Selective Tumor Hypoxia Targeting by Hypoxia-Activated Prodrug TH-302 Inhibits Tumor Growth in Preclinical Models of Cancer

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Abstract

Purpose: Tumor hypoxia underlies treatment failure and yields a more aggressive, invasive, and metastatic cancer phenotype. TH-302 is a 2-nitroimidazole triggered hypoxia-activated prodrug of the cytotoxin bromo-isophosphoramide mustard (Br-IPM). The purpose of this study is to characterize the antitumor activity of TH-302 and investigate its selective targeting of the hypoxic cells in human tumor xenograft models.

Experimental Design: Antitumor efficacy was assessed by tumor growth kinetics or by clonogenic survival of isolated cells after tumor excision. Hypoxic fractions (HF) were determined by immunohistochemistry and morphometrics of pimonidazole staining. Tumor hypoxia levels were manipulated by exposing animals to different oxygen concentration breathing conditions. The localization and kinetics of TH-302 induced DNA damage was determined by γH2AX immunohistochemistry.

Results: TH-302 antitumor activity was dose-dependent and correlated with total drug exposure. Correlation was found between antitumor activity and tumor HF across 11 xenograft models. Tumor-bearing animals breathing 95% O2 exhibited attenuated TH-302 efficacy, whereas those breathing 10% O2 exhibited enhanced TH-302 efficacy, both compared with air (21% O2) breathing. TH-302 treatment resulted in a reduction in the volume of the HF 48 hours after dosing and a corresponding increase in the necrotic fraction. TH-302 induced DNA damage as measured by γH2AX was initially only present in the hypoxic regions and then radiated to the entire tumor in a time-dependent manner, consistent with TH-302 having a "bystander effect."

Conclusions: The results show that TH-302 has broad antitumor activity and selectively targets hypoxic tumor tissues.

Introduction

The existence of hypoxic regions is a common feature of tumors (1, 2). Tumor hypoxia has been characterized clinically by a variety of different techniques, including polarographic needle-based electrodes, magnetic resonance and positron emission imaging based approaches (3), immunohistochemistry of tumor biopsies using exogenous (e.g., pimonidazole) or endogenous (e.g., CA-IX) hypoxia biomarkers (4), microRNA biomarkers (5), and circulating protein (6). The magnitude and extent of tumor hypoxia have been shown in numerous studies to correlate directly with poor clinical prognosis (7). The basis for this correlation between tumor hypoxia and prognosis has been ascribed to both therapeutic treatment failure (8–10) and hypoxia-induced prometastatic potential (11–13). The hypothesis that the efficacy of anticancer therapies is limited by the presence of hypoxic tumor cells has resulted in a variety of therapeutic approaches designed to eliminate hypoxic areas, to kill hypoxic cells, or to reduce the treatment resistance of hypoxic cells (7, 14, 15). The development of hypoxia-targeted therapies has included bioreductive prodrugs, HIF-1 targeting, and genetic engineering of anaerobic bacteria (16–18). Among these approaches, hypoxia-activated prodrugs (HAPs) are especially promising, because they act via a mechanism involving activation of a nontoxic prodrug selectively in the hypoxic regions of tumors. Design criteria for an optimized HAP include pharmacokinetic properties to ensure adequate tumor delivery and penetration; stability to oxygen concentration-independent activating or inactivating reductases; high hypoxia selectivity with activation only in severely hypoxic tumor tissues and not moderately hypoxic normal tissues;
and a bystander effect, where adjacent tumor cells, not hypoxic enough to activate the prodrug, are nonetheless targeted by the diffusible effector moiety (19).

Early HAP efforts focused on quinone reductive drugs such as porfimycin, N-oxides such as tirapazamine, and nitroaromatic agents such as CI-1010. The major drawback of quinone-based prodrugs is that they are often good substrates for oxygen-insensitive 2-electron reductases (20). CI-1010 did not proceed to clinical trials because of the irreversible retinal toxicity observed in preclinical testing (21). HAPs that have advanced to clinical trials include tirapazamine (22, 23), PR104 (24, 25), AQ4N (26, 27), and TH-302 (28–31). TH-302 (1-methyl-2-nitro-1H-imidazole-5-yl) N,N'-bis (2-bromoethyl) diamidophosphate is a 2-nitroimidazole-linked prodrug of a brominated version of isophosphoramide mustard (Br-IPM; ref. 29). The 2-nitroimidazole moiety of TH-302 is a substrate for intracellular 1-electron reductases and, when reduced in deeply hypoxic conditions, releases Br-IPM. In vivo cytotoxicity and clonogenic assays employing human cancer cell lines show that TH-302 has little cytotoxic activity under normoxic conditions and greatly enhanced cytotoxic potency under hypoxic conditions (28–30). The clinically validated warhead, a bis-alkylator, acts as a DNA cross-linking agent and is able to kill both nondenuding and dividing cells.

Here we report the in vivo mechanism of action and antitumor activity of TH-302 with a panel of 11 human tumor xenograft models.

Materials and Methods

Compound

TH-302 (1-methyl-2-nitro-1H-imidazole-5-yl) N,N'-bis (2-bromoethyl) diamidophosphate, manufactured by Syn-

Translational Relevance

The hypoxia-activated prodrug TH-302 is currently in clinical trials for the treatment of cancer, which include a randomized controlled Phase 2 pancreatic cancer trial and a pivotal Phase 3 sarcoma trial. Over 500 patients have been treated to date.

