Anti-CD24 Antibody–Nitric Oxide Conjugate Selectively and Potently Suppresses Hepatic Carcinoma
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Abstract
Nitric oxide (NO) has a wide range of potential applications in tumor therapy. However, a targeted delivery system for NO donors has remained elusive, creating a bottleneck that limits its druggability. The antibody–drug conjugate (ADC) is a targeted drug delivery system composed of an antibody linked to an active cytotoxic drug. This design may compensate for the weak targeting ability and various biological functions of the NO donor. In this study, we designed the NO donor HL-2, which had a targeted, cleaved disulfide bond and an attachable maleimide terminal. We conjugated HL-2 with an antibody that targeted CD24 through a thioether bond to generate an ADC-like immunoconjugate, antibody-nitric oxide conjugate (ANC), which we named HN-01. HN-01 showed efficient internalization and significantly increased the release of NO in hepatic carcinoma cells in vitro. HN-01 induced apoptosis of tumor cells and suppressed tumor growth in hepatic carcinoma-bearing nude mice through antibody-dependent co-toxicity; HN-01 also increased NO levels in tumor cells. Collectively, this study expands the concept of ADC and provides an innovative NO donor and ANC to address current challenges in targeted delivery of NO. This new inspiration for an ANC design can also be used in future studies for other molecules with intracellular targets.

Significance: This study is the first to expand the concept of ADC with an antibody-nitric oxide conjugate that suppresses hepatic carcinoma in vitro and in vivo.

Introduction
The endogenous free radical nitric oxide (NO) is one of the smallest and extensively distributed signaling messengers that arbitrates numerous physiologic processes, which include neurotransmission, immune response, and vasodilation (1–3). For tumor, NO is a double-edged sword. Commonly, a proper concentration of NO is preferable for the proliferation of cancer, but a very low intracellular level of NO that is induced by NOS inhibition, and high levels of NO released by NO donors, can induce inhibition of cancer cell growth (4–7). However, due to the various biological functions of NO in the entire body and dichotomous effects on cancer biology, the ideal NO-based tumor-targeting agents could store efficiently and release therapeutic concentrations of NO controllably for the desired period at the target sites with minimal side effects.

Although many NO-based small molecules were developed as selective and potent anticancer agents, there is increasing interest in developing NO-releasing nanomaterials that include polymeric nanoparticles (8–10), dendritic polymers (11), liposomes (12), and silica nanoparticles (13). Compared with classical NO donors, the NO-releasing nanomaterials load a higher level of NO onto the high surface area and deliver NO to the desired sites, but it also increases the stability of the NO donor, which avoids the side effects of the off-target release of NO. However, some artificial nanoparticle materials could be accumulated in the body, which would be difficult to excrete after long-term administration. Additionally, the complexity of preparing nanoparticles limits their druggability.

Antibody–drug conjugates (ADC) display an encouraging therapeutic approach for tumor therapy by utilizing antigen-specific monoclonal antibodies to deliver cytotoxic chemotherapeutic drugs (14). The FDA-approved brentuximab vedotin and trastuzumab have validated this design of an ‘armed’ antibody, which forms the next generation of antitumor antibodies (15–19). The development of ADC technology has attracted the attention of researchers in the biological and chemical synthesis fields in recent years, which has resulted in more than 50 ADCs in clinical trials (20, 21). Improved conjugation methods have shown a remarkable advantage over traditional techniques; various types of toxin payloads have emerged, which are crucial components of ADCs.

Inspired by the superiority of accurate targeting, internal stability, and appropriate half-life of ADCs compared with nanoparticles, we hypothesized that a NO-donor molecule could be conjugated to an antibody and then cleaved inside targeted tumor cells. The combination of the NO donor and antibody might be...
beneficial for NO delivery that is targeted at cancer cells and for its druggability. We designed and synthesized a new NO-donor compound, HL-2, which has the following characteristics: (i) the maleimide terminal reacts readily with the reduced cysteine of antibodies to form a thioether bond and (ii) the disulfide bond is stable in the circulatory system and can be triggered by high concentrations of glutathione (GSH) in tumor cells to generate diazeniumdiolate anion by a 1,6-elimination reaction, which could release two molecules of NO spontaneously in situ. In addition, a cluster of differentiation 24 (CD24) is expressed rarely in normal cells, but it is overexpressed in many solid tumors, such as hepatocellular carcinoma (HCC; refs. 22–24), in which the chemotherapy drugs and targeted drugs have only limited effects. Antibody G7mAb can target the human CD24 specifically, and it can induce efficient internalization, which is an appropriate antibody for ADC design (25–27). Using this approach, HL-2 was conjugated to G7mAb to provide a previously unknown antibody–nitric oxide conjugate (ANC). HN-01. We proposed that after being injected, HN-01 would exhibit stability in circulation and would target CD24+ tumor cells selectively. After internalization into tumor cells that were mediated by CD24, HN-01 was triggered in the presence of high concentrations of GSH. Subsequent cleavage of the disulfide bond liberated a diazeniumdiolate anion through a 1,6-elimination reaction, which released two molecules of NO spontaneously in situ; this exerted antiproliferative and apoptosis-inducing activity to generate anticancer effects synergistically in vitro and in vivo (Fig. 1).

