, 13). Furthermore, the ability of both vehicles to form micelles leads to drug entrapment, significantly altering the disposition of the formulated drugs (14, 15). The current study examined the influence of Cremophor EL and Tween 80, as well as binding proteins, on angiogenesis inhibition by paclitaxel and docetaxel.

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Materials and Methods

Rat Aortic Ring Assay. Twelve-well tissue culture plates were coated with 250 μl of Matrigel (Becton-Dickinson, Bedford, MA) and allowed to gel for 30 min at 37°C and 5% CO_2. Thoracic aortas were excised from 8- to 10-week-old male Sprague Dawley rats. After removal of fibroadipose tissues, the aortas were cut into 1-mm-long cross-sections, placed on Matrigel-coated wells and covered with an additional 250 μl of Matrigel. After the second layer of Matrigel had set, the rings were covered with EGM-II and incubated overnight at 37°C and 5% CO_2. EGM-II consists of endothelial cell basal medium (EBM-II; Cambrex, Walkersville, MD) plus endothelial cell growth factors provided as the EGM-II Bulletkit (Cambrex). The culture medium was subsequently changed to EBM-II supplemented with 2% fetal bovine serum, 0.25 μg/ml amphotericin B, and 10 μg/ml gentamicin. To test whether formulation vehicles alone would influence angiogenesis, aortic rings were treated with EBM-II containing 0.01–0.5% (0.1–5 μl/ml) of Cremophor EL (Sigma, St. Louis, MO) or Tween 80 (Sigma) for 4 days and photographed on the fifth day using a ×2.5 objective. In a second series of experiments, aortic rings were treated with EBM-II or human serum containing 0.5% DMSO, 0.05% Cremophor EL, 0.05% Tween 80, paclitaxel (0.5–4 nM) solubilized in DMSO or Cremophor EL, or docetaxel (0.01–1 nM) solubilized in DMSO or Tween 80 for 4 days and photographed on the fifth day as described above. Human serum used in all of the experiments was prepared by mixing fresh donor and commercial (Gemini Bioproducts, Woodland, CA) serum to yield reasonably good microvessel outgrowth (16). The concentrations of paclitaxel and docetaxel were selected with reference to previous publications (7, 9). Carboxyamidotriazole, a known antiangiogenic agent, was used at higher than clinically achievable concentration (12 μg/ml in EBM-II and 60 μg/ml in serum) as positive control. Experiments were repeated four times using aortas from four different rats. The area of angiogenic sprouting, reported in square pixels, was quantified using Adobe Photoshop.

Endothelial Cell Proliferation Assay. Human umbilical vein endothelial cells (HUVECs; Cambrex) were maintained in EGM-II at 37°C and 5% CO_2. HUVECs were seeded onto 12-well plates at a density of 30,000 cells/well and were allowed to attach overnight. The culture medium was then aspirated, and fresh culture medium containing 0.5% DMSO, 0.05% Tween 80, 0.05% Cremophor EL, paclitaxel (0.5–4 nM) dissolved in DMSO or Cremophor EL, or docetaxel (0.01–1 nM) dissolved in DMSO or Tween 80 was added to each well. After 48 h, cells were trypsinized and counted with a Coulter Z1 counter (Coulter Corp., Hialeah, FL). All experiments were repeated three times.

Statistics. All of the results were presented as mean ± SE. Comparisons were made with Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's test with $P < 0.05$ as the criterion for statistical significance.

Results

Effects of Formulation Vehicles on Rat Aortic Angiogenesis. Cremophor EL significantly inhibited angiogenesis by greater than 80% at concentrations ≥1 μl/ml (0.1%; Fig. 1, *C–F*) relative to the EBM-II control (Fig. 1*A*). Significant inhibition of microvessel outgrowth by Tween 80 was not observed until the concentration reached 5 μl/ml (0.5%; Fig. 1, *G–J*).

