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Activity of the hypoxia-activated pro-drug TH-302 in hypoxic and perivascular regions of solid tumors and its potential to enhance therapeutic effects of chemotherapy

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Many chemotherapy drugs have poor therapeutic activity in regions distant from tumor blood vessels because of poor tissue penetration and low cytotoxic activity against slowly-proliferating cells. The hypoxia-activated pro-drug TH-302 may have selective toxicity for hypoxic and neighboring cells in tumors. Here we characterize the spatial distribution and ability of TH-302 to selectively target hypoxic regions and complement the effect of doxorubicin and docetaxel by modifying biomarker distribution. Athymic nude mice bearing human breast MCF-7 or prostate PC-3 tumors were treated with doxorubicin or docetaxel respectively and TH-302 alone or in combination. Biomarkers of drug effect including γH2aX (a marker of DNA damage), cleaved caspase-3 or -6 (markers of apoptosis) and reduction in Ki-67 (a marker of cell proliferation) were quantified in tumor sections in relation to functional blood vessels (recognized by DiOC7) and hypoxia (recognized by EF5) using immunohistochemistry. γH2aX expression at 10 min and cleaved caspase-3 or -6 at 24 hr after doxorubicin or docetaxel decreased with increasing distance from tumor blood vessels, with minimal expression in hypoxic regions; maximum reduction in Ki67 levels was observed in regions closest to vasculature at 24 hr. TH-302 induced maximal cell damage in hypoxic and neighboring regions, but was also active in tumor regions closer to blood vessels. TH-302 given 4 hr before doxorubicin or docetaxel increased DNA damage and apoptosis throughout the tumor compared to chemotherapy alone. When given with doxorubicin or docetaxel, TH-302 complements and enhances anticancer effects in both perivascular and hypoxic regions but also increases toxicity.

Most solid tumors contain hypoxic regions, and hypoxic cells may be important in limiting the effects of both radiotherapy and chemotherapy. The presence of oxygen increases the effects of radiation via increased fixation of free radicals on DNA strands and hypoxic cells are relatively radio-resistant.[1,2] Hypoxic cells in tumors are deficient in other nutrients and are slowly or non-proliferating: they may be spared by chemotherapy because of poor penetration of drugs to hypoxic tumor regions,[3] and because most anticancer drugs are more effective against rapidly proliferating cells. In addition to their radio-resistance, the presence of hypoxia leads to a more aggressive phenotype due to the expression of pro-survival genes (including mutated p53), increased levels of proteins favoring an epithelial-to-mesenchymal transition, increased metastasis, angiogenesis and inhibition of apoptosis.[4] For these reasons, hypoxic cells may be an important cause of treatment failure in solid tumors and may repopulate the tumor. Therapies that target hypoxic tumor cells therefore have potential to augment the effects of radiotherapy and chemotherapy.[1] Also, hypoxic cells occur rarely in normal tissues, so that targeting them might offer tumor selectivity. Hypoxia-targeted therapies such as gene therapy (using a promoter highly responsive to Hypoxia Inducible Factor-1 [HIF-1]), recombinant anaerobic bacteria (whose spores activate in necrotic centers of tumors and kill tumor tissue) and hypoxia-activated pro-drugs (HAPs) are all under investigation.[1,5]

HAPs are administered in an inactive form and are reduced to their active metabolites within hypoxic regions of tumors.[1] Once activated they may diffuse to act against cells in neighboring tumor regions ("the bystander effect"). The first HAP to undergo clinical testing was tirapazamine; and although activity against hypoxic cells in human tumors could be shown,[6] large randomized trials evaluating tirapazamine in combination with radiotherapy or chemotherapy did not establish therapeutic benefit.[7–9] Tirapazamine had

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What's new?

When tumor cells are far from blood vessels and low on oxygen, they can evade the reach of therapeutic drugs that focus on the most rapidly-dividing cells and rely on the presence of oxygen. A drug that selectively targets hypoxic cells could help. In this paper the authors measured the tissue distribution of a drug, TH-302, which can help enhance the killing ability of some chemotherapeutic drugs. The authors found that administering TH-302 in conjunction with doxorubicin or docetaxel helped spread the drug activity throughout the tumor, to hypoxic regions as well as cells near blood vessels.

several properties that limited its effectiveness including poor tissue penetration,[10] and toxicity unrelated to its hypoxic activation, which led to declining interest in this strategy.

Newer HAPs such as TH-302 have been designed with more favorable properties than tirapazamine. TH-302 is a 2-nitroimidazole containing a toxic alkylating moiety that is released only when its attached nitro-heterocyclic trigger molecule fragments in hypoxic cells to release the DNA crosslinking agent bromo-isophosphoramide mustard.[11] Traditional chemotherapy combined with TH-302 can lead to greater cell kill and growth delay of various rodent tumors and human xenografts,[12] and promising results have been reported from early clinical trials evaluating TH-302 combined with gemcitabine for treatment of pancreatic carcinoma.[13] However, there have been few studies evaluating the microenvironmental distribution of activity of TH-302 within solid tumors and the mechanisms which lead to antitumor effects remain uncertain.