Materials and Methods

HN-01 preparation

Hybridoma technology was used to generate the anti-CD24 monoclonal antibody G7mAb. Hybridoma cells were injected intraperitoneally into mice. About 1 week later, ascites was collected and purified by a protein G affinity chromatography column (GE Healthcare), as previously performed (25). Mouse IgG1 control antibody (GenScript Biotech Corporation, Catalog No. 18K001612) was used as the isotype antibody. Please refer to the Supplementary Materials for the method for synthesizing NO donor HL-2. G7mAb was reduced partially with TCEP·HCl (ThermoFisher Scientific) to 1 mL G7mAb at 10 mg/mL, dissolved in PBS solution, and 33.8 µL TCEP at 1 µg/µL was added. This was followed by incubation on ice for 1 hour, and the TCEP was removed through Ultrafree-15 centrifugal filter devices (molecular weight cutoff of Mr 30,000; Millipore). The reaction was stopped and the partially reduced G7mAb was stored in 1 mL PBS that included antioxidants. Next, 47.6 µL HL-2 (10 mg/mL in DMEM) was added slowly, then reacted on ice for 30 minutes, and the excess HL-2 was removed by centrifugal ultrafiltration. The conjugated product HN-01 was filtered through a 0.2 µm filter under sterile conditions, stored in PBS at −80°C, and identified by SDS-PAGE electrophoresis. Accordingly, an isotype antibody-nitric oxide conjugate (IgG-NO) was generated and used as an isotype control group in subsequent experiments.

Analysis of drug-to-antibody ratio

The drug-to-antibody ratio (DAR) of HN-01 was analyzed by hydrophobic interaction chromatography (HIC) as previously performed (28). 2.5 µm particle size in a dimension of 4.6 mm × 35 mm was packed in the nonporous TSK-gel Butyl-NPR column ( Tosoh Bioscience). We equilibrated the column with mobile phase A at 0.5 mL/min flow rate until the baseline (monitored at 280 nm) was achieved. Then, we injected 10 µL of the HN-01 (10 mg/mL) sample and eluted with the gradient mix of mobile phase A (25 mmol/L sodium phosphate, 1.5 M ammonium sulfate, pH 6.95) and mobile phase B (75 % (v/v) aqueous solution of 25 mmol/L sodium phosphate (pH 6.95) and 25 % (v/v) isopropyl alcohol. The eluted results were detected at 280 nm, and DAR was calculated according to the percentage peak area.

Cell culture and animals

Hepatocellular carcinoma BEL-7402, Huh7, and normal human liver HL-7702 cell lines were bought from Cobioer Biosciences Co., Ltd., and preserved in our lab between passages 2 and 20. The cells were cultured in DMEM medium that contained 10% (v/v) FBS, 5% CO2, 37°C. All cells were authenticated by short tandem repeat (STR) profiling and examined Mycoplasma routinely by Mycoplasma Detection Kit (ThermoFisher Scientific, Catalog No. M7006). The human peripheral blood mononuclear cells (PBMC) were collected from 15 healthy donors. Written informed consents were obtained from donors. The BALB/c nude mice (female) of 5 weeks old were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou, China). All animals were raised and treated according to the standards of the Comparative Medicine Centre of Yangzhou University.

