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ORIGINAL ARTICLE

TH-302, a hypoxia-activated prodrug with broad in vivo preclinical combination therapy efficacy: optimization of dosing regimens and schedules

Qian Liu · Jessica D. Sun · Jingli Wang · Dharmendra Ahluwalia · Amanda F. Baker · Lee D. Cranmer · Damien Ferraro · Yan Wang · Jian-Xin Duan · W. Steve Ammons · John G. Curd · Mark D. Matteucci · Charles P. Hart

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Abstract
Purpose Subregional hypoxia is a common feature of tumors and is recognized as a limiting factor for the success of radiotherapy and chemotherapy. TH-302, a hypoxia-activated prodrug selectively targeting hypoxic regions of solid tumors, delivers a cytotoxic warhead to the tumor, while maintaining relatively low systemic toxicity. The antitumor activity, different dosing sequences, and dosing regimens of TH-302 in combination with commonly used conventional chemotherapeutics were investigated in human tumor xenograft models.
Methods Seven chemotherapeutic drugs (docetaxel, cisplatin, pemetrexed, irinotecan, doxorubicin, gemcitabine, and temozolomide) were tested in combination with TH-302 in eleven human xenograft models, including non-small cell lung cancer (NSCLC), colon cancer, prostate cancer, fibrosarcoma, melanoma, and pancreatic cancer.
Results The antitumor activity of docetaxel, cisplatin, pemetrexed, irinotecan, doxorubicin, gemcitabine, and temozolomide was increased when combined with TH-302 in nine out of eleven models tested. Administration of TH-302 2–8 h prior to the other chemotherapeutics yielded superior efficacy versus other sequences tested. Simultaneous administration of TH-302 and chemotherapeutics increased toxicity versus schedules with dosing separations. In a dosing optimization study, TH-302 administered daily at 50 mg/kg intraperitoneally for 5 days per week in the H460 NSCLC model showed the optimal response with minimal toxicity.
Conclusions TH-302 enhances the activity of a wide range of conventional anti-neoplastic agents in a broad panel of in vivo xenograft models. These data highlight in vivo effects of schedule and order of drug administration in regimen efficacy and toxicity and have relevance to the design of human regimens incorporating TH-302.

Keywords TH-302 · Tumor hypoxia · Hypoxia-activated prodrug · Combination chemotherapy · Human tumor xenograft

Introduction

Hypoxic regions are common in tumors and contribute to malignant progression, metastasis and often resistance to both chemotherapy and radiotherapy [12, 43]. Major reasons for hypoxic tumor tissue resistance to chemotherapy include their distance from blood vessels and slower rate of proliferation [4, 26]. Many conventional chemotherapeutic agents exhibit only limited penetration from tumor capillaries to distal regions, e.g., doxorubicin and mitoxantrone [31, 38]; others exhibit low activity on slowly proliferating cells, e.g., gemcitabine [16, 36]. Most standard chemotherapeutics are cytotoxic only to the normoxic part of the tumors [1, 36, 37].

The magnitude of tumor hypoxia measured in patients' tumor tissue correlates with reduced survival [40].

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Q. Liu · J. D. Sun · J. Wang · D. Ahluwalia · D. Ferraro · Y. Wang · J.-X. Duan · W. S. Ammons · J. G. Curd · M. D. Matteucci · C. P. Hart (✉)
Threshold Pharmaceuticals, 170 Harbor Way, Suite 300, South San Francisco, CA 94080, USA
e-mail: chart@thresholdpharm.com

A. F. Baker · L. D. Cranmer
Arizona Cancer Center, University of Arizona, 1515 N. Campbell Ave., Tucson, AZ 85724, USA

Selective killing of hypoxic tumor cells is hypothesized to slow tumor progression [reviewed in 4, 12, 43]. Low oxygen levels found in tumor subregions are rarely observed in normal tissues. Therefore, tumor hypoxia can serve as the basis for selective, microenvironmentally targeted cancer therapy. Exploitation of this target is possible through prodrugs activated by enzymatic reduction under hypoxic conditions to release cytotoxic effectors ("warheads"). Hypoxia-activated compounds that have progressed to clinical trials for the treatment of cancer include tirapazamine [32], AQ4N [29], PR-104 [17], and TH-302 [41].

TH-302 is a hypoxia-activated prodrug (HAP) composed of 2-nitroimidazole conjugated to bromo-isophosphoramide mustard (Br-IPM) [8]. The 2-nitroimidazole moiety of TH-302 acts as an oxygen concentration sensor, releasing the DNA-alkylating Br-IPM within hypoxic regions of tumors. TH-302 is more potent under hypoxic conditions versus aerobic conditions in cancer cell lines in vitro [8, 14, 27] and specifically targets hypoxic tumor cells in xenograft tumors in vivo [35].

Combining a normoxic compartment-selective conventional chemotherapeutic with a hypoxia compartment-selective agent, such as TH-302, should provide a complementary approach to eliminate all tumor cell subpopulations, without a corresponding increase in systemic toxicity [6, 30, 42]. However, the combination of a conventional chemotherapeutic and TH-302 may not act on the tumor subcompartments independently of each other. The sequence and schedule of the administration of the two agents could affect the therapeutic index of the combination. If doses are staggered, pharmacologic effects of the first agent could affect the activity of the second agent, either pharmacokinetically or pharmacodynamically. Peculiar to this particular approach to cancer therapy, one of the agents could affect the compartmental specificity of the other agent, relative to single-agent administration [7, 10, 15, 21]. For example, an effect on the tumor vasculature could increase or decrease the magnitude and distribution of tumor hypoxic regions [15]. Cytostatic or cytotoxic effects in the normoxic compartment could reduce the rate of oxygen consumption in the cells and affect the magnitude or volume of the hypoxic compartment [7].

Here, we investigated the combination therapy efficacy of TH-302 in different xenograft models with different companion chemotherapeutics. We also investigated whether there was dosing sequence and regimen dependence on the efficacy and toxicity observed in the combination therapy groups. The studies were carried out to help guide decisions about which diseases and chemotherapy combinations to explore in subsequent clinical trials. Criteria for the prioritization of diseases and chemotherapeutics in combination with TH-302 are by the considerations of unmet medical need, and settings in which the conventional chemotherapeutics are approved as single agents.