Previous studies from our laboratory using immunohistochemistry (IHC) have shown that the combination of mitoxantrone with another HAP, AQ4N, resulted in effective drug exposure throughout solid tumors and improvement in therapeutic index when used to treat human breast tumor xenografts.[14] These studies were possible because both mitoxantrone and AQ4N are fluorescent. It is important for the development of TH-302 to characterize its distribution in tumor tissue, including its ability to diffuse to hypoxic regions, where it is activated, and for its active metabolite(s) to diffuse to neighboring better-nourished and oxygenated tumor regions (*i.e.*, the bystander effect). We have validated techniques to analyze the pharmacodynamic changes that occur after treatment with non-fluorescent drugs by using the biomarkers γH2Ax (a marker of DNA damage), cleaved caspase-3 or -6 (markers of apoptosis) and reduction in Ki-67 (a marker of cell proliferation).[15] Here we quantify the distribution of these biomarkers following treatment of two human xenografts with TH-302 alone or with doxorubicin or docetaxel.

Material and Methods

Cell lines

Experiments were conducted using human breast carcinoma (MCF-7) and human prostate cancer (PC-3) cell lines. Cell lines were purchased from the American Type Culture Collection (Manassas, VA). MCF-7 cells were cultured in α-minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). PC-3 cells were cultured in Ham's F-12K medium (Life Technologies Inc.) supplemented with 10% FBS. Cells were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Routine tests to exclude mycoplasma were performed. Short tandem repeat analysis was performed to characterize the origin of these cells. MCF-7 cells were chosen to represent estrogen receptor positive breast cancer and PC-3 cells to represent prostate cancer.

To generate MCF-7 tumors, 4-6 week old female athymic nude (nu/nu) mice (Harlan Sprague-Dawley, Madison, WI) were implanted with 17β-estradiol tablets (60-day release; Innovative Research of America, Sarasota, FL) and then injected subcutaneously with 5×10^6 MCF-7 cells per side. To generate PC-3 tumors, 4–6 week old male athymic nude (nu/nu) mice (Jackson, Bar Harbor, Maine) were injected subcutaneously in both flanks with 2×10^6 PC-3 cells. There were five mice per treatment group (8–10 tumors) in each experiment and all experiments were repeated.

Drugs and reagents

Doxorubicin (Pharmacia, Mississauga, Ontario, Canada) and docetaxel (Sanofi-Aventis, Laval, Quebec, Canada) were purchased from the Princess Margaret Cancer Centre pharmacy; they were provided as solutions with concentration of 2 mg/mL and 40 mg/mL, respectively. TH-302 was provided by Threshold pharmaceuticals (San Francisco) as a powder and then dissolved in 0.9% saline before use.

EF5 was provided by the National Cancer Institute as a powder and then dissolved in distilled water supplemented with 2.4% ethanol and 5% dextrose to make a 10-mM stock solution that was stored at room temperature. Cy5-conjugated mouse anti-EF5 antibody was purchased from Dr. Cameron Koch, University of Pennsylvania, PA. DiOC7 was purchased from AnaSpec (San Jose, CA) and a stock solution (2.5 mg/mL) was made by dissolving in dimethyl sulfoxide; this stock was diluted 1:10 in phosphate-buffered saline and 10% Solutol HS 15 (Sigma-Aldrich, Oakville, ON, Canada). γH2aX was recognized with a rabbit anti-human γH2aX primary antibody (Cell Signaling, Danvers, MA). Cleaved caspase-3 was recognized with primary rabbit anti-human cleaved caspase-3 antibody (Cell Signaling, Danvers, MA) and cleaved caspase-6 with rabbit anti-human cleaved caspase-6 antibody (Novus Biologicals, Oakville, ON,

Canada). Ki-67 was identified with primary rabbit anti-human Ki-67 antibody (NovusBiologicals, Oakville, ON, Canada). Application of all primary antibodies was followed by Cy3-conjugated goat anti-rabbit IgG secondary antibody and visualized using the Olympus fluorescent upright microscope.

Effect of drugs on biomarker distributions

Animals bearing tumors with a mean cross-sectional area 0.7–0.8 cm^2 were given a single intravenous injection of doxorubicin (8 mg/kg) or intraperitoneal injection of docetaxel (15 mg/kg) and/or TH-302 (150 mg/kg) or saline (controls); these doses were used based on the maximum tolerated dose established and used to provide therapeutic benefit in previous studies.[11,16–18] In most experiments evaluating drug combinations, TH-302 was administered 4 hr before doxorubicin or docetaxel, but we also evaluated γH2aX expression following concurrent delivery of TH-302 and doxorubicin in MCF-7 tumors; this was done to test observations of a previous study that TH-302 administration 4 hr before chemotherapy was superior to concurrent administration.[19] Mice were killed and tumors excised from saline (control) and treated animals at 10 min and 24 hr following drug injection; EF5 and DiOC7 were administered 2 hr and 1 min, respectively, before animal death.

Samples were embedded immediately in OCT compound, flash frozen in liquid nitrogen and stored at −70°C before tissue sectioning and IHC staining. Cryostat sections (10 μm) were cut from each tumor. Whole tumor sections were imaged and analyzed with artifacts and regions of necrosis omitted. At least six tumors were analyzed per treatment group.

Functional blood vessels were identified in tumors by injection of DiOC7—a fluorescent carbocyanine dye that stains cells immediately adjacent to functional vasculature.[20] Hypoxic regions were identified by binding of EF5.

