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ORIGINAL ARTICLE

Targeting acute hypoxic cancer cells by doxorubicin-immunoliposomes directed by monoclonal antibodies specific to RON receptor tyrosine kinase

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Abstract
Purpose Hypoxia contributes to acquired drug resistance in various cancer cells. The underlying mechanism is cellular insensitivity regulated by hypoxia-inducible factors (HIF), which impairs drug uptake, transport, and metabolism. The current study determines anti-RON antibody-directed cytotoxicity of doxorubicin (Dox)-immunoliposomes (IL) in hypoxic colon cancer cells.
Methods Cells were cultured under hypoxia (1% O_2, 5% CO_2, and 96% N_2) for 24 h. Dox-loaded IL were formulated followed by post-insertion of monoclonal antibody Zt/g4 specific to RON. Western blotting was used to detect HIF-1α and RON expression. Cellular uptake of Zt/g4-conjugated IL was determined by confocal and internalization assays. Cell viability was assessed by the MTT assay.
Results RON and HIF-1α expression were observed in hypoxic colon HCT116 and SW620 cells. Resistance to Dox-induced cytotoxicity was acquired in hypoxic cells with increased IC_{50} values. However, acquired resistance was attenuated by Zt/g4-directed Dox-IL, which displays increased cytotoxic activities. IL binding and uptake revealed that hypoxic RON expression is functional, which mediates high levels of Zt/g4-Dox-IL binding and cytoplasmic internalization. Zt/g4-Dox-IL is effective in killing hypoxic HCT116 and SW620 cells with reduced IC_{50} values compared to Dox and pegylated-liposomal Dox. These effects were dependent on hypoxic RON expression. HCC1937 cells with diminished RON expression under hypoxia were insensitive to Zt/g4-Dox-IL-induced cytotoxic effect.
Conclusions RON expressed by hypoxic colon cancer cells is thus a potential targeting molecule for delivery of chemotherapeutics. The ability of anti-RON mAb to direct Dox-IL cytotoxicity could be developed for attenuating hypoxia-acquired drug resistance in various cancer cells.

Keywords Monoclonal antibody · Receptor tyrosine kinase · Immunoliposome · Hypoxia · Drug delivery · Colon cancer cells

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Introduction

Hypoxia, characterized by reduction in the levels of tissue oxygen tension (up to 1%) in certain regions of a tumor mass [1], is a key regulatory factor in tumor growth and malignant progression [2, 3]. The cause of tumor hypoxia is poorly structured neovasculature during the expansion of a tumor mass [2, 3]. Under hypoxia, tumor cells initiate a cellular/genetic program featured by expression of

hypoxia-regulated genes such as transcription factor hypoxia-inducible factors (HIFs) [4] and angiogenic vascular endothelial growth factor (VEGF) [5]. The products of these genes modulate critical pathophysiological processes, which facilitate adaptation of tumor cells to harsh conditions for survival and growth [1–3]. There is convincing evidence indicating that hypoxic tumor cells are highly malignant with increased resistance to apoptosis, enhanced matrix invasiveness, and aggressive angiogenesis [6]. Hypoxic tumor cells also acquire resistance to chemotherapeutics and radiation therapy [7–9]. Clinically, hypoxia-induced acquisition of drug resistance is associated with poor prognosis and frequent treatment failure [7–9]. Clearly, hypoxia provides an opportunity for tumor cells to undergo adaptive response to hostile environment and to advance their malignancy for aggressive behavior. Thus, understanding the biological nature of hypoxic tumor cells should provide insight into the mechanisms underlying tumorigenic progression. Moreover, targeting hypoxic cancer cells with novel therapeutics should lead to the development of an innovative approach to achieve maximal therapeutic efficiency.

The RON receptor tyrosine kinase is a 180-kDa heterodimeric protein belonging to the MET proto-oncogene family [10]. The receptor is first synthesized as a single-chain precursor (pro-RON) and then processed into biologically active α/β two-chain mature RON, which is recognized by ligand macrophage-stimulating protein [11]. The members of this family including the MET receptor, hepatocyte growth factor receptor have been implicated in pathogenesis and carcinogenesis of various types of cancers [12]. RON is expressed by epithelial cells and activated by macrophage-stimulating protein (MSP) [11, 13]. Aberrant expression of RON occurs in various tumors including colon, breast, and pancreatic cancers [13–15]. Immunohistochemical analyses show that RON is overexpressed in large amounts of primary colon, breast, pancreatic and other epithelial cancer samples [13–15]. The consequence of RON overexpression is activation of multiple signaling cascades that increase migration, invasion, and malignant progression of cancerous cells [16, 17]. Overexpression of RON has also been linked to increased resistance to chemotherapeutics [18]. Clinically, RON and MET overexpression is associated with disease progression and worsened prognosis in colon and breast tumors [15, 19].

Because of its unique expression pattern and its role in oncogenesis, RON has been considered as a potential drug target for cancer therapy [20–22]. Currently, small molecule tyrosine kinase inhibitors (SMI) and therapeutic monoclonal antibodies (mAb) specific to RON are under intensive investigation [20–22]. In vitro studies using cell lines have shown that RON-specific tyrosine kinase inhibitors such as PHA665752 or compound 1 inhibit MSP-dependent or independent RON phosphorylation and disrupt RON-mediated downstream signaling cascades [22, 23]. In vivo experiments using tumor xerograph models further demonstrate that RON-specific SMI inhibit tumor growth and increase survival rate [22, 23]. Similarly, RON-specific mAb-induced reduction in tumor growth in mouse tumor models has also been confirmed [21]. We recently developed a novel approach to enhance cytotoxic effect of doxorubicin (Dox) on various tumor cells [24]. This is achieved by using a RON-specific mAb Zt/g4 in conjugation with Dox-immunoliposome (IL) for targeted cell cytotoxicity. In vitro studies have demonstrated that anti-RON antibody-directed drug delivery is effective for increased uptake of cytotoxic drugs in cancer cell under normoxic conditions [24]. These studies provide a basis to further study antibody-based RON targeting as a potential therapeutic mean for treatment of malignant cancers.