The results described in this paper show the in vivo proof-of-concept that TH-302 selectively targets the hypoxic regions of human xenograft tumors. Multiple complementary lines of evidence are presented, which support selective tumor hypoxia targeting by TH-302. In addition, the results also show the potential utility for the diagnostic use of pimonidazole as a hypoxia biomarker to guide the selection of patients for TH-302 treatment. The strong correlation between the magnitude of tumor hypoxia as measured by pimonidazole staining and TH-302 efficacy has served as the basis for ongoing and new clinical trial proposals that include the analysis of patient biopsies stained with pimonidazole and the subsequent comparison with TH-302 efficacy.

Cell lines

All cell lines were obtained from the American Type Culture Collection (ATCC) except for the patient-derived melanoma line Stew2 (Dr. Lee Cranmer, University of Arizona Cancer Center, AZ) and have not been reauthenticated. Cells were cultured in the ATCC-suggested media (Stew2 in RPMI media) with 10% FBS added and maintained in a 5% CO2 humidified environment at 37°C.

Xenograft models

NCI SCID female mice (Taconic) were used for the PLC/PRF/5, hepatocellular carcinoma (HCC) xenograft model. Specific pathogen-free homozygous female nude mice (Nu-Foxn 1<sup>st</sup> NU/NU) were used for the other xenograft models, except males were used for the prostate cancer models (Charles River Laboratories). Mice were given food and water ad libitum and housed in microisolator cages. Four- to 6-week-old animals were tagged with microchips (Locus Technology) for identification. All animal studies were approved by the Institutional Animal Care and Use Committee of Threshold Pharmaceuticals, Inc.

Cell suspensions were mixed 1:1 with Matrigel (BD Bioscience), except for the H460 non-small-cell lung cancer (NSCLC) cells, which were mixed 2:1 with Matrigel. Cell number for inoculation (0.2 mL per mouse to the subcutaneous area of the flank) was determined by the specific tumor growth kinetics for each xenograft model: 1 × 10<sup>5</sup> for H460 and Calu-6 (NSCLC); 3 × 10<sup>5</sup> for PC-3 (prostate cancer); 5 × 10<sup>5</sup> for H82 (small cell lung cancer; SCLC); A375 (melanoma), Stew2 (melanoma), 786-O (renal cell carcinoma, RCC), PLC/PRF/5, Hs766t (pancreatic cancer), BxPC-3 (pancreatic cancer), and SU.86.86 (pancreatic cancer).

For xenograft experiments employing controlled oxygen concentration breathing chambers, groups of mice were placed in a controlled atmospheric chamber and exposed to 95% O2 (carbogen with 5% CO2), 21% O2 (air), or 10% O2, for 30 minutes followed by dosing of TH-302 or vehicle control, and then, the animals remained in the controlled breathing chambers for 2 additional hours. The chambers were flushed continuously with gases at 5 L/min.

In vivo antitumor activity

Tumor growth and body weight were measured twice a week after cell implantation. Tumor volume was calculated as (length × width<sup>2</sup>) / 2. When the mean value of tumor volume was approximately 100 to 150 mm<sup>3</sup>, mice were randomized into 10 mice per group, and TH-302 or vehicle control treatment started (Day 1). Antitumor activity was assessed by tumor growth inhibition (TGI) and tumor growth delay (TGD). TGI was defined as (1 − DT/ΔC) × 100, where DT/ΔC is the ratio of the change in mean tumor volume of the treated group (DT) and the control group (ΔC). Animals were called when individual tumor size was
over 2,000 mm$^3$ or mean tumor volume exceeded 1,000 mm$^3$ in the group. TGI was determined on the last measurement when all the animals in the vehicle group still survived. TGD$_{500}$ or TGD$_{1,000}$ was determined as the increased time (days) for the treated tumor's size on average to reach 500 or 1,000 mm$^3$ as compared with the vehicle group.

Maximum tolerated dose (MTD) for TH-302 was determined by dose escalations in a small number of nontumor bearing nu/nu mice. The MTD was defined as the highest possible dose resulting in no animal deaths, less than 20% weight loss for any one animal in an experimental group, no significant changes in general clinical signs, and no abnormal gross anatomical findings after necropsy. General clinical signs included respiratory rate, behavior, and response to normal stimuli (32). The doses of TH-302 used in all studies were no higher than MTD.

**Tumor excision assays with clonogenic survival endpoints**

Twenty-four hours post TH-302 treatment, tumors were excised, weighed, minced, and then digested with an enzyme cocktail containing 0.2 mg/mL each of collagenase (Sigma-Aldrich), DNaseI (Sigma), and pronase (Sigma) in sterile buffered salt solution (Sigma) for 15 minutes at 37°C with stirring, and the resulting cell suspension counted and plated to determine clonogenicity fractions. Cell colonies (>50 cells per colony) were counted, and the surviving fraction (SF) was calculated as the plating efficacy (PE) of treated divided by the PE of vehicle control.

**Histology and immunohistochemistry**

To determine the magnitude of the hypoxic fraction (HF) for each of the different xenograft tumor models, when tumor size reached 400 to 600 mm$^3$ pimonidazole (Natural Pharmacia International) at 60 mg/kg was intraperitoneally (i.p.) injected 1 hour before animal was killed. To label regions of blood perfusion, Hoechst 33342 (Invitrogen) at 10 mg/kg was peritoneally (i.p.) injected 1 hour before animal was killed (33). Tumors were recovered immediately and either embedded and frozen in OCT or fixed in 10% neutral buffered formalin and embedded in paraffin. A total of 8-μm-thick frozen tissue sections or 5-μm-thick paraffin sections were cut and adhered to poly-l-lysine-coated glass microscope slides. Frozen sections were stored at −80°C, and paraffin sections were placed at room temperature (RT) until use.