Separate tissue sections were stained for γH2aX, cleaved caspase-3 (or -6) and Ki-67 with the appropriate antibodies. We evaluated cleaved caspase-6 expression in MCF-7 tumors because MCF-7 cells do not express caspase-3 due to a deletion in exon 3 that encodes the caspase-3 gene[21]; the effector caspase-6 processes caspases-8 and -10 leading to apoptosis.[22] Tumor sections were imaged for biomarkers using the Cy3 filter set (530–560 nm excitation/573–647 nm emission).

Media Cybernetics Image Pro PLUS software was used for image analysis and quantification. A minimal threshold for detection (below the level of detection of drug or biomarker) was determined for each tumor to minimize noise due to auto-fluorescence.

Biomarkers were analyzed using a protocol described previously.[15,23] Briefly, binarized DiOC7 or EF5 images were created and then used to create distance maps. Biomarker images (γH2aX, cleaved caspase-3 or -6 and Ki-67) were used to create binary masks which were then combined with the distance map to form a composite image with distance measurements that corresponded only to the biomarker. The data are represented graphically as the percent of pixels positive for any given biomarker at a given distance from the nearest functional blood vessel or hypoxic region in the section; a cut-off distance of 60 μm was used to minimize interference from neighboring blood vessels or hypoxic regions that are out of the plane of the section.

Growth delay

MCF-7 or PC-3 tumor-bearing mice were assigned randomly to treatment groups of 5–6 mice. Treatments with saline (control), standard chemotherapy (doxorubicin, 8 mg/kg or docetaxel, 15 mg/kg), TH-302 (150 mg/kg) or TH-302 in combination of chemotherapy were administered weekly for three weeks: doses were selected based on previous experience[12,16,17] to minimize animal weight loss. The longest and perpendicular tumor diameters were estimated using calipers three times per week until tumors reached a maximum diameter of 1 cm or tumors became necrotic, when animals were killed humanely. Tumor volume was estimated by the formula: (longest tumor diameter) × (smallest tumor diameter)^2/2. Body weight of the mice was also measured.

Statistical analysis

Biomarker distributions and growth delay. A one-way ANOVA was used to determine the statistical differences between treatment groups. Tests were two-sided and no corrections were applied for multiple significance testing. A p-value of less than 0.05 was used to indicate statistical significance. Tumor volumes and biomarker distributions are represented as mean values ± SEM.

Results

Distribution of biomarkers in tumor sections

Expression of the biomarkers γH2aX, cleaved caspase-3 or -6 and Ki-67 were recognized with specific antibodies as shown in Figures 1*a*–1*c* in control, docetaxel, TH-302 or combination-treated PC-3 tumors. Expression of γH2aX was evaluated at 10 min after treatment with TH-302 alone or after chemotherapy (given alone or 4 hr after TH-302), while increase in the apoptotic markers and decrease in Ki-67 was evaluated at 24 hr after these treatments; these time points have been shown to give optimal expression of the biomarkers after drug treatment.[15]

The distribution of biomarkers after treatment of MCF-7 tumors with doxorubicin, TH-302 and the combination in relation to the nearest patent functional vessel (left panels) and the nearest region of hypoxia (right panels) is shown in Figure 2. In control tumors expression of γH2aX and cleaved caspase-6 was low and Ki67 expression decreased with increasing distance from blood vessels (Fig. 2, left panels) to low levels near hypoxic regions (Fig. 2, right panels). Following treatment with doxorubicin, expression of γH2aX and cleaved caspase-6 was increased significantly ($p < 0.05$) above control levels with maximal expression in regions closest to blood vessels (Figs. 2*a* and 2*b*); expression of these markers declined in