The present study determines the effectiveness of antibody-directed RON targeting as a drug-delivery pathway for Dox-IL-induced cytotoxicity against hypoxic cancer cells. Since hypoxic cells exist in a tumor mass, our goal is to determine whether anti-RON IL will also target hypoxic cells. Using hypoxic colon and breast cancer cell lines, acquiring resistance to chemotherapeutic-induced cytotoxicity [25, 26], as the model, we analyzed RON expression, drug uptake, and IL cytotoxicity against hypoxic colon cancer cells. Results from these studies demonstrate that RON is a suitable moiety for delivery of chemodrugs to hypoxic cancer cells for enhanced drug cytotoxicity. Such a targeted approach is also able to partially overcome the resistance of tumor cells acquired under acute hypoxic conditions.

Materials and methods

Cell lines, antibodies, and reagents

Human colon HCT116, SW620, SW837, and breast T-47D, HCC1937 cancer cell lines were from ATCC (Manassas, VA). Human mature MSP [13] was from Dr. E. J. Leonard (National Cancer Institute, Bethesda, MD). Mouse mAb Zt/g4 and rabbit polyclonal antibodies R5029 to RON were used as previously described [24]. Antibodies to phosphotyrosine, Erk1/2 (p44/42), and AKT were from Cell Signaling Inc. (Beverly, MA) and Santa Crutz Biotechnology (Santa Crutz, CA). Enhanced chemiluminescence reagents were from Thermo Fisher Scientific (Waltham, MA).

Cell culture under hypoxia

For hypoxia, cells (1×10^6 cells/ml $\times$ 5 ml in a 60 mm diameter culture dish) in DMEM with 10% fetal bovine serum (FBS) were cultured at 37°C overnight and then

subjected to hypoxia (1% O_2, 5% CO_2, and 94% N_2) for various intervals. Normoxic cell culture was performed under 21% O_2 with 5% CO_2 as previously described [13].

Formulation of Dox-loaded LS and IgG-conjugated IL

Chemicals and phospholipids (PPL) including cholesterol, hydrogenated soya phosphatidyl choline (HSPC), mPEG-DSPE, Mal-PEG-DSPE, and rhodamine (RD) were from Avanti Polar Lipids (Birmingham, AL). Free Dox was from Alexis Biochemicals (San Diego, CA). Pegylated-liposomal Dox (PLD) was from Ortho Biotech Products (Horsham, PA). Preparation of liposome loaded with Dox (Dox-LS) and incorporation of fluorescent dye RD into LS (RD-LS) were performed as previously described [24]. Incorporation of Zt/g4 into Dox-LS (Zt/g4-Dox-IL) was carried out by post-insertion technique as detailed previously [27]. IgG incorporation on LS was determined by 10% SDS–PAGE analysis using IgG standards followed by densitometry analysis [24]. Dox-IL conjugated with normal mouse IgG (NIg-Dox-IL) were used as the control. Zt/g4-dox-IL stability in serum was determined at 37°C by measuring leaked Dox. The % of leaked Dox at 1, 4, and 8 h were 0.3, 20.6, and 30.2%, respectively, which are similar to labeled PLD reported previously [28].

Western blot, immunoprecipitation, and protein phosphorylation assays

Western blot analysis was performed as previously described [13]. Rabbit polyclonal antibody R5029 to synthetic RON C-terminus was used to detect RON expression [13]. To measure RON phosphorylation, cells were stimulated with MSP for 15 min at 37°C, lysed in lysis buffer, and then immunoprecipitated with Zt/g4 [24]. The phosphorylated RON was detected by Western blotting using PY100 specific to phospho-tyrosine [13]. The levels of actin were used as loading controls.

Methods for measuring cellular uptakes of Zt/g4-conjugated IL

Two methods were used to determine cellular IL uptake. The first was the confocal microscopic method, in which RD is used as an indicator of cellular IL uptake [24]. Briefly, cells were cultured overnight under normoxic or hypoxic conditions on slide chambers and then treated at 37°C with Zt/g4-RD-IL for 60 min. Cells treated with NIg-RD-IL served as the control. After incubation, cells were washed with PBS, fixed with 4% paraformaldehyde solution, and then observed under Zeiss confocal microscope. The second method is to use PPL as an indicator to evaluate cellular IL uptake [29]. In this assay, the fluorescent intensity of RD was quantitatively measured and then converted into the amount of PPL incorporated into IL [29]. Cells were incubated with various amounts of Zt/g4 or NIg-RD-IL at 4°C or 37°C for 60 min and then washed with PBS. The fluorescent intensity from cell lysates was measured by a microplate fluorescent reader. The uptake was calculated and converted to amount of PPL in the cells [29]. The uptake of IL was also determined by directly measuring the amount of cytoplasmic Dox as previously described [24].

Cell viability assay

The MTT assay was used to monitor the effect of drugs on viability of normoxic or hypoxic cells as previously described [24]. Briefly, cells (0.8×10^4 cells per well) in a 96-well plate were cultured in triplicate in DMEM with 10% FBS and treated with different amounts of drugs for 72 h. Cell viability was determined by the MTT assay [24].

Results

Effect of acute hypoxia on HIF and RON expression by colon and breast cancer cells

Five cancer cell lines were subjected to hypoxic conditions for 24 h. HIF-1α and RON expression from cellular proteins was determined by Western blot analysis. HIF-1α was barely expressed in normoxic cells but dramatically increased under hypoxia as evident by specific antibody detection (Fig. 1a). RON was constitutively expressed at high levels by all five normoxic cell lines. Under hypoxia for 24 h, RON expression was not significantly affected in three colon cancer cell lines tested. The levels of expression were maintained in HCT116, SW620, and SW837 cells. However, in hypoxic HCC1937 cells, RON expression was dramatically diminished. In T-47D cells, levels of mature RON β-chain were maintained although pro-RON was reduced (Fig. 1a). RT–PCR analysis indicated that diminished RON mRNA expression attributes to reduced RON expression (our unpublished data). Thus, RON expression by HCT116, SW620 and SW837 cells is not subjected to regulation under acute hypoxia even though HIF-1α expression is increased. However, RON expression declined at variable extents in breast HCC1937 and T-47D cells.