Materials and methods

Human cancer cell lines

H460 and Calu-6 non-small-cell lung cancer cells, HT29 colon cancer cells, PC3 prostate cancer cells, HT1080 fibrosarcoma cancer cells, Hs766t, SU.86.86, BxPC-3, and MIA PaCa-2 pancreatic cancer cells, and A375 melanoma cells were from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were grown as monolayer cultures under standard conditions in ATCC recommended media supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) in a humidified atmosphere at 37°C under 95% air and 5% CO_2. Stew2 melanoma cancer cells were from Dr. Lee Cranmer Arizona Cancer Center (Tucson, AZ) and grown in RPMI1640 supplemented with 10% fetal bovine serum.

Chemotherapeutics and dosing regimens

Cisplatin and irinotecan were from Sigma Chemical Co. (St. Louis, MO), docetaxel was from Sanofi Aventis (Bridgewater, NJ), gemcitabine was from Synchem OHG (Felsberg- Altenburg, Germany), doxorubicin was from Bridge Bioservice LLC (Daly City, CA), temazolomide and pemetrexed were from AK Scientific Inc (Mountain View, CA). TH-302 was synthesized at Syngene (Bangalore, India). Docetaxel was dissolved in 5% ethanol and 5% cremophor EL in water. All other drugs were dissolved in saline (0.9% NaCl). TH-302 dosing solutions were made either fresh or stored at 4°C for no more than 5 days. The other dosing solutions were made fresh before dosing. All the dosing solutions were filtered through a sterile 0.2-μm filter before administration. Docetaxel was dosed at 10 mg/kg Q7Dx2 or Q7Dx3, iv; Pemetrexed was dosed at 100 mg/kg QDx3/wk x2wks, ip; Cisplatin was dosed at 6 mg/kg Q7Dx2, iv; Irinotecan was dosed at 20 mg/kg QDx5/wk x2wks, ip; Doxorubicin was dosed at 4 mg/kg Q7Dx2, iv; Temozolomide was dosed at 25 mg/kg QDx5/wk x2wks, ip; Gemcitabine was dosed at 60 mg/kg Q3Dx5, ip.

Animals

Five to six weeks old female homozygous nude mice (Nu-Foxn 1^{nu} NU/NU), except for SCID in the MIA PaCa-2 xenograft study, were used in the studies. The mice (Charles River, Wilmington, MA) were housed in sterilized

filter-topped cages and maintained in sterile conditions with constant temperature and humidity and 12-h light and dark cycles. Aseptic animal handling techniques were employed. All study protocols were approved by Threshold Pharmaceutical's Institutional Animal Care and Use Committee (IACUC) or the University of Arizona Cancer Center's IACUC.

Ectopic xenograft models

1×10^6 H460, HT1080, Calu-6, or MiaPaCa-2 cells, 3×10^6 HT29, or PC3 cells, 5×10^6 Hs766t, SU.86.86, BxPC-3, A375 or Stew2 cells in 0.2 ml were implanted subcutaneously into the right flanks of the immunodeficiant mice. All of the cell suspensions were prepared in 50% Matrigel (BD Biosciences, Franklin Lakes, NJ) mixed with 50% serum-free medium, except for H460 that was prepared in 30% Matrigel and 70% serum-free medium. Tumors were measured with calipers twice weekly, and tumor volume was calculated using the following formula: tumor volume (mm^3) = [length × (width)^2]/2. Animals were monitored for signs of toxicity daily, and animal body weight was measured at least twice weekly until the study end. Each animal was euthanized when its body weight loss was greater than 20% as compared with the body weight on the 1st day of treatment, its tumor size reached 2,000 mm^3, or its tumor became ulcerated, whichever came first. The end points for evaluating antitumor efficacy were tumor growth inhibition (TGI) or tumor growth delay to 500 mm^3 (TGD_{500}). TGI was calculated using the following formula: $TGI = (1 - \Delta T/\Delta C)$ %. The ratio of the change in mean tumor volume of the treated groups (ΔT) versus the change in mean tumor volume of the vehicle control group (ΔC) was calculated as $\Delta T/\Delta C = (T_n - T_i)/(C_n - C_i)$, where T_n is tumor volume in the treatment group on day n, when the 1st animal in the study had to be euthanized due to tumor size limitation or when the tumor volume of vehicle mice reached 1,000 mm^3 on average; T_i is the tumor volume in the treatment group on the 1st day of treatment; C_n is the tumor volume in the vehicle control group at day n; and C_i is the tumor volume in the vehicle control group on the 1st day of treatment. TGD_{500} was determined as the difference in time required for the mean tumor size to reach 500 mm^3 between treated group and the vehicle control group. The median and range in days of individual animals to reach a tumor size of 500 mm^3 were also determined. Statistical comparisons were performed with Dunnett's Test using Prism GraphPad software.

Orthotopic/metastatic xenograft model

The model was established following the procedure described by Kraus-Berthier et al. [23]. In brief, 1×10^6 H460 cells suspended in 100 µl PBS were implanted through the chest wall into the left pleural space of nude mice. The hypoxic cell marker pimonidazole hydrocholoride (Natural Pharmacia International, Burlington, MA) was administered via ip injection at 60 mg/kg 1 h before animal killing. Immediately after animal killing, the lung and tumor tissues were removed and washed with PBS before freezing and embedding in OCT. The 8-µm-thick tissue sections were cut using a Microm HM500 cryostat microtome. The tissue sections were stained with H and E and imaged by light microscopy (Eclipse 90i, Nikon, Japan). Immunofluorescent staining was performed to visualize hypoxia using FITC-conjugated anti-pimonidazole monoclonal antibody (Natural Pharmacia International, Burlington, MA). The stained tumor sections were viewed using fluorescence microscopy under corresponding filters. Drug treatments were initiated seven days after tumor cell implantation. Each treated group consisted of 11–12 mice. Animal mortality was checked daily. The Kaplan–Meier plots were constructed as the percentage animals surviving in each group as a function of time. Median survival time (MST) is the time at which half the animals are still alive in the group. The antitumor activity was evaluated as follows: $T/C\%$ = MST of treated group/MST of control group × 100. Results were also expressed as the percentage of increased life span (ILS, T/C of treated group − 100). Statistical significance between the treated groups versus vehicle control group was evaluated by the log-rank test using Prism GraphPad software.