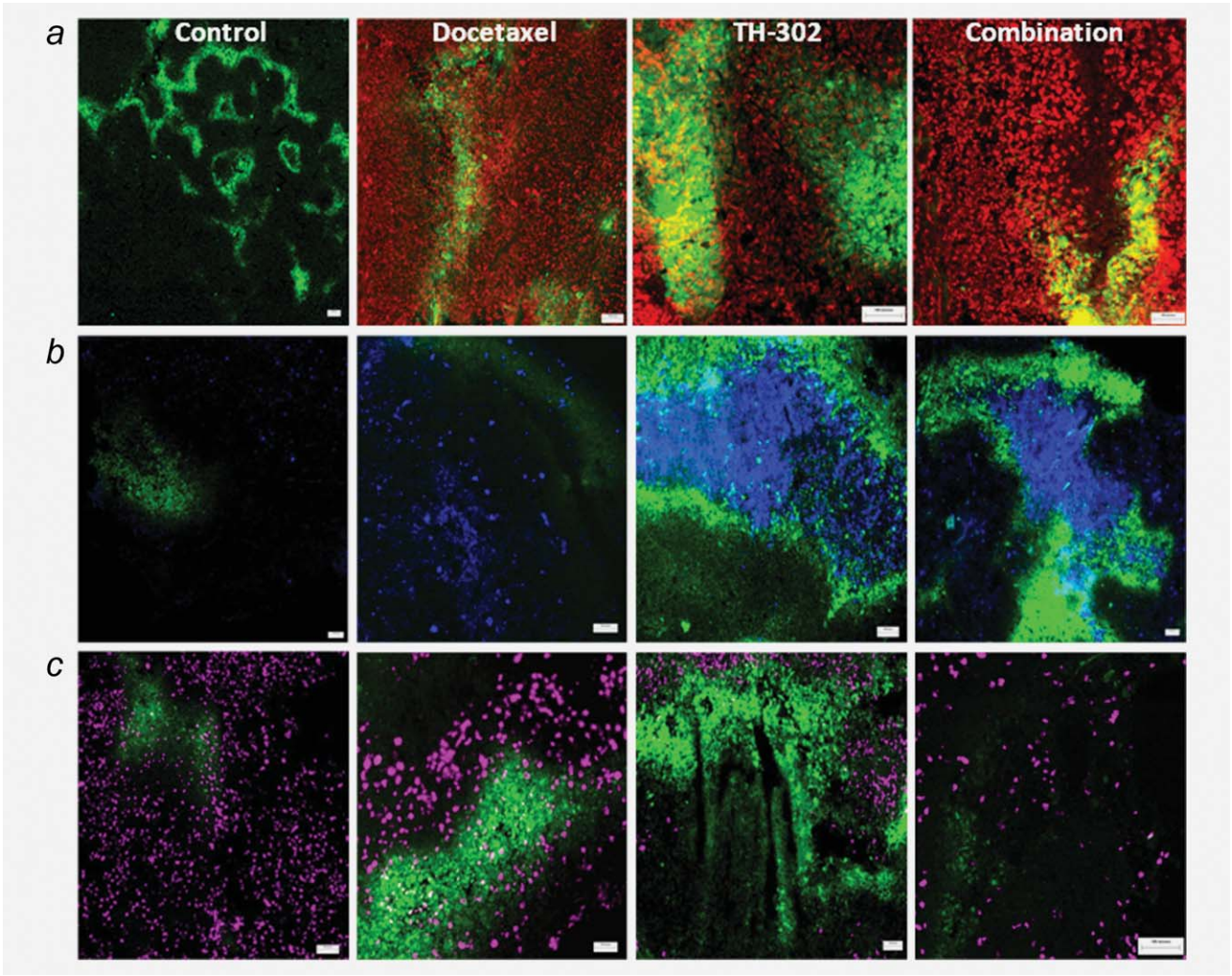

Figure 1. Photomicrographs of (*a*) γH2aX (red), (*b*) Cleaved caspase-3 (blue) and (*c*) Ki-67 (magenta) in PC-3 tumor xenografts following treatments: saline (control), docetaxel, TH-302 or docetaxel preceded 4 hr earlier by TH-302 (combination). γH2aX was evaluated at 10 min post-treatment and cleaved caspase-3 and Ki-67 24 hr post-treatment; co-localization with hypoxia (green) produces yellow. Scale bar = 100 μm.

regions distal to blood vessels with levels similar to control in hypoxic regions (Figs. 2*d* and 2*e*). Cell proliferation following treatment with doxorubicin was reduced in regions close to blood vessels (Figs. 2*c*) but was near (already low) control levels in regions close to hypoxia (Fig. 2f). TH-302 treatment alone caused an increase in γH2aX at 10 min and this effect was augmented at 4 hr following treatment. TH-302 treatment alone also increased expression of cleaved caspase-6 and while these effects were maximal adjacent to hypoxic regions they were observed in all tumor regions. Ki-67 was suppressed throughout the tumor. Combination therapy with doxorubicin and TH-302 led to the greatest increase in expression of γH2aX and cleaved caspase-6 in regions both close to blood vessels (Figs. 2*a* and 2*b*) and hypoxia (Figs. 2*d* and 2*e*), with greater increase in γH2aX levels seen when TH-302 was administered 4 hr before doxorubicin as compared to concurrent treatment. Ki-67 expression was suppressed throughout the tumor following combined treatment (Figs. 2*c* and 2*f*).

Results for PC-3 tumors treated with docetaxel and/or TH-302 were qualitatively similar to those obtained in MCF-7 tumors and are shown in Figure 3. There was low expression of γH2aX and caspase-3 in control tumors, and the distribution of cell proliferation (Ki67) decreased with distance from the nearest blood vessel (Fig. 3, left panels) with slow proliferation near to hypoxic regions (Fig. 3, right panel). Following treatment with docetaxel alone, the expression of γH2aX and of caspase-3 was increased significantly ($p < 0.05$) above that in the control, but it decreased with increasing distance from a functional blood vessel, and was low in hypoxic regions. Suppression of Ki67 followed a similar pattern. As expected, treatment with TH-302 alone increased expression of biomarkers further from blood vessels and near to hypoxic regions, but also led to significantly increased expression of the biomarkers and suppression of cell proliferation in all regions compared to controls ($p < 0.05$). The combination of docetaxel and TH-302 produced the most