Flow cytometry assays of HN-01

A total of 5 × 10^5 BEL-7402, Huh7, or HL-7702 cell cells were suspended in PBS and then incubated with 200 nmol/L isotype antibody (Genscript Biotech Corporation, Catalog No. 18K001612) for 15 minutes, followed by 200 nmol/L G7mAb, HN-01, or IgG-NO at 4°C for 1 hour; the control groups were incubated with 200 nmol/L isotype antibody. All the cells were collected and washed three times with PBS. After washing, the cells were stained with FITC conjugated goat anti-mouse IgG (Nanjing Sunbio Technology Co., Catalog No.: L330) in the dark for 30 minutes and detected by flow cytometry (FACSCalibur; BD Biosciences).
Internalization assay
A total of $5 \times 10^3$ BEL-7402 or Huh7 cells were collected in PBS and then incubated with G7mAb or HN-01 (200 nmol/L each antibody) on ice for 1 hour, and after incubation, the cells were washed to remove the unbound antibodies. A group of cells remained on ice to stop the internalization, and the remainder of the cells was incubated at $37^\circ C$ for different periods of time from 8 minutes to 2 hours. Next, all those cells were fixed in 2% paraformaldehyde for 20 minutes and then stained with goat anti-mouse IgG, FITC conjugated (Nanjing Sunbio Technology Co.). The mean fluorescent intensity (MFI) was analyzed by flow cytometry and the receptor–antibody complex internalization was calculated as percent MFI loss at $37^\circ C$ relative to that on ice.

To observe the dynamic process of internalization, we tested labeled antibodies and cells at different time points by a confocal microscope. Cy5 Conjugation Kit (Fast; Abcam, Catalog No.: ab188288) was used to conjugate the HN-01 to Cy5 (HNP1-Cy5). HL-7702 or Huh7 cells were plated in the cell culture dish. After 24 hours, the cells were covered with 300 nmol/L DAPI stain solution (ThermoFisher Scientific, Catalog No. D1306), incubated for 5 minutes, and then washed. Cy5-labeled HN-01 were added into cells, incubated at $37^\circ C$ for 5 minutes or 1 hour and imaged by a confocal microscope.

Measurement of intracellular NO
4-Amino-5-(methylamino)-2',7'-difluorescein diacetate (DAF-FM DA; Beyotime) can react with NO in cells to produce a fluorescent compound, so we used it as a NO fluorescent probe. Huh7 or BEL-7402 cells were plated in the 96-well plates. When cells reached 85% confluence, they were cocultured with different antibodies or compounds (G7mAb 500 nmol/L, HN-01 500 nmol/L, HL-2 1500 nmol/L, JSK 1500 nmol/L) at $37^\circ C$ for 8 hours. Then, cells were fixed with paraformaldehyde (4%, 20 minutes) and Triton X-100 permeabilized (0.1%, 15 minutes). 2 μg/mL anti-3-nitrotyrosine antibody (Abcam, Catalog No. ab110282) was incubated with cells overnight at 4°C. Goat anti-mouse IgG, FITC conjugated (Nanjing Sunbio Technology Co., Catalog No. L3302) was added into cells for 1 hour. Cells were covered with 300 nmol/L DAPI stain solution (DAF-FM DA; Beyotime) can react with NO in cells to produce a fluorescent compound, so we used it as a NO fluorescent probe.

Antibody-dependent cellular cytotoxicity
The Non-Radioactive Cytotoxicity Assay (Promega, Catalog No.: G1780) was performed to detect the antibody-dependent cellular cytotoxicity (ADCC). PBMCs served as effector cells, BEL-7402 or Huh7 cells were cocultured with various amounts of effector cells in the presence or absence of HL-2, HN-01, or G7mAb (4 hours, 37°C). After centrifugation, 50 μl of the supernatant from the coculture medium was analyzed for LDH release according to the manufacturer’s protocol. The following equation was used to calculate the percentage of cytotoxicity: % Cytotoxicity = 100% × ([Experimental – Effector Spontaneous – Target Spontaneous]/[Target Maximum – Target Spontaneous]).

Proliferation of hepatocellular carcinoma cells
Proliferation and viability of BEL-7402, Huh7, or HL-7702 cells treated with ANC were detected using an MTT assay (Vybrant MTT Cell Viability Assay; ThermoFisher Scientific). Briefly, the cells were plated at $4 \times 10^3$/well into 96-well plates overnight. After overnight culture, the cells were washed and then treated with different concentrations of HN-01, G7mAb, or HL-2 for 72 hours. An MTT assay was performed and the inhibition rate was reported as inhibition % = (1 – untreated control cells%)/100%.