Hematoxylin and eosin (H&E) staining was conducted for tumor morphology and to quantify tumor necrotic fraction. Cryostat sections were air dried and fixed in cold acetone for 10 minutes. After washing with PBS and incubation with Peroxidazed 1 (Biocare Medical) to quench the endogenous peroxidase, sections were incubated with Background Sniper (Biocare Medical) at RT for 15 minutes to block the nonspecific binding site. Sections were then incubated 1 hour at RT with the appropriate dilutions of primary antibodies: anti-pimonidazole rabbit polyclonal at 1:500 (Natural Pharmacia International), or anti-CD31 rat polyclonal at 1:250 (Biocare Medical). HRP-conjugated goat anti-rabbit IgG (Epitomics) served as secondary antibody for pimonidazole adducts, and Rat Probe HRP Polymer Detection Kit (Biocare Medical) was used for CD31. The colorimetric reaction was developed by DAB chromogen (Biocare Medical) system with hematoxylin used as a counter stain. Hoechst 33342 was observed under UV light with blue 465/30 nmol/L band-pass filter on cryostat sections before immunostaining. Hypoxia (by pimonidazole), and TH-302 induced DNA damage (by γH2AX immunostaining), were characterized in consecutive paraffin sections. For pimonidazole staining, sections were incubated with Pronase Reagent (GeneTex) at 37°C for 40 minutes to retrieve the antigen. For γH2AX (1:2,000, rabbit monoclonal, Epitomics) detection, antigen retrieval was conducted by incubating the slides with Heat Induced Epitope Retrieval Buffer (Biocare Medical) in a Decloaker Chamber (Biocare Medical).

**Image analysis**

At 20× magnification, digital images of H&E stained or pimonidazole stained sections were captured and assembled to compose a whole tumor image. Pimonidazole-positive and necrotic regions were extracted using Image-Pro Plus v6.0 (MediaCybernetics). The necrotic fraction (NF) in the individual tumor was calculated as the percentage of the necrotic area of the tumor tissue in the whole tumor. The HF was evaluated in the viable tissue with the necrotic area excluded. To compare the volume of the hypoxic compartment in the tumors after TH-302 treatment, pimonidazole-positive hypoxic areas in the whole tumor were analyzed as well. Hoechst 33342 positivity was only evaluated in the viable tissue by subtracting the necrotic area in the section. At 200× magnification, 10 to 15 images per section were randomly selected. All images were captured under consistent illumination and exposure for their respective stains. No image postprocessing was carried out. Scripts were developed in Image Pro-Plus to analyze the target signals using color and morphologic segmentation tools. For semi-quantification of CD31-positive areas and microvessel density, point counting was carried out. A total of 15 to 20 fields at 400× magnification, per section, were counted in each animal on a 1-cm$^2$ eyepiece graticule with 10 equidistant grid lines. The percentage of fractional area (percentage of positive area per total area counted of the section) was calculated using the following formula: percentage of positive fractional area = number of grid intersections with positive staining/total number of grid intersections multiplied × 100%. γH2AX positive cells were counted at 400× magnification. Five fields per section were used. Normoxic compartment was identified by cell morphology, and hypoxic compartment was identified by pimonidazole staining in serial sections. The percentage of γH2AX positive cells in each compartment was calculated as number of γH2AX positive...
cells per number of total cells in the compartment × 100.

Statistical analysis

Data are expressed as the mean ± SEM. A Student t test was used to find the significance between 2 groups. Comparison of the multiple groups was done using One-way analysis of variance with Dunnett test (GraphPad PRISM 4). Correlation was calculated by linear regression. A P level less than 0.05 was considered statistically significant.

Results

Antitumor activity of TH-302 as a single agent

Using the H460 NSCLC xenograft model, we investigated the dose-dependent antitumor activity of TH-302. When TH-302 was administered at 6.25, 12.5, 25, or 50 mg/kg, QD × 5/wk × 2 wks (once a day for 5 days per week for 2 weeks) i.p., the TGI at Day 22 was 43%, 51%, 75%, and 89%, respectively (Table 1). A dose of 50 mg/kg yielded modest body weight loss of 2.9% on average. With this dosing regimen, 50 mg/kg was considered as MTD, as

Table 1. Comparison of different doses and regimens of TH-302 on tumor growth in the H460 human NSCLC xenograft model

