Use of Molecular Biomarkers to Quantify the Spatial Distribution of Effects of Anticancer Drugs in Solid Tumors

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Abstract

Poor distribution of anticancer drugs within solid tumors may limit their effectiveness. Here, we characterize the distribution within solid tumors of biomarkers of drug effect. γ-H2AX, cleaved-caspase-3 or –6, and Ki67 were quantified in tumor sections in relation to blood vessels (recognized by CD31) using monoclonal antibodies and immunohistochemistry. To validate their use, we compared their time-dependent distribution with that of (i) fluorescent doxorubicin and (ii) a monoclonal antibody that detects melphalan-induced DNA adducts. The biomarkers were then used to quantify the distribution of docetaxel in relation to tumor blood vessels. Activation of γ-H2AX was evaluated following in vitro exposure of tumor cells to multiple drugs. Distributions of doxorubicin in MDA-MB-231 and MCF-7 xenografts and of melphalan-induced DNA adducts in MCF-7 and EMT-6 tumors decreased with distance from blood vessels, similar to the distributions of (i) γ-H2AX at 10 minutes, (ii) cleaved caspase-3 or –6, and (iii) change in Ki67 at 24 hours following treatment. The distribution of these biomarkers following treatment with docetaxel also decreased with increasing distance from tumor blood vessels. Activation of γ-H2AX occurred within 1 hour after exposure to several drugs in culture. Multiple anticancer drugs show a decrease in activity with increasing distance from tumor blood vessels; poor drug distribution is an important cause of drug resistance. The above biomarkers may be used in designing strategies to overcome therapeutic resistance by modifying or complementing the limited spatial distribution of drug activity in solid tumors. Mol Cancer Ther; 12(4); 1–11. ©2013 AACR.

Introduction

Solid tumors develop an imperfect vasculature. Although tumor cells release factors that stimulate angiogenesis (such as VEGF), the resultant tumor vasculature lacks structure, and as tumor cells proliferate, blood vessels may be separated by longer distances than in normal tissues and blood flow becomes irregular (1, 2). As a result, some regions of the tumor become deficient in oxygen and other nutrients, whereas poor clearance of cellular breakdown products, including lactic and carbonic acid, leads to regions of low pH (3, 4). The disorganized vascular architecture also causes problems of limited drug penetration and drug delivery throughout the solid tumor. Tumor regions distal to patent blood vessels may receive lower amounts of drug than those cells located proximal to tumor blood vessels, whereas changes in blood flow may lead to intermittent delivery of drugs to some tumor regions. Within nutrient-deprived and hypoxic tumor regions, the rate of cell proliferation is relatively low and slowly proliferating cells are resistant to most currently used anticancer drugs, including many targeted agents (5–7). As a result of these factors, tumor cells within poorly nourished regions of solid tumors are likely to survive drug therapy, regardless of their intrinsic sensitivity to the drugs that are used. This important mechanism of drug resistance has been rather neglected in comparison with molecular causes of resistance operative at the level of the single cell, which dominate when cells are exposed to drugs under optimal conditions in tissue culture.

The spatial and temporal distribution of clinically used drugs in tumor tissue are important factors in determining tumor response to therapy, whereas modifying or complementing such distributions with other agents represent potential strategies to improve therapeutic index. In previous studies, our group has shown limited distribution from tumor blood vessels of doxorubicin and mitoxantrone using immunohistochemistry to quantify these fluorescent drugs in tumor sections (8, 9). However, most anticancer drugs are not fluorescent and antibodies that recognize them in tissue are not generally available. While autoradiography has been used to show limited distribution of other drugs such as paclitaxel (10, 11), this technique is cumbersome. Moreover, it would be preferable to quantify the distribution not only of the native drugs but of the pharmacodynamic effects that they and their metabolites have on tumor cells. The aim of the present study was to characterize the spatial distribution...
within solid tumors of biomarkers of drug effect: changes in the phosphorylated histone γ-H2AX (a marker of DNA damage), cleaved caspase-3 or -6 (a marker of apoptosis), and Ki67 (a marker of cell proliferation) in relation to blood vessels (recognized by an antibody to CD31). We elected to first study immunohistochemical methods for quantifying markers of effect for doxorubicin, which is fluorescent, and for melphalan, where adducts with DNA can be recognized by an antibody; this strategy enables a comparison between drug concentration and changes in the pharmacodynamic markers of drug effect in relation to tumor blood vessels. We then extended these techniques to study a widely used nonfluorescent agent (docetaxel) and to evaluate expression of γ-H2AX after treatment of cultured cells with a range of anticancer drugs.