Results

Antitumor efficacy of TH-302 in combination with conventional chemotherapeutic agents: different sequences of administration

The effect of changing the sequence of drug administration in combination therapy on efficacy was explored in three different ectopic xenograft models: H460 NSCLC, HT1080 fibrosarcoma and PC3 prostate cancer. The dosing regimens of the standard chemotherapeutic agents were based on published studies (Supplemental Table 1). In the H460 model, TH-302 was given 2 h before, 2 h after, or simultaneously with cisplatin on the day when combination of both drugs was scheduled. Administration of TH-302 prior to cisplatin showed higher tumor growth inhibition versus the other two sequences (Table 1). The effect on efficacy from varying the dosing sequence of docetaxel and TH-302 was also studied in the H460 model. TH-302 was given 1, 4, 24, and 48 h before docetaxel or 4, 24, and 48 h after docetaxel. The efficacy observed with TH-302 4 h before docetaxel was higher than that observed for any of the

Table 1 Effect of dosing sequence on the antitumor efficacy of TH-302 in combination with different chemotherapeutic drugs against ectopic human tumor xenograft models

Tumor model	Combination chemotherapy drug regimen	TH-302 regimen	Dosing sequence[a]	Time interval (h)	TGD_{500}[b] (days)	TGI[c] (%)	MBL[d] (%)	Death[e] (*n*/total)
H460 NSCLC	Cisplatin 6 mg/kg iv Q7Dx2	50 mg/kg ip QDx5/wk x2wk	TC	2	16	87	7.4	0/10
			TC	0	16	84	11.3	0/10
			CT	2	13	75	3.6	0/10
	Docetaxel 10 mg/kg iv once	150 mg/kg ip once	TC	48	12	76	4.1	0/10
			TC	24	12	73	0.7	0/10
			TC	4	19	88	2.9	0/10
			TC	1	8	58	2.6	0/10
			CT	4	12	70	2.2	0/10
			CT	24	12	73	2.5	0/10
			CT	48	9	60	3.7	0/10
	Gemcitabine 60 mg/kg ip Q3Dx5	100 mg/kg ip Q3Dx5	TC	24	30	76	15.7	2/10
			TC	8	35	87	14.8	1/10
			TC	4	33	86	13.8	2/10
			TC	2	30	75	14.4	1/10
			TC	0	30	77	15.9	4/10
HT1080 fibrosarcoma	Doxorubicin 4 mg/kg iv Q7Dx2	100 mg/kg ip Q7DX2	TC	24	12	90	2.6	0/10
			TC	8	14	100	2.1	0/10
			TC	4	21	106	1.8	0/10
			TC	2	21	106	3.3	0/10
			CT	0	17	102	2.0	0/10
			CT	2	15	102	3.5	0/10
PC3 prostate cancer	Docetaxel 10 mg/kg iv once	150 mg/kg ip once	TC	24	22	86	7.9	0/10
			TC	4	24	88	12.5	0/10
			TC	2	16	71	7.4	0/10
			TC	0	15	66	15.8	6/10
			CT	2	15	70	8.3	1/10
			CT	4	21	80	4.7	0/10
			CT	24	19	76	5.8	1/10

[a] T, TH-302; C, Chemotherapeutic drugs

[b] TGD_{500}, tumor growth delay to 500 mm^3

[c] TGI, tumor growth inhibition

[d] MBL, maximal body weight loss due to drug treatment as compared with the first day of treatment

[e] Mice were euthanized due to body weight loss greater than 20%

other examined schedules. TH-302 4 h before docetaxel significantly enhanced antitumor activity compared with TH-302 1 h before docetaxel, i.e., TGI was 88 and 58%, respectively, ($p < 0.05$). TH-302 in combination with gemcitabine was examined in the H460 model, in which TH-302 was given simultaneously with or prior to gemcitabine (from 2 to 24 h). The efficacy observed with a 4- or 8-h interval was superior to 2, 24 h, or simultaneous administration. In the HT1080 model, TH-302 was given simultaneously with or prior to doxorubicin (from 2 to 24 h) or vice versa (doxorubicin administered 2 h before TH-302). Tumor inhibition (TGI) ranged from about 90–106% among all schedules examined, while TGD_{500} favored TH-302 given 2 or 4 h before doxorubicin (21 days versus less than 17 days in the other groups). In PC3, with 2-, 4-, and 24-h intervals, TH-302 was administered before or after docetaxel. TH-302 given 4 h prior to docetaxel showed the best efficacy of the schedules examined.

In three out of four dose-sequencing studies performed, the simultaneous dosing groups showed much more toxicity compared with other schedules (Table 1). When TH-302 was administered simultaneously with cisplatin or docetaxel, mice lost more body weight on average than other schedules tested (Table 1). When TH-302 was administered simultaneously with gemcitabine, 4 out of 10

