Differences in Therapeutic Indexes of Combination Metronomic Chemotherapy and an Anti-VEGFR-2 Antibody in Multidrug-resistant Human Breast Cancer Xenografts

Giannoula Klement, Ping Huang, Barbara Mayer, Shane K. Green, Shan Man, Peter Bohlen, Daniel Hicklin, and Robert S. Kerbel

Sunnybrook and Women’s College Health Sciences Centre, Molecular and Cellular Biology, Toronto, Ontario, M4N 3M5 Canada [G. K., P. H., B. M., S. K. G., S. M., R. S. K.], and ImClone Systems, Inc., New York, New York 10014 [P. B., D. H.] and Department of Medical Biophysics, University of Toronto [R. S. K.]

ABSTRACT

One of the greatest barriers to the treatment of cancer with chemotherapeutic drugs is acquisition of drug resistance. This includes multidrug resistance mediated by P-glycoprotein (Pgp) to multiple lipophilic natural compounds such as taxanes, doxorubicin (Adriamycin), and vinblastine. The considerable efforts made thus far to reverse this and other types of drug resistance have had very limited success. We report here that a variety of orthotopic human breast cancer xenografts selected for high levels of Pgp and multidrug resistance respond in a significant and durable manner to different continuous low-dose (e.g., one-tenth the maximum tolerated dose of chemotherapy) chemotherapy regimens, when used in combination with an antivascular endothelial cell growth factor (anti-VEGF) receptor-2 (flk-1)-neutralizing antibody (DC101). The Pgp substrates paclitaxel (Taxol), Adriamycin, and vinblastine were all effective using this type of combination treatment, although the chemotherapy protocols showed little or no effect as monotherapies. Similar results were also obtained using cisplatinum (a non-Pgp substrate drug) against cisplatinum-resistant tumors. Evidence of significant tumor cell death by the combination treatment was detected within 3 weeks of initiation of therapy by histopathological analysis, in the absence of shrinkage of tumor mass. There were, however, marked differences in the cumulative toxicity of long-term regimens of Adriamycin and cisplatinum, where toxicity was observed, when compared with the tubulin inhibitors, vinblastine and Taxol, where it was not. We conclude that vascular-targeting protocols involving frequent administration of very low doses of certain chemotherapeutic drugs can provide a stable and safe way to circumvent multidrug resistance in established orthotopically growing tumors, as long as these are used in combination with a second antiangiogenic drug, in this case, anti-VEGFR-2 blocking antibodies.

INTRODUCTION

One of the major factors that account for the limited advances made in cancer treatment is acquired drug resistance. Cancers that respond to chemotherapeutic drugs such as ovarian, breast, colon, and non-small cell lung cancer, or to antihormonal therapies, e.g., breast and prostate cancer, almost always relapse in a resistant form some time later. Information is also emerging that even the newest generation of anticancer drugs such as the signal transduction inhibitors (e.g., the bcr-abl antagonist, STI571) may be compromised by acquired drug resistance (1–3). The numerous and diverse genetic instabilities of cancer cells are thought to be a major factor in explaining the propensity of cancer cells to give rise to such drug-resistant mutant or variant subpopulations (4, 5).

Various strategies to circumvent or reverse acquired resistance to chemotherapeutic drugs have had little clinically significant success thus far, with respect to the treatment of the common adult solid malignancies. Examples of such strategies include the use of combination chemotherapy, multimodality therapies, and the use of drugs such as Pgp3 antagonists to block the function of this particular mediator of multidrug resistance to natural lipophilic compounds (6). A new and seemingly counterintuitive preclinical strategy to combat drug resistance in cancer was developed recently (7), which exploits “chemotherapeutics as antiangiogenic agents,” i.e., the property of such drugs to damage or kill the genetically stable, host endothelial cells of a tumor’s newly formed neovasculature (8, 9). It involves the use of various chemotherapeutic drugs, e.g., cyclophosphamide (8) or vinblastine (9), given frequently and in a chronic manner (with no significant rest periods), at doses lower than the MTD. The administration of chemotherapeutic drugs in...
this manner has been termed “antiangiogenic” or “metronomic” chemotherapy (8, 10).

A rationale for this type of therapeutic approach is that the dividing ECs of newly forming tumor vessels (11) should be sensitive to chemotherapeutic drugs, similar to other types of normal dividing cells such as hair follicle, bone marrow, or gut mucosal cells (4). However, because such cells lack the genetic instabilities of tumor cells, their ability to mutate and acquire resistance properties would be expected to be much more limited (4, 5, 12). Indeed, Browder et al. (8) have shown that chemotherapeutic drugs given at the MTD can cause EC apoptosis of tumor-associated vessels in ectopically growing mouse tumors, but this damage can be repaired rapidly during the prolonged recovery periods necessary for myeloid recovery following MTD chemotherapy. Hence, by giving chemotherapy more frequently (13–15), chemotherapy respond to the same drug at a lower dose, but clinical cases in which patients not responsive to standard MTD chemotherapy enhanced (8).

These preclinical results may provide an explanation of the clinical cases in which patients not responsive to standard MTD chemotherapy respond to the same drug at a lower dose, but administered more frequently (13–15). In the past such schedules were used primarily for palliation, because of the less severe side effects (14, 15). The availability of oral chemotherapeutic drugs (16) makes chemotherapy administered in this manner a more practical possibility.