Materials and Methods

Cell lines

Studies were conducted using the following cell lines: human breast carcinomas MDA-MB-231 and MCF-7, mouse mammary EMT-6, human vulvar epidermoid carcinoma A-431, and human prostate cancer PC-3. EMT-6 cells were originally provided by Dr. Peter Twetmyman (University of Cambridge, Cambridge, United Kingdom), and all other cell lines were purchased from the American Type Culture Collection in 2011. MCF-7, MDA-MB-231, A-431, and EMT-6 cells have been maintained in our laboratory and were grown in α-minimum essential medium supplemented with 10% FBS (Hyclone). PC-3 cells were grown in Ham’s F-12K medium (Life Technologies Inc.) supplemented with 10% FBS. All cells were grown in a humidified atmosphere of 95% air/5% CO2 at 37°C. Routine tests to exclude Mycoplasma in all cell lines were conducted several times each year. Short tandem repeat analysis was conducted to ensure cells (PC-3, MCF-7, MDA-MB-231, and A-431) were of human or (EMT-6) murine origin in August, 2012. These cell lines were used to test the efficacy of drugs on tumor models relevant to their clinical use e.g., the use of doxorubicin for human (MCF-7, MDA-MB-231) and murine (EMT-6) breast tumors and docetaxel for human prostate (PC-3) and cervical (A-431) tumors.

To generate tumors, 4- to 6-week-old male athymic nude mice (Jackson Laboratory) were injected subcutaneously in both flanks with 2 × 106 PC-3 cells, and 4- to 6-week-old female athymic nude mice (Harlan Sprague-Dawley) with implanted 17β-estradiol tablets (60 day release; Innovative Research of America) were injected subcutaneously with 5 × 103 MCF-7 cells per side; non-estradiol-implanted female athymic nude mice were injected with 5 × 106 MDA-MB-231 cells or 1 × 106 A-431 cells. Female balb/c mice were injected with 1 × 106 EMT-6 cells. There were 6 mice per treatment group (~10–12 tumors) and each experiment was repeated twice.

Drugs and reagents

Doxorubicin (Pharmacia), melphalan (Glaxo Wellcome Inc.), docetaxel (Sanofi-Aventis), 5-fluorouracil (5-FU), cabazitaxel (Sanofi-Aventis), gemcitabine (Eli Lilly), methotrexate (Pfizer), topotecan (GlaxoSmithKline), paclitaxel (Bristol Myers Squibb), and vinblastine (Mayne Pharma) were purchased from the Princess Margaret Hospital (Toronto, Canada) pharmacy; they were provided as solutions with concentrations of 2, 5, or 40 µg/mL.

Purified rat anti-mouse CD31 (platelet/endothelial cell adhesion molecule 1) monoclonal antibody was purchased from BD PharMingen and the Cy3-conjugated goat anti-rat immunoglobulin G (IgG) secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. The MP5 monoclonal antibody that recognizes melphalan/DNA adducts (12) was generously provided by Dr. M.J. Tilby (University of Newcastle, Newcastle, UK) as a hybridoma culture supernatant, dilution 1:100. γ-H2AX was stained with a rabbit anti-human γ-H2AX antibody [Cell Signaling; horseradish peroxidase (HRP) chromogen], cleaved caspase-3 with rabbit anti-human cleaved-caspase-3 antibody (Cell Signaling; HRP chromogen), cleaved caspase-6 with rabbit anti-human cleaved caspase-6 antibody (Novus Biologicals; HRP chromogen), and Ki67 with rabbit anti-human Ki67 antibody (Novus; HRP chromogen).

Effect of anticancer drugs on biomarkers

In vitro studies were conducted in chamber slides with 1 chamber per slide (Lab-Tek II) using a protocol described elsewhere (12). Briefly, PC-3 cells were grown in chamber slides and then treated with 50 nmol/L docetaxel for 0, 10, 30, 60, and 90 minutes at 37°C. Following the removal of culture media, cells were washed with PBS and then fixed for 10 minutes at room temperature in 0.3% H2O2 solution supplemented with acetone to block endogenous peroxidase. Cells were subsequently air-dried for 10 minutes and stored at −20°C while awaiting immunohistochemical staining with γ-H2AX antibody.