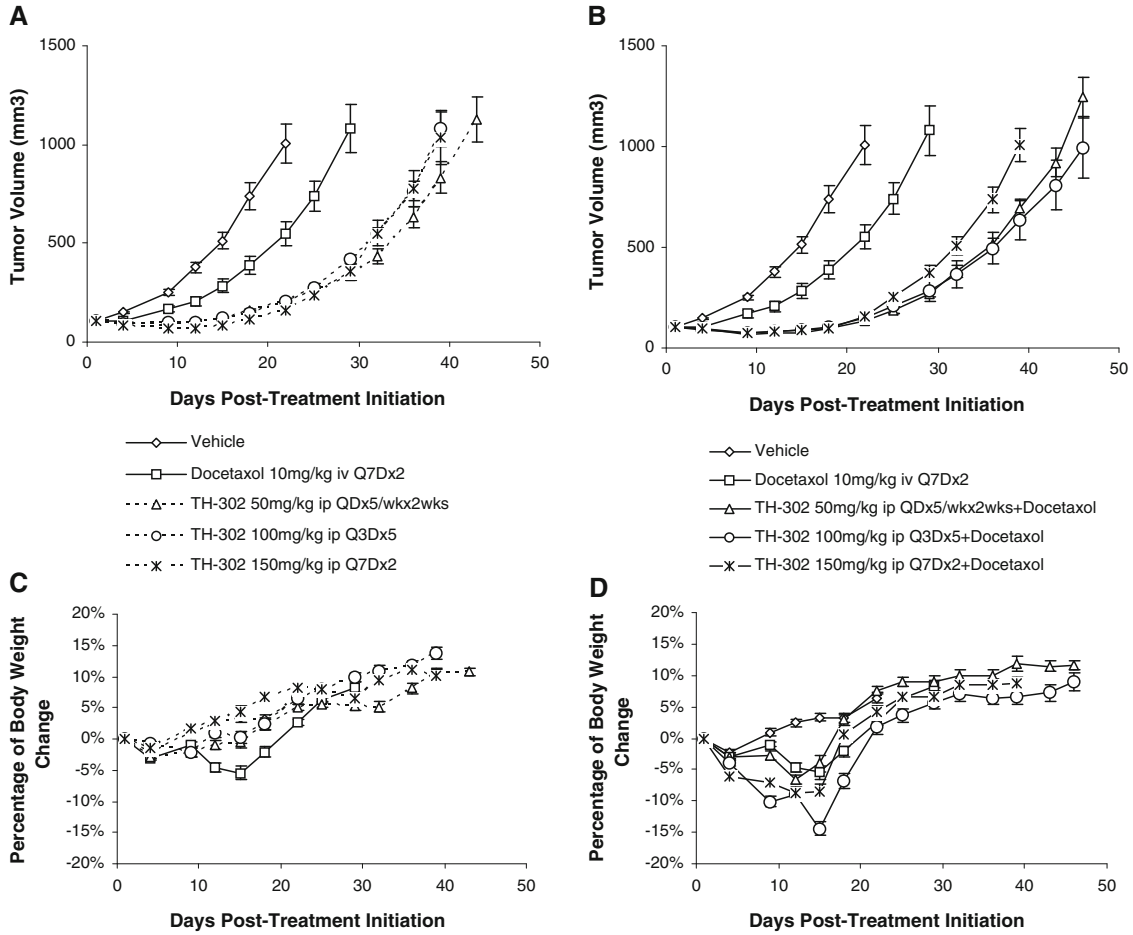


Fig. 1 Effect of dosing regimens on the antitumor efficacy of TH-302 alone and in combination with docetaxel in the ectopic H460 xenograft model. TH-302 was given 4 h prior to docetaxel when the two drugs were given on the same day. Means and standard errors of tumor volumes from the 10 mice per group are presented. Effect of different regimens of TH-302 alone (**a**) and in combination with docetaxel on tumor growth (**b**). Animal body weight change is plotted as a percentage change of that on the first day of treatment with TH-302 alone (**c**) or in combination with docetaxel (**d**)

E

Drugs	Monotherapy				Combination Therapy		
	Regimen	TGI (%)	TGD_{500} (d)	MBL (%)	TGI (%)	TGD_{500} (d)	MBL (%)
Docetaxel	10mg/kg Q7Dx2	51	6	5.5			
TH-302	50mg/kg QDx5/wk x2wk	89	18	2.9	96	21	6.6
	100mg/kg Q3Dx5	89	16	2.3	95	21	14.4
	150mg/kg Q7Dx2	94	16	1.4	95	17	8.7

mice were euthanized due to body weight loss greater than 20%. In addition, two separate hematology studies showed that simultaneous administration of TH-302 with either doxorubicin or gemcitabine led to lower white blood cell counts versus other sequences (Supplemental Figure 1).

In five separate studies employing different companion chemotherapeutics and xenograft models, administration of TH-302 2–8 h before the conventional chemotherapeutic agent showed consistently superior efficacy compared with other sequences. In the following combination studies, the TH-302 was administered 4 h before the conventional chemotherapeutic drug.

TH-302 in combination with docetaxel in the ectopic H460 model: dosing regimen dependence

To identify the optimal dosing regimen for in vivo antitumor efficacy of TH-302 in the H460 model, we performed

a study employing different dosing regimens of TH-302 in combination with docetaxel. Tumor-bearing mice were treated ip with TH-302 according to three different regimens: 50 mg/kg QDx5/wk x2wk; 100 mg/kg Q3Dx5; and 150 mg/kg Q7Dx2. The tumor growth curves following monotherapy with TH-302 at different dosing regimens or docetaxel are presented in Fig. 1a. During TH-302 monotherapy, tumor growth was inhibited 89, 89 and 94%, and the TGD_{500} compared to vehicle control was 18, 16 and 16 days, with these three regimens, respectively (Fig. 1e). Body weight changes observed in the different regimen groups are presented in Fig. 1c. TH-302 monotherapy showed less than 3% body weight loss in all three groups. The docetaxel monotherapy group exhibited maximal 5.5% body weight loss on average, with 3 out of 10 mice exhibiting greater than 10% body weight loss.

The tumor growth curves from combination therapy of TH-302 with docetaxel are presented in Fig. 1b. TGI was similar among all regimens examined. The 50 mg/kg QD and 100 mg/kg Q3D regimens showed the longest tumor growth delays, with a TGD_{500} of 21 days compared with vehicle control observed in both regimens. However, the TH-302 100 mg/kg Q3D combination regimen showed much more toxicity, as evidenced by body weight loss, compared to the other two regimens. Mice in the 100 mg/kg Q3D group lost 14% body weight on average, with 8 out of 10 mice having more than 10% body weight loss 15 days post-treatment (Fig. 1d). The TH-302 50 mg/kg, QD combination group did not show much additive toxicity, compared with docetaxel alone, in which maximal body weight loss was 6.6% in combination versus 5.5% in docetaxel monotherapy. The superior efficacy and lower toxicity results demonstrated that TH-302 50 mg/kg QD was an optimal regimen for combination with docetaxel in the H460 model. Therefore, the TH-302 50 mg/kg, QD regimen was applied to a number of xenograft models, including H460, Calu-6, PC3, Stew2 ectopic and H460 pleural models, for combination studies.

Antitumor efficacy of TH-302 in combination with docetaxel, pemetrexed, cisplatin, and irinotecan in H460 xenograft models

Animals bearing subcutaneous H460 tumors were treated with TH-302 alone or in combination with docetaxel, pemetrexed, cisplatin, or irinotecan. The regimens and doses employed for the companion chemotherapeutic drugs were based on published studies (Supplemental Table 1). The dose of cisplatin and pemetrexed was the optimal dose reported in the literature. Based on the body weight loss, the MTD of irinotecan was 20 mg/kg. To investigate the combination efficacy of TH-302 and docetaxel, the 10 and 20 mg/kg of docetaxel were employed. In both docetaxel alone and combination groups, the efficacy was similar between 10 and 20 mg/kg groups. However, the body weight loss was almost twice high in the docetaxel 20 mg/kg groups (Supplemental Table 3). Therefore, docetaxel 10 mg/kg was determined as an optimal dose.

As monotherapy, TH-302 yielded an average TGI of 74%, compared with 42, 38, 61, and 82% tumor growth inhibition by docetaxel, pemetrexed, cisplatin, and irinotecan monotherapy, respectively (Fig. 2). TH-302 enhanced the efficacy of all of the tested chemotherapeutic compounds when assessed in combination. TH-302 combination therapy delayed tumor growth 2- to 4-fold compared with the chemotherapy-only groups (24 vs. 8 days, 14 vs. 3 days, 17 vs. 4 days and 26 vs. 13 days, respectively).