Apoptosis assay
BEL-7402, Huh7, or HL-7702 cells were incubated with HN-01 (100 or 500 nmol/L), HL-2 (300 or 1500 nmol/L) or G7mAb (100 or 500 nmol/L) at $37^\circ C$ for 48 hours. They were then stained with Annexin V-FLICA and propidium iodide (PI; Sangon Biotech) and detected by flow cytometer. The percentage of early apoptotic cells (Annexin V+/PI–) and the percentage of late apoptotic cells (Annexin V+/PI+) were summed as the total percentage of apoptotic cells.

Measurement of protein-bound 3-nitrotyrosine
Nitration can react with protein tyrosine residues or free tyrosine nitration to form a stable metabolite of 3-nitrotyrosine (3-NT). The nitration can thus be determined by the detection of 3-NT. JSK was a cancer drug candidate, which belongs to the diazeniumdiolate class. It can react with glutathione to generate two moles of NO at physiological pH, which has some similarities to HL-2 (29, 30). So, JSK was used as the positive control in this experiment. BEL-7402, Huh7, or HL-7702 cells were incubated with different antibodies or compounds (G7mAb 500 nmol/L, HN-01 500 nmol/L, HL-2 1500 nmol/L, JSK 1500 nmol/L) at $37^\circ C$ for 8 hours. Then, cells were fixed with paraformaldehyde (4%, 20 minutes) and Triton X-100 permeabilized (0.1%, 15 minutes). 2 μg/mL anti-3-nitrotyrosine antibody (Abcam, Catalog No. ab110282) was incubated with cells overnight at 4°C. Goat anti-mouse IgG, FITC conjugated (Nanjing Sunbio Technology Co., Catalog No. L3302) was added into cells for 1 hour. Cells were covered with 300 nmol/L DAPI stain solution (ThermoFisher Scientific, Catalog No. D1306), incubated for 5 minutes, and then washed. 3-NT was detected with a laser scanning confocal microscope (LSCM). An FV10-ASW 3.0 Viewer was used to calculate the MFI.

Antitumor efficacy of HN-01 in vivo
A hepatocellular carcinoma xenograft model was established by subcutaneously injecting Huh7 or BEL-7402 cells (1 × 10^3) into right flanks of BALB/c nude mice (Yangzhou University Comparative Medicine Centre Yangzhou, China). When the average tumor volume reached 100 mm^3, tumor-bearing mice were divided randomly into seven groups (six mice/group) and treated with HN-01 (1) (2.5 mg/kg), HN-01 (M) (5 mg/kg), HL-01 (H) (10 mg/Fisher Scientific, Catalog No. D1306), G7mAb (10 mg/kg), HL-2 (0.25 mg/kg), G7mAb+HL-2, or saline intravenously every 3 days. Each in vivo experiment was repeated three times. Tumor growth was measured using a digital caliper periodically and survival data of mice was recorded. And the tumor volume was calculated as $V = (length \times width^2)/2$. All mice were sacrificed on day 33 for the collection of their tumors.

Western blotting for cytochrome c
BEL-7402 tumor tissue from mice was minced on ice in lysis buffer that included phosphatase inhibitors, protease inhibitors, and 100 mmol/L PMSF, and tissues were homogenized to collect the mitochondrial proteins. The protein samples were detected by Western blotting. Anti-cytochrome c (Cyt c) antibody (ab133504) was used as the first antibody. Images were taken using the ChemiDoc XRS system (Bio-Rad).

Statistical analysis
The data were calculated by GraphPad Prism software and expressed as means ± SD. The level of significance was estimated
using the Student $t$ test, and $P$ values of 0.05 or less were considered to be statistically significant.

**Results**

**Chemistry**

Target compounds 1–7 were synthesized (Fig. 2A). Diazeniumdiolates, which is an important class of NO donor, can liberate two molecules of NO spontaneously at physiologic conditions with a range of half-lives from a few seconds to several minutes. Nevertheless, the $O^2−$-derived diazeniumdiolates generated stable precursors that were cleaved enzymatically in tumor cells to produce a diazeniumdiolate anion and, subsequently, to release NO in situ, which exhibited potent and selective antiproliferative activity. The 1,6-elimination-based linker is often used in self-immolative prodrug designs, and the triggers at the para position of the benzyl group supply various strategies to liberate the active drug from the prodrug molecule, which include diazeniumdiolates $O^2−$-anion moieties. Disulfide linkage, which is tolerant in blood plasma and sensitive in a tumor microenvironment, has been used widely as a potential strategy in the discovery of drugs that target tumors. In this regard, we used 1,2-bis(4-methylphenyl) disulfane as a redox-triggered linker to conjugate $N,N$-diethylamino)diazen-1-ium-1,2-diolate with an antibody. The disulfide linkages were broken in the high GSH tumor microenvironment, and the diazeniumdiolates subsequently released large amounts of NO in situ (Fig. 2B).

