Chemotherapy Rescues Hypoxic Tumor Cells and Induces Their Reoxygenation and Repopulation—An Effect That Is Inhibited by the Hypoxia-Activated Prodrug TH-302

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Abstract

**Purpose:** Chemotherapy targets rapidly proliferating tumor cells, but spares slowly proliferating hypoxic cells. We hypothesized that nutrition of hypoxic cells would improve in intervals between chemotherapy, and that hypoxic cells destined to die without treatment would survive and proliferate.

**Experimental Design:** We therefore evaluated repopulation and reoxygenation following chemotherapy, and the effects of the hypoxia-activated prodrug TH-302 on these processes. Tumor-bearing mice were treated with doxorubicin or docetaxel ± TH-302. Pimonidazole (given concurrent with chemotherapy) and EF5 (given 24 to 120 hours later) identified hypoxic cells. Proliferation (Ki67) and oxygen status (EF5 uptake) of formerly hypoxic (pimo positive) cells were quantified by immunohistochemistry.

**Results:** Chronically hypoxic cells had limited proliferation in control tumors. After chemotherapy, we observed reoxygenation and increased proliferation of previously hypoxic cells; these processes were inhibited by TH-302.

**Conclusions:** Chemotherapy leads to paradoxical sparing of hypoxic cells destined to die in solid tumors in absence of treatment, and their reoxygenation and proliferation: TH-302 inhibits these processes. Clin Cancer Res; 1–8. ©2015 AACR.

Introduction

Hypoxia in tumors can occur due to limited perfusion of blood vessels (acute hypoxia) or because of limited oxygen diffusion and its consumption by tumor cells (chronic hypoxia) that leads to hypoxic regions at distances of approximately 100 to 200 μm from functional blood vessels (1). Hypoxic cells in tumors have a limited lifespan, and chronically hypoxic regions in untreated tumors appear to be in a state of dynamic equilibrium, where new hypoxic cells produced by proliferation and migration of cells from regions closer to blood vessels is matched by cell death from the hypoxic compartment (2). Hypoxic cells are resistant to radiotherapy because its toxic effects are mediated in part by oxygen-dependent free radicals (1). Chemotherapy targets rapidly proliferating cells and there is poor penetration of many drugs to tumor cells that are located distal to functional blood vessels, including tumor cells in hypoxic regions (3–7); hence, hypoxic cells are also likely to be resistant to chemotherapy. Both chemotherapy and radiotherapy are delivered in multiple doses, with chemotherapy often scheduled in 3-week cycles to allow for the repopulation and recovery of critical normal tissues such as bone marrow. During the intervals between treatments, repopulation of surviving tumor cells can occur, and is an established mechanism that reduces the effectiveness of fractionated radiotherapy (8–10). Repopulation in the longer intervals between cycles of chemotherapy has also been implicated to limit the effectiveness of treatment (11, 12), but few studies have focused on repopulation following chemotherapy as it relates to the tumor microenvironment. Because hypoxic cells are likely to be resistant to chemotherapy, they might reoxygenate and proliferate, thereby contributing to treatment failure. As hypoxia is associated with poor clinical outcome, there is a need for the development of hypoxia-targeted agents capable of killing these cells.

Hypoxia-activated prodrugs (HAP) are administered in an inactive form, and are activated via chemical reduction in hypoxic regions to a toxic moiety that damages DNA (13). HAPs are oxidized to their native form in oxygenated cells and can diffuse to distantly located hypoxic regions. The HAP TH-302 is in clinical trials and consists of a nitroimidazole conjugated to the cytotoxic bromo-isophosphoramide mustard that is released in hypoxic tissues (14), so that TH-302 targets hypoxic cells selectively (15, 16). The combination of chemotherapy to target the oxygenated compartment of solid tumors and TH-302 to target the hypoxic compartment might inhibit survival and/or repopulation of hypoxic cells and augment the effects of chemotherapy.

In the present study, we evaluated the hypotheses that (i) cells in hypoxic regions of solid tumors are destined to die in the absence of chemotherapy, (ii) nutrition of hypoxic cells would...
Translational Relevance
Chemotherapy targets rapidly dividing cells and thus spares hypoxic tumor cells that contribute to chemotherapy treatment failure. Here, we demonstrate that hypoxic tumor cells reoxygenate and proliferate after chemotherapy that would have otherwise died in the absence of treatment. TH-302 is a novel hypoxia-activated prodrug that is activated in hypoxic cells and can inhibit reoxygenation and proliferation of formerly hypoxic cells. Pretreatment of tumors with TH-302 followed by chemotherapy increased DNA damage and cell death in addition to inhibiting tumor cell repopulation. This research elucidates the rationale for the use of TH-302 with chemotherapy and is currently in clinical trials.