The long-term antitumor efficacy of antiangiogenic/metronomic chemotherapy protocols in ectopic syngeneic mouse or human tumor xenograft models can be increased, sometimes substantially so, by combination with a second, antiangiogenic drug such as D(chloroacetyl-carbamoyl) Fumagillol (8) or blocking monoclonal antibodies to VEGFR-2 (9). The rationale for this combination is that anti-VEGFR-2- or anti-VEGF-targeting drugs (17), agents capable of specific blockade of activated EC cell survival mechanisms (18), will selectively enhance the damaging or cytotoxic effects of continuous low-dose chemotherapy on newly formed blood vessels (9).

The purpose of the present paper was to address several important questions that the previous preclinical studies on antiangiogenic/metronomic chemotherapy have raised. First, do different classes of chemotherapeutic agents have similar potentials in terms of both efficacy and toxicity, when used alone or in combination with a second drug such as anti-VEGFR-2 antibodies? Would this type of therapeutic approach work as well on orthotopically transplanted tumors? Finally, would the approach have efficacy on multidrug-resistant tumors with high levels of resistance attributable to mechanisms such as overexpression of Pgp? Our results show that, indeed, various low-dose antiangiogenic chemotherapy regimens are highly effective against orthotopically grown multidrug-resistant human breast cancers in SCID mice, but usually only when the drugs are used in combination with the antiangiogenic, VEGFR-2-inhibiting antibody. Furthermore, at least in our study, some drugs [for example, paclitaxel (Taxol) and vinblastine] exhibit better therapeutic profiles than others [cisplatinum or doxorubicin (Adriamycin)] mainly because of their lack of cumulative toxicity.

**MATERIALS AND METHODS**

**Cells and Culture Conditions.** Two human breast cancer cell lines, MDA-MB-231 and MDA-MB-435, and a number of multidrug-resistant, Pgp-positive variants selected from these lines were used in the studies: MVB9, MD22, MPAHS, and TO.1. MVB9 was selected for resistance in vitro to vinblastine sulfate and maintained in 5 ng/ml vinblastine in culture, MD22 was selected for resistance to doxorubicin and maintained in 12 ng of doxorubicin/ng, and MPAHS was isolated by transfection of MDA-MB-231 with mdrl-1 gene and maintained in 10 ng/ml vincristine sulfate and 400 μg of G418. All three were originally obtained from Dr. Jeff Lemontt (Genzyme Corporation, Framingham, MA). The derivation of these lines is similar to that described by Lemontt et al. (19), and expressed stable and high levels of cross-resistance to drugs such as colchicine, vinblastine, vincristine, and Adriamycin4. TO.1, a Pgp-positive multidrug-resistant variant of MDA-MB-435 breast carcinoma, was obtained from Dr. Dalia Cohen (Novartis, East Hanover, NJ) and was derived by progressive serial in vitro exposure of the cell line to increasing concentrations of Taxol; these cells express 100-fold resistance to Taxol relative to the parental line (20). Finally, a cisplatinum-resistant variant called MDA-CDDP-S4 was selected from MDA-MB-231 by serial in vivo drug exposure, in our laboratory. This variant does not express Pgp. To select this variant, 4 × 106 MDA-MB-231 cells were implanted into the mammary fat pads of 8-week-old female athymic nude mice, tumors were grown to ~150 mm3, and the mice were treated with 5 mg/kg cisplatin every second day three times. The tumors were then removed, adapted to culture, and grown in vitro for 3 weeks, reimplanted into the mammary fat pad of a new group of athymic nude mice, and treated in the same manner; the selection process was repeated three more times.

All cell lines were expanded as monolayer cultures by serial passage on tissue culture plates (Nalge Nunc International, Naperville, IL) in DMEM, 5% fetal bovine serum (Invitrogen, Carlsbad, CA) with the addition of 22 nM (12 ng/ml) Adriamycin (Pharmacia Upjohn, Mississauga, Canada) for MD22, 12.2 nM (10 ng/ml) vincristine sulfate (Sigma-Aldrich Chemical Co., Canada, Oakville, Ontario, Canada) for MPAHS, 6 nM (5 ng/ml) vinblastine sulfate (Calbiochem, La Jolla, Ca) for MVB9, and 0.1 μM Taxol (Abbott Laboratories, North Chicago, IL) for TO.1. HUVECs (Clonetics, San Diego, CA) were expanded on 1% gelatin-coated tissue culture plates in MCDB131 culture medium (JRH Biosciences, Lenexa, KS) supplemented with 5 ng/ml bFGF (R & D Systems, Minneapolis, MN), 10 units/ml heparin (Wyeth-Ayerst Laboratories, Philadelphia, PA), 10 ng/ml epidermal growth factor (Upstate Biotechnology, Lake Placid, NY), and 10% fetal bovine serum.