**Preparation and identification of HN-01**

The free thiol group of the partially reduced antibody reacted with the maleimide of HL-2, which generated HN-01. Then, HN-01 was confirmed by SDS-PAGE electrophoresis (Fig. 3A). The SDS-PAGE analysis showed that G7mAb and HN-01 were similar with the molecular weight of 150KD, because the molecular weight of HL-2 is only 588Da, and the purity of G7mAb decreased slightly after being reduced by TCEP.

**Detection of DAR**

HIC was used to analyze the drug-loaded species of HN-01. The addition of hydrophobic HL-2 to the G7mAb increased its hydrophobicity. Elution with a gradient of high concentration of a salt solution and an increasing organic modifier changed the column retention, which resulted in drug-loaded species with the least hydrophobic, unconjugated form to be eluted first and the most hydrophobic, 4-drug form to be eluted last (Fig. 3B). Based on the HIC results, the percentage in the peak area represented the relative proportion of a particular drug-loaded format, and G7mAb conjugated with two drug-loaded forms was the main component. The average DAR was 3.327, which was calculated from the formula $\text{DAR} = \Sigma(\text{weighted peak area})/100$. 

![Figure 2](https://example.com/figure2.png)

**Figure 2.** The synthetic and degradation route of HN-01. A, The synthetic route for HN-01. Reagents and conditions: a, NaHCO$_3$, H$_2$O, 20 minutes; b, NBS, benzene, room temperature, 6 hours, then NBS/AIBN, benzene/reflux, 80°C, 12 hours; c, diazeniumdiolates, DMF, N$_2$, 0°C, 4 hours; d, compound 2, acetone/DMF (2:1), N$_2$, 4°C, 3.5 hours; and e, G7mAb, DMSO, TCEP, room temperature, 2 hours and degradation route of HN-01. B, GSH triggered NO release from anti-CD24 antibody-nitric oxide conjugate HN-01.
In vitro selectivity of HN-01

A significantly overexpressed CD 24 was detected by western blot assay in Huh7 and BEL-7402 cell lines (Supplementary Fig. S1), and the cells were then pre-incubated with G7mAb, HN-01, or IgG-NO and stained with a fluorescent antibody. The selective binding capacity of HN-01 to the CD24 overexpressing tumor cell lines or a normal liver cell line was performed by flow cytometry (Fig. 3C). The specific percentages of binding of HN-01 to BEL-7402 (77.0%) and Huh7 (90.8%) were obtained, but there was no binding with normal liver cell HL-7702. The isotype control IgG-NO showed no binding with BEL-7402/Huh7 cells (less than 4%). The percentages of binding of HN-01 was nearly the same with naked G7mAb, which indicated that HN-01 retained the high specific binding capacity of G7mAb to hepatic carcinoma cell lines.

Internalization assay

To examine whether HN-01 induced the CD24 receptors on the membrane surface to mediate internalization, we treated BEL-7402 or Huh7 cells with G7mAb or HN-01 and then detected the cell surface level of the antibody by flow cytometry. HN-01 or G7mAb elicited a high level of receptor-mediated internalization, and the speed of internalization was faster in Huh7 than in BEL-7402 (Fig. 4A,i and ii). Conjugation with HL-2 was less significant to the internalization capacity that was induced by G7mAb. To further illustrate that the dynamic process of internalization was induced by HN-01, we used a confocal microscope. In the beginning, ANC was gathered around the edge of the cell membrane. After 1 hour of incubation, ANC internalized into the Huh7 cells and abundant HN-01 was located in the cytoplasm of the CD24-overexpressed tumor cells (Fig. 4B,i). However, ANC showed no internalization into the human normal liver cell HL-7702 (Fig. 4B,ii).