The effect of MSP stimulation was studied using HCT116 and HCC1937 as model. MSP-induced RON phosphorylation in hypoxic HCT116 cells showed no difference when compared to that in normoxic cells (Fig. 1b). Similar results were also seen with SW620, SW837, and T-47D cells (data not shown). Since RON declined significantly in HCC1937 cells, MSP-induced RON phosphorylation was not observed, indicating the

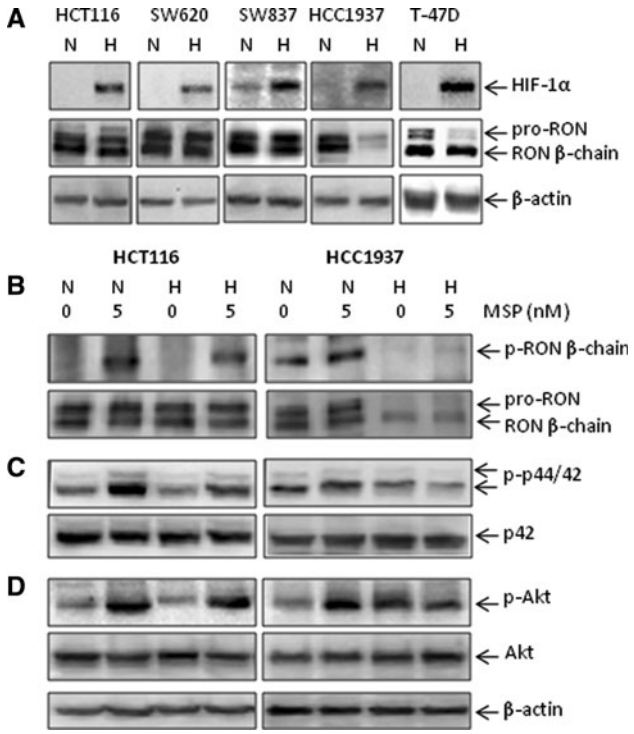

Fig. 1 Effect of hypoxia on RON expression and its activation by MSP in a panel of colon and breast cancer cells: **a** Expression of HIF-1α and RON under acute hypoxia. Five cell lines (2×10^6cells/culture dish) were cultured under hypoxia for 24 h, and cellular proteins (50 μg/sample) were subjected to Western blot analysis using antibodies specific to HIF-1α or RON. Levels of actin were used as the loading control. **b**, **c** and **d** Effect of MSP on phosphorylation of RON and its downstream signaling proteins. Cellular proteins were prepared from HCT116 and HCC1937 cells cultured as described in **a**. Cells were stimulated with MSP (5 nM) in serum-free media at 37°C for 15 min. Phosphorylated RON (**b**) was detected by Western blotting using antibody to phospho-tyrosine after immunoprecipitation of RON by Zt/g4 from cellular proteins (250 μg/sample). Phosphorylated Erk1/2 (**c**) and AKT (**d**) were analyzed by Western blot analysis using antibodies specific to p-Erk1/2 and p-AKT, respectively. Levels of actin were used as the loading control. N, normoxia; H, hypoxia. One of three experiments with similar results

requirement of RON for induced activation. MSP-induced phosphorylation of downstream proteins Erk1/2 and AKT was observed in normoxic and hypoxic HCT116 cells. The levels of phosphorylation showed no major difference between culture conditions (Fig. 1c, d). These effects were also seen in SW620, SW837, and T-47D cells (data not shown). However, the effect of MSP on hypoxic HCC1937 cells was minimal as evident by the baseline levels of Erk1/2 and AKT phosphorylation. Taken together, results in Fig. 1 demonstrate that under hypoxia, HIF-1α expression is dramatically increased in all cancer cell lines tested. However, RON expression by three colon cancer cells is not affected, and their responsiveness to MSP stimulation and activation of downstream signaling molecules is maintained. In contrast, RON expression was significantly reduced in HCC1937 cells, which impairs their responsiveness to ligand stimulation.

Acquired resistance of hypoxic cancer cells to doxorubicin-induced cytotoxicity

To determine the effect of hypoxia on Dox sensitivity, cells were incubated with various amounts of free Dox for 72 h followed by the cell viability assay. Under normoxia, all five cell lines showed dose-dependent sensitivity towards Dox (Fig. 2a). The individual IC_{50} values, as shown in Table 1, indicated that sensitivities among five cells lines were at the comparable levels. In contrast, hypoxic cells were insensitive to Dox. Three colon cancer cell lines displayed a significant resistance towards Dox with IC_{50} values ranging from 423 μg/ml in HCT116, 41.9 μg/ml in SW620, and 100.0 μg/ml in SW837 cells. These cell lines were considered as highly resistant to Dox based on their IC_{50} ratio (Table 1). Increased Dox resistance by breast HCC1937 and T-47D cells was also observed, although their resistant levels are relatively low (low resistant) (Table 1). These results demonstrate that colon and breast cancer cells acquire resistance under hypoxia to Dox-induced cytotoxicity at variable levels.

The sensitivity of cancer cells to PLD was shown in Fig. 2b. PLD is long circulating liposomes, and cells are generally less sensitive to their cytotoxicity [27]. PLD showed certain amount of cytotoxicity against normoxic HCT116 cells but had no effect on hypoxic cells. Normoxic SW620 cells were sensitive to PLD; however, cells were resistant to PLD under hypoxia. SW837 cells were highly insensitive to PLD under both normoxic and hypoxic conditions. The sensitivity of these two breast cancer cell lines to PLD under hypoxia did not change significantly. Hypoxic HCC1937 showed similar sensitivity to PLD compared to normoxic cells. T-47D cells showed moderate levels of resistance to PLD. These results demonstrate that hypoxic colon and breast cancer cells are insensitive in variable levels to PLD-induced cytotoxicity.

Effect of anti-RON antibody on RD-IL internalization by hypoxic cancer cells

To determine whether hypoxic RON expression facilitates anti-RON mAb-induced IL internalization, cytoplasmic internalization of IL was studied by monitoring intracellular fluorescence intensity of IL labeled with RD. Preliminary studies have found no differences in Zt/g4-binding affinity to RON expressed by hypoxic and normoxic colon cancer cells (our unpublished data). Binding of Zt/g4-RD-IL to hypoxic HCT116 cells was performed at 37°C followed by washing with acidic buffer to eliminate any cell surface-bound antibody molecules [24]. Confocal image analysis