<table>
<thead>
<tr>
<th>Dose, mg/kg</th>
<th>Regimen</th>
<th>Drug exposure, mg/kg</th>
<th>TGI%</th>
<th>TGD500, d</th>
<th>TGD1,000, d</th>
<th>Max. BW loss% (day &quot;x&quot;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>QD × 5/wk × 2 wks</td>
<td>62.5</td>
<td>43</td>
<td>4.5</td>
<td>6</td>
<td>1.5 (4)</td>
</tr>
<tr>
<td>12.5</td>
<td>QD × 5/wk × 2 wks</td>
<td>125</td>
<td>51</td>
<td>7</td>
<td>8.5</td>
<td>1.7 (4)</td>
</tr>
<tr>
<td>25</td>
<td>QD × 5/wk × 2 wks</td>
<td>250</td>
<td>75</td>
<td>11</td>
<td>12</td>
<td>2.5 (4)</td>
</tr>
<tr>
<td>50</td>
<td>QD × 5/wk × 2 wks</td>
<td>500</td>
<td>89</td>
<td>18</td>
<td>19</td>
<td>2.9 (4)</td>
</tr>
<tr>
<td>100</td>
<td>Q7D × 2</td>
<td>200</td>
<td>72</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>Q3D × 5</td>
<td>500</td>
<td>89</td>
<td>16</td>
<td>16</td>
<td>2.3 (9)</td>
</tr>
<tr>
<td>150</td>
<td>Once</td>
<td>150</td>
<td>58</td>
<td>8</td>
<td>10</td>
<td>1.9 (4)</td>
</tr>
</tbody>
</table>

NOTE: TH-302 was administered intraperitoneally. TGI, tumor growth inhibition was defined as (1 − ΔT/ΔC) × 100, where ΔT/ΔC presented the ratio of the change in mean tumor volume of the treated group and of the vehicle group. TGI of all the groups were calculated at Day 22. TGD500 or TGD1,000 was determined as the increased time (days) for the treated tumors’ size on average to reach 500 or 1,000 mm³ as compared with the vehicle group using the tumor growth curves. Data were obtained from 10 mice per group; Drug exposure (in mg/kg) = dose (in mg/kg) × number of doses; Max. BW loss%, percentage of mean maximal body weight lost compared with pretreatment with the time point (day "x") when reached.

Table 2. TH-302 exhibits broad antitumor activity in a panel of xenograft tumors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>Dose</th>
<th>Drug exposure</th>
<th>TGD500</th>
<th>TGD1,000</th>
<th>TGI% (day &quot;x&quot;)</th>
<th>P value vs. vehicle</th>
<th>Maximal BW loss% (day &quot;x&quot;)</th>
<th>HF% (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H460</td>
<td>NSCLC</td>
<td>50</td>
<td>500</td>
<td>19</td>
<td>18</td>
<td>89 (22)</td>
<td>&lt;0.001</td>
<td>2.9 (4)</td>
<td>16.3 (8)</td>
</tr>
<tr>
<td>Calu-6</td>
<td>NSCLC</td>
<td>50</td>
<td>500</td>
<td>11</td>
<td>12</td>
<td>51 (33)</td>
<td>&lt;0.01</td>
<td>1.2 (11)</td>
<td>10.2 (6)</td>
</tr>
<tr>
<td>H82</td>
<td>SCLC</td>
<td>50</td>
<td>500</td>
<td>5</td>
<td>5</td>
<td>50 (15)</td>
<td>&lt;0.01</td>
<td>0.1 (4)</td>
<td>6.9 (7)</td>
</tr>
<tr>
<td>Stew2</td>
<td>Melanoma</td>
<td>50</td>
<td>500</td>
<td>14</td>
<td>NA</td>
<td>31 (39)</td>
<td>&lt;0.05</td>
<td>0</td>
<td>6.4 (6)</td>
</tr>
<tr>
<td>A375</td>
<td>Melanoma</td>
<td>50</td>
<td>500</td>
<td>5</td>
<td>7</td>
<td>48 (18)</td>
<td>&lt;0.05</td>
<td>0</td>
<td>5.0 (5)</td>
</tr>
<tr>
<td>PLC/PRF/5</td>
<td>HCC</td>
<td>50</td>
<td>500</td>
<td>5</td>
<td>NA</td>
<td>29 (39)</td>
<td>&gt;0.05</td>
<td>5.6 (22)</td>
<td>4.7 (7)</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate</td>
<td>50</td>
<td>500</td>
<td>13</td>
<td>NA</td>
<td>47 (36)</td>
<td>&lt;0.01</td>
<td>1.2 (11)</td>
<td>4.2 (8)</td>
</tr>
<tr>
<td>786-O</td>
<td>RCC</td>
<td>50</td>
<td>500</td>
<td>10</td>
<td>NA</td>
<td>10 (64)</td>
<td>&lt;0.05</td>
<td>0.1 (8)</td>
<td>1.4 (8)</td>
</tr>
<tr>
<td>Hs766t*</td>
<td>Pancreas</td>
<td>75</td>
<td>375</td>
<td>16.5</td>
<td>&gt;25</td>
<td>75 (11)</td>
<td>&lt;0.01</td>
<td>3.4 (11)</td>
<td>14.8 (8)</td>
</tr>
<tr>
<td>BxPC-3*</td>
<td>Pancreas</td>
<td>75</td>
<td>375</td>
<td>1</td>
<td>6</td>
<td>40 (15)</td>
<td>&lt;0.01</td>
<td>1 (8)</td>
<td>7.2 (11)</td>
</tr>
<tr>
<td>SU.86.86*</td>
<td>Pancreas</td>
<td>75</td>
<td>375</td>
<td>1.5</td>
<td>3.5</td>
<td>28 (18)</td>
<td>&gt;0.05</td>
<td>0</td>
<td>4.8 (8)</td>
</tr>
</tbody>
</table>