Interference of Taxane-Mediated Antiangiogenesis by Formulation Vehicles. As shown in Fig. 2*A*, paclitaxel in DMSO inhibited microvessel outgrowth in a concentration-dependent manner relative to the DMSO vehicle control, reaching statistical significance at 4 nM

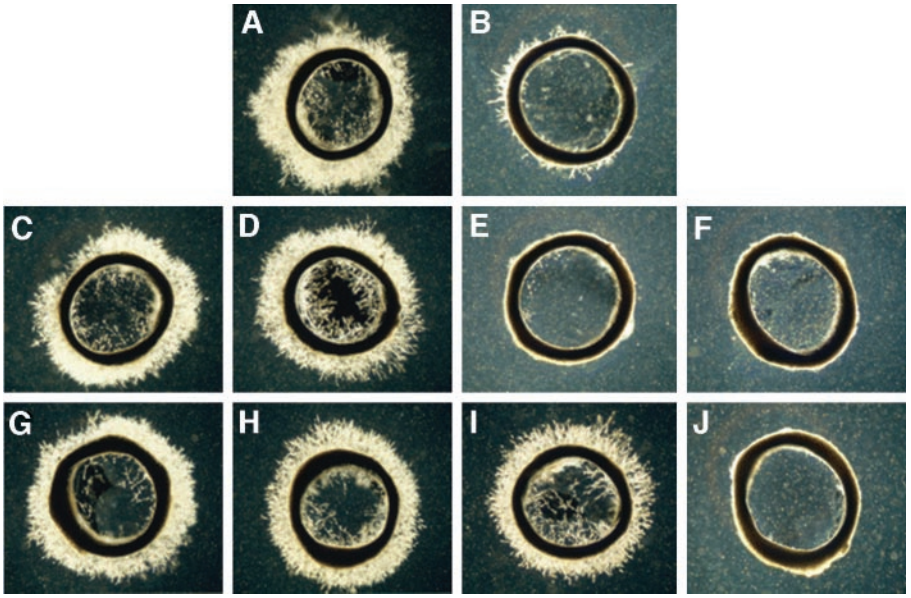

Fig. 1. Representative images of rat aortic rings treated with EBM-II alone (*A*); carboxyamidotriazole at 12 μg/ml as positive control (*B*); Cremophor EL at 0.01% (*C*), 0.05% (*D*), 0.1% (*E*), or 0.5% (*F*); or Tween 80 at 0.01% (*G*), 0.05% (*H*), 0.1% (*I*), or 0.5% (*J*).

(~55% inhibition). However, the antiangiogenic effect of similar concentrations of paclitaxel disappeared when the drug was solubilized in Cremophor EL (Fig. 2*A*). Docetaxel in DMSO significantly blocked angiogenesis compared with the DMSO control at much lower concentrations, with ~80% inhibition observed at only 1 nM (Fig. 2*B*). Similar concentrations of docetaxel in Tween 80 failed to inhibit microvessel outgrowth relative to the Tween 80 vehicle control. DMSO (0.5%), Cremophor EL (0.05%), and Tween 80 (0.05%) alone, used as vehicle controls, had no effect on angiogenesis compared with EBM-II (Fig. 2, *A* and *B*).

HUVEC Proliferation in Response to Taxanes in the Absence and Presence of Formulation Vehicles. HUVEC proliferation was significantly inhibited by paclitaxel in DMSO, with ~36% reduction at 4 nM compared with the DMSO control (Fig. 3*A*). Docetaxel in DMSO significantly decreased HUVEC proliferation in a concentration-dependent fashion, with ~55% inhibition at only 1 nM (Fig. 3*B*). However, the antiproliferative activity of paclitaxel and docetaxel was completely lost in the presence of Cremophor EL and Tween 80, respectively, relative to the corresponding vehicle control (Fig. 3, *A* and *B*). DMSO (0.5%), Cremophor EL (0.05%), and Tween 80 (0.05%) alone, used as vehicle controls, did not affect HUVEC proliferation compared with culture medium alone (data not shown).