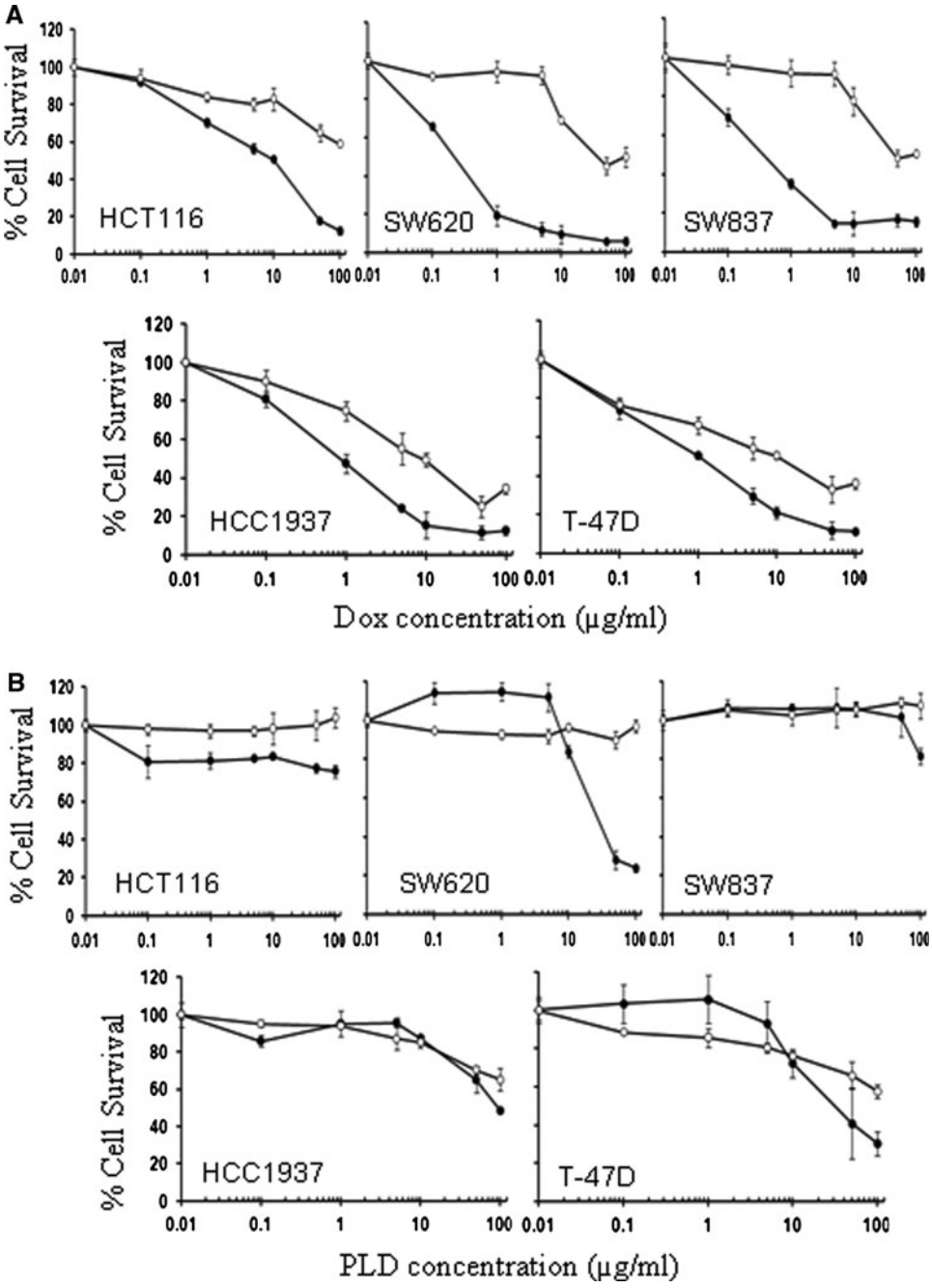

Fig. 2 Effect of hypoxia on sensitivity of cancer cells to doxorubicin or PLD-mediated cytotoxicity. **a** Increased resistance of cancer cells to Dox under hypoxia**:** Cells (1×10^4 cells/well in a 96-well plate) were treated in triplicate with various amounts of Dox under hypoxic conditions. Normoxic cells with similar treatment were used as the control. Cell viability was determined 72 h after treatment by the MTT assay as previously described [24]. **b** Effect of hypoxia on sensitivity of cancer cells to PLD. Cells were cultured as described in **a** and treated with various amounts of PLD. The MTT assay was used to determine the cell viability as described in **a**. (*open circles*), hypoxic cells; (*closed circles*), normoxic cells. One of two experiments with similar results

indicated the presence of intracellular RD fluorescence in Zt/g4-RD-IL-treated hypoxic HCT116 cells (Fig. 3a). The levels of cytoplasmic fluorescence intensity were comparable to those from normoxic cells. No intracellular fluorescence was detected in normoxic and hypoxic cells incubated with control NIg-RD-IL. Similar results were also seen in SW620 and SW837 cells (data not shown). To verify whether RON expression is required for internalization, hypoxic HCC1937 cells were used as the model. As shown in Fig. 3b, cytoplasmic fluorescence was detected after normoxic HCC1937 cells were incubated with Zt/g4-RD-IL. However, fluorescence was barely observed in hypoxic HCC1937 cells treated with Zt/g4-RD-IL, which had diminished RON expression (Fig. 1a). These results demonstrate that the RON expression by hypoxic colon cells is functional in mediating Zt/g4-induced IL internalization. Also, the levels of Zt/g4-induced IL internalization in hypoxic cells are comparable to those in normoxic cells.

Table 1 Cytotoxic effect of doxorubicin on hypoxic colon and breast cancer cells

Cell lines	IC_{50} of Dox (μg/ml)		IC_{50} ratio (hypoxia/normoxia)
	Normoxia	Hypoxia	
HCT116	10.8 ± 1.5	423 ± 21.2	40.2
SW620	0.4 ± 0.1	41.9 ± 5.2	104.7
SW837	0.6 ± 0.1	100.0 ± 12.4	166.7
HCC1937	2.6 ± 0.5	9.4 ± 1.8	3.6
T-47D	1.2 ± 0.2	11.0 ± 2.1	10.4

* Cells were cultured under normoxic or hypoxic conditions and then treated with various amounts of Dox (from 0 to 500 μg/ml of Dox) for 60 min. After washing, cells were incubated for an additional 72 h followed by the MTT assay to determine % of cell viability. The IC_{50} values of Dox for individual cell lines were calculated from data of two experiments using statistical software