NOTE: TH-302 was administered intraperitoneally. and the regimens were QD × 5/wk × 2 wks except *, Q3Dx5 in Hs766t, BxPc3, and SU.86.86; Drug exposure (in mg/kg) = dose (in mg/kg) × number of doses. TGI was obtained from 10 animals per group. Data are presented as mean value for each group. P value of tumor growth between TH-302-treated and vehicle-treated groups was analyzed by t test when TGI was calculated. The hypoxic fraction (HF) was evaluated in the viable tumor tissue with necrotic area excluded. day “x”, time points at which TGI and weight loss was determined. n, number of tumors samples analyzed; NA, not available before the study was terminated.
Figure 1. A, representative images of tumor hypoxia detected by pimonidazole binding and antitumor activity of TH-302 in the 8 xenograft tumor models. TH-302 was dosed at 50 mg/kg, QD x 5/wk × 2 wks, intraperitoneally (i.p.) in all the models. Dotted lines with open circles are the vehicle-treated groups, and solid lines with closed circles are the TH-302-treated groups. Data are presented as mean ± SEM of 10 animals in each group; *, P < 0.05 or **, P < 0.01 versus vehicle-treated group by t test. B, correlation between hypoxic fraction (HF) in the 8 xenograft tumor models and the corresponding antitumor activity of TH-302 expressed as tumor growth inhibition (TGI). HF was obtained as mean ± SEM from 5 to 11 animals for each tumor type, TGI was obtained as mean ± SEM from 10 animals of each tumor type.
75 mg/kg resulted in severe body weight loss (>20%) in 1 of 10 animals and led to a maximal body weight loss on average 9.3%. TH-302 was also tested at the higher doses of 100 and 150 mg/kg with a less frequent dosing regimen (once, i.p.) or 100 mg/kg with an intermittent regimen (Q3D/C2, i.p.). Under all these regimens, there was no organ abnormalities noted after necropsy at treatment end. Furthermore, hematologic toxicity of TH-302 was investigated in the immunocompetent CD-1 animals 3 or 7 days after TH-302 treatment. As was observed in the nude mice, there was no significant body weight loss observed among the groups, TH-302 50 mg/kg, QD × 5/wk × 2 wks did not show any hematologic toxicity as evaluated by white blood cell, neutrophil, or lymphocyte counts on either 3 or 7 days after treatment end. TH-302 at 100 mg/kg showed a decrease in blood cell counts 3 days after treatment end, but was totally recovered 7 days posttreatment (Supplementary Fig. S1). TH-302 under all tested regimens exhibited efficacy metrics ranging from 58% to 89% TGI (Table 1). In the H460 NSCLC model, when TH-302 was tested at no higher than MTD with different regimens, there was a total drug exposure (= dose × number of injections) dependent TGD to 500 or 1,000 mm³. In addition, significant correlation between antitumor activity, evaluated as TGI and total drug exposure was observed. R² = 0.80, P < 0.01. As TH-302 50 mg/kg QD × 5/wk × 2 wks i.p. yielded the highest drug exposure, exhibited minor body weight loss, and showed a safe hemato-logic profile, this regimen was employed in many of the preclinical models tested.

TH-302 antitumor activity was then evaluated in 11 different ectopic xenograft models, including NSCLC, SCLC, melanoma, prostate cancer, HCC, RCC, and pancreatic cancer (Table 2). The same dose and regimen of TH-302, 50 mg/kg, QD × 5/wk × 2 wks, i.p. was used in these xenografts, except the pancreatic cancer models (75 mg/kg, Q3D × 5, i.p.). Under the same drug exposure, all the animals tolerated TH-302 well, and there was no apparent drug induced toxicity determined by body weight change. TGI across all models ranged from approximately 10% to approximately 89%. Antitumor activity was reflected in TGD as well, with from 1 day to more than 25 days delay observed. Compared with vehicle control–treated groups, TH-302 significantly inhibited tumor growth in 8 of 11 xenograft tumor models examined.
Antitumor activity of TH-302 as a function of tumor hypoxia

Hypoxic regions in the tumors were detected by pimonidazole immunostaining. Representative pimonidazole staining images in 8 xenograft tumors are presented in Fig. 1A. Semiqualitative morphometric analysis of the hypoxic compartments was conducted with tumor samples from 5 to 8 animals per xenograft tumor model. The HF was determined in the viable area only for the tumor models profiled (Table 2 and Fig. 1A). HF varied in the models, from 16.3% (in the H460 xenografts) to 1.4% (in the 786-O xenografts). The corresponding tumor growth kinetics in each xenograft model, and the antitumor activity of TH-302 as a monotherapy is presented in Fig. 1A. Under the same dose and regimen of TH-302 the HF and TH-302 efficacy (TGI) in 8 xenograft models showed a good correlation ($R^2 = 0.77$, $P = 0.004$) by linear regression (Fig. 1B). Hypoxia regions were detected in the 3 pancreatic xenograft tumors as well (Fig. 1C). The H8766, BxPc3, and SU.86.86 models showed hypoxia levels ranging from high to low, with HF of 14.8%, 7.2%, and 4.8%, respectively (Fig. 1C). The antitumor activity of TH-302 significantly correlated with HF in the 3 pancreatic models ($R^2 = 1.0$, $P = 0.001$; Fig. 1D). In the PLC/PRF/5 HCC, 786-O RCC, and SU.86.86 pancreatic cancer xenograft models, where the antitumor effect did not achieve statistical difference compared with the vehicle treatment, the pimonidazole-positivity was relatively low, with HF less than 5%.