To determine the distribution of drugs and/or pharmacodynamic biomarkers in vivo, mice bearing tumors of mean cross-sectional area 0.7 to 0.8 cm2 were given a single intravenous injection of doxorubicin (25 mg/kg) or intraperitoneal injection of melphalan (6 mg/kg) or docetaxel (15 mg/kg). These doses were selected as the maximum-tolerated doses that caused minimal weight loss in mice. Animals were killed and tumors were excised from control (untreated) mice and from treated animals at varying times after drug injection. Samples were embedded immediately in optimum cutting temperature compound and tissue sectioning and immunohistochemical staining. Single cryostat sections (10-µm thick) were then cut from each tumor. Whole tumor sections were analyzed and artifacts and regions of necrosis were omitted; a minimum of 10 tumors were analyzed per treatment group.

Tumor sections were stained for blood vessels using the rat anti-CD31 primary antibody and Cy3-conjugated goat anti-rat IgG secondary antibody; they were stained for γ-H2AX, cleaved caspase-3 (or -6), and Ki67 with the appropriate antibodies. Because MCF-7 cells contain a
deletion in the exon 3 that encodes the caspase-3 gene, they do not express caspase-3 (13). A study by Inoue and colleagues found that the effector caspase-6 processes caspases-8 and -10 leading to apoptosis, and we therefore chose to study the expression of cleaved caspase-6 (14). Tumor sections were imaged for CD31 using the Cy3 (530–560 nm excitation/573–647 nm emission) filter set. γ-H2AX, cleaved caspase-3 (or -6), and Ki67 were imaged using transmitted light. Photomicrographs of these biomarkers in relation to blood vessels are illustrated for control and docetaxel-treated A-431 xenografts in Fig. 1.

**Image analysis and quantification**

Image analysis and quantification were conducted using Media Cybernetics Image Pro PLUS software. To minimize noise due to tumor autofluorescence, a minimal threshold for detection (below the level of detection of doxorubicin) was determined for each tumor. Doxorubicin was quantified according to the method described by Primeau and colleagues (8).

A novel protocol developed by Fung and colleagues (15) was developed to analyze composite images based on the method described by Primeau and colleagues (8). Briefly, binarized CD31 images were created and then used to create a distance map such that each pixel is represented by its distance to the nearest functional blood vessel in the section. Distributions of the pharmacodynamic biomarkers γ-H2AX, cleaved caspase-3 (or -6), and Ki67 were determined by creating binary masks (black and white images). The biomarker mask was then combined with the blood vessel distance map to form a composite image with distance measurements that corresponded only to the biomarker (γ-H2AX, cleaved caspase-3, and Ki67)–positive pixels. The data are represented graphically as the percentage of pixels that are biomarker positive at any given distance from the nearest blood vessel.

![Figure 1](image-url)

**Figure 1.** Photomicrographs of γ-H2AX (A), cleaved caspase-3 (B), and Ki67 (C) in untreated (control) or docetaxel treated A-431 tumor xenografts.
vessel in the section; a cutoff of 60 μm was used to minimize interference from neighboring blood vessels that are out of the plane of the section. For in vitro studies, γ-H2AX-positive pixels were counted and expressed as a percentage of the total number of pixels.

**Statistical analysis**

A one-way ANOVA, followed by Tukey post hoc test, determined statistical differences between treatment groups. *P < 0.05* was used to indicate statistical significance; all tests were two-sided and no corrections were applied for multiple significance testing. Drug and biomarker distributions are represented as mean ± SEM.

**Results**

**Quantification of biomarkers in tumor sections by immunohistochemistry**

The biomarkers γ-H2AX, cleaved caspase-3 or -6, and Ki67 could be recognized and quantified by applying appropriate antibodies as shown in Fig. 1A–C. γ-H2AX and the apoptotic markers cleaved caspase-3 or -6 increased after drug treatment (see later for time course), whereas proliferation as indicated by Ki67 decreased.