Dose and time-dependent uptake of Zt/g4-RD-IL by hypoxic cancer cells

The effectiveness of hypoxic cancer cell uptake of IL was determined by measuring the amounts of internalized Zt/g4-RD-ILs using PPL as the indicator [24]. Results in Fig. 4a illustrate dose-dependent uptake of Zt/g4-RD-IL by hypoxic HCT116 and SW620cells. HCC1937 cells were used as the control. The levels of Zt/g4-RD-IL uptake by hypoxic HCT116 and SW620 cells upon incubation for 60 min were comparable to those by normoxic cells, although a slight reduction (24 or 12% reduction in HCT116 or SW620 cells, respectively) was observed. Normoxic HCC1937 cells showed increased Zt/g4-Rd-IL uptake; however, the uptake was hardly observed under hypoxia due to diminished RON expression. The effect of time-dependent uptake was shown in Fig. 4b. The increased uptake of Zt/g4/RD-IL by hypoxic HCT116 and SW620 cells was observed with an increased incubation up to 3 h. At that time point, the levels of Zt/g4-RD-IL uptake by hypoxic cancer cells were comparable to those from normoxic conditions. Again, a slight reduction was observed (25 and 15% reduction in HCT116 and SW620 cells, respectively). It is reasoned that impairment of receptor-mediated endocytosis under hypoxia could be the cause for the observed uptake reduction. Again, control hypoxic HCC1937 cells hardly show the time-dependent uptake of Zt/g4-RD-IL. We also confirmed the uptake by direct measurement of intracellular Dox upon Zt/g4-Dox-IL treatment. The amount of Dox in cytoplasm of hypoxic HCT116 cells was about 8.6 ± 3 ng per one million cells, which is comparable to the amounts in normoxic cells (10.7 ± 4 ng per one million cells). These results demonstrate that relatively higher levels of Zt/g4-directed RD-IL uptake by colon cancer cells are maintained under hypoxic conditions. Such uptake is dependent on RON expression.

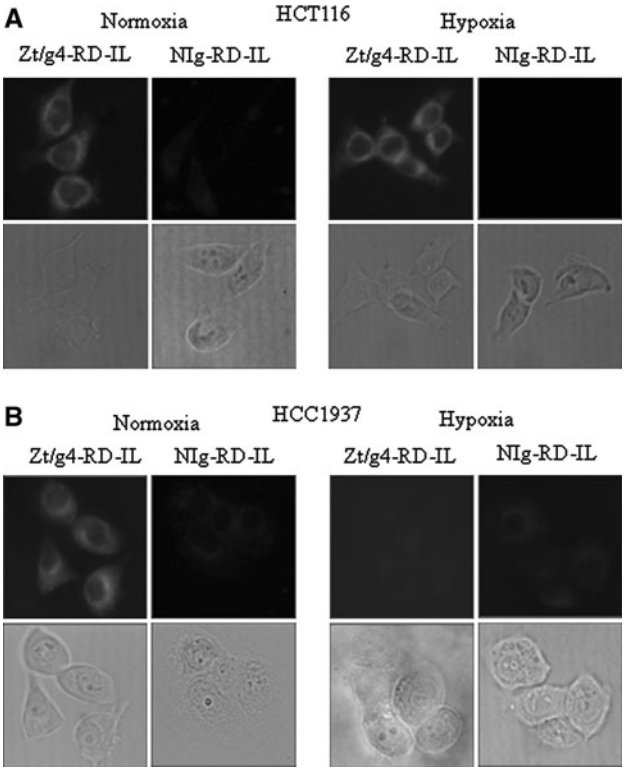

Fig. 3 Effect of Zt/g4 on hypoxic RON-mediated IL internalization: HCT116 (**a**) and HCC1937 (**b**) cells (1×10^4 cells/slide) under normoxic or hypoxic conditions were incubated with Zt/g4-RD-IL (equivalent to 1 μM PPL) at 37°C for 60 min. Cells treated with NIg-RD-IL served as the control. RD was used as the fluorescence indicator. After incubation, cells were washed with acidic buffer to eliminate cell surface bound IgG [24] and then fixed with 4% formaldehyde. Cell morphologies were observed and photographed under bright field. Intracellular fluorescence was documented by using the Olympus DSU confocal microscope

Cytotoxic effect of anti-RON mAb-directed Dox-IL on hypoxic colon cancer cells

Hypoxic HCT116 and SW620 cells were used as the model to validate the effectiveness of Zt/g4-Dox-IL. Cells were treated with various amounts of Zt/g4-Dox-IL. Free Dox, PLD, and NIg-Dox-IL were used as the controls. Results in Fig. 5a and b show the cytotoxic effects observed in normoxic and hypoxic cancer cells. Normoxic HCT116 and SW620 cells were highly sensitive to Zt/g4-Dox-IL-induced cytotoxicity. The IC_{50} values of Zt/g4-Dox-IL (51.6 μg/ml for HCT116 and 8.8 μg/ml for SW620) were at the comparable levels to free Dox (10.8 μg/ml for HCT116 and 0.4 μg/ml for SW620) but significantly lower than that of PLD (>1,000 μg/ml for HCT116 and 70.7 μg/ml for SW620 cells). The significant effect of Zt/g4-Dox-IL was observed in hypoxic HCT116 and SW620 cells, both showing acquired resistance to free Dox. When used at 100 μg/ml, Zt/g4-Dox-IL caused a significant reduction of cell viability