Materials and Methods
Cell lines
Human breast carcinoma (MCF-7) and prostate cancer (PC-3) cell lines were purchased in 2012 from the American Type Culture Collection. Cells were cultured in α-MEM (MCF-7 cells) or Ham’s F-12K (PC-3) media (Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone) and incubated in a humidified atmosphere of 95% air/5% CO2 at 37°C. Routine tests to exclude mycoplasma and characterize the origin of the cells (short tandem repeat analysis) were performed every 6 months (date of last test, August 2014).

Female athymic nude mice (4- to 6-weeks old; Harlan Sprague-Dawley) were implanted with 17β-estradiol tablets (60-day release; Innovative Research of America) and injected subcutaneously with 5 × 106 MCF-7 cells per flank. Male athymic nude mice (4- to 6-week old; Jackson ImmunoResearch) were injected subcutaneously in both flanks with 2 × 106 PC-3 cells. There were four mice per treatment group (6–8 tumors) in each experiment and each experiment was repeated. Analysis of each tumor was based on two 10-μm thick sections.

Drugs and reagents
Doxorubicin (Pharmacia) and docetaxel (Sanofi-Aventis) were purchased from the Princess Margaret Cancer Centre pharmacy and provided as solutions with concentrations of 2 mg/mL and 40 mg/mL, respectively. TH-302 was provided by Threshold Pharmaceuticals as a powder and then dissolved in 0.9% saline before use.

Pimonidazole (pimo) and a FITC-conjugated mouse IgG1 monoclonal antibody that recognizes pimo adducts were purchased from Hypoxyprobe. The National Cancer Institute provided EF5 as a powder, which was prepared by dissolving it in distilled water supplemented with 2.4% ethanol and 5% dextrose to make a 10-mmol/L stock solution that was stored at room temperature. Cy5-conjugated mouse anti-EF5 antibody was purchased from Dr. Cameron Koch, University of Pennsylvania, PA. Ki67 was identified with primary rabbit anti-human Ki67 antibody (Novus Biologicals) followed by Cy3-conjugated goat anti-rabbit IgG secondary antibody and visualized using an Olympus fluorescent upright microscope.

Effect of drugs on tumor reoxygenation and repopulation
Tumor-bearing mice (mean cross-sectional area 0.7–0.8 cm²) were given a single intraperitoneal injection of pimo (60 mg/kg) at the same time as chemotherapy: i.v. injection of doxorubicin (MCF-7, 8 mg/kg) or i.p. injection of docetaxel (PC-3, 15 mg/kg) or saline (controls). Previous studies have established these doses as the maximum tolerated (5, 6, 16, 17). In combination groups, TH-302 (150 mg/kg) was administered i.p 4 hours before chemotherapy; this was based on previous studies establishing greater tumor cell death, DNA damage (7), and increased growth delay (18) in this administration. Mice were injected with a second marker of tumor hypoxia, EF5, to quantify hypoxic regions at 24, 48, 72, 96, or 120 hours following initial treatment and animals were killed 2 hours later. Tumors were excised and embedded immediately in OCT compound and then flash frozen in liquid nitrogen and stored at −70°C before tissue sectioning and immunohistochemical staining. Ten-micrometer thick cryostat sections were cut from each tumor. Whole-tumor sections were imaged and analyzed; artifacts and regions of necrosis were omitted.

Originally, hypoxic cells were identified by binding of pimo and visualized using FITC; current hypoxia was recognized using EF5 and visualized with a Cy-5 far-red filter set. Hypoxia changes were elucidated by identifying cells that were pimo+/EF5– (formerly hypoxic cells) and pimo+/EF5+ (chronically hypoxic cells). Ki67 was detected using the Cy-3 filter set.

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 hypoxia) was determined for each tumor to minimize noise due to autofluorescence.

Regions of hypoxia were analyzed as described previously (3, 7). Binarized pimo and EF5 images were used to make a composite displaying regions of only pimo staining (pimo+/EF5–) or EF5 staining (EF5+/pimo–): these composites represented the reoxygenated or newly hypoxic regions, respectively, and were reported as a percentage of the initially hypoxic [pimo+] region. The Ki67 biomarker image was then used to identify proliferating cells within the reoxygenated region and quantified and reported as a percentage of that in the total formerly hypoxic (pimo+/EF5–) region (i.e., to represent change in proliferation of the reoxygenated cells) or in chronically hypoxic region (pimo+/EF5+). For the assessment of Ki67 distribution in relation to current hypoxia in tumors, a binarized EF5+ image (black and white image with white corresponding to only biomarker positive pixels) was created, and used to create a distance map such that each pixel was represented by its distance to the nearest hypoxic region (EF5+) in the section. The data are represented graphically as the percentage of pixels positive for Ki67 at a given distance from the nearest currently hypoxic region in the section; a cutoff distance of 60 μm was used to minimize interference from neighboring hypoxic regions that are out of the plane of the section.