**Time-dependent distribution of doxorubicin and biomarkers in tumors**

To determine the time-dependent distribution of the biomarkers, we studied previously frozen, doxorubicin-treated MDA-MB-231 tumors. These tumors were excised at 10 minutes, 3, 6, 24, and 48 hours after doxorubicin treatment or control (untreated). The fluorescent distribution of doxorubicin at all time points displayed decreased levels of drug at greater distances from blood vessels (Fig. 2A). As seen in Fig. 2B, γ-H2AX was maximal at 10 minutes after treatment and then decreased to undetectable levels. Cleaved caspase-3 levels increased with time up to 24 hours and decayed by 48 hours (Fig. 2C). The Ki67 distribution remained suppressed from 3 to 48 hours (Fig. 2D). We chose to evaluate subsequently γ-H2AX distribution at 10 minutes, and cleaved caspase-3 or change in Ki67 distributions at 24 hours, after drug treatment.

Results for MCF-7 tumors were qualitatively similar to the above with highest doxorubicin concentration adjacent to blood vessels (Fig. 2E). γ-H2AX expression was increased compared with control at 10 minutes and maximal in regions closest to blood vessels (Fig. 2F). There was increased apoptosis in regions close to blood vessels at 24 hours compared with control (Fig. 2G). Ki67 staining was highest in regions closest to blood vessels with a marked reduction 24 hours after doxorubicin treatment (Fig. 2H).

Maximum (close to blood vessels) and minimum levels (at 60 μm, largest distance from blood vessels evaluated) of doxorubicin fluorescence and of the biomarkers (in relation to values in control tumors) are shown in Table 1. Reductions in γ-H2AX at 10 minutes and cleaved caspase-3 or -6 at 24 hours reflect the distribution of doxorubicin fluorescence. Interpretation of change in Ki67 is more complex because of 2 effects: drug-induced decrease in proliferation proximal to blood vessels and low proliferation in distant regions even in control tumors.

**Distribution of melphalan-induced DNA adducts and biomarkers in tumors**

Melphalan-induced DNA adducts were assessed in MCF-7 xenografts and murine EMT-6 tumors by staining with the MP5 antibody, which recognizes adducts formed as a result of melphalan binding to N7 of guanine (12). Such adducts were evident at 10 minutes after injection and decayed only slightly at 24 hours (Fig. 3A and E). The frequency of adducts decreased with increasing distance from tumor blood vessels.

At 10 minutes after administration of melphalan, there was a marked increase in γ-H2AX compared with control and γ-H2AX decreased with increasing distance from blood vessels; this distribution reflected that of melphalan-induced DNA adducts in both tumor types (Fig. 3B and F). There were few apoptotic cells in untreated tumors, but at 24 hours after treatment there was increased staining for cleaved caspase-3 or -6 and this was most pronounced in regions proximal to blood vessels (Fig. 3C and G). Control tumors showed decreased proliferation with increasing distance from blood vessels in both tumors. There was a reduction in Ki67 levels at 24 hours following treatment with melphalan, more pronounced in regions close to blood vessels (Fig. 3D and H). As shown in Table 1, the decrease in levels of γ-H2AX at 10 minutes and of cleaved caspase-3 at 24 hours reflect quite closely the distribution of melphalan–DNA adducts.

**Effect of docetaxel on biomarker distribution in xenografts**

Staining of the biomarkers in relation to blood vessels in control and docetaxel-treated A-431 tumors is shown in Fig. 1. At 10 minutes after docetaxel treatment, there was marked activation of γ-H2AX in PC-3 and A-431 xenografts and γ-H2AX decreased with increasing distance from blood vessels (Fig. 4A and D). There was a corresponding increase of cleaved caspase-3 over control levels at 24 hours after treatment, again with more marked apoptosis in proximal as compared with distal regions (Fig. 4B and E). There was a decrease in Ki67 at 24 hours after docetaxel treatment, especially in regions close to blood vessels (Fig. 4C and F). The distribution of biomarkers after docetaxel treatment is quite similar to that after treatment with doxorubicin and melphalan (Table 1).

**γ-H2AX expression and decay following chemotherapy**

To evaluate the expression of γ-H2AX following a range of anticancer drugs with different mechanisms of action, we treated PC-3 cells for 1 hour with: 5-FU (10 μmol/L), cabazitaxel (100 nmol/L), gemcitabine (100 nmol/L), methotrexate (400 nmol/L), topotecan (50 nmol/L),
paclitaxel (50 nmol/L), or vinblastine (50 μmol/L). As seen in Fig. 5A, all chemotherapeutic agents induced expression of γ-H2AX.