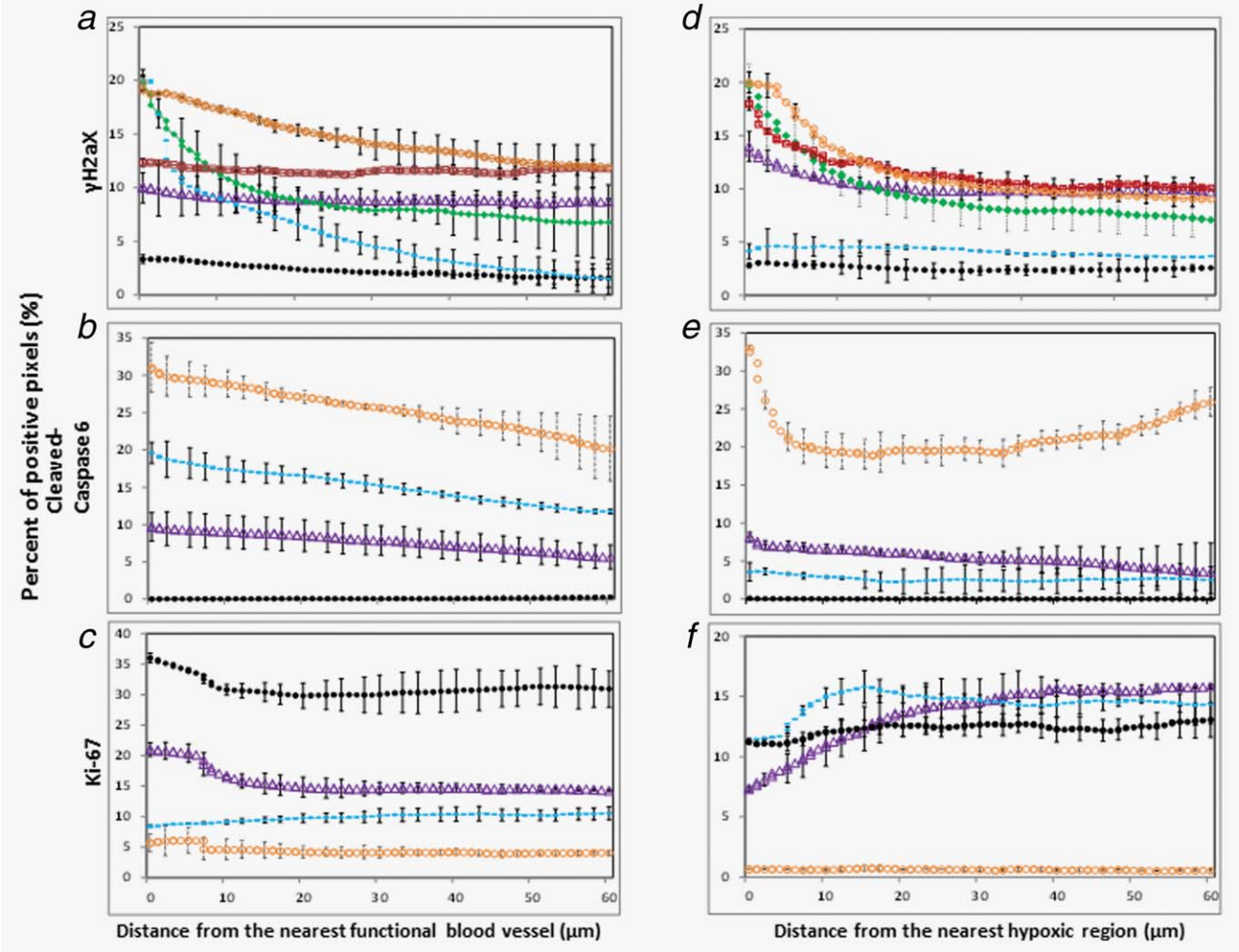

Figure 2. MCF-7 tumors treated with: saline control (●), doxorubicin (8 mg/kg; –), TH-302 (150 mg/kg at 10 mins △ or 4 hr □) or TH-302 + doxorubicin combination (concurrent administration ◆ or TH-302 4 hr before doxorubicin ○). The distributions of biomarkers of drug effect in relation to the nearest functional blood vessel (panels *a–c*) or region of hypoxia (panels *d–f*) are shown. Distribution of γH2aX at 10 min and 4 hr after treatment (panels *a* and *d*); cleaved caspase-3 (panels *b* and *e*) and Ki67 at 24 hr after doxorubicin treatment (panels *c* and *f*) are compared to untreated controls. Points indicate mean for 5–6 mice per group; bars, SE. Treatments in panels *a–f* are statistically significantly different from one another ($p < 0.05$).

profound effects on the biomarkers and on suppression of cell proliferation ($p < 0.001$), with increased evidence of activity both in regions proximal to and distal from blood vessels (including hypoxic regions).

Effects of doxorubicin, docetaxel and TH-302 on the growth of xenografts

Doxorubicin had minimal effects to inhibit growth of MCF-7 xenografts as compared to controls and TH-302 treatment alone also had limited effect. The combination of TH-302 with doxorubicin led to the greatest delay in growth (Fig. 4*a*). Docetaxel treatment of PC-3 xenografts resulted in growth delay that was increased when combined with TH-302 (Fig. 4*b*).

Animal body weights were also measured. Chemotherapy treatment alone led to a significant 4% (doxorubicin) and 1.5% (docetaxel) decrease in body weight compared to control mice ($p < 0.05$) but TH-302 alone had no significant effect (Table 1). Combined treatment led to a significant 11% (mice with MCF-7 tumors) and 8.4% (mice with PC-3 tumors) decrease in body weight compared to controls ($p < 0.0001$) and a 6.8% (MCF-7 tumors) and 7.0% (PC-3 tumors) decrease in body weight compared to doxorubicin or docetaxel alone treated animals ($p < 0.05$) (Table 1).