**In Vitro Determination of Drug Sensitivity.** Analysis of in vitro drug sensitivity was assessed on cells grown in monolayer as well as three-dimensional multicellular spheroids. For monolayer analysis, 3,000 cells in 200 μl of growth medium were plated per well in 96-well flat-bottomed tissue culture plates (Nunc) and incubated at 37°C, 5% CO2 for 24 h prior to

---

4 J. Lemontt, personal communication.
were then pulsed with 2 \text{ng}/\text{ml} of either vinblastine sulfate (Calbiochem, San Diego, CA), cisplatinum (Faulding Canada Inc., Vaudreuil, Quebec, Canada), or Taxol (Abbot) for 24, 48, and 72 h. The cells were harvested onto a filtermat using a Titertek cell harvester.

Radioactivity was measured on a Wallac 1205 BetaPlate scintillation counter. The formation of spheroids was initiated by a gentle 10-min spin (1,000 rpm/min) of the freshly detached cells in suspension, followed by a 24- to 48-h incubation at 37 °C, 5% CO$_2$. To assess the inhibitory activity of chemotherapeutic agents, tumor cells, grown either as spheroids or as a monolayer, were exposed to 0–12 \mu M (0–10,000 ng/ml) of either vinblastine sulfate (Calbiochem, San Diego, CA), Adriamycin (Pharmacia & Upjohn Inc. Mississauga, Ontario, Canada), cisplatinum (Faulding Canada Inc., Vaudreuil, Quebec, Canada), or Taxol (Abbot) for 24, 48, and 72 h. The cells were then pulsed with 2 \muCi/well of \text{[methyl-]H}thymidine (Amersham Life Science, Buckinghamshire, United Kingdom), incubated at 37 °C for 6 h to allow for incorporation of \text{[H]}thymidine into their DNA, frozen, and thawed; then, the DNA was harvested onto a filtermat using a Titertek cell harvester. Radioactivity was measured on a Wallac 1205 BetaPlate scintillation counter (Wallac Oy, Turku, Finland), and proliferation was expressed as absolute counts of \text{[H]}thymidine per minute or as percentage of untreated control. Each dose concentration was done in sextuplicate and repeated twice. Because the most significant effect was observed at 24 h for the monolayer culture and at 72 h for the spheroid culture, only these points are presented in Table 1 and Fig. 1.

**Table 1** Differences in sensitivity of cancer cell lines and HUVECs to chemotherapeutic agents

<table>
<thead>
<tr>
<th>Cell population tested</th>
<th>Agent</th>
<th>IC$_{50}$ (nM) at 24 h</th>
<th>at 72 h</th>
<th>Spheroid at 24 h</th>
<th>at 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVECs</td>
<td>Vinblastine</td>
<td>0.55</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Adriamycin</td>
<td>1.65</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Cisplatinum</td>
<td>4</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Taxol</td>
<td>0.4</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>MDA-MB-231 parental tumor</td>
<td>Taxol</td>
<td>2</td>
<td>4.5</td>
<td>3,434</td>
<td>848</td>
</tr>
<tr>
<td>MD22 variant</td>
<td>Adriamycin</td>
<td>79.4</td>
<td>41.5</td>
<td>1,610</td>
<td>260</td>
</tr>
<tr>
<td>MPAHS variant</td>
<td>Vinblastine</td>
<td>15.7</td>
<td>7.1</td>
<td>1,349</td>
<td>&gt;123,301</td>
</tr>
<tr>
<td>MVB9 variant</td>
<td>Taxol</td>
<td>27.4</td>
<td>19</td>
<td>10,084</td>
<td>1,059</td>
</tr>
<tr>
<td>CDDP-S4 variant</td>
<td>Cisplatinum</td>
<td>2581</td>
<td>2083</td>
<td>27,773</td>
<td>2,380</td>
</tr>
</tbody>
</table>

*All of the variants are drug-resistant sublines of MDA-MB-231 human breast carcinoma cell line.

Initiation of treatment. For analysis in three-dimensional culture, multicellular tumor spheroids were formed using the liquid overlay technique, as described previously (21). Ninety-six-well round-bottomed tissue culture plates (Nunc) were covered with 2% Poly-Hema in 100% ethanol (Aldrich Chemical Co., Milwaukee, WI) to prevent attachment to the tumor cells to the plastic of the dish. The formation of spheroids was initiated by a gentle 10-min spin (1,000 rpm/min) of the freshly detached cells in suspension, followed by a 24- to 48-h incubation at 37 °C, 5% CO$_2$. To assess the inhibitory activity of chemotherapeutic agents, tumor cells, grown either as spheroids or as a monolayer, were exposed to 0–12 \mu M (0–10,000 ng/ml) of either vinblastine sulfate (Calbiochem, San Diego, CA), Adriamycin (Pharmacia & Upjohn Inc. Mississauga, Ontario, Canada), cisplatinum (Faulding Canada Inc., Vaudreuil, Quebec, Canada), or Taxol (Abbot) for 24, 48, and 72 h. The cells were then pulsed with 2 \muCi/well of \text{[methyl-]H}thymidine (Amersham Life Science, Buckinghamshire, United Kingdom), incubated at 37 °C for 6 h to allow for incorporation of \text{[H]}thymidine into their DNA, frozen, and thawed; then, the DNA was harvested onto a filtermat using a Titertek cell harvester. Radioactivity was measured on a Wallac 1205 BetaPlate scintillation counter (Wallac Oy, Turku, Finland), and proliferation was expressed as absolute counts of \text{[H]}thymidine per minute or as percentage of untreated control. Each dose concentration was done in sextuplicate and repeated twice. Because the most significant effect was observed at 24 h for the monolayer culture and at 72 h for the spheroid culture, only these points are presented in Table 1 and Fig. 1.