Effects of Binding Proteins on Taxane-Mediated Antiangiogenesis. In human serum, the antiangiogenic activity of paclitaxel (0.5−4 nM) in DMSO previously observed in EBM-II was no longer evident (Fig. 4*A*). On the contrary, docetaxel (0.01−1 nM) in DMSO retained its antiangiogenic activity in human serum with 56% inhibition at 1 nM (Fig. 4*B*), similar to that in EBM-II. Both paclitaxel and docetaxel failed to inhibit microvessel outgrowth when solubilized in Cremophor EL and Tween 80, respectively, compared with their respective vehicle controls (Fig. 4, *A* and *B*). DMSO (0.5%), Cremophor EL (0.05%), and Tween 80 (0.05%) alone, used as vehicle controls, had no effect on angiogenesis compared with serum (Fig. 4, *A* and *B*).

Discussion

Our results demonstrate that the antiangiogenic property of taxanes can be significantly impaired by their formulation vehicles Cremophor EL and Tween 80, as well as serum binding proteins. Cremophor EL and Tween 80 are biologically active compounds the intrinsic activity of which has been shown to alter the disposition of solubilized

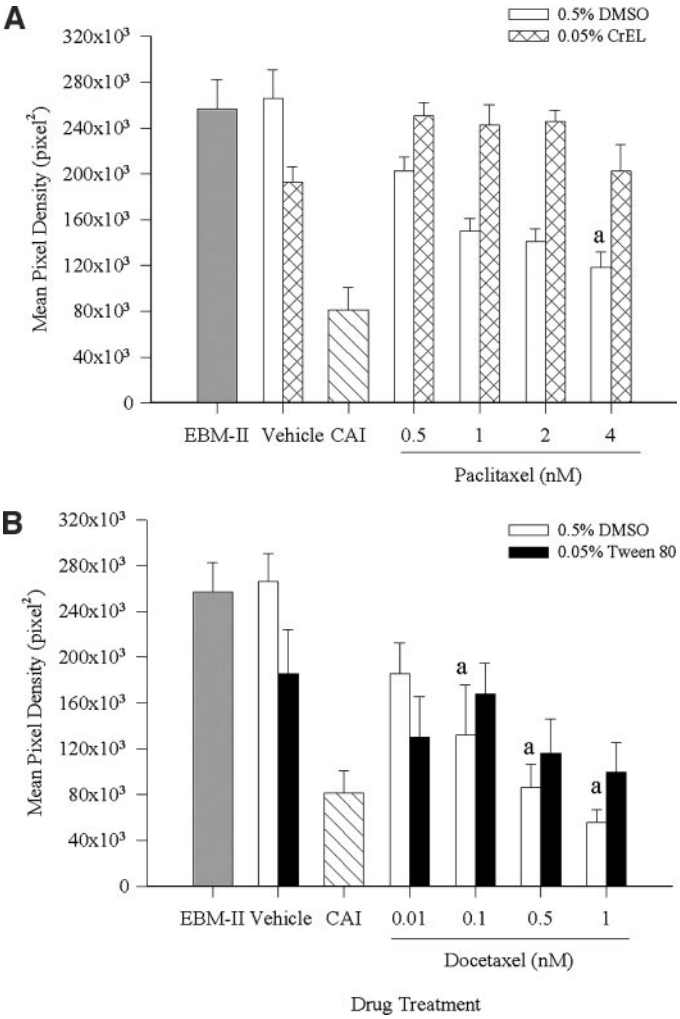

Fig. 2. Bar graphs of the total area of microvessel outgrowth measured as square pixel in response to culture medium EBM-II alone, the vehicle controls, carboxyamidotriazole (*CAI*; 12 μg/ml), or increasing concentrations of paclitaxel (*A*) or docetaxel (*B*) in 0.5% DMSO (*white bar*) or 0.05% Cremophor EL (*CrEL*; *cross-hatched bar*) or 0.05% Tween 80 (*black bar*). All of the experiments were done in EBM-II. *Bars*, mean ± SE. *a*, significantly ($P < 0.05$) different from the respective vehicle control.