Antitumor efficacy of TH-302 combined with chemotherapeutic drugs in additional human xenograft models

In the HT29 colon cancer model, TH-302 was dosed at 100 mg/kg Q3Dx5. TH-302 increased the TGD_{500} of cisplatin monotherapy by 3-fold (5 vs. 15 days, cisplatin monotherapy vs. cisplatin and TH-302 combination therapy, respectively) (Fig. 3a).

The efficacy of TH-302 in combination with doxorubicin was studied in both the HT1080 fibrosarcoma model and the Calu-6 NSCLC model (Fig. 3b, c). TH-302 monotherapy using 50 mg/kg QDx5 for 2 weeks in the HT1080 model inhibited tumor growth by only 20%, while the 100 mg/kg Q7Dx2 regimen inhibited tumor growth by 75% (Table 2). Combination therapy in the HT1080 model with doxorubicin yielded 106% TGI (Fig. 3b). The HT1080 model exhibited great sensitivity to doxorubicin monotherapy, which inhibited tumor growth by 95%. However, after drug treatment stopped, the tumors treated with doxorubicin alone regrew rapidly, while tumor regrowth was slower after discontinuation of TH-302 combination therapy. The TGD_{500} was 12 and 21 days for doxorubicin alone and in combination with TH-302, respectively.

A similar pattern was observed in the Calu-6 NSCLC model; where the doxorubicin-treated tumors grew more rapidly after treatment discontinuation as compared to TH-302 alone or TH-302 and doxorubicin combination treatment (Fig. 3c). TGD_{500} of doxorubicin or TH-302 monotherapy was 9 days, and TGD_{500} of TH-302 combined with doxorubicin was 14 days. Doxorubicin alone inhibited tumor growth by 32%, while combination therapy with TH-302 inhibited tumor growth by 64% (Table 2).

The PC3 prostate cancer xenograft was very sensitive to docetaxel treatment. Adding TH-302 to docetaxel did not significantly alter the efficacy. TGI of docetaxel alone and

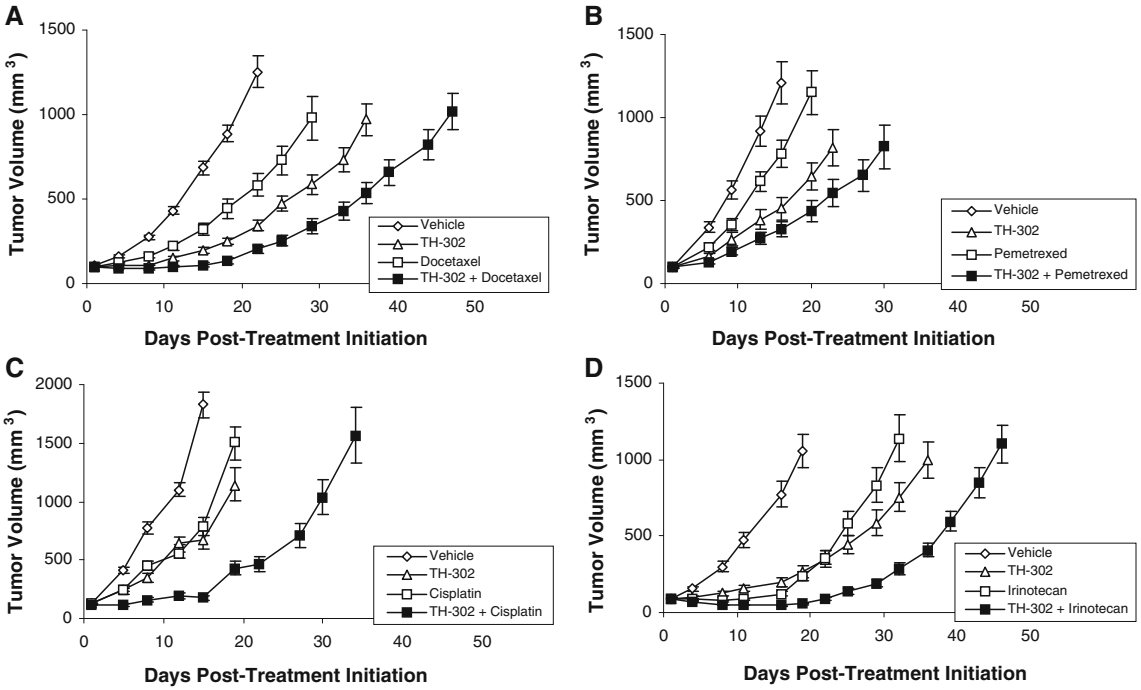


Fig. 2 Antitumor efficacy of TH-302 alone and in combination with different chemotherapeutics in the ectopic H460 xenograft model. TH-302 was administered ip at 50 mg/kg QDx5 per week for 2 weeks. **a** Effect of TH-302 and docetaxel alone and in combination on tumor growth. Docetaxel was administered iv at 10 mg/kg Q7Dx3. **b** Effect of TH-302 and pemetrexed alone and in combination on tumor growth. Pemetrexed was administered ip at 100 mg/kg QDx3 per week for 2 weeks. **c** Effect of TH-302 and cisplatin alone and in combination on tumor growth. Cisplatin was administered iv at 6 mg/kg Q7Dx2. **d** Effect of TH-302 and irinotecan alone and in combination on tumor growth. Irinotecan was administered ip 20 mg/kg QDx5 per week for 2 weeks

in combination with TH-302 were 94 and 105%, respectively. TGD_{500} of docetaxel alone and in combination with TH-302 were 38 and 40 days, respectively (Fig. 3d)