Antitumor activity of TH-302 as a function of breathing oxygen concentration

To further investigate the relationship between tumor hypoxia and TH-302 activity, we modified tumor hypoxia by exposing H460 NSCLC tumor-bearing animals exposed to different oxygen concentration levels in controlled atmospheric breathing chambers gassed with 95%, 21%, and 10% $O_2$. Antitumor activity was assessed by clonogenic assay of tumors excised 24 hours after a single dose of TH-302 at 50 mg/kg, i.v. The SF of individual tumors is presented in Fig. 2A, where the mean SFs were 50.4%, 5.8%, and 2.6% for 95%, 21%, and 10% $O_2$, respectively. The results showed that TH-302 induced cell killing was breathing oxygen concentration dependent, with the greatest cytotoxicity occurring when the tumor-bearing mice were exposed to low oxygen concentrations. On the basis of these results, we further evaluated differential TH-302 antitumor activity with multiple dosing under varied breathing oxygen concentration conditions using TGI and TGD as read-outs. H460 tumor-bearing animals were treated with vehicle or TH-302 at 50 mg/kg, i.p., QD $\times 5$/wk $\times 2$ wks, when tumor size reached 100 mm$^3$. On treatment days, all mice were exposed to the different levels of oxygen in the controlled atmosphere chamber for 30 minutes before and 2 hours after each dose. As shown in Fig. 2B, tumor growth rates in the vehicle control group were not affected by the different oxygen level breathing conditions. However, after TH-302 treatment, TGI was clearly dependent on oxygen level breathing conditions, with superior efficacy achieved in the lower breathing oxygen concentration groups. Tumor growth was significantly reduced by TH-302 in animals breathing 10% $O_2$ compared with 95% $O_2$ breathing. The TGI on day 29 were 90% in 10% $O_2$, as compared with 50% in the 95% $O_2$ group. $P < 0.05$. TGD$_{500}$ was 20, 16, and 7 days in animals breathing 10%, 21%, and 95% $O_2$, respectively. Animal body weight was not affected...
by different breathing conditions. All vehicle and TH-302-treated groups showed less than 3% body weight loss during the study.

**TH-302 reduces the volume of the hypoxic compartment**

To determine TH-302 effects on the tumor microenvironment, vascular, hypoxic, and necrotic staining of H82 SLC1C xenograft tumor sections was employed at various posttreatment times after TH-302 dosing. Representative images are shown in Fig. 3A, and morphometric analysis results are shown in Fig. 3B–E. CD31 endothelial cell staining was used to identify blood vessels, and Hoechst 33342 was used to detect functional blood perfusion. In the vehicle-treated control tumors, blood vessels were evenly distributed in the tumor and appeared as distinct clusters of endothelial cells with intact lumen. After 150 mg/kg TH-302 i.p. treatment, there was no apparent loss of vessel integrity or any evidence of hemorrhaging or loss of CD31 staining. By semiquantitative morphometric analysis, tumor vessel density (Fig. 3B) and vascular perfusion (Fig. 3C) in the viable tumor tissue showed no significant difference between vehicle and TH-302–treated tumors at any of the time points examined. The HF in the tumors was assessed by pimonidazole immunostaining (Fig. 3D). After TH-302 treatment, the pimonidazole-positive area was significantly decreased at 48 hours after dosing (6.3 ± 1.2% in vehicle vs. 1.8 ± 1.1% in the TH-302 treatment group, P < 0.05). The percentage of pimonidazole-positive area in the whole tumor remained at this reduced level at 72 hours after dosing. The tumor necrotic fraction (NF, Fig. 3E) exhibited a corresponding time-dependent increase, where the NF was increased from 7.3% in vehicle to 37.9% after TH-302 treatment. In addition, 48 hours after TH-302 treatment at 150 mg/kg, i.p., pimonidazole-positive area in the whole tumor was also investigated in the H460 (NSCLC), Hs766t (pancreatic), and A375 (melanoma) xenograft tumors. Representative images are shown in Fig. 3F, and the morphometric analysis showed significant pimonidazole-positivity reduction in all 3 models (Fig. 3G). The reduced pimonidazole-positive area in H460 xenograft tumors was recovered and was back to normal 7 days after TH-302 150 mg/kg, i.p., treatment (Supplementary Fig. S2).

**Intratumoral distribution of TH-302 induced DNA damage**

γH2AX foci formation was employed to assay TH-302 induced DNA damage. Animals bearing H460 tumors were treated with TH-302 at 100 or 25 mg/kg, i.p., or vehicle when the tumor size reached 400 to 600 mm³. Tumors were excised at 6 or 24 hours later, and γH2AX and pimonidazole immunostaining was carried out on consecutive formalin-fixed, paraffin-embedded sections (Fig. 4A). In the vehicle-treated H460 xenografts, few γH2AX positive cells were present and were mainly located near the necrotic regions. Six hours after 100 mg/kg of TH-302, i.p., treatment, there was a significant increase of γH2AX-positive cells. Those γH2AX-positive cells were predomi-