Discussion

Causes of resistance to chemotherapy include not only molecular changes in cancer cells but also mechanisms related to the tumor microenvironment. We have shown previously that γH2aX, cleaved caspase-3 (or -6) and Ki-67 are useful biomarkers to quantify the distribution of drug activity within solid tumors.[15] By using these markers and other methods, our group and others have shown limited

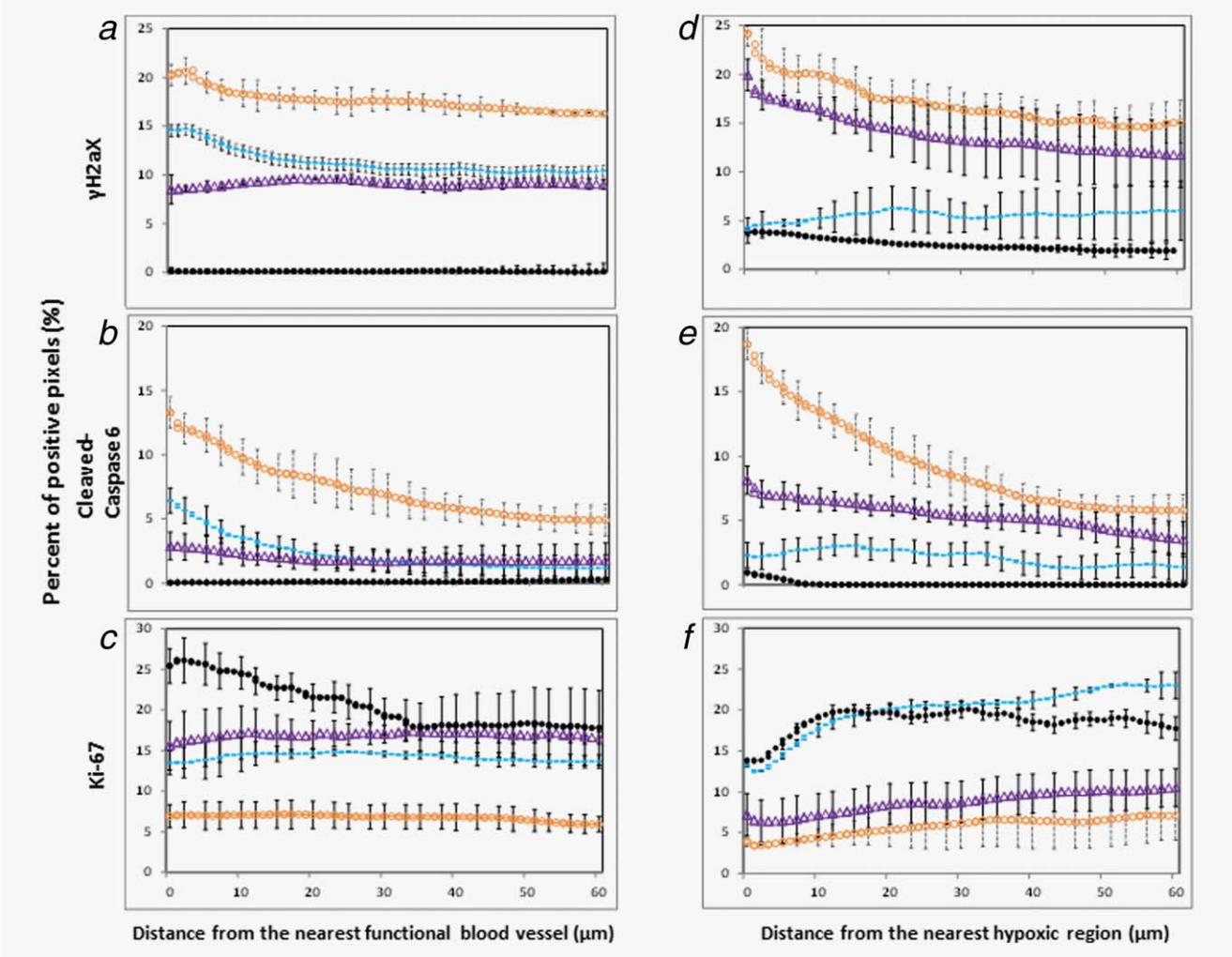

Figure 3. PC-3 tumors treated with: saline control (●), docetaxel (15 mg/kg; –), TH-302 (150 mg/kg; Δ) or TH-302 4 hr before docetaxel (○). The distribution of biomarkers of drug effect in relation to the nearest functional blood vessel (panels *a–c*) or region of hypoxia (panels *d–f*) are shown. Distribution of γH2aX at 10 min after treatment (panels *a* and *d*); cleaved caspase-3 (panels *b* and *e*) and Ki67 at 24 hr after docetaxel treatment (panels *c* and *f*) are compared to untreated controls. Points indicate mean for 5–6 mice per group; bars, SE. Treatments in panels *a–f* are statistically significantly different from one another ($p < 0.05$).

distribution of activity of multiple anticancer drugs from blood vessels.[14,15,17,18,24] Thus anticancer drugs are often effective against cells adjacent to vasculature but penetrate poorly through tumor tissue so that they are not delivered in effective concentrations to tumor cells in regions distal to functional blood vessels; moreover, such cells have low rates of cell proliferation and are resistant to cycle-active chemotherapy. Therefore the development of drugs that can kill cells located distant from vasculature, including hypoxic cells, is of clinical importance. Here, we have evaluated the ability of the HAP TH-302 to enhance the effects of doxorubicin and docetaxel against human tumor xenografts, and have studied the distribution of its activity within the tumor microenvironment.