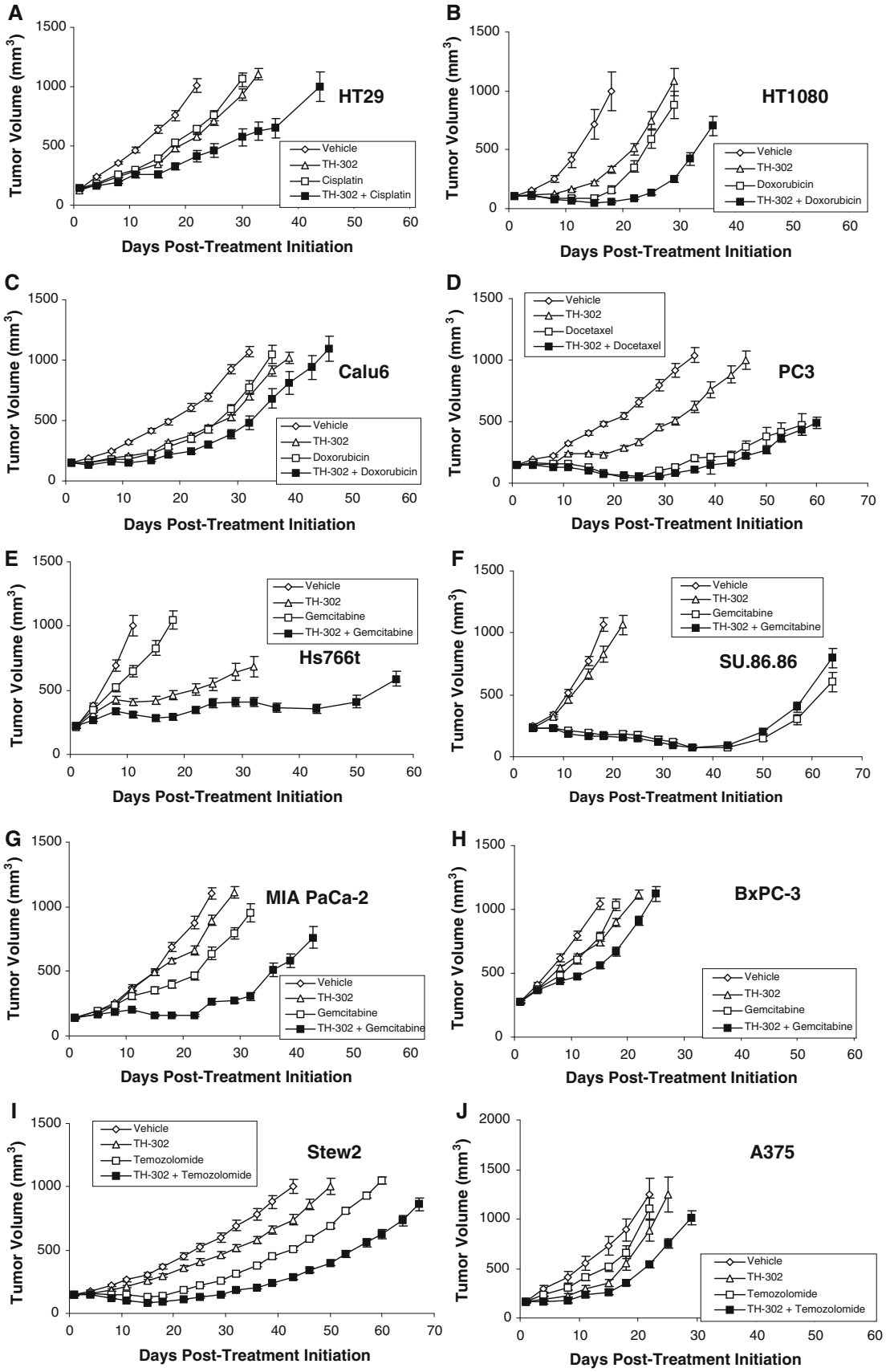
TH-302 in combination with gemcitabine was tested in four pancreatic xenograft models: Hs766t, SU.86.86, MIA PaCa-2, and BxPC-3 (Fig. 3e–h). TH-302 and gemcitabine were dosed Q3Dx5 at 75 and 60 mg/kg, respectively. This combination regimen was designed to model dosing regimens used in ongoing pancreatic cancer clinical trials of TH-302 (http://www.clinicaltrials.gov: NCT00743379, NCT01144455). TH-302 enhanced gemcitabine's efficacy in vivo in three out of four pancreatic models, but not in model SU.86.86 (Table 2). In the SU.86.86 model, TH-302 monotherapy had only slight activity and the high efficacy of gemcitabine monotherapy made the detection of an additional TH-302 effect difficult. In the other three pancreatic models, TH-302 added to gemcitabine delayed tumor growth 3- to 16-fold compared with gemcitabine alone (48 vs. 3 days, 22 vs. 8 and 6 vs. 2 days in the Hs766t, MIA PaCa-2 and BxPC-3 models, respectively).

In the Stew2 and A375 melanoma models, temozolomide alone inhibited tumor growth by 58 and 34%, respectively. TH-302 in combination with temozolomide inhibited Stew2 and A375 tumor growth in these models by 84 and 63%, respectively. The TGD_{500} of temozolomide alone and in combination with TH-302 in the Stew2 model was 18 versus 30 days, respectively. The TGD_{500} of temozolomide alone and in combination with TH-302 in the A375 model was 15 versus 21 days, respectively.

TH-302 in combination with docetaxel in the intrapleural H460 xenograft model

H460 cells were implanted in the pleural cavity of nude mice to obtain an orthotopic/metastatic tumor model, following the method of Kraus-Berthier and co-workers [24]. H460 cells proliferated in the pleural cavity and invaded contiguous lung parenchyma. Treatments started seven days after cell implantation, when tumor nodules were able to be observed in the lungs; large metastases were observed by 14 days. Hypoxic regions, identified by pimonidazole staining, were detectable as early as day 4. In general, micrometastases (diameter <1 mm) exhibited severe hypoxia, while larger metastases exhibited less hypoxia, consistent with the observations of others in experimental models of metastasis [24].

Efficacy of monotherapy and combination therapy was assessed by differential survival and analyzed by Kaplan–Meier analysis. The MST in the vehicle group was 24 days, whereas MSTs in the TH-302 monotherapy, docetaxel


A
HT29
Vehicle
TH-302
Cisplatin
TH-302 + Cisplatin
B
HT1080
Vehicle
TH-302
Doxorubicin
TH-302 + Doxorubicin
C
Calu6
Vehicle
TH-302
Doxorubicin
TH-302 + Doxorubicin
D
PC3
Vehicle
TH-302
Docetaxel
TH-302 + Docetaxel
E
Hs766t
Vehicle
TH-302
Gemcitabine
TH-302 + Gemcitabine
F
SU.86.86
Vehicle
TH-302
Gemcitabine
TH-302 + Gemcitabine
G
MIA PaCa-2
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TH-302
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TH-302 + Gemcitabine
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Stew2
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Temozolomide
TH-302 + Temozolomide
J
A375
Vehicle
TH-302
Temozolomide
TH-302 + Temozolomide
Tumor Volume (mm3)
Days Post-Treatment Initiation