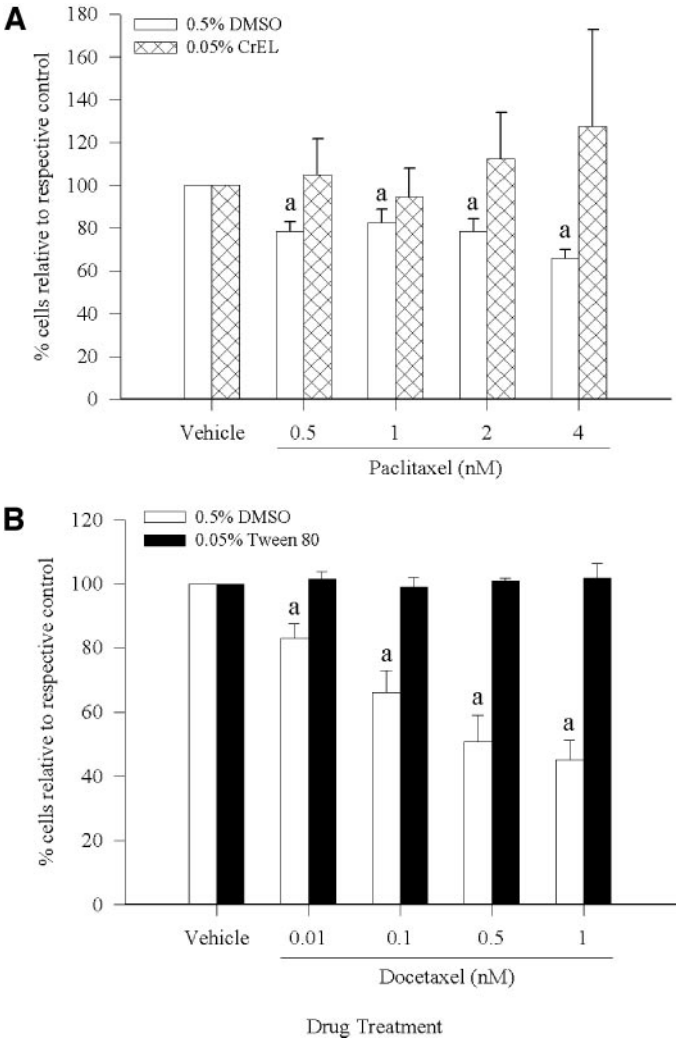

Fig. 3. Bar graphs of the percentage of human umbilical vein endothelial cells (HUVECs) relative to the respective vehicle control *versus* treatment with increasing concentrations of paclitaxel (*A*) or docetaxel (*B*) in 0.5% DMSO (*white bar*) or 0.05% Cremophor EL (*CrEL; cross-hatched bar*), or 0.05% Tween 80 (*black bar*) for 48 h. All of the experiments were done in EBM-II. *Bars*, mean ± SE. *a*, significantly ($P < 0.05$) different from the respective vehicle control.

drugs (14). We showed in the present study that Cremophor EL and Tween 80 cause significant inhibition of angiogenesis at ≥1 μl/ml and ≥5 μl/ml, respectively, both of which are clinically achievable concentrations. Loos *et al.* (15) previously indicated that the expected clinically relevant range for Tween 80 was 0.01–1 μl/ml. Similarly, clinically relevant concentrations of Cremophor EL in patients receiving a 3-h i.v. infusion of paclitaxel at doses ranging from 100 to 225 mg/m^2 were shown to be 3.40−6.58 μl/ml (17). These reported concentrations of Cremophor EL and Tween 80 were based on the use of taxanes in cytotoxic chemotherapy. Antiangiogenic chemotherapy requires that antineoplastic agents like paclitaxel and docetaxel to be administered at much lower doses; the corresponding plasma concentrations of Cremophor EL and Tween 80 would likely be much lower and incapable of exerting any antiangiogenic effects by themselves. Furthermore, tumor delivery of Cremophor EL and Tween 80 was believed to be insignificant because of its extremely low volume of distribution (17).