**In Vitro Confirmation of Drug Resistance.** MDA-MB-231 and its drug-resistant variants, grown and expanded in DMEM with 5% FCS, were washed with PBS twice and incubated in fresh medium with 5 \mu M rhodamine chloride (Sigma-Aldrich Canada) or without either 5 \mu M cyclosporin A (Novartis Pharmaceuticals, Canada Inc., Dorval, Quebec, Canada) or 30 \mu M verapamil hydrochloride (Sigma-Aldrich, Canada) for 30 min at 37 °C. Cells were then trypsinized at room temperature, washed, and resuspended in PBS and analyzed for intracellular fluorescence by flow cytometry (excitation 488 nm, emission 550 nm) with an EPICS Elite V flow cytometer (Coulter Electronics, Ltd., Luton, United Kingdom).

**Effect of Antitubulin Agents in Combination with Anti-VEGFR-2 Antibody on HUVECs.** Passage two of HUVECs was seeded on 1% gelatin-coated sterile microscope slides and allowed to grow to approximately 80% confluence before staining. HUVECs treated with medium containing 0.5 or 1 ng/ml vinblastine, 25 \mu g/ml 1C11 (monoclonal antibody against the VEGFR-2/KDR; Ref. 22), or the combination of the two for 4 h were stained for \beta-tubulin as follows. After a thorough wash with PBS, the monolayer was covered with ice-cold methanol for 10 min at −20 °C, rinsed with ice cold acetone twice for 10 s, washed twice with PBS and rehydrated in fresh PBS for at least 30 min prior to labeling with antibody. The anti-\beta-tubulin/Cy-3 conjugate (Sigma-Aldrich Chemical Co., St. Louis, MO) was then diluted to 1:100 in PBS, 1% BSA and added to the slides for 60 min at room temperature. After rinsing the excess antibody off with three 5-min washes with PBS, the tissues were mounted, coverslipped, and evaluated using a Zeiss confocal microscope. The same procedure was done using Taxol at 0.6 nM (data not shown).

**In Vivo Tumor Growth Assessment.** Each of the cell lines was harvested on the day of injection using 1% trypsin-EDTA (Invitrogen), and a single-cell suspension of 2 × 10$^6$ cells in 0.05 ml of serum-free growth medium was injected into the mammary fat pad of 4- to 6-week-old CB-17 SCID mice (Charles River, St.-Constant, Quebec, Canada). Approximately 3 weeks later, when most of the tumors had grown to 300 mm$^3$, the mice were randomized into groups of five animals. Two independent experiments were done for each xenograft, each totaling 30 animals in six groups. The treatment groups were as follows: **group I** (Control): 0.2 ml of PBS (DC101 vehicle) i.p. every 3 days and 0.2 ml injectable saline (vinblastine vehicle) i.p. every 3 days; **group II**: 0.4 ml of 2 mg/ml DC101 antibody (800 \mu g/mouse) i.p. every 3 days and 0.2 ml of injectable saline i.p. every 3 days; **group III**: 0.5 mg/kg (1.5 mg/m$^2$) vinblastine sulfate i.p. every 3 days in the case of MPAHS and MVB9, 1 mg/kg (3 mg/m$^3$) Adriamycin i.p. every 3 days in the case of MD22, 1 or 2 mg/kg (3 and 6 mg/m$^3$) cisplatinum i.p. every 3 days in the case of CDDP-S4, and 0.4 ml of PBS i.p. every 3 days; **group IV** (Pgp inhibitor control): cyclosporin A, 10 mg/kg, i.p. every 3 days in the case of MPAHS and MVB9, 1 mg/kg (3 mg/m$^3$) Adriamycin i.p. every 3 days in the case of MVB9; **group V**: chemotherapy as in group II combined with Pgp inhibitor in group IV; **group VI**: chemotherapy as in group II combined with DC101. Body weight and tumor size were assessed weekly, and general clinical status of the animals was assessed every day. Perpendicular tumor diameters were meas-
ured using a vernier scale caliper and tumor volume estimated using the formula for ellipsoid: \( \text{volume} = \frac{4}{3} \pi \text{width}^2 \times \text{length} / 2 \). Growth curves were analyzed statistically using repeated measures ANOVA.

For histological comparison, a separate group of animals was treated and sacrificed at 3 weeks of therapy, at which time tumors were excised and fixed in 10% (v/v) formalin or cryopreserved in Tissue-Tek O.C.T. compound (Bayer Corp., Elkhart, IN) until processed for histochemical analysis. Animal care was in accordance with institutional guidelines.

**Immunohistochemistry of Tumor Tissues.** Formalin-fixed paraffin-embedded sections were cut to 5-\(\mu\)m sections and stained with H&E according to standard protocols.