◀ **Fig. 3** Antitumor activity of TH-302 in combination with different chemotherapeutics in various ectopic xenograft models. **a** TH-302 as monotherapy and in combination with cisplatin in the HT29 colon carcinoma xenograft model. TH-302 was administered ip at 100 mg/kg Q3Dx5. Cisplatin was administered iv at 6 mg/kg Q7Dx2. **b** TH-302 efficacy as monotherapy and in combination with doxorubicin in the HT1080 fibrosarcoma xenograft model. TH-302 was administered ip at 100 mg/kg Q7Dx2. Doxorubicin was administered iv at 4 mg/kg Q7Dx2. **c** TH-302 alone and in combination with doxorubicin efficacy on Calu6 NSCLC xenograft. TH-302 was administered ip at 50 mg/kg daily for 5 days per week for 2 weeks. Doxorubicin was administered iv at 4 mg/kg Q7Dx2. **d** TH-302 efficacy as monotherapy and in combination with docetaxel in the PC3 prostate cancer xenograft model. TH-302 was administered ip at 50 mg/kg daily for 5 days per week for 2 weeks. Docetaxel was administered iv at 10 mg/kg Q7Dx2. (**e–h**) TH-302 monotherapy and in combination with gemcitabine efficacy on Hs766t, SU.86.86, MIA PaCa-2, and BxPC-3 pancreatic xenografts. TH-302 and gemcitabine was administered ip at 75 mg/kg and 60 mg/kg Q3Dx5, respectively. **i–j** TH-302 monotherapy and in combination with temozolomide efficacy in the Stew2 and A375 melanoma xenograft model. TH-302 was administered ip at 50 mg/kg daily for 5 days per week for 2 weeks. Temozolomide was administered ip at 25 mg/kg and po at 50 mg/kg daily for 5 days per week for 2 weeks on Stew2 and A375 melanoma xenografts, respectively

monotherapy and the combination groups was 43, 35, and 56 days, respectively (Fig. 4). The increase in life span compared with the vehicle group was 77, 46, and 133% by TH-302 alone, docetaxel alone, and combination therapy, respectively. Combination therapy significantly prolonged the survival time compared with vehicle ($p < 0.05$) or either monotherapy ($p < 0.05$)

Discussion

The narrow therapeutic index (TI) of most anti-cancer agents is one of the major limitations of cancer chemotherapy. Hypoxia is commonly found in subregions of solid tumors. TH-302 is relatively inactive in normal tissue oxygenation levels but is activated to release a toxic DNA crosslinker in areas of reduced oxygenation. This enables TH-302 to target tumor tissue and spare normal tissue, reducing systemic toxicity and improving the TI. Combination therapy with hypoxia-selective TH-302 and normoxic-selective conventional chemotherapeutics is intended to yield complementary antitumor activity (Supplemental Figure 2).

The characteristic hypoxic fractions (HF) for a broad range of xenograft models were reported by Sun et al. [35]. For example, HF was more than 15% (high) in the H460 model, 5–10% (medium) in the Calu6, A375, and Stew2 models and less than 5% (low) in the PC3 model. In the pancreatic cancer models employed, the HF in Hs766t, BxPC-3, and SU.86.86 was 15, 7, and 5%, respectively. We have shown that the antitumor activity of TH-302 as a monotherapy correlates with the magnitude of tumor hypoxia in a given model [35]. In this study, we have demonstrated that the addition of TH-302 to commonly used chemotherapeutic agents enhanced in vivo antitumor efficacy. We also demonstrate that the sequence and schedule of co-administration can impact both the efficacy and toxicity of the combination therapies.

Specific drug combination regimens can be employed using different dosing sequences and schedules, leading to different efficacies and toxicities [11, 13, 33, 44]. These differences are determined by the different pharmacokinetic properties and different mechanisms of action of the agents being combined. These interactions may inhibit or enhance the efficacy or toxicity of the drugs under study. In the present study, we investigated the effect of changing the administration sequence of TH-302 in combination with cisplatin, docetaxel, gemcitabine or doxorubicin in three xenograft models: H460 NSCLC, PC3 prostate cancer, and HT1080 fibrosarcoma. Our results indicate that administration of TH-302 2–8 h before the companion chemotherapeutic yielded superior antitumor efficacy, as opposed to administration after, or simultaneously with, the companion chemotherapy. Statistical analysis showed significant difference was only obtained between the TH-302 4 h before docetaxel compared with 1 h before docetaxel in the H460 ectopic model. However, the trend appears clear and suggests that TH-302 administered 2–8 h prior to administration of the companion chemotherapeutic is an optimal sequence. Others have shown that for the hypoxia-activated prodrug tirapazamine, the maximal antitumor response was observed when tirapazamine was administered 2–3 h before cisplatin in the RIF-1 fibrosarcoma model [5]. Greater tumor inhibition was also observed when tirapazamine was administered 3 h before irinotecan, compared with 3 h after irinotecan [2]. It is possible that administration of the conventional chemotherapeutic before TH-302 may cause re-oxygenation of the hypoxic compartment by the conventional chemotherapeutic activity on the normoxic cells resulting in a reduction of their consumption of oxygen [7, 19]. This may thereby decrease the hypoxic fraction susceptible to TH-302. Thus, when TH-302 is administered after chemotherapy, the activity of TH-302 may be reduced due to a smaller hypoxic compartment. In addition, TH-302 simultaneously administrated with chemotherapeutic drugs yielded higher toxicity than other schedules (Table 1). Whether or not there is drug–drug interaction between TH-302 and the chemotherapeutic compounds tested is not clear. There is no evidence for TH-302 drug–drug interaction in the clinical setting [3, 9]. But notably, the plasma half-life of TH-302 in mice is short (8–10 min), and therefore, after the 4 h delay, plasma levels of TH-302 are very low [18].