Statistical analysis
A one-way ANOVA was used to determine statistical differences among 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.

Results

**Hypoxia markers in tumor sections**

Photomicrographs displaying uptake of pimo and EF5 in MCF-7 xenografts following concurrent administration (Fig. 1, A–C). Spatial redistribution of Ki67-positive cells within the formerly hypoxic region (i.e., the reoxygenated cell population) following treatments are illustrated for MCF-7 xenografts in Fig. 2, A–D, respectively. Note that doxorubicin results in the greatest visible increase in Ki67 cells.

**Flux through the hypoxic compartment in untreated tumors**

The proportion of viable tumor that was originally hypoxic in control samples (i.e., pimo+) and the generation of new hypoxic (i.e., EF5+/pimo−) regions is illustrated in Figs. 4A and C (MCF-7 tumors) and Figs. 4B and D (PC-3 tumors). The fraction of originally hypoxic pimo-labeled cells decreased over time from 24 to 120 hours in both MCF-7 and PC-3 tumors, consistent with death of cells from the hypoxic compartment. In MCF-7 tumors, close to half of the originally (1.5%) pimo-labeled cells were lost (0.8%) by the 96-hour point and were further reduced by 120 hours (0.3%; $P < 0.01$ (Fig. 4A). Accumulation of new hypoxia (EF5+/pimo−) increased over time, by 24 hours more than half (56%) of the currently hypoxic cells were due to the flux of previously oxygenated cells into hypoxia and this increased to nearly 100% by 120 hours, $P < 0.01$ between groups (Fig. 4C). PC-3 xenografts had a similar response, the pimo+ fraction in the entire viable tumor at 24 hours (1.7%) dropped almost by half at
96 hours (0.7%) and further reduced to 0.58% at 120 hours, $P < 0.01$ between groups (Fig. 4B). Flux of previously oxygenated cells into hypoxia as determined by uniquely EF5+ labeling, increased over time from 52% at 24 hours to 76% and 80% by 96 and 120 hours, respectively ($P < 0.01$; Fig. 4D).

**Effects of treatment on reoxygenation and proliferation**

Reoxygenation of hypoxic tissues occurred in untreated MCF-7 control tumors (consistent with some fluctuating hypoxia) at all times observed (Fig. 5A and Supplementary Figs. S1–S4, A), however, proliferation as assessed by the presence of Ki67 ranged from 5% to 7% in these reoxygenated cells (Fig. 5B and Supplementary Figs. S1–S4, B). Reoxygenation of formerly hypoxic cells (pimo+/EF5−) in chemotherapy and/or TH-302-treated MCF-7 tumors is shown in Fig. 5A and Supplementary Figs. S1–S4, A. Although all treatments resulted in significant reoxygenation as compared with that in untreated control tumors ($P < 0.05$), treatment with doxorubicin alone led consistently to the highest rate of reoxygenation ($P < 0.0001$). Furthermore, proliferation in the reoxygenated compartment was increased following treatment with doxorubicin (Fig. 5B and Supplementary Figs. S1–S4, B) at all tested time points ($P < 0.0001$). Treatment with TH-302 alone increased reoxygenation compared with controls, but resulted in reduced cell proliferation in the formerly hypoxic region, and this effect was enhanced when combined with chemotherapy. Combined treatment resulted in the greatest inhibition of proliferation ($P < 0.05$, compared with all other groups).

Reoxygenation in PC-3 tumors is similar to that observed in MCF-7 tumors (Fig. 6 and Supplementary Figs. S5–S8, A). All treatments resulted in greater reoxygenation compared with control tumors with maximum reoxygenation in tumors treated with docetaxel alone ($P < 0.05$). Docetaxel alone resulted in increased...
proliferation of reoxygenated cells above that seen in the control treatment at each time observed (P < 0.05) and TH-302 reduced cell proliferation in the reoxygenated region and this effect was maximal upon combination with docetaxel (Fig. 6 and Supplementary Figs. S5–S8, A).