**RESULTS**

**In Vitro Drug Sensitivity Determination.** Prior to undertaking any in vivo experiments, drug levels at which significant toxicity against endothelial, but not tumor cells, might be achieved were established. A difference in relative chemotherapeutic drug sensitivities between human neuroblastoma cells and activated endothelial cells has been reported previously for vinblastine (9), and we asked whether similar differential sensitivities could be detected with other chemotherapeutic drugs and human breast cancer cells. To address this question, we subjected monolayer and spheroid cultures to increasing dose concentrations of one of four different drugs: Adriamycin, vinblastine, cisplatinum, or Taxol. To optimize growth conditions and achieve comparable baseline growth levels in tumor cells and HUVECs, tumor cell lines were grown in DMEM with 10% bovine serum, but HUVECs were grown on gelatinized plates, and in the presence of growth factors. The untreated controls showed similar levels of [\(^3\)H]thymidine incorporation for all three cell lines at the baseline drug level, eliminating the concern that the differences in proliferation may be attributable to inherent factors (Fig. 1). The chemotherapeutic drug sensitivities were found to be remarkably different between HUVECs
and the tumor cells tested. Whereas the proliferation of HU-VECs was inhibited by dose concentrations <0.5 nm (0.45 ng/ml) vinblastine, <0.4 nm (0.33 ng/ml) Taxol, <1.7 nm (0.9 ng/ml) Adriamycin, and <4 nm (1.2 ng/ml) cisplatinum, there was no significant growth inhibition of the parental (“drug-sensitive”) mammary carcinoma cell line MDA-MB-231 until dose concentrations greater than 3000 nm (3,000 ng/ml) were reached. This was further enhanced by culturing cancer cells as three-dimensional spheroids, which mirrored the tumor growth in vitro. The chemotherapeutic resistance of cancer cells grown in three-dimensional spheroid-like structures is, interestingly, further enhanced and enabled the cancer cell to withstand concentrations of chemotherapeutic agent 800–10,000-fold higher than those of ECs. In fact, all of the selected drug-resistant cell lines were resistant to a degree that would preclude clinical relevance, because these dose concentrations are unachievable in patients. For example, the growth rate of the MDA-MB-231-derived, cisplatinum-resistant variant CDDP-S4 was not affected until dose concentrations of 2,600 nm (770 ng/ml) were reached in monolayer, and 27,773 nm (8,330 ng/ml) in spheroid culture. This in vitro “screen” suggests that actively proliferating endothelial cells may be sensitive to a much lower range of dose concentrations of chemotherapeutic agents than actively proliferating tumor cells. If so, there is at least a theoretical possibility of substantially lowering the present clinically used MTDs of chemotherapeutic drugs to specifically target dividing endothelial cells present in tumors.

The Effect of Tubulin Inhibitors and Anti-VEGFR-2 Antibodies on HUVECs Grown in Monolayer Culture. Despite the limitations of in vitro culture as a model, including the use of (large vein) HUVECs rather than microvascular endothelial cells, it provides an approach for direct observation of the effects of tubulin inhibitors, a monoclonal antibody against the VEGFR-2, or a combination of the two on endothelial cells. We observed no appreciable effect of IMC-1C11, a monoclonal neutralizing antibody against the human VEGFR-2/KDR receptor, when used alone (Fig. 2). However, in combination with low-dose concentration vinblastine, the effects were striking. For example, in combination with 0.5 nm (0.5 ng/ml) vinblastine, IMC-1C11 caused retraction of the cellular membrane and full coagulation of the cytoskeleton (Fig. 2). Interestingly, a potent effect was observed with the lower dose concentration, and doubling the dose concentration to 1 ng/ml provided no additional benefits (Fig. 2). This suggests that, at least in combination with a specific inhibitor of EC survival and other functions, lower doses of tubulin inhibitors may produce adequate anti-endothelial effects.

Drug Resistance Testing. Before proceeding to study the efficacy of low-dose continuous chemotherapy in vivo against multidrug-resistant tumors, we verified the functional presence of the Pgp membrane pump. Using rhodamine chloride (a Pgp substrate that is easily detected by flow cytometry), we found a clear shift of the fluorescent population to the left (Fig. 3), confirming the presence of Pgp-mediated efflux of rhodamine chloride from the MVb9, MPAHS, MD22, and TO.1 cells, but not, as expected, from the Pgp-negative parental cell line, MDA-MB-231, or the cisplatinum-resistant CDDP-S4 variant. As expected, we also found that this drug efflux is reliably inhibited, at least in vitro, by Pgp reversal agents such as cyclosporin A or verapamil (data not shown).

In Vivo Tumor Growth Assessment. Building upon the striking in vitro differences in relative chemotherapeutic drug sensitivities between the tumor and endothelial cells tested, we evaluated the effects of a continuous low-dose regimen of each of these agents in inhibiting growth of orthotopic tumor xenografts in vivo. The cell lines were intentionally selected for their high levels of acquired resistance, e.g., up to 100-fold, to negate the possibility that any antitumor effects were due to direct antitumor cell activity.

The first group, treated with the neutralizing antibody directed against mouse VEGFR-2/flk-1 (DC101) shown previously to inhibit the s.c. growth of many different kinds of human xenograft in immune-deficient mice (23), displayed the expected effectiveness in inhibiting tumor growth (Fig. 4, green line, filled circles in all panels). Over the first weeks of therapy the effectiveness of DC101 was comparable with that of the combination treatment group, such that if this experiment had been terminated at 30–60 days, erroneous conclusions might have been drawn regarding the apparent equal efficacy of the two treatment groups. In most of the long-term experiments, the tumor volume curves diverge beyond 60–70 days and the benefit of adding the chemotherapeutic agent becomes apparent. Further confirmation of this delayed divergence effect was seen upon analysis of histological specimens at 3 weeks (see below and Fig. 5).