TH-302 enhanced the antitumor effect of chemotherapeutic drugs in various xenograft models. An interesting

Table 2 Antitumor activity of TH-302 in combination with different chemotherapeutics in human tumor xenograft models

Tumor model	Chemotherapeutic in combination[a]	Chemotherapeutic monotherapy			TH-302 monotherapy[f]			Combination therapy		
		TGI (%)	TGD_{500} (days)	MBL (%)	TGI (%)	TGD_{500} (days)	MBL (%)	TGI (%)	TGD_{500} (days)	MBL (%)
H460 NSCLC	Docetaxel[b]	42**	8	4.1	79**	15	3.7	91**, #	24	6.0
	Pemetrexed[b]	38	3	0.0	68**	9	4.0	80**	14	6.2
	Cisplatin[b]	61**	4	1.9	68**	4	3.1	97**, #	17	0.8
	Irinotecan[b]	85*	13	10.4	82**	15	2.5	103**	26	8.7
Calu6 NSCLC	Doxorubicin[b]	32	9	0.0	41*	9	1.4	64**	14	6.2
HT29 colon	Cisplatin[c]	43*	5	3.3	48**	7	1.8	69**	15	6.8
HT1080 sarcoma	Doxorubicin[b]	79**	9	3.1	20	3	4.5	93**	14	2.9
	Doxorubicin[d]	95**	12	0.0	75*	10	1.2	106**	21	1.8
PC3 prostate	Docetaxel[b]	94**	38	3.8	47**	13	1.2	105**	40	10.8
MIA PaCa-2 pancreatic	Gemcitabine[e]	48**	8	0.0	21	0	1.9	87**, ##	22	9.1
Hs766t pancreatic	Gemcitabine[e]	45*	3	2.4	75**	17	3.4	89**, #	48	6.0
SU.86.86 pancreatic	Gemcitabine[e]	104**	50	0.0	28	1	0.0	106**	47	3.4
BxPC-3 pancreatic	Gemcitabine[e]	34*	2	0.0	40**	1	1.0	63**	6	7.9
Stew2 melanoma	Temozolomide[b]	58**	18	0.0	31*	7	1.2	84**	30	1.8
A375 melanoma	Temozolomide[b]	32	5	5.6	46	7	1.8	74	11	9.4

[a] Doses and regimens of chemotherapeutics are included in "Materials and methods"

[b] TH-302 was administered at 50 mg/kg ip QDx5 per week for 2 weeks

[c] TH-302 was administered at 100 mg/kg ip Q3Dx5

[d] TH-302 was administered at 100 mg/kg ip Q7Dx2

[e] TH-302 was administered at 75 mg/kg ip Q3Dx5

[f] TH-302 monothearpy data for PC3, Hs766t, SU.86.86, BxPC-3, and Stew2 are from [35]

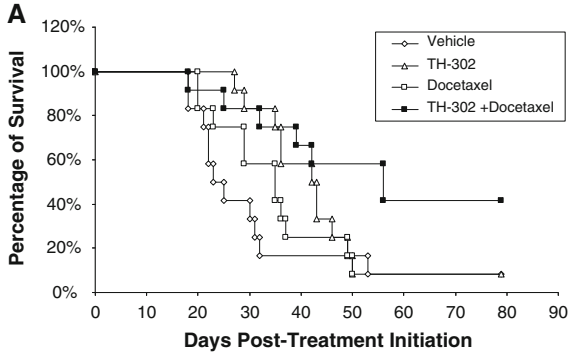
* $p < 0.05$, ** $p < 0.01$ as compared with vehicle

# $p < 0.05$, ## $p < 0.01$ as compared with monotherapy of chemotherapeutic

observation was that tumor regrowth after treatment with several of the conventional chemotherapeutic drugs alone was faster than after treatment with TH-302 and the other chemotherapeutic in combination, as observed with Calu6 and HT1080 tumors treated with doxorubicin, HT29 tumor treated with cisplatin, H460 tumor treated with irinotecan, and Hs766t tumor treated with gemcitabine.

Many studies have explored the mechanistic basis for the resistance against conventional cytotoxic therapy of cells in the hypoxic compartment of tumors. Doxorubicin is a poor tissue penetrator, and multiple previous studies have documented its inability to reach the hypoxic tumor compartment efficiently [28, 31]. Grau and Overgaard showed that cisplatin produced little or no effect on hypoxic cells in tumors, despite killing a large proportion of normoxic tumor cells [10]. In HCT-116 human colon cancer xenografts, cells located at the border of the necrotic, and presumably hypoxic, compartment, were the first cells to proliferate after gemcitabine treatment [16]. Hypoxic cells have also demonstrated resistance to the cytotoxic effects of gemcitabine [45]. In the A253 human head and neck squamous cell cancer xenograft, 5- to 7-fold less irinotecan was present in hypoxic regions versus well-vascularized regions of the same tumor [2]. In addition, cancer cells with stem cell properties may be preferentially localized to hypoxic regions of tumors and serve to repopulate the tumor after chemotherapy [20]. It is not known whether this is true for all cancer cell line-derived xenografts. The faster tumor regrowth kinetics that we observed with doxorubicin, cisplatin, irinotecan, and gemcitabine may be due to the selective sparing of stem cells in the hypoxic regions of xenografts [22, 39]. Slower regrowth in the combination groups is consistent with TH-302 selectively reducing the population of hypoxic cells.

Orthotopic xenografts of human tumors in nude mice have been reported to reproduce the histology and metastatic pattern of human tumors [23]. In this study, the antitumor efficacy of TH-302 in combination with docetaxel in the intrapleural H460 NSCLC xenograft model was evaluated. TH-302 combined with docetaxel significantly increased the life span as compared to docetaxel alone or TH-302 alone. Like others, we have observed high hypoxic fractions in micrometastases [25, 34]. The increased life span observed in the combination group of TH-302 and docetaxel might be due to TH-302's elimination of hypoxic cells in micrometastases resistant to traditional



B

Groups	MST (d)	T/C (%)	ILS (%)
Vehicle	24		
TH-302	43	177	77
Docetaxel	35	146	46
TH-302 + Docetaxel	56	233	133[*, a, b]

[*] p 0.05 *vs.* vehicle, [a] p 0.05 *vs.* TH-302 monotherapy, [b] p 0.05 *vs.* docetaxel monotherapy

Fig. 4 Antitumor efficacy of TH-302 alone and in combination with docetaxel in the intrapleural H460 NSCLC xenograft. Kaplan–Meier curve of animals treated TH-302, docetaxel alone and in combination. TH-302 was administered at 50 mg/kg ip, QDx5 per week for 2 weeks. Docetaxel was administered at 10 mg/kg, iv, Q7Dx2 ($n = 12$ per group)

chemotherapy. An alternate, although not exclusive, explanation is the elimination of tumoral stem cells from the hypoxic sanctuary regions, yielding more durable disease control and longer survival in the combination therapy-treated animals. While speculative, these are testable hypotheses.

In summary, the hypoxia-activated prodrug TH-302 enhanced the antitumor efficacy of several standard chemotherapeutic agents when tested in combination therapy regimens in multiple preclinical xenograft tumor models. In addition to guiding the ongoing clinical development of TH-302, these results further develop our understanding of microenvironmentally targeted therapy, focusing on tumor hypoxia, as a potentially compelling addition to available cancer treatments.

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