In the present study, paclitaxel (0.5−4 nM) and docetaxel (0.01−1 nM) in DMSO were observed to inhibit rat aortic angiogenesis and HUVEC proliferation, with the latter being more potent than the former, in accordance with previous findings (7, 9). The presence of clinically relevant concentration (0.05% or 0.5 μl/ml) of Cremophor EL and Tween 80 was shown to interfere with the antiangiogenic effects of paclitaxel and docetaxel, respectively. This can be attributed to the ability of these formulation vehicles to form micelles with a highly hydrophobic interior, leading to drug entrapment and, therefore, reduced cellular uptake (14, 18).

Preclinical angiogenesis assays often use either serum-free or 2−10% fetal bovine serum culture conditions, which may underestimate protein binding effects. Indeed, it was previously demonstrated that the extent of angiogenesis inhibition by the polysulfonated naphthylurea suramin at 50 μg/ml in human serum was markedly diminished compared with that in culture medium EBM-II (16). Another antiangiogenic agent carboxyamidotriazole at 12 μg/ml was shown to inhibit microvessel outgrowth almost completely in EBM-II (19) but only by ≤30% in human serum (16). The impairment of taxane-mediated inhibition of angiogenesis observed in the present study further indicates the significance of protein–drug interactions. However, it is unclear why docetaxel but not paclitaxel in DMSO retained its antiangiogenic activity. One would, in fact, expect the opposite because the *in vitro* binding of docetaxel to serum proteins (~93% bound) was reported to be higher than that of paclitaxel (~87% bound) in the absence of their respective formulation vehicles (15,

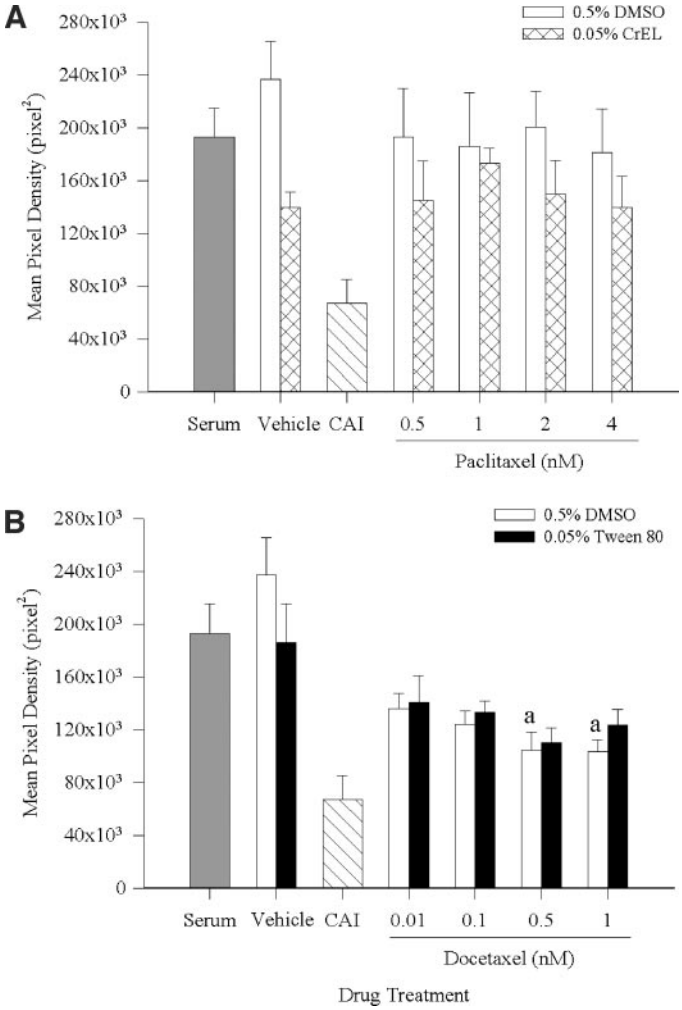

Fig. 4. Bar graphs of the total area of microvessel outgrowth measured as square pixels in response to human serum alone, the vehicle controls, carboxyamidotriazole (*CAI*; 60 μg/ml), or increasing concentrations of paclitaxel (*A*) or docetaxel (*B*) in 0.5% DMSO (*white bar*) or 0.05% Cremophor EL (*CrEL; cross-hatched bar*) or 0.05% Tween 80 (*black bar*). All of the experiments were done in human serum. *Bars*, mean ± SE. *a*, significantly ($P < 0.05$) different from the respective vehicle control.