Consistent with previous results from our group and others, docetaxel and doxorubicin alone induced γH2aX, cleaved caspase-3 (or -6) and reduced Ki-67 expression and this effect occurred predominantly in regions close to functional blood vessels; there were minimal effects in and near to hypoxic regions.[15,23]

TH-302 is a pro-drug that is activated specifically in hypoxic cells, and has demonstrated promising results both in preclinical studies and in early clinical trials.[11–13,25,26] However, since TH-302 is non-fluorescent, its direct visualization in tumor tissue is not possible. Therefore, we extended our techniques of assessing biomarker distribution following drug treatment to evaluate the effects of TH-302 in hypoxic and vascular regions of solid tumors. As expected we found that TH-302 induced expression of γH2aX, cleaved caspase-3 (or -6) and reduced Ki-67 expression immediately in and adjacent to hypoxic regions, supporting the mechanism of action that TH-302 is activated in hypoxic areas and diffuses to nearby tissues (*i.e.*, consistent with its bystander effect). More surprisingly, our data show that TH-302 also

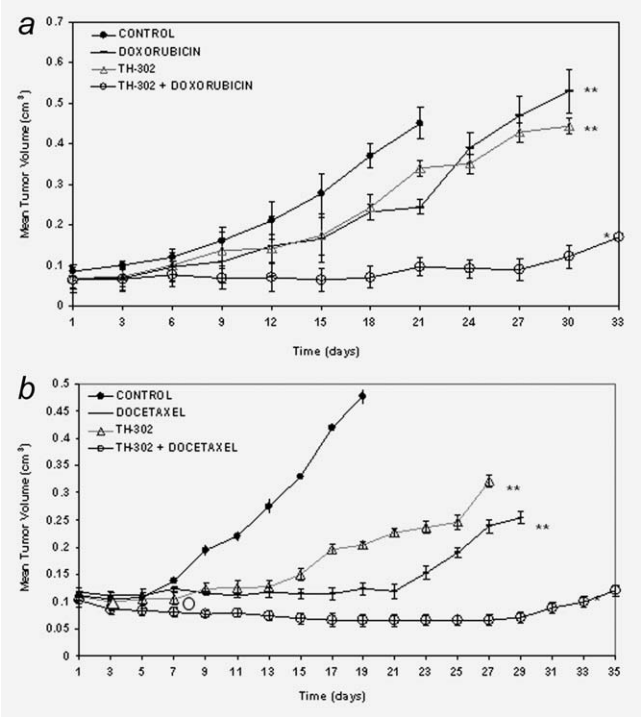

Figure 4. (*a*) Growth delay of MCF-7 xenografts in nude mice treated with: saline control, doxorubicin (8 mg/kg i.v.), TH-302 (150 mg/kg) or the combination of doxorubicin preceded 4 hr earlier by TH-302 (treatments were given once weekly for three weeks). (*b*) Growth delay of PC-3 xenografts in nude mice treated with: saline control, docetaxel (15 mg/kg i.p.), TH-302 (150 mg/kg) or combination docetaxel preceded 4 hr earlier by TH-302 (treatments were given once weekly for 3 weeks). Points indicate mean for 5 mice per group; bars, SE. * and ** signify statistical significance ($p < 0.05$). Legend: • Control, - Doxorubicin or Docetaxel, ◆ TH-302, ◯ TH-302 4 hr + Doxorubicin or Docetaxel.

Table 1. Mean loss in body weight (%) of MCF-7 or PC-3 tumor bearing animals treated with either: saline (control), chemotherapy (doxorubicin [DOX] or docetaxel [DOC]), TH-302 or combination (TH-302 4 hr before DOX or DOC)

Treatment	MCF-7 tumors (%)	*p*-value	PC-3 tumors (%)	*p*-value
Control	–	–	–	–
DOX or DOC	−4	$p \leq 0.05$	−1.5	$p \leq 0.05$
TH-302	+0.8	$p \geq 0.05$	−0.7	$p \geq 0.05$
TH-302 + DOX or DOC[1]	−11	$p \leq 0.0001$	−8.4	$p \leq 0.0001$

p values compared to control are displayed.
[1]Combination treatment group is also statistically significant compared to DOX or DOC alone: −6.8% (MCF-7) and −7% (PC-3) loss in body weight ($p \leq 0.05$).