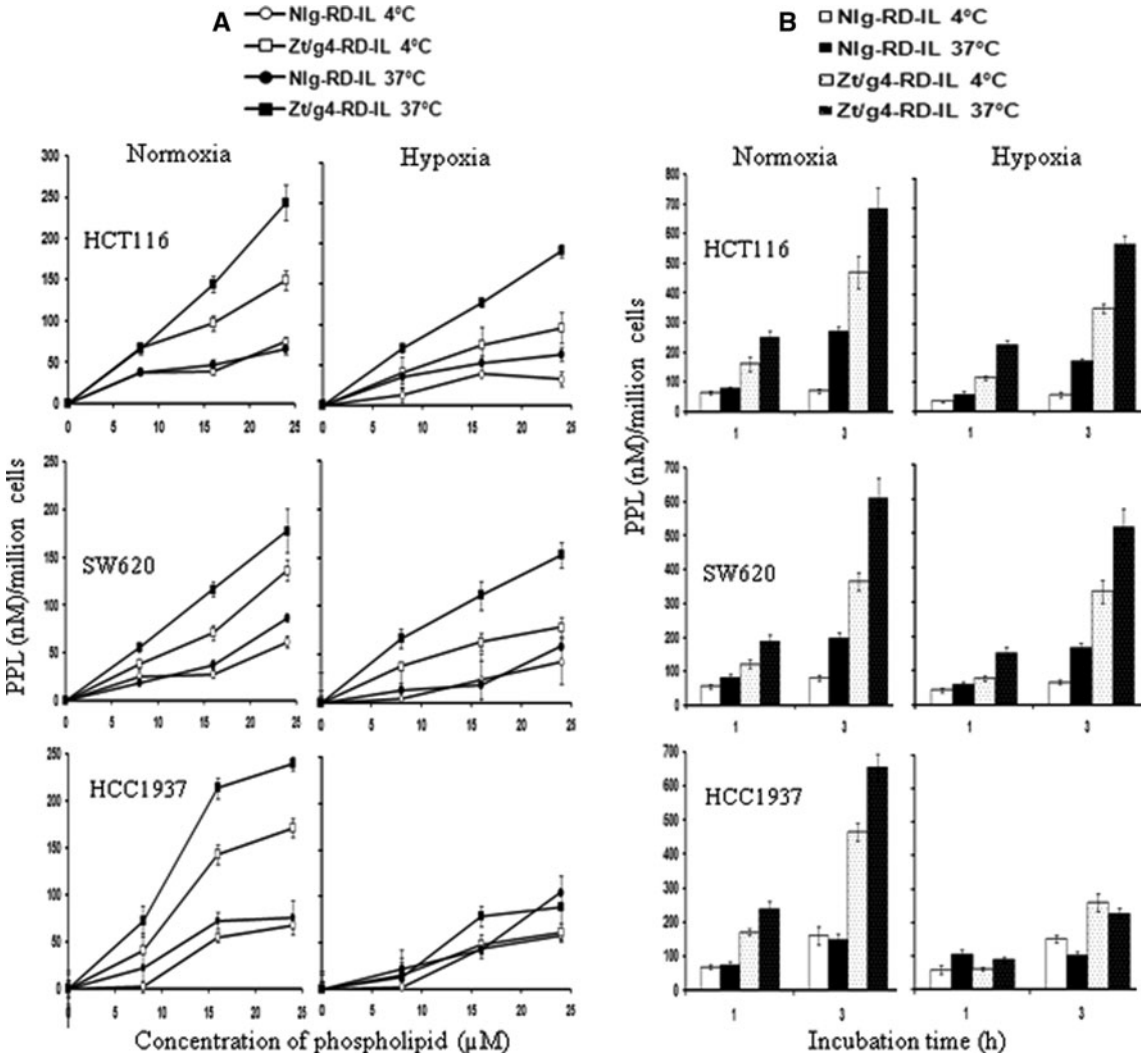

Fig. 4 Effect of hypoxia on dose- and time-dependent uptake of Zt/g4-directed IL by hypoxic cancer cells. **a** Dose-dependent uptake of Zt/g4-RD-IL by hypoxic cells. HCT116, SW620 and HCC1937 Cells (2×10^6 cells/sample) under normoxia or hypoxia were treated in triplicate with various amounts of Zt/g4-RD-IL (equivalent up to 25 μM of PPL) for 60 min. The purpose of performing assays at 4°C was to determine the levels of Zt/g4-RD-IL binding to cells without internalization. After washing, RD fluorescence was determined. The amount of IL bound (in nmol of PPL) was calculated from the initial specific activities of RD-IL. **b** Kinetic effect of Zt/g4-RD-IL uptake by hypoxic cells. HCT116, SW620, and HCC1937 cells (2×10^6 cells/sample) under normoxic or hypoxic conditions were incubated with Zt/g4-RD-IL (adjusted to 25 μM of PPL/sample) for 1 or 3 h. After incubation, cells were washed and subjected to fluorescent analysis as described previously [24]. One of two experiments with similar results

in HCT116 (42% reduction) and SW620 (33% reduction) cells, although the effectiveness is less than that under normoxic conditions. Acquired resistance under hypoxia seems to be the cause for the reduced effectiveness [30]. The control PLD and NIg-Dox-IL showed only a marginal effect on hypoxic HCT116 and SW620 cells. We also tested the toxic effect of Zt/g4-IL not loaded with Dox in HCT116 and SW620 cells. Under similar conditions, viabilities of HCT116 and HCC1937 cells were 96.4 and 98.1%, respectively, after cells were treated with Dox-free Zt/g4-IL equivalent to the amounts of lipids in Zt/g4-IL loaded with 100 μg/ml of Dox. Thus, results in Fig. 5 demonstrate that Zt/g4-directed Dox-IL is effective in killing hypoxic HCT116 and SW620 cells.

To further verify the effect of Zt/g4-Dox-IL, hypoxic HCC1937 cells with diminished RON expression were treated with various amounts of Zt/g4-Dox-IL and control drugs. Results in Fig. 5c show the cytotoxic effect of Zt/g4 on both normoxic and hypoxic cells. Zt/g4-Dox-IL were effective in normoxic cells with the IC_{50} values at 7.8 μg/ml, similar to free Dox. However, the effect of Zt/g4-Dox-IL on hypoxic cells was not significant. The percentage of killing was at comparable levels to control PLD and NIg-Dox-IL. These results demonstrate that the effect of