NO release

The intracellular NO release by HN-01 in Huh7 or BEL-7402 cells was detected by DAF-FM DA (31). Compared with the MFI of the control group (125.5k) and G7mAb group (127.5k), MFI of HN-01 was 313.0k and was higher than the HL-2 group (298.0k) in Huh7 cells. Similar results were seen in BEL-7402 cells. HN-01 produced more NO than HL-2 in Huh7 and BEL-7402 cells. In sharp contrast, almost no NO release was detected in Huh7 and BEL-7402 cells treated with G7mAb, because it was not linked with HL-2 (Fig. 4C and D).
Antibody-dependent cellular cytotoxicity

To detect the ability of the Fc fragment of HN-01 to induce lysis of effector cell-mediated target cells, an ADCC study on BEL-7402 or Huh7 cells was performed, as described previously (32). Approximately 12.26% and 11.16% of the BEL-7402 and Huh7 cells, respectively, were lysed when exposed to an effector cell:target cell (E:T) ratio of 30:1 with 100 μg/mL HN-01. When the E:T ratio was 100:1, HN-01 triggered 21.75% lysis of BEL-7402 and 26.81% of Huh7 cells. Moreover, the lysis rate of G7mAb groups was higher than the corresponding groups of HN-01 (Fig. 5A). Overall, data indicated that HN-01 retained the Fc-mediated ADCC effect.

HN-01 inhibited proliferation

The selective antiproliferative activity of HN-01 was evaluated based on CD24-positive cell lines and normal liver cell lines by a 72-hour MTT assay. HL-2 (3,000 nmol/L) and G7mAb (1,000 nmol/L) showed a slight inhibition of Huh7 cell lines (38.2% and 24.8%, respectively). However, HN-01 exhibited significant antiproliferative activity on Huh7 in a dose- and time-dependent manner (60.4%; Fig. 5B,ii). The results were consistent with that of BEL-7402 cells (Fig. 5B,ii). HL-2 exhibited indiscrinate anti-proliferative activity on HL-7702 (inhibition%: 34.5% in 1,000 nmol/L; Fig. 5B,iii). In contrast, HN-01 did not affect the proliferation of normal cell line HL-7702, which suggested that HN01 exhibited selective cytotoxicity on target cells. Meanwhile, compared with control group, few colonies were observed following treatment with 50 nmol/L HN-01 for a 7-day colony formation experiment (Supplementary Fig. S2).

HN-01 promoted apoptosis

To explore the reason for inhibition of proliferation on BEL-7402/Huh7/HL-7702 cells, apoptosis assay was performed. Basically, the cells were treated with different concentrations of HN-01, G7mAb, or HL-2 for 48 hours and examined by flow cytometry (Fig. 5C and D). At 500 nmol/L, the apoptosis rates of HN-01 treated Huh7 and BEL-7402 cells were 21.43% and 25.81%, respectively, which was significantly increased compared with the control group. As a contrast, no obvious apoptosis was found in HN-01 treated HL-7702 cells, whereas significantly increased apoptosis was detected in HL-2 treated HL-7702 cells.
These results proved that HL-2 non-targeted apoptosis in normal cells. With antibody conjugation, HN01 improved the targeted toxicity of NO donor part. HN-01 nitrated mitochondrial proteins of hepatocellular carcinoma cells.

NO can react with reactive oxygen species to form reactive nitrogen species, which can lead to nitration of the tyrosine residue at position 3 in mitochondrial protein Cyt c, forming a nitrated Cyt c (3-NT Cyt c; refs. 33–36). 3-NT was used as an extensive measure of the content of nitrated protein (34). HN-01 released a relatively large amount of NO and exerted an effective anti-proliferative effect. Compared with the MFI of the G7mAb-treated group (137.0 ± 29.8) and HL-2 group (393.5 ± 98.8), HN-01 treatment generated relatively high levels of fluorescence (700.7 ± 138.8), which meant that the HN-01 group generated relatively high levels of mitochondrial 3-NT in Huh7 cells. The positive control JSK treatment led to higher levels of 3-NT than