induced these biomarkers in all regions of the tumors, including those close to functional blood vessels, and that it augmented the effects of chemotherapy in all tumor regions. The ability of TH-302 to induce expression of γH2aX has also been reported by Sun *et al.*,[11] who administered TH-302 to mice as well as pimonidazole (to detect hypoxia) given an hour before animal death. These authors showed that induction of γH2aX was located preferentially near pimonidazole positive cells. Our results support the findings of Sun *et al.*[11] with respect to γH2aX expression near hypoxic regions (here identified by EF5); however, our evaluation of γH2aX and other biomarkers (cleaved caspase -3 or -6 and reduction of Ki-67) in regions close to functional blood vessels identified by DiOC7 revealed that TH-302 can also change the expression of these biomarkers in well-oxygenated regions of tumors. We have evaluated previously biomarker distributions following treatment with another nitrogen mustard melphalan, and found increased γH2aX, cleaved caspase and reduced Ki-67 expression close to blood vessels. Although both melphalan and TH-302 are nitrogen mustards, differences in their distributions may be related to their activation: TH-302 is a pro-drug and is designed to become activated in hypoxic cells and is able to penetrate into these areas as it is not activated in oxic cells, whereas melphalan is not a pro-drug and readily attacks DNA in cells proximal to the vasculature, thus reducing the amount of drug available to diffuse to distantly located hypoxic regions.

It is possible that the combination of TH-302 with doxorubicin or docetaxel led to greater DNA damage and cell death due to activation of TH-302 in hypoxic cells and its resulting bystander effect—*i.e.*, the ability of the activated drug to diffuse to surrounding non-hypoxic tissues.[27] The 2-nitroimidazole portion of TH-302 undergoes electron rearrangement forming a radical anion in regions with less than about 0.5% oxygen.[27] The radical anion can then fragment to the toxic bromo-isophosphoramide mustard which can diffuse to surrounding tissues. Therefore, the diffusible toxic bromo-isophosphoramide coupled with doxorubicin or docetaxel may have a heightened effect on the expression of biomarkers γH2aX, cleaved caspase-3 (or -6) and reduction in Ki-67.

Sun *et al.* have reported that TH-302 delivery 4 hr before chemotherapy results in greater tumor growth inhibition than does concurrent administration of these agents.[19] Our findings support this conclusion in that we found greater γH2aX expression in both perivascular and hypoxic regions when TH-302 was given 4 hr before doxorubicin than following concurrent treatment, thus providing further evidence for the use of this schedule.

Interestingly, TH-302 treatment alone resulted in an increase in γH2aX, cleaved caspase-3 (or -6) and reduction in Ki-67 in regions close to blood vessels. Under aerobic conditions, TH-302 is fragmented to its radical anion and then back oxidized to its pro-drug form.[27] A byproduct of this reaction is the generation of superoxide via the reduction of oxygen. Superoxides can react with free iron generating hydroxyl radicals; these radicals in turn can act as oxidants and damage DNA.[28] It is possible that the production of excess hydroxyl radicals leads to the relatively uniform expression of γH2aX as a function of distance from blood

vessels that is observed following TH-302 treatment alone. While this effect might further augment the antitumor effects of chemotherapy, it is less likely to be tumor-specific since it might also occur in well-oxygenated normal tissues. TH-302 not only enhances the antitumor effects of chemotherapy but also increases toxicity in normal tissues. It is also possible that TH-302 is activated and diffuses to surrounding oxygenated tumor tissue within seconds, and that the relatively uniform expression of γH2aX at 10 min after injection of TH-302 is due to the bystander effect. Weiss *et al.*[26] reported the presence of both TH-302 and its toxic metabolite bromoisophosphoramide in the plasma following drug administration in patients; it is possible that this toxic moiety that was activated during circulation (*i.e.*, via hepatic activation) then caused activation of the biomarkers in perivascular regions in our study. TH-302 has also exhibited activity as monotherapy in a phase I clinical trial[26]; our results including elevated levels of apoptosis in tumors provide further validation for its activity when used alone.

A phase I clinical trial that combined TH-302 with doxorubicin in soft tissue sarcomas suggested added benefit in the form of a response rate of 33% compared to the projected 12-23% in patients treated with doxorubicin alone.[29] The enhanced effects on DNA damage, apoptosis and reduction in cell proliferation that we found when TH-302 was combined with doxorubicin or docetaxel in the present study may provide some explanation for the enhanced effect found in the clinic.

Our study has limitations. Assessment of biomarker distributions was undertaken in two-dimensional tumor sections, whereas solid tumors are three-dimensional. We limited our analysis to a distance of 60 μm from blood vessels or hypoxic regions to decrease the confounding effects of undetected blood vessels or hypoxic regions outside of the tumor section, but a three-dimensional analysis would be preferred. Also, although the biomarkers evaluated show consistent changes, none of them provide direct evaluation of loss of reproductive activity of tumor cells, the most important endpoint of cell death. However, the elevated biomarkers after combined treatment correspond to significant increases in tumor growth delay at the cost of added toxicity.

In summary, our evaluation of biomarkers of drug effect following chemotherapy with doxorubicin or docetaxel applied to two human xenografts showed greatest drug activity proximal to functional blood vessels, and decreased activity within or close to regions of hypoxia. The distribution of cellular damage was modulated by the addition of TH-302 4 hr before doxorubicin or docetaxel and resulted in greater activation of biomarkers in hypoxic regions, but also in regions closer to blood vessels. The activity of both doxorubicin and docetaxel can be complemented and enhanced by the activity of the HAP TH-302, resulting in better distribution of drug activity throughout the tumor but also increased toxicity as well. Our results support the hypothesis that use of HAPs with cycle-active chemotherapy might reduce drug resistance and treatment failure in solid tumors.

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