The findings in the second treatment group (chemotherapeutic agent alone) showed minimal to no growth inhibition (Fig. 4, blue lines, filled squares), and combining the chemotherapeutic agent with a Pgp pump inhibitor failed to improve this lack of an antitumor effect (Fig. 4, mauve diamonds).

Regardless of the lack of effect of the chemotherapeutic agent alone, in most instances addition of the anti-flk-1 antibody, DC101, produced tumor growth suppressions over and above those observed with either agent alone (Fig. 4, red line, filled squares in all panels). Most importantly, this combination therapy did not result in detectable drug resistance to the treatment even after >100 days of continuous treatment. With the exception of those treated with Adriamycin and cisplatinum, the mice remained healthy throughout the course of treatment, and in all cases the tumor remnants, when assessed by histology, appeared to contain mainly fibrous scar tissue.

Histopathological Analysis. In the multiple in vivo preclinical trials undertaken in our laboratory, we have frequently observed that even through significant differences can be seen with tumor volume measurements, the true degree of tumor regression is not always appreciated. However controlled, the gross measurement of tumor volume reflects a delayed response, and resorption, of the tissues and not necessarily the actual degree of the tissue damage inflicted. We have therefore evaluated representative samples of tumor tissues from all groups at arbitrary time points and observed, surprisingly, that significant differences were apparent as early as 3 weeks post initiation of therapy. Care was taken to include reference mammary gland tissue in all tumor samples (visible on the left in Fig. 5, a–e), to avoid concerns about sampling bias. The MPAHS tumor histology, shown in Fig. 5, represents a typical example of the histopathology of all of the different tumor subtypes and their
respective changes. No easily appreciated differences were seen in mice treated with saline or vinblastine alone. Healthy looking cancer cells with high nuclear:cytoplasmic ratio, high mitotic: karyorrhetic index, and high invasive potential are the prevalent component in tissues from these two groups (Fig. 5, a, f–h, k–m). In contrast, cells with pyknotic nuclei and a high degree of cytoplasmic blebbing, features consistent with apoptosis, predominate in the combination group (Fig. 5e) and are prevalent in the DC101 alone group (Fig. 5d). In fact, it appears that the degree of tissue necrosis and apoptosis is over and above that expected on the basis of tumor size measurements at 3 weeks (Fig. 4, day 51 of MPAHS panel). Similar differences are evident for mitotic:karyorrhetic indexes. Whereas a typical field in the control and vinblastine alone group may manifest many mitotic figures (Fig. 5, f and g), these are hard to find in samples treated with DC101 (Fig. 5i), and none were found in the

Fig. 2 Sensitivity of HUVECs grown in monolayer to vinblastine alone or in combination with IMC-1C11 (a monoclonal antibody to human VEGFR-2/KDR). HUVECs grown on glass slides coated with 1% gelatin, and supplemented with growth factors, were treated with either 0.5 ng/ml vinblastine or a combination of vinblastine and 1C11(25 μg/ml), a monoclonal antibody against the VEGFR-2. The culture was then fixed and stained with anti-β-tubulin/Cy-3 conjugate. The mild polymerization of tubulin evident in the cultures treated with 0.5 ng/ml vinblastine is visibly enhanced by combining the agent with IMC-1C11, and no further escalation of this effect is achieved by doubling the vinblastine dose concentration.
combination treatment group (Fig. 5j). Also shown are blood vessels filled with invasive tumor cells, a frequent, but difficult to quantitate observation. Invading cells were prevalent and easily noticeable in the vessels of this highly metastatic tumor when treated with vehicle, vinblastine alone, or cyclosporin combination (Fig. 5, k–m) but were not detectable in the combination treatment group (Fig. 5o), where the same vessels are devoid of any viable cellular component, or, as in the DC101 group, are filled with RBCs or clots (Fig. 5n). Because these data are presented as collaborative evidence to the tumor growth curves, no attempt at quantification of apoptosis was made.

**Toxicity Evaluation.** The mainly antivascular mechanism of this therapy suggests that minimal toxicity would be the case in adult animals, which we confirmed. Weight was plotted at regular intervals and considered a surrogate for evaluation of systemic well-being, anorexia, or failure to thrive. A summary of these results is presented in Fig. 6. Mice treated with the combination of Pgp substrate (vinblastine, Taxol, or Adriamycin) and a Pgp inhibitor (verapamil or cyclosporin A) experienced significant drug toxicities despite our having lowered the chemotherapy dose to half. In the case of Adriamycin, the toxicity of Adriamycin and cyclosporin was such that mice had to be sacrificed at only 2 weeks of treatment. Except for this, there were minimal differences in weight loss in the vinblastine alone, DC101, or vinblastine/DC101 group (Fig. 6). In contrast, the growth retardation in the treatment groups in which Adriamycin or cisplatinum were used is significantly different (Fig. 6, MD22 and CDDP-S4 panels), reaching weight loss of up to 25% of body weight. Other evidence of toxicity in the mice (ruffled fur, anorexia with subsequent cachexia, skin tenting due to dehydration, skin ulcerations, and toxic deaths; Ref. 24) was not seen.