**Distribution of Ki67 in tumor sections**

Ki67 expression as a function of distance from the nearest hypoxic region in the tumors was evaluated at 24 (Figs. 5 and 6, C) and 48, 72, 96, and 120 hours (Supplementary Figs. S1–S8, C) in controls and following chemotherapy, TH-302 or the combination. Cell proliferation increases with increasing distance from hypoxic regions. Ki67 expression was significantly (P < 0.001) increased above that found in the control tumors at every time point (24, 48, 72, 96, and 120 hours) following treatment of MCF-7 tumors treated with doxorubicin and PC-3 tumors treated with docetaxel. TH-302 alone reduced Ki67 proliferating cells to a level below that of the control (P < 0.001) and this effect was amplified when combined with chemotherapy producing the lowest levels of proliferating cells near hypoxic regions (P < 0.001).

**Discussion**

Chemotherapy is delivered frequently in 3-week cycles where intervals between cycles allow for the repopulation of cells in the peripheral blood through proliferation and maturation of precursors in the bone marrow. Unfortunately, bone marrow cells are not the only ones to proliferate as surviving tumor cells can also proliferate. Repopulation of tumor cells following radiotherapy is well documented but repopulation following chemotherapy has not been studied extensively. Limited studies have found that the rate of proliferation of surviving tumor cells may increase following chemotherapy (19–22). The objectives of the present study...
were to quantify the flux of tumor cells through the hypoxic region in two solid tumor xenografts in the absence of treatment, the reoxygenation and repopulation that occurs in tumor cells that were formerly hypoxic following chemotherapy, and the potential of TH-302 to inhibit these processes.

We addressed the above questions by using two sequentially administered markers of hypoxia: this method allowed identification of cells that were hypoxic at time of treatment and then underwent reoxygenation following treatment with two widely used chemotherapeutic agents: doxorubicin and docetaxel. In control tumors, the originally hypoxic fraction in the viable tumor (as labeled by pimo) decreased over time, consistent with loss of pimo-labeled cells due to cell death or possibly to some dilution of the pimo-labeled adducts. We were also able to determine rates of reoxygenation by identifying the population of pimo+/EF5− cells at each time point. We report the flux of cells into hypoxia by determining the population of EF5+/pimo− cells in control tumors and this steadily increases over time, suggesting that in untreated tumors, cells continuously enter the hypoxic compartment, and eventually undergo cell death. There were substantial differences in rates of reoxygenation and repopulation between the MCF7 and PC3 tumors (see Figs. 5 and 6), but reoxygenation levels following drug treatment were highest following chemotherapy in both tumor types. This effect may be due to clearance of dead cells closest to blood vessels, and/or to increased perfusion of blood vessels following treatment.

We used Ki67 to investigate changes in proliferation among the chronically hypoxic and reoxygenated cells. Proliferation rates were low in chronically hypoxic cells and decreased further over time, indicating that cells that remain hypoxic for extended periods of time have markedly reduced proliferative ability. Proliferation of cells within newly reoxygenated tissues was contingent upon drug treatment. In both MCF7 and PC3 tumors, chemotherapy alone resulted in the greatest proliferation of reoxygenated tissue at all times following treatment compared with untreated controls. It is probable that following chemotherapy-induced cell death, surviving tumor cells benefit from an enhanced supply of nutrients (including oxygen) resulting from interrupted metabolism and/or clearance of dead cells that were located closer to blood vessels, thus leaving more nutrients for cells that survive therapy and undergo proliferation (3, 23). It is also possible that chemotherapy improves blood flow in tumor vessels, perhaps related to reduced interstitial fluid pressure following removal of killed cells (24). Durand and Aquino-Parsons tracked SiHa cells that were pimo labeled under transient hypoxic conditions in spheroids over 9 days using flow cytometry (25). Returning spheroids to normoxic (air) conditions resulted in a higher pimo+ cell turnover rate compared with the innermost (and presumably most hypoxic) layer (25); this was attributed to rapid cell turnover in the outermost layers presumably due to reoxygenation and rapid cell proliferation. Durand & Aquino-Parsons also investigated biopsies from patients treated with pimo before chemoradiotherapy and found that before treatment, pimo+ cells were primarily in nonproliferative G0/G1 phases of the cell cycle as assessed using flow cytometry; following treatment, the pimo+ label disappeared as cells entered proliferation (25), indicating that chemotherapy induces hypoxic cells to proliferate.