Figure 2. MDA-MB-231 (A–D) and MCF-7 (E–H) tumors were treated with doxorubicin (25 mg/kg) or untreated controls. A and E, doxorubicin fluorescence in relation to tumor blood vessels at different times after injection. Corresponding changes in biomarkers of drug effect: γ-H2AX (B and F), cleaved caspase-3 (MDA-MB-231, C) or 6 (MCF-7, G), and Ki67 (D and H). The figures represent percentage of positive pixels as a function of distance from the nearest blood vessel. Points indicate average for 10 mice per group; bars, SE. ○, control; ■, 10 minutes; ▲, 3 hours; +, 6 hours; ×, 24 hours; ♦, 48 hours.

PC-3 cells were treated with 50 nmol/L of docetaxel and samples were taken at different time points. As seen in Fig. 5B, γ-H2AX expression is highest at 10 minutes following
treatment and then decays to near basal levels by 90 minutes.

Discussion

Our group and others have shown that the distribution of doxorubicin (8, 16), mitoxantrone, and topotecan (9, 17) decrease as a function of distance from the nearest blood vessel in solid tumors. However, most anticancer agents are not fluorescent and their visualization and quantification in solid tumors is difficult. Moreover, the activity of many drugs depends on metabolites as well as the parent drug, so that evaluation of the distribution in tumors of cellular damage caused by drugs is more relevant than the distribution of the drugs themselves. We have therefore evaluated the use of biomarkers of drug effect, which can be recognized by fluorescent- or chromogen-tagged monoclonal antibodies, to determine the distribution of activity of anticancer drugs. Using fluorescent doxorubicin (25 mg/kg) to initiate our studies, we have shown that not only does the distribution of doxorubicin decrease at increasing distances from the vasculature, but also markers of drug effect mimic drug distribution in different solid tumors. We chose a high dose of doxorubicin to visualize the autofluorescence of the drug in tumor tissue; however, previous studies from our laboratory have shown that even a single smaller dose (8 mg/kg) of doxorubicin resulted in delayed growth delay of various tumor types including EMT-6 and MCF-7 (8, 16).

We chose to evaluate γ-H2AX, cleaved caspase-3 (or -6), and Ki67. The 3 drugs that we evaluated are all approved for use in patients and proven to have a therapeutic effect. Each treatment led to stimulation of cleaved caspase-3 or -6 and reduction of Ki67 at 24 hours after treatment, and these could be used as biomarkers of drug distribution and activity. Huxham and colleagues (18) evaluated the distribution of cell proliferation (using bromodeoxyuridine) following treatment of human colon cancer xenografts with gemcitabine (240 mg/kg) and found complete inhibition of uptake of the S-phase marker at 24 hours following treatment. Gemcitabine is a nucleoside analog that was administered at a very high dose in these experiments and has a different mechanism of action from those that we studied in vivo. Although we observed a reduction in Ki67–positive cells after treatment with the 3 drugs evaluated, we did not find complete inhibition of proliferation. Also, this assay is complicated because low proliferation in regions of control tumors distal from blood vessels makes it difficult to assess drug activity in those regions.

Biomarkers of drug activity reflect accumulated drug activity and not drug distribution at a fixed time. Changes