**DISCUSSION**

Our results have revealed several new and important aspects of continuous low-dose (also called antiangiogenic or metronomic) chemotherapy (8–10). These include the success of the treatment strategy on multidrug-resistant tumors when combined with anti-VEGFR-2-blocking antibodies (even when the low-dose chemotherapy regimen on its own is ineffective), the variable toxicity profiles of different drugs used in this
chronic manner, and, finally, the success in treating orthotopically growing tumors.5

As our in vitro drug sensitivity screen showed (Fig. 1), there is a marked difference in relative sensitivity to chemotherapeutic agents between endothelial and tumor cells. Whereas there are some differences in the ability of specific anticancer agents to inhibit EC growth, these are probably not large enough to compensate for the differences in sensitivity between EC and tumor cells; moreover, the differences are further enhanced by testing drug-resistant variants as well as by culturing the cells as spheroids. On the basis of these differences, we feel there is at least a theoretical possibility of substantially lowering the present clinically used MTDs of chemotherapeutic drugs so as to specifically target dividing endothelial cells present in tumors.

For the majority of the studies reported here we chose human breast cancers selected by different methods to express a MDR phenotype to drugs such as Taxol, vinblastine, and Adriamycin, by virtue of Pgp overexpression (6, 25). The MDR phenotype is known to be easily reversible in vitro by exposing Pgp-positive cells to Pgp-blocking agents (26), and the same appears to be the case preclinically for leukemias or ascites tumors in vivo (6, 25). However, for a variety of reasons, reversal of the MDR phenotype in solid tumors treated in the clinical setting has proven to be much more difficult (27). Hence, our results showing that a combination of empirical low-dose/metronomic regimens using either Taxol (data not shown), vinblastine, or Adriamycin, in combination with a VEGFR-2-blocking antibody, induces significant and durable antitumor responses is encouraging. It is noteworthy that the various low-dose chemotherapy protocols we studied usually were not effective on their own and, indeed, in some cases, actually stimulated tumor growth, for reasons that are as yet unclear. Low-dose cisplatinum chemotherapy can cause such changes as an increase in endothelial cell VEGF (28) or enhanced expression of tumor cell-associated epidermal growth factor receptors (29), either of which could promote tumor growth. However, the addition of the DC101 anti-VEGFR-2 (anti-flk-1) antibody not only eliminated such growth stimulation, but resulted in enhanced antitumor effects in comparison with those observed with the antibody alone. Also interesting in this regard was the striking discordance between tumor volume measurements observed soon after combination therapy was

Fig. 4 Growth of orthotopic human breast cancer tumor xenografts in SCID mice. Drug-resistant variants were implanted in the mammary fat pad of 4–6-week-old SCID mice, grown to 300 mm3, and treated with a low dose of the chemotherapeutic drug to which they were resistant. There is no evidence of significant tumor growth suppression in the mice treated by the low-dose chemotherapeutic regimens alone and a partial regression by the DC101 antibody alone. However, the combination of the two agents causes a reliable and reproducible tumor growth suppression/regression in all treated cell lines. Each point on the graphs is presented as mean ± S.E. and represents an average of two different experiments with five mice per group in each experiment.

5 See note added in proof.
initiated (e.g., 3 weeks) on established tumors, where no apparent evidence of a response (i.e., tumor shrinkage) was detected, and histological analysis of the same tumors, which revealed clear evidence of significant tumor cell loss. The basis for this discrepancy may be the presence of necrotic tissue and the delayed resorption of such tissue in the treated tumors, especially those exposed to both the low-dose chemotherapy (e.g., vinblastine) protocol and the DC101 antibody. Such a discrepancy strengthens the argument for the use of tumor models incorporating secreted, soluble surrogate serum/urinary tumor markers as a means of more accurately quantitating true tumor burden and the effects of various anticancer therapies such as those described here (30).

It is also encouraging that the results we have obtained using orthotopic tumor models confirm results published previously using s.c. growing (ectopic) models (8, 9). This is because it has been shown that treatments that show efficacy in an ectopic model may not necessarily do so in an orthotopic model using the same tumor (31, 32). The next step for such studies will be to evaluate the effects of the therapeutic strategy on distant metastases growing in sites such as the liver, brain, bones, or lungs, after orthotopic injection of tumor cells (33), or in transgenic mouse models of tumor development and progression (34, 35). In this regard, it is encouraging that we noted the DC101 antibody, or DC101, in combination with low-dose vinblastine had an obvious suppressive effect on intravascular invasion of drug-resistant MDA-MB-231 (MPAHS) tumor cells (Fig. 5).