Our study enhances the current understanding of how chemotherapy contributes to treatment failure by identifying hypoxic cells that have been rescued (i.e., they may have died in the absence of treatment) and contribute to repopulation. Administration of other treatments can inhibit repopulation of tumor cells between courses of chemotherapy but it is important that such treatments be relatively specific for tumors. Due to our observations of reoxygenation and proliferation of formerly hypoxic cells following chemotherapy, we chose to evaluate the potential of TH-302 to inhibit these processes and thereby increase therapeutic efficacy by reducing the proliferation of reoxygenated, formerly hypoxic, cells.

TH-302 is a hypoxia activated prodrug that contains an oxygen-sensing 2-nitroimidazole group conjugated to a bromo-isophosphoramid mustard, which undergoes fragmentation releasing the toxic mustard moiety that binds to DNA and causes cross-linkage (15). We hypothesized that TH-302 would be able to inhibit proliferation of formerly hypoxic cells. Indeed, we found that although treating tumors with TH-302 did not appear to inhibit the process of reoxygenation, it reduced proliferation of the reoxygenated cells. It is possible that many cells in the reoxygenated region might have been lethally damaged by TH-302. In a previous study (7), we evaluated the distribution of TH-302 in relation to vascular and hypoxic regions and reported maximum DNA damage (as measured by γH2AX), cell death (as measured by cleaved caspases -3 or -6) and reduced cell proliferation in regions closest to hypoxia in MCF-7 and PC-3 xenografts, consistent with this hypothesis. Furthermore, we noted that the combination of TH-302 with either doxorubicin or docetaxel resulted in the greatest tumor growth delay and cell death and reduced proliferation compared with either treatment alone in both perivascular and hypoxic regions. In addition, chemotherapy treatment alone resulted in reduced cell proliferation closest to blood vessels but led to increased proliferation closest to blood vessels, presumably due to an increased oxygen and nutrient supply to these regions following the clearance of cells located closest to the blood vessel (7). In the present study, we found that combined treatment inhibited proliferation in the reoxygenated compartment—an important finding as it provides support for the activation of TH-302 in hypoxic tissues and its ability to reduce repopulation from this region. Huxham and colleagues reported that gemcitabine treatment of HCT-116 tumors induced proliferation of cells located at the border of necrotic (presumably hypoxic) regions (19). Fung and colleagues also reported that cell proliferation (repopulation) occurred in regions close to hypoxia following chemotherapy (3). Thus in our present study, the additive anticancer effects of combined TH-302 and chemotherapy may be attributed to the complementary roles of these agents. For example, chemotherapy will target rapidly dividing cells in perivascular regions while TH-302 predominantly attacks hypoxic tissues, although combined therapy did not abolish all Ki67 proliferating cells. This might be due to the multifactorial nature of repopulation, which can be attributed to several reasons aside from reoxygenation of hypoxic tissues, including loss of p53 activity leading to reduced apoptosis (26) or a need to increase the dosage and frequency of TH-302. We chose to give a single high dose of TH-302 4 hours before chemotherapy that yielded superior antitumor activity based on our previous study (7) as well as data from others (16, 18).

The current observation of the greatest reduction in cell proliferation in the formerly hypoxic area as well as the greatest inhibition in proliferation of cells in regions closest to hypoxia in the combined treatment group may be due to several factors. TH-302 produces free radicals as it is back-oxidized to its prodrug form in the presence of oxygen as it diffuses through the tumor.
Furthermore, TH-302 is activated in extremely hypoxic areas (<0.1% oxygen) and releases its cytotoxic moiety that is capable of diffusing to surrounding tissue that may have a higher oxygen content and kill these cells, thus having a bystander effect—an ability that is crucial in maximizing the effects of a hypoxia-targeted agent (15).

Our study has limitations, including our inability to assess changes in cell proliferation as it relates to the formerly hypoxic compartment beyond 5 days due to the limited stability of pimozone (27). Also, we assessed cell proliferation and hypoxia using a two-dimensional approach although tumors are three-dimensional. It was difficult to assess actual cell death at the time points we measured as we have previously demonstrated that cleaved caspase-3 or -6 signal—is most robustly measured at 24 hours following treatment; given our time course, we were impeded in this measurement.

Overall, our data demonstrate the effects of chemotherapeutic agents (doxorubicin and docetaxel) to induce repopulation by rescuing previously hypoxic tumor cells that were destined to die in the absence of treatment, and allowing their reoxygenation and proliferation. This effect can be inhibited with the use of TH-302, which thereby has potential to improve therapeutic outcome.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Authors’ Contributions

Conception and design: J. Saggar, I.F. Tannock
Development of methodology: J. Saggar, I.F. Tannock
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Saggar, I.F. Tannock
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Saggar, I.F. Tannock
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Saggar, I.F. Tannock
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