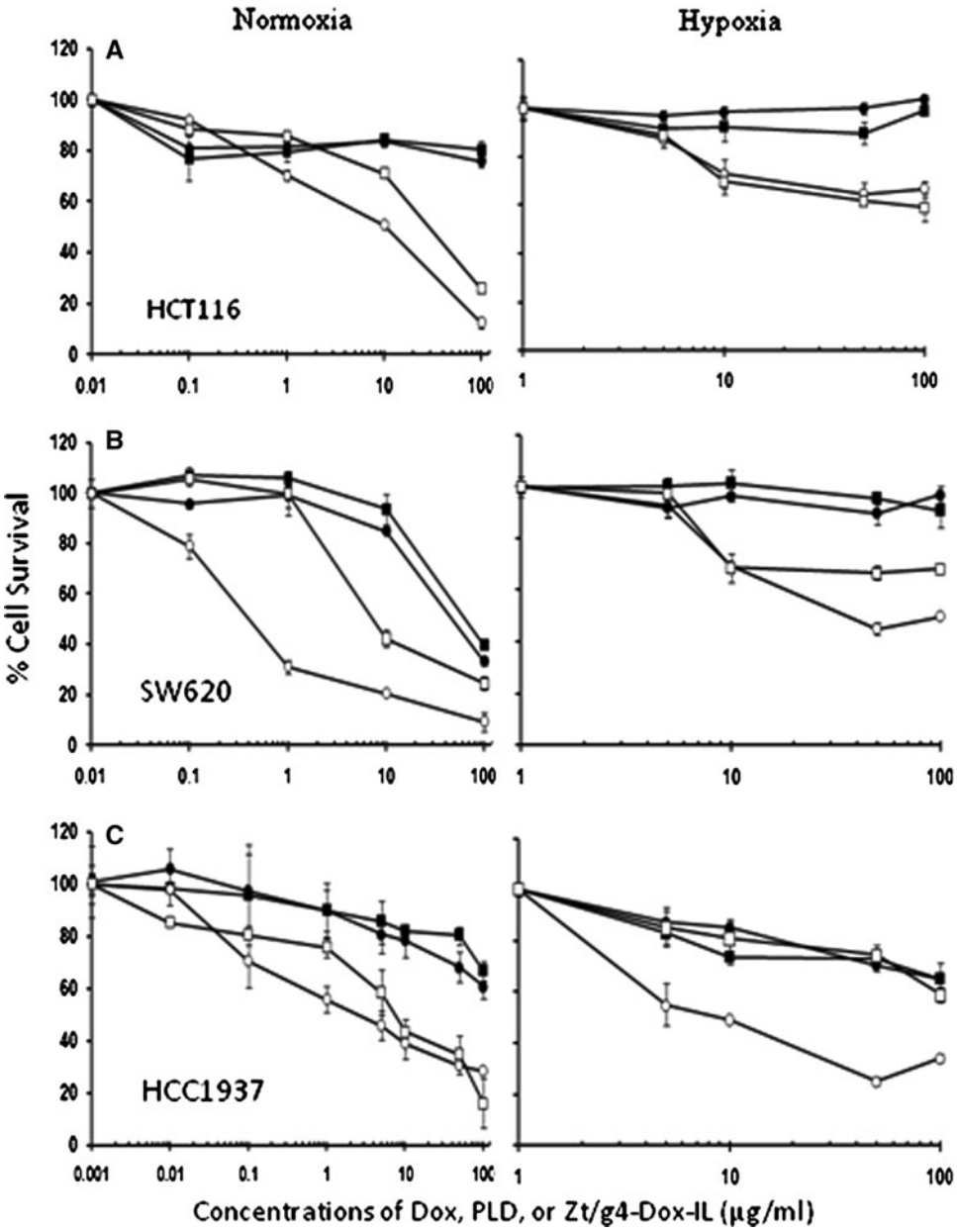

Fig. 5 Cytotoxic effect of Zt/g4-Dox-IL on hypoxic cancer cells. Cells (1×10^4 cells/well in a 96-well plate) were treated in triplicate with various amounts of Zt/g4-dox-IL, Dox, PLD, and NIg-Dox-IL under hypoxic conditions. Normoxic cells with similar treatment were used as the control. After incubation for 72 h, cell viability was determined by the MTT assay as previously described [24]. (*open circles*), treated with free Dox; (*filled circles*), treated with PLD; (*open square*), treated with Zt/g4-Dox-IL; and (*filled square*), treated with NIg-Dox-IL. One of three experiments with similar results

Zt/g4-Dox-IL is dependent on RON expression. The declined activity of Zt/g4-Dox-IL in hypoxic HCC1937 cells is related to the diminished RON expression.

Discussion

Hypoxic cancer cells are a group of distinguished cells with unique biological and phenotypic features [3, 4]. Various studies have confirmed that hypoxic cancer cells are highly resistant to apoptotic death and are malignant with aggressive behavior [3–6]. Acquisition of drug resistance by hypoxic cancer cells is a pathogenic feature that causes failure of various chemotherapeutics [7–9]. Clinically, acquired resistance has emerged as a challenge for effective chemotherapy [31]. Currently, development of strategies to overcome drug resistance by hypoxic or other malignant tumor cells is under intensive investigation. The findings in this report demonstrate that antibody-directed RON targeting is a rational approach for delivery of chemoagents such as Dox for enhanced cytotoxicity against hypoxic cancer cells. Using specific anti-RON mAb Zt/g4, we have previously formulated IL loaded with Dox and tested their effectiveness in normoxic colon cancer cells [24]. Zt/g4 is a monoclonal antibody recognizing the RON extracellular domain [32]. Binding of Zt/g4 to RON is highly specific and causes RON dimerization followed by internalization into the cytoplasm [24]. Moreover, it is observed that the Zt/g4

whole molecule is required to induce RON internalization. The Zt/g4 Fab fragment is inefficient in delivering Dox to cancer cells [24]. These properties provide a basis for using this antibody to target RON for delivery of chemoagents for enhanced cytotoxicity in normoxic cancer cells [24]. Our in vitro results have demonstrated that under acute hypoxia, cancer cells acquired resistance to Dox- and PLD-induced cytotoxicity. Also, colon cancer cells such as HCT116 and others maintained high levels of RON expression under acute hypoxia. Increased HIF-1α expression does not seem to modulate RON expression in these colon cancer cells. However, reduced RON expression was observed in two breast cancer cell lines. These results indicate that maintaining RON expression under hypoxia may be variable with different types of cancer cells. In hypoxic colon cancer cells, the RON receptor responds to MSP stimulation with increased tyrosine phosphorylation. RON-mediated binding and internalization of Zt/g4-Dox-IL were also maintained under hypoxic conditions, which results in significant amounts of Dox-IL uptake. Moreover, Zt/g4-directed delivery of Dox-IL partially overcomes acquired resistance and shows effective cytotoxicity against hypoxic cancer cells. Thus, anti-RON mAb directed delivery of chemodrugs has the potential to be developed into novel therapeutics with implications in targeted therapy of normoxic and hypoxic cancer cells.