20). Not surprisingly, we also found that both taxanes solubilized in formulation vehicles failed to inhibit microvessel outgrowth in human serum. Previous findings demonstrated that the fractions of unbound paclitaxel and docetaxel in the presence of (0.5 μl/ml) formulation vehicles and serum proteins decreased by >10% and increased by ~8%, respectively (15, 20). It appears that such reported increase in unbound docetaxel was insufficient to restore angiogenesis inhibition seen in docetaxel in DMSO.

In conclusion, the data show that clinically relevant concentrations of formulation vehicles and binding proteins that are present in human serum significantly reduce the antiangiogenic activity of paclitaxel and docetaxel. This suggests that these agents and possibly several other anticancer drugs that are either formulated in Cremophor EL or Tween 80 or coadministered with taxanes in combination regimens may need to be used at much higher doses than anticipated to achieve effective antiangiogenic chemotherapy. In this case, the advantage of the lack of undesirable side effects associated with low-dose taxane regimens compared with conventional maximum-tolerated-dose chemotherapy may be compromised. Taxane formulations free of these excipients could potentially alleviate the problem. Indeed, ABI-007, a novel Cremophor EL-free, protein-stabilized, nanoparticle formulation of paclitaxel, has been recently reported to exhibit improved antitumor efficacy compared with the Cremophor EL-containing formulation in preclinical and clinical studies (21, 22). It is proposed that consideration of potential antagonistic antitumor effects of drug formulation vehicles is urgently needed as an integral component of the preclinical development of any new investigational anticancer agent. Future studies will focus on the potential clinical significance of the present observations in addition to unraveling the underlying mechanistic basis.