Another important aspect of our results relates to the toxicity associated with chronic low-dose treatment of the chemotherapeutic drugs that we studied. Confirming previous results (9), very low doses of vinblastine given twice weekly were not found to be overtly toxic to mice, using body weight as a surrogate marker, even after months of continuous therapy. The same was true for Taxol (data not shown). However, both Adriamycin and cisplatinum eventually resulted in significant loss of body weight, up to 25%, after several months of continuous therapy. In all cases the dose of drug used for the injection was in the range of one-tenth, or lower than, the MTD for mice, and was given two or three times a week. Thus the total amount of drug administered was still well below the MTD given once every 3 weeks. The basis for these differences is unknown but clearly could be a factor in the decision of what drugs to use for this type of chronic therapeutic approach. It must be acknowledged, however, that different dosing regimens and administration schedules and/or use of different drugs may improve both the efficacy and toxicity profiles of the chemotherapeutic drugs used in our studies. Indeed, we have found that oral cyclophos-
phamide given continuously in the drinking water at approximately 25 mg/kg/day can have substantial long-term (e.g., 3–4 months) antitumor effects as a nontoxic monotherapy against established PC3 human prostate tumor xenografts in SCID mice, results that are consistent with those of Browder et al. (8), who found that cyclophosphamide given weekly (at one-third the MTD) as a monotherapy could cause substantial antitumor effects in several tumor models, as well as the confirmatory results of other groups who have tested cyclophosphamide using dosing and administration schedules similar to Browder et al. (37, 38).

Finally, our results should also be viewed in the context of clinical studies in which various regimens of continuous or metronomic low-dose chemotherapy have been administered to patients, but without the addition of an antiangiogenic drug such as TNP-470 or VEGFR-2-blocking inhibitors. Some of these results, although clearly preliminary, are encouraging and may, in retrospect, have an antiangiogenic basis; the extent of the effect could most likely be improved by inclusion of cytostatic antiangiogenic drugs. For example, 4 of 10 non-small cell lung cancer patients who failed to respond to MTD etoposide given i.v. responded to the same drug given p.o. in a metronomic, low-dose fashion (14). Similarly, some breast cancer patients, who stopped responding to MTD dosing of Taxol given every 3 weeks were found to respond to the same drug given at one-third the MTD every week (15, 36). Similar results in breast cancer patients have been noted using cyclophosphamide at very low doses given on a daily basis, combined with low-dose oral methotrexate given twice a week (37). Large, prospective, randomized clinical trials are clearly needed to substantiate the survival benefit of the less toxic low-dose/continuous chemotherapy protocols over the standard MTD protocols.

Even if the survival benefits of continuous low-dose chemotherapy-alone protocols are marginal, it could be speculated, on the basis of our results, that the addition of a cytostatic antiangiogenic drug in a manner described herein (9) may significantly improve the results. Similarly, the treatment of...

---

6 S. Man and R. S. Kerbel, unpublished observations.

---

Fig. 6 Toxicity evaluation. Weight was plotted at regular intervals and considered a surrogate for evaluation of systemic well-being, anorexia, or failure to thrive. Mice treated with the combination of Pgp substrate (vinblastine, Taxol, and Adriamycin) and the Pgp inhibitor (verapamil or cyclosporin A), experienced significant drug toxicities despite reduction of chemotherapy doses to half. Except for this toxicity, there were minimal differences in weight loss in the vinblastine alone, DC101, or vinblastine/DC101 group MPAHS (c and MVB9). In comparison, there is significant growth retardation in the Adriamycin or cisplatinum treatment groups (MD22 and CDDP-S4), with weight loss reaching up to 25% of the body weight. Note that 1 mg/kg cisplatinum twice weekly, although it has very comparable effectiveness, is significantly less toxic.
children with acute lymphoblastic leukemia has been greatly improved since the introduction of therapeutic regimens that use initial MTD-based induction therapy, followed by 2–3 years of long-term, low-dose, continuous “maintenance” chemotherapy. The latter involves frequent daily/weekly administration of low doses of chemotherapeutic drugs such as 6-mercaptopurine and methotrexate. Might this maintenance therapy have, at least in part, an antiangiogenic basis? If so, could it be improved by combination with a cytostatic antiangiogenic drug? Clinical trials, and further preclinical studies, will hopefully provide answers to these and similar questions surrounding the use of continuous low-dose antiangiogenic therapies for the treatment of malignancies, especially those that are drug resistant.

ACKNOWLEDGMENTS

We thank Cassandra Cheng and Lynda Woodcock for excellent secretarial assistance, and James Huber and Angel Santiago for assistance in producing the DC101 antibody. We also thank Dr. Jeff Lemontt and Dr. Dalia Cohen for some of the cell lines used in this study.

Note Added in Proof: A recent study was published showing superior antitumor effects on orthotopically grown gliomas using combination low-dose chemotherapy and an antiangiogenic drug compared to either treatment used alone (L. Bello et al., Cancer Res., 61: 7501–7506, 2001).

REFERENCES

28. Wild, R., Ghash, K., Dings, R., and Ramakrishnan, S. Carboplatin differentially induces the VEGF stress response in endothelial cells:


Differences in Therapeutic Indexes of Combination Metronomic Chemotherapy and an Anti-VEGFR-2 Antibody in Multidrug-resistant Human Breast Cancer Xenografts

Giannoula Klement, Ping Huang, Barbara Mayer, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/1/221

Cited articles
This article cites 38 articles, 18 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/1/221.full.html#ref-list-1

Citing articles
This article has been cited by 35 HighWire-hosted articles. Access the articles at:
/content/8/1/221.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.