| Table 1. Maximal and minimal levels of doxorubicin fluorescence and biomarkers (γ-H2AX, cleaved caspases, change in Ki67) in various tumors |
| Drug | Tumor type | MDA-MB-231 | | | MCF-7 | | | |
| | | Max | Min | Min value (% max.) | Max | Min | Min value (% max.) |
| Doxorubicin | | | | | | | |
| Doxorubicin, 10 min | 7.7 | 5.8 | 75 | 7.8 | 5.5 | 70 |
| Doxorubicin, 24 h | 4.1 | 3.7 | 92 | 4.6 | 3.3 | 72 |
| γ-H2AX, 10 min | 9.3 | 6.7 | 71 | 3.7 | 1.6 | 44 |
| Cleaved caspase-3 (or-6) 24 hrs | 1.8 | 1.2 | 63 | 3.5 | 2.4 | 67 |
| Reduction in difference of Ki67, 24 h | 6 | 0.02 | 0.3 | 4 | 0.08 | 2 |
| Melphalan | | | | | | | |
| Melphalan–DNA adducts, 10 min | 0.80 | 0.26 | 33 | 0.39 | 0.24 | 62 |
| Melphalan–DNA adducts, 24 h | 0.49 | 0.20 | 41 | 0.38 | 0.23 | 61 |
| γ-H2AX, 10 min | 1.37 | 0.63 | 46 | 5.0 | 2.6 | 52 |
| Cleaved caspase-3 (or-6) 24 hrs | 0.77 | 0.25 | 33 | 1.1 | 0.6 | 54 |
| Reduction in difference of Ki67, 24 h | 3.7 | 0.1 | 2.7 | 3.6 | 0.3 | 8.3 |
| Docetaxel | | | | | | | |
| γ-H2AX, 10 min | 5.4 | 2.6 | 48 | 7.5 | 5.0 | 66 |
| Cleaved caspase-3, 24 h | 4.7 | 2.2 | 48 | 4.7 | 3.2 | 45 |
| Reduction in difference of Ki67, 24 h | 7.8 | 0.5 | 6.4 | 9.6 | 1.5 | 15 |

NOTE: Values represent percentage of positive pixels at a given distance from blood vessels. Maximum values are at or very close to the blood vessel at 0 μm and minimal values are at 60 μm.
in drug distribution, loss of damaged cells, and changes in the spatial distribution of surviving or damaged but intact cells can occur during the 24-hour period that is required to evaluate the distribution of cleaved caspase, or changes in Ki67. γ-H2AX is formed rapidly in response to induction of double-strand DNA breaks following ionizing

Figure 3. MCF-7 (A–D) and EMT-6 tumors (E–H) treated with melphalan (6 mg/kg) or untreated controls. A and E, changes in melphalan–DNA adducts in relation to tumor blood vessels at 10 minutes and 24 hours after injection. Corresponding changes in biomarkers of drug effect: γ-H2AX (B and F), cleaved caspase-6 (MCF-7, C) or 3 (EMT6, G), and Ki67 (D and H). The figures represent percentage of positive pixels as a function of distance from the nearest blood vessel. Points indicate average for 10 mice per group; bars, SE. ◊, control; □, 10 minutes; △, 24 hours.
radiation or treatment with some drugs, due to phosphorylation of serine 139 of H2AX (19). γ-H2AX expression has been detected as soon as 3 minutes following ionizing radiation (20) and then gradually decreases (presumably due to repair of DNA double-strand breaks or lysis of lethally damaged cells) and returns to basal levels (21). Other studies have suggested that γ-H2AX may be a more general sensor of DNA damage following treatment with agents that are not known to cause double-strand DNA breaks (21, 22); this is consistent with our results, which indicate that γ-H2AX is formed within 10 minutes following treatment with a variety of anticancer drugs in vitro. In single cells, γ-H2AX levels fell to near basal levels by 90 minutes after treatment with docetaxel, whereas in solid

Figure 4. PC-3 (A–C) and A431 tumors (D–F) treated with docetaxel (15 mg/kg) or untreated controls. Changes in biomarkers of drug effect are shown at 10 minutes for γ-H2AX (A and D) and 24 hours for cleaved caspase-3 (B and E) and Ki67 (C and F) compared with untreated controls. The figures represent percentage of positive pixels as a function of distance from the nearest blood vessel. The figures represent percentage of positive pixels as a function of distance from the nearest blood vessel. Points indicate average for 10 mice per group; bars: SE. ◯, control; ■, 10 minutes; ◊, 24 hours.
tumors treated with doxorubicin, melphalan, or docetaxel (structures shown in Fig. 6), it diminished to pretreatment control levels at 3 hours. The distribution of γ-H2AX as a biomarker of cellular damage at 10 to 60 minutes after drug administration has the advantage that the time interval is sufficiently short to minimize any changes in the spatial distribution of the tumor cells between drug administration and the assay.

Doxorubicin, a DNA topoisomerase II inhibitor can intercalate with DNA, which leads to DNA damage and production of γ-H2AX (23). Our results show that the distribution of γ-H2AX decreased with increasing distance from the nearest blood vessel following a single treatment of doxorubicin in 2 human xenografts (MDA-MB-231 and MCF-7). The pattern of distribution of induced γ-H2AX was similar to that observed for drug distribution. Furthermore, subsequent changes in cleaved caspase-3 or -6 showed similar effects, despite possible kinetic changes within 24 hours. These results show that not only is there poor distribution of doxorubicin in solid tumors, but also poor distribution of drug activity.