nantly found in the pimonidazole-positive hypoxic areas (47% vs. 11% in vehicle in the hypoxic compartment [HC], P < 0.001; 19% vs. 2%, P < 0.001 in the oxic compartment [OC], Fig. 4B). Twenty-four hours after TH-302 treatment, γH2AX positive cells were significantly increased in the OC, which were evenly distributed; however, the percentage of positive cells was reduced in the HC. Six hours after a lower dose of TH-302 (25 mg/kg), there was a significant increase of γH2AX-positive cells in both the OC (4%) and the HC (19%), P < 0.05 versus vehicle. Twenty-four hours after treatment, the percentage of γH2AX-positive cells in OC was 5%, but there was a significant reduction in the HC. The other exogenous hypoxia biomarker marker employed, F6, was administered sequentially with pimonidazole to identify regions with changes in hypoxia. Immunostaining of pimonidazole (PIMO), F6, γH2AX, and Ki-67 (a marker of proliferation) were carried out in consecutive sections. PIMO+/F6+/cells were scored as chronically hypoxic cells, which were predominant in this model. PIMO+/F6− or PIMO−/F6− cells were scored as acutely hypoxic cells, and were usually found proximal to blood vessels. Six hours after TH-302 (100 mg/kg) treatment, γH2AX-positive cells were significantly increased. γH2AX-positive cells were observed in both PIMO+/F6+/ (chronic) and PIMO+/F6− (acute) hypoxia regions. The DNA damage was predominantly located in the PIMO-dark and Ki-67 negative areas. Ki-67 positive proliferating cells were dramatically decreased in both acute and chronic hypoxic regions (Supplementary Fig. S3).

**Discussion**

Preclinical mouse models of cancer have been shown to recapitulate the architecture of the tumor microenvironment and the prevalence of subregional hypoxia. The use of human tumor xenografts in immunocompromised mice for the preclinical evaluation of new anticancer drugs is an integral part of drug discovery and optimization strategies in which specific characteristics of tumor lines can be used to orient future clinical development (34, 35). Selective targeting of hypoxic tumor regions with HAPs designed to activate or release a cytostatic or cytotoxic effector selectively in hypoxic tumor regions is a promising therapeutic strategy for the treatment of cancer. To show the hypoxic selectivity of TH-302 in vivo, we utilized a panel of 11 human tumor xenografts and characterized TH-302 activity with different mechanistic antitumor read-outs.

Employing the H460 NSCLC xenograft model, we have showed that the antitumor activity of TH-302 is solely dependent on total drug exposure regardless of how frequently the drug was administered (Table 1). The ability of the TH-302 toxin (Br-IPM) and other DNA cross-linking agents to yield long-lived DNA lesions and exhibit cell cycle independent cytotoxicity underlies their utility in killing the hypoxic cells in tumors. This mechanism of action feature is consistent with our finding of the relative regimen independence of TH-302 as monotherapy. TH-302 show antitumor activity as a single agent across a broad panel of different tumor type xenograft models. Significant TGI was
observed in 8 of 11 xenograft models examined. The low activity of TH-302 observed in the 786-O RCC, PLC/PRF/5 HCC, and SU.86.86 pancreatic xenograft models was consistent with their tumor microenvironment phenotypes exhibiting little hypoxia as measured by pimonidazole staining. A key finding described here is the correlation...
between TH-302 antitumor activity and the corresponding magnitude of tumor hypoxia present in a xenograft model (Fig. 1). Significant correlation was found between the relative volume of hypoxia and TH-302 activity in the 8 xenografts at equal total drug exposure. A higher HF usually yielded greater antitumor effect of TH-302. The HAP tirapazamine also show a correlation between hypoxic volume (as measured by EF5 staining) and DNA damage (as measured by comet assay) between individual SCCVII tumor-bearing animals (22). However, other cell autonomous factors may also be involved in the antitumor activity of TH-302. For example, although the xenograft models, Calu-6 (NSCLC) and H82 (SCLC), exhibited different HFs (10.2% in Calu-6 vs. 6.9% in H82), they exhibited very similar TH-302 antitumor activity profiles, with TGIs of 50%, after the same TH-302 treatment regimen (50 mg/kg, daily). Factors affecting TH-302 antitumor activity could be the difference in DNA repair machinery; for example, H82 has wild-type p53 (36) and Calu-6 has mutant p53 (37). Others have shown mutant p53 elevated DNA damage repair led to drug resistance (38). Another factor responsible for the different antitumor activity could be differential prodrug activating reductase expression (39).

To extend the observation of a significant correlation between the volume of the HF in xenografts and the corresponding TH-302 antitumor activity, we examined whether lower oxygen level breathing conditions (10% O₂ breathing) would yield greater efficacy, because more of the tumor would be hypoxic and more TH-302 would be activated, and conversely, whether higher oxygen concentration breathing conditions (95%) would yield less efficacy for TH-302. Previous published studies have showed the effect of modified oxygen concentration breathing conditions on the HF of xenograft tumors (40–43) and differential efficacy of an HAP (43). Both acute (single dose) and chronic (daily dosing for 2 weeks) TH-302 dosing regimens were employed. In both acute and chronic breathing condition and dosing regimen models, the results show TH-302 induced cytotoxicity in an oxygen concentration breathing level dependent manner.

We investigated the spatial distribution of TH-302 induced changes in tumor microenvironment architecture with a focus on characterizing selective effects on the hypoxic compartment. In the 4 xenograft tumor models examined (H82 SCLC, H460 NSCLC, Hs766t pancreatic cancer, and A375 melanoma), the volume of the hypoxic regions was significantly reduced after TH-302 treatment. We have also shown the reduction of hypoxic regions after TH-302 treatment was not because of the disruption of tumor vessel perfusion that prevented the pimonidazole hypoxia probe from reaching the hypoxic areas. In contrast to the reduced hypoxia region, the volume of the necrotic compartment was correspondingly increased. Given that the necrotic fraction increased to over 30% of the tumor volume in the H82 model (compared with 7% in the vehicle-treated tumors), whereas the HF at the time of dosing was usually less than 8% in this tumor type, the relative change in the 2 compartments (the hypoxic compartment decreasing from 6% to 2% and necrotic compartment increasing from 8% to 30%) is consistent with a bystander effect occurring, where adjacent normoxic cells are also killed by the released cytotoxic warhead, although the possibility that normoxic activation of TH-302 was high enough to kill normoxic cells cannot be excluded under the test conditions employed.