Existence of tumor hypoxia provides an opportunity for cancer-selective therapy [33]. Various approaches have been studied, which include prodrugs activated by hypoxia, hypoxia-specific gene therapy, and small chemicals targeting HIF-1 transcription factor [33–37]. We took advantage to use the antibody-directed RON targeting as a suitable method for delivery of cytotoxic drugs to hypoxic cancer cells. Targeting cell surface proteins for intracellular delivery of chemoagents has been successful in the treatment of tumors such as leukemia and lymphoma [38]. The efficacy of this approach for solid tumors has also improved significantly. The clinical use of therapeutic antibodies to EGFR or HER2 has provided a platform for further development of novel therapeutics [39–41]. The combinational approach of antibody specificity with long circulating liposome loaded with chemoagents is a logical step towards improvement on therapeutic efficiency [42, 43]. One such example is targeting EGFR or HER2 receptors by mAb such as Cetuximab or trastuzumab, respectively, in conjugation with IL [39–41]. By targeting tumor cells overexpressing EGFR or HER2, Cetuximab or trastuzumab-directed drug delivery significantly enhances the efficacy of multiple chemoagents against cancer cells [39–41]. We have previously selected RON as a drug delivery moiety by using Zt/g4 to formulate Dox-IL for enhanced cytotoxic activities [24]. Altered RON expression exists in large amounts of primary cancers including colon, breast, pancreatic tumors [13–15]. The enhanced effectiveness of Zt/g4-Dox-IL has been validated in various types of cancer cells using in vitro models [24]. The results in this study extend these observations showing that RON-mediated uptake of Zt/g4-Dox-IL has potential for drug delivery in hypoxic cancer cells. This conclusion is supported by the following evidence. First, high levels of RON expression are maintained under acute hypoxic conditions by colon cancer cells, although reduced expression was seen in breast cancer HCC1937 and T-47D cells. A previous report has shown that increased HIF-1α expression engineered by plasmid transfection regulates RON expression in normoxic breast cancer cells [44]. However, under our experimental conditions, we did not observe such an increase in these two hypoxic breast cancer cell lines. Regardless of underlying mechanisms, our data suggest that anti-RON-directed drug delivery is suitable for certain types of tumors such as colon cancer. Selection of tumors with high levels of RON expression under hypoxia would be of crucial importance in achieving the therapeutic effect. Second, hypoxic RON expression by colon cancer cells is functional in terms of ligand binding, receptor internalization, and uptake of antibody-directed IL. As shown in Figs. 3 and 4, high levels of Zt/g4-induced Dox-IL uptake are achieved, which provide the biochemical basis for using RON as the targeting molecule. Third, treatment of hypoxic colon cancer cells with Zt/g4-Dox-IL showed increased cytotoxicity. Although Zt/g4-Dox-IL does not completely overcome acquired resistance, a significant toxicity to hypoxic cells was obtained at certain concentrations. Considering these facts, it is believed that hypoxic RON expression is a strong candidate molecule suitable for targeted delivery of chemotherapeutics for enhanced cytotoxicity against hypoxic cancer cells.

Acquired resistance to chemotherapeutics under hypoxic conditions is widely observed in various types of cancer cells [2, 7]. Our results in Fig 1 confirm that colon cancer cells such as HCT116 acquire doxorubicin resistance with a shift in IC_{50} value from 10.8 ± 1.5 μg/ml to 423 μg/ml. Acquired resistance is also observed in additional four cell lines showing variable increases in IC_{50} values. RON overexpression is associated with increased drug resistance [18]. Interestingly, sustained RON expression by colon cancer cells under hypoxia seems to correlate with increased chemoresistance as shown in Figs. 1 and 2. Currently, the mechanisms underlying hypoxia-induced resistance are complex. HIF-dependent and independent activation of signaling pathways that regulate cell apoptosis, proliferation, and senescence contributes to acquired resistance [2, 7]. Extracellular acidification and impairment of drug transporter activity such as p-glycoprotein under hypoxia have also been emphasized [45–47]. The observed resistance in our hypoxic colon cancer model could be as a result of combination of several factors. We showed that

hypoxia leads to profound extracellular acidosis in cell cultured environment (pH 6.8 after 24 h of hypoxia as compared to pH 7.3 under normoxia), which limits the uptake of Dox by hypoxic cells [45–47]. These results are consistent with those reported previously showing that acidification is a factor that impairs the uptake of cellular Dox and leads to drug resistance [45]. To address this reduction, we tested the Zt/g4-directed delivery method. The obtained results demonstrate that Zt/g4-directed uptake of Dox-IL is not significantly affected under hypoxic conditions. We showed by confocal microscopic analysis that fluorescence intensity of Zt/g4-RD-IL treated hypoxic cells is at comparable level to that from normoxic cells. Moreover, analysis of dose-dependent internalization confirmed that hypoxic colon cancer cells maintain high levels of Zt/g4-RD-IL uptake. More than 80% of uptake was achieved when compared to normoxic levels. The uptake is further increased in a time-dependent manner. Thus, anti-RON mAb-directed internalization is an effective method for delivery of cytotoxic chemoagents.

Different strategies have been tested to overcome acquired resistance or to increase the drug sensitivity of hypoxic cancer cells [33, 37]. Although still in the early stages of development, results from these studies have shown promising cytotoxic activities in hypoxic cancer cells, although the efficacy is still lower as compared to chemoagents. Nevertheless, these studies open an avenue for testing additional methods to overcome hypoxia-induced drug resistance. Studies using antibody-directed immunoliposome loaded with chemodrugs for hypoxic cancer cells have not yet been reported. Our current experiment is the first attempt to explore the effectiveness of such a possibility. Analysis of cytotoxicity from our studies indicates that anti-RON mAb directed delivery of IL-loaded chemodrugs could be a novel approach for targeted therapy of hypoxic colon cancer cells. As shown in Fig. 5, a large fraction of hypoxic HCT116 and SW620 cells were killed upon treatment with Zt/g4-Dox-IL. At the defined concentrations, the percentage of cell viability from both cell lines treated with Zt/g4-Dox-IL was reduced significantly. However, it needs to be noted that Zt/g4-Dox-IL do not completely overcome the resistance acquired by hypoxic cells. The resistance to Zt/g4-Dox-IL still exists. Yet Zt/g4-directed delivery approach causes increased cytotoxicity against hypoxic cancer cells resistant to Dox. Such an increase in hypoxic cytotoxicity provides a pharmaceutical basis for further improvement in future.

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