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References

1. Polverini, P. J., and Novak, R. F. Inhibition of angiogenesis by the antineoplastic agents mitoxantrone and bisantrene. Biochem. Biophys. Res. Commun., *140:* 901–907, 1986.
2. Miller, K. D., Sweeney, C. J., and Sledge, G. W., Jr. Redefining the target: chemotherapeutics as antiangiogenics. J. Clin. Oncol., *19:* 1195–1206, 2001.
3. Browder, T., Butterfield, C. E., Kraling, B. M., Shi, B., Marshall, B., O'Reilly, M. S., and Folkman, J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res., *60:* 1878–1886, 2000.
4. Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D. J., Bohlen, P., and Kerbel, R. S. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J. Clin. Investig., *105:* R15–R24, 2000.
5. Choy, H. Taxanes in combined-modality therapy for solid tumors. Oncology (Huntingt.), *13:* 23–38, 1999.
6. Belotti, D., Vergani, V., Drudis, T., Borsotti, P., Pitelli, M. R., Viale, G., Giavazzi, R., and Taraboletti, G. The microtubule-affecting drug paclitaxel has antiangiogenic activity. Clin. Cancer Res., *2:* 1843–1849, 1996.
7. Vacca, A., Ribatti, D., Iurlaro, M., Merchionne, F., Nico, B., Ria, R., and Dammacco, F. Docetaxel versus paclitaxel for antiangiogenesis. J. Hematother. Stem Cell Res., *11:* 103–118, 2002.
8. Hotchkiss, K. A., Ashton, A. W., Mahmood, R., Russell, R. G., Sparano, J. A., and Schwartz, E. L. Inhibition of endothelial cell function in vitro and angiogenesis in vivo by docetaxel (Taxotere): association with impaired repositioning of the microtubule organizing center. Mol. Cancer Ther., *1:* 1191–1200, 2002.
9. Grant, D. S., Williams, T. L., Zahaczewsky, M., and Dicker, A. P. Comparison of antiangiogenic activities using paclitaxel (Taxol) and docetaxel (Taxotere). Int. J. Cancer, *104:* 121–129, 2003.
10. Bertoncello, I., Kriegler, A. B., Woodcock, D. M., Williams, B., Barber, L., and Nilsson, S. K. Haematopoietic radioprotection by Cremophor EL: a polyethoxylated castor oil. Int. J. Radiat. Biol., *67:* 57–64, 1995.
11. Drori, S., Eytan, G. D., and Assaraf, Y. G. Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability. Eur. J. Biochem., *228:* 1020–1029, 1995.
12. Witek, R., Krupa, S., and Kubis, A. Cytotoxic action of diethanolamine oleate on Ehrlich exudative carcinoma in mice, compared with the action of polyoxyethylene sorbitan mono-oleate (Tween 80). Arch. Immunol. Ther. Exp. (Warsz), *27:* 321–324, 1979.
13. Crispens, C. G., Jr., and Sorenson, J. R. Treatment of reticulum cell sarcoma in SJL/J mice with Tween 80. Anticancer Res., *8:* 1341–1343, 1988.
14. ten Tije, A. J., Verweij, J., Loos, W. J., and Sparreboom, A. Pharmacological effects of formulation vehicles: implications for cancer chemotherapy. Clin. Pharmacokinet., *42:* 665–685, 2003.
15. Loos, W. J., Baker, S. D., Verweij, J., Boonstra, J. G., and Sparreboom, A. Clinical pharmacokinetics of unbound docetaxel: role of polysorbate 80 and serum proteins. Clin. Pharmacol. Ther., *74:* 364–371, 2003.
16. Kruger, E. A., and Figg, W. D. Protein binding alters the activity of suramin, carboxyamidotriazole, and UCN-01 in an *ex vivo* rat aortic ring angiogenesis assay. Clin. Cancer Res., *7:* 1867–1872, 2001.
17. Sparreboom, A., Verweij, J., van der Berg, M. E., Loos, W. J., Brouwer, E., Vigano, L., Locatelli, A., de Vos, A. I., Nooter, K., and Gianni, L. Disposition of Cremophor EL in humans limits the potential for modulation of the multiple resistance phenotype *in vivo*. Clin. Cancer Res., *4:* 1937–1942, 1998.
18. Sparreboom, A., van Zuylen, L., Brouwer, E., Loos, W. J., de Bruijn, P., Gelderblom, H., Pillay, M., Nooter, K., Stoter, G., and Verweij, J. Cremophor EL-mediated alteration of paclitaxel distribution in human blood: clinical pharmacokinetic implications. Cancer Res., *59:* 1454–1457, 1999.
19. Bauer, K. S., Cude, K. J., Dixon, S. C., Kruger, E. A., and Figg, W. D. Carboxyamido-triazole inhibits angiogenesis by blocking the calcium-mediated nitric-oxide synthase-vascular endothelial growth factor pathway. J. Pharmacol. Exp. Ther., *292:* 31–37, 2000.
20. Brouwer, E., Verweij, J., De Bruijn, P., Loos, W. J., Pillay, M., Buijs, D., and Sparreboom, A. Measurement of fraction unbound paclitaxel in human plasma. Drug Metab. Dispos., *28:* 1141–1145, 2000.
21. Ibrahim, N. K., Desai, N., Legha, S., Soon-Shiong, P., Theriault, R. L., Rivera, E., Esmaeli, B., Ring, S. E., Bedikian, A., Hortobagyi, G. N., and Ellerhorst, J. A. Phase I and pharmacokinetic study of ABI-007, a Cremophor-free, protein-stabilized, nanoparticle formulation of paclitaxel. Clin. Cancer Res., *8:* 1038–1044, 2002.
22. Hawkins, M., Desai, N., and Soon-Shiong, P. Rationale, preclinical support, and clinical proof-of-concept for formulating water-insoluble therapeutics as albumin-stabilized nanoparticles: experience with paclitaxel. Proc. Am. Assoc. Cancer Res.-National Cancer Inst.-Eur. Org. Res. Treat. Cancer., 50 (A3), Boston, MA, November 17–21, 2003.