In the H82 SCLC xenograft model, TH-302 treatment did not exhibit any apparent effect on the microvasculature or blood perfusion during the 72-hour observation period. There was no decrease of microvessel density in the viable zone as assessed by endothelial cell surface marker CD31 staining. Moreover, there was no significant reduction of functional blood perfusion detected by Hoechst 33342.
Figure 4. Representative images of immunostaining of pimonidazole and γH2AX on consecutive 10% formalin-fixed paraffin-embedded sections. A total of 60 mg/kg of pimonidazole was i.p. injected 1 hour before TH-302 was administered at 100 or 25 mg/kg, i.p. Tumors were recovered 6 and 24 hours after the TH-302 treatment compared with vehicle. C, capillary; O, oxic (normoxic) compartment; H, hypoxic compartment; and N, necrotic region. Magnification scale bar, 100 μm. B, morphometric analysis of percentage of γH2AX positive cells in oxic and hypoxic compartments. Each bar represents mean ± SEM of 3 to 4 animals per group, *P < 0.05 versus vehicle group; †, ††P < 0.05 versus TH-302 6-hour-treated group.

Figure 4. Representative images of immunostaining of pimonidazole and γH2AX on consecutive 10% formalin-fixed paraffin-embedded sections. A total of 60 mg/kg of pimonidazole was i.p. injected 1 hour before TH-302 was administered at 100 or 25 mg/kg, i.p. Tumors were recovered 6 and 24 hours after the TH-302 treatment compared with vehicle. C, capillary; O, oxic (normoxic) compartment; H, hypoxic compartment; and N, necrotic region. Magnification scale bar, 100 μm. B, morphometric analysis of percentage of γH2AX positive cells in oxic and hypoxic compartments. Each bar represents mean ± SEM of 3 to 4 animals per group, *P < 0.05 versus vehicle group; †, ††P < 0.05 versus TH-302 6-hour-treated group.

staining, which suggested little or no vessel disruption after treatment. Similar results were obtained in the H460 NSCLC xenograft model. It has been previously shown that tirapazamine causes extensive, central vascular dysfunction in HCT-116 xenograft tumor within 24 hours of dosing (44). Given that the xenograft models are different, it is not clear whether these different effects on the vasculature of these 2 different HAPs indicate a functional difference between the HAPs. One possible mechanism underlying tirapazamine as a vascular disrupting agent is activity on moderately hypoxic endothelial cells or perivascular cells (45). Tirapazamine is activated at relatively higher oxygen concentration compared with TH-302 (28). Therefore, only severely hypoxic tumor cells may be targeted by TH-302 but not the moderately hypoxic microvessel cells in the tumor. This feature of TH-302 exhibiting little effect on tumor microvasculature may facilitate the delivery of both TH-302 itself and a companion coadministered conventional chemotherapeutic drug when used in combination with TH-302 and, therefore, maximize antitumor activity.

The phosphorylation of the variant histone H2AX at Ser139 (γH2AX) is a sensitive marker of DNA damage responses (46). In vehicle control–treated H460 tumors, there were low levels of γH2AX positive cells, mainly located in the areas adjacent to the necrotic areas. This observation is consistent with previous published studies (47). Six hours after TH-302 treatment, γH2AX-positive cells were dramatically increased in the hypoxic regions of the tumors in a dose-dependent manner. Using 2 different exogenous markers of hypoxia administered at different times show increased γH2AX in both double stained (chronic hypoxia) and single stained (acute hypoxia) regions. This pattern of DNA damage by TH-302 treatment was consistent with the preferential toxicity of TH-302 for hypoxic cells. A similar pattern has also been reported after HAP tirapazamine treatment in SiHa xenograft tumors (48). At 24 hours after TH-302 treatment, however, DNA damage had radiated throughout the tumor, consistent with TH-302 having a bystander effect. A bystander effect could also be responsible for the high antitumor efficacy of TH-302 as a single agent in the broad panel of xenograft models.

Taken together, the results presented here on (1) the correlation of HF and TH-302 antitumor activity; (2) the enhanced antitumor activity in tumor-bearing animals after breathing lower oxygen concentration atmospheres; (3) the selective reduction in the volume of the hypoxic compartments in xenograft tumors and corresponding increase in the necrotic compartments volumes in xenograft tumors; and (4) the colocalization of γH2AX positive immunostaining in the pimonidazole-positive hypoxic regions of xenograft tumors, are all consistent with the selective targeting of the hypoxic compartments of tumors by TH-302. The extension of TH-302 induced DNA damage to the normoxic regions indicates an accompanying bystander effect. TH-302 is currently in clinical trials for the treatment of cancer (31). The results described here support the hypoxic selectivity of TH-302 in vivo, and the use
of hypoxia biomarkers to profile patient biopsies and guide patient selection for therapy with TH-302.

Disclosure of Potential Conflicts of Interest

All authors are current or former employees and stockholders of Threshold Pharmaceuticals, Inc.

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