Melphalan is a bifunctional alkylating agent that forms adducts with DNA leading to DNA damage (24). Melphalan–DNA adducts expressed a pattern of damage such that tumor cells located closest to blood vessels were affected preferentially. The spatial distribution of γ-H2AX was similar to that observed for drug distribution. Furthermore, subsequent changes in cleaved caspase-3 or -6 showed similar effects, despite possible kinetic changes within 24 hours. These results show that not only is there poor distribution of doxorubicin in solid tumors, but also poor distribution of drug activity.

Finally, we studied changes in biomarker distribution following treatment with docetaxel, a drug that is used...
widely in the clinic and is nonfluorescent. We chose a dose of 15 mg/kg that our laboratory has previously shown to cause delayed growth delay of PC-3 tumors. Docetaxel binds to microtubules, preventing their disassembly and turnover, leading to accumulation of cells in the G2–M phase of the cell cycle and to cell death. Kolfschoten and colleagues have reported that p53 expression increases in ovarian cells expressing wild-type p53 following treatment with docetaxel. H2AX and p53 are both substrates for phosphorylation by the ATM kinase, which is involved in repairing DNA damage, and we found higher levels of γ-H2AX following docetaxel treatment. Zhang and colleagues also observed increased levels of γ-H2AX assessed by flow cytometry in non–small cell lung cancer (A549) cells following docetaxel treatment, supporting the hypothesis that γ-H2AX as a general marker of DNA damage.

γ-H2AX expression at 10 minutes, increased apoptosis and reduced proliferation at 24 hours following docetaxel treatment was highest proximal to blood vessels and decreased at greater distances. Thus, the activity of docetaxel also decreases substantially at increasing distance from tumor blood vessels. We are unaware of direct assessment of the distribution of the drug itself, but studies of radiolabeled paclitaxel by autoradiography have shown a similar distribution in hypopharyngeal tumor xenografts.

To evaluate γ-H2AX as a general marker of activity of anticancer drugs, we evaluated its expression at 1 hour after treating cultured cells with a range of chemotherapeutic agents with differing mechanisms of action: antimitotic, paclitaxel, cabazitaxel, and docetaxel, nucleoside analog (gemcitabine), antifolate activity (methotrexate), and topoisomerase I inhibition (topotecan). Increased γ-H2AX expression after drug treatment of PC-3 cells supports the use of γ-H2AX to study the distribution of activity of many anticancer agents in solid tumors.

Our study has limitations. We assessed distributions of biomarkers and drugs in 2-dimensional tumor sections, whereas solid tumors are 3-dimensional. We limited our studies to a distance of 60 μm from blood vessels because of unknown effects from blood vessels out of the plane of the tumor section, and cells at greater distances, some of which are likely to be hypoxic, are likely to have even lower levels of activity than those documented here. In addition, we did not use a flow marker to indicate patent blood vessels.

In summary, we have used the induction of biomarkers γ-H2AX and cleaved caspase-3, and inhibition of Ki67, to show that the effects of 3 quite different anticancer drugs decrease with increasing distance from blood vessels of a variety of solid tumors. These results suggest that limited delivery of drugs to regions distal to blood vessels, and/or limited response of cells within them because of their low proliferative rate or microenvironment, is a general problem that is likely to be an important cause of clinical drug resistance. We observed that γ-H2AX serves as an early marker of DNA damage that is induced within 10 minutes following treatment with different types of drugs. This early induction is an advantage as this interval is too short to be influenced by changes in drug distribution and by the kinetics of the tumor cells that may occur at later times after drug administration. We are evaluating the distribution of biomarkers in biopsies from patients; our techniques require 10-μm sections, which are easily cut from biopsies. Our results suggest that induction of γ-H2AX might be used more generally to predict the spatial distribution of drug activity within solid tumors. This will be essential in designing strategies to overcome therapeutic resistance by modifying or complementing the limited spatial distribution of drug activity in solid tumors.

Disclosure of Potential Conflicts of Interest
I.F. Tannock has commercial research grants from Threshold and Sanofi. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: J.K. Saggar, I.F. Tannock
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