Figure 5.
The ant-hepatic carcinoma effect of HN-01 in vitro. A, HN-01 enhanced cytotoxicity of PBMCs. HN-01 maintained the ADCC in vitro. Huh7 or BEL-7402 cells were used as target cells, and PBMCs were used as effect cells. Cell lysis remained essentially unchanged after treatment with HN-01 compared with G7mAb. B, The cell viability was assessed by MTT assay at 72 hours after treatment with HN-01, G7mAb, or HL-2 (each at 0.8–1000 nmol/L). Cell death induced by HN-01, G7mAb, or HL-2 was plotted relative to the viability of untreated controls set at 100%. Data are given as mean ± SD (n = 5). C(i) and D(i), HN-01 induced apoptosis of Huh7 and BEL-7402 cells. Huh7 or BEL-7402 cells were treated separately with G7mAb (100 or 500 nmol/L), HL-2 (300 or 1500 nmol/L), and HN-01 (100 or 500 nmol/L) for 48 hours and then analyzed by flow cytometry after staining with Annexin V-FITC and PI. The percentage of cells in each quadrant is indicated. C(ii) and D(ii), Quantitative analysis of apoptosis assay. Data are presented as the mean ± SD (n = 3). . . , P < 0.001; **, P < 0.01; *, P < 0.05; ns, no significance.
that of HN-01 (Fig. 6A,i), which was similar in BEL-7402 cells (Fig. 6A,ii). Consistent data were presented in BEL-7402 cells (Fig. 6A,ii). However, no mitochondrial 3-NT was detected in HN-01-treated HL-7702 cells, although significantly increased mitochondrial 3-NT was generated in HL-2 and JSK-treated HL-7702 cells. Overall, the data proved that without targeted delivery, the small-molecules like HL-2 and JSK led to nonspecific toxicity.

Antitumor efficacy of HN-01 in tumor-bearing mice

BEL-7402 cells were inoculated in the armpit of BALB/c nude mice subcutaneously, and tumor volumes were measured during the treatment. Tumor growth in the high dose HN-01-treated group was significantly more inhibited than in the control group ($P < 0.0001$) and the G7mAb group ($P = 0.0054$; Fig. 6B,i). At the end of the treatment, the growth of tumors was inhibited in HN-01(H) group (81.15%). Middle doses and low doses of HN-01 reduced tumor burdens by 71.4% and 48.96%, respectively, although G7mAb + HL-2 groups (36.65%) did not display a prominent anti-tumor effect in the tumor-bearing model (Fig. 6B). To further illustrate the antitumor efficacy in vivo, we compared HN-01 treatment groups with its naked G7mAb groups and HL-2 groups, which reduced tumor burdens by 33.43% and 19.5% at comparable doses, respectively. These results suggested that HN-01 targeted tumor cells and released NO after being
internalized, and it exhibited better antitumor efficacy compared with all control groups. Furthermore, BEL-7402 cells bearing mice treated with HN-01 showed longer survival than those treated with G7mAb and HL-2. These results suggested that HN-01 showed better antitumor efficacy compared with G7mAb and HL-2 (Fig. 6B). In Huh7 cells bearing mouse model, HN-01 also was superior to the treatment with G7mAb and HL-2. (Supplementary Fig. S4).

Meanwhile, the immunohistochemistry staining was used to detect the effect of HN-01 on the cellular proliferation marker Ki67 and apoptosis marker cleaved caspase-3 (CC-3). After treatment with HN-01, a significant increase in CC-3 and a decrease in Ki67 levels were observed, which indicated that ANC inhibited proliferation of and induced apoptosis in tumor cells (Supplementary Fig. S5). The near-infrared (NIR) imaging results indicated that HN-01 effectively targeted CD24+ HCC cells in vivo. (Supplementary Fig. S6A).

**Assay of cytochrome c in tumor tissues**

The effect of HN-01 on the levels of Cyt c in tumor tissues was analyzed by Western blot analysis. The relative levels of Cyt c in the tumor tissues of mice that had been treated with HN-01 were dose-dependent, and they were significantly higher than that in the control group, G7mAb group, G7mAb cotreatment with HL-2 group and HL-2 group (Fig. 6C). The results showed that NO released by HN-01 may be the cause of the nitration, which exerted strong inhibitory activity on HCC cells in vivo.

**Discussion**

As a gaseous signaling molecule, NO plays a significant role in regulating the functions of cardiovascular, neurological, and immune systems (37). Meanwhile, the functions of NO in tumorogenesis and development have gained increasing attention. Available evidence suggests that relatively high levels of NO could act as a cytotoxic and apoptosis-inducing agent against tumor cells (6, 7, 37–39). A number of GST-reactive O2- (sulfonylidyethyl derived) diazeniumdiolates were generated as the NO donors (40, 41). Even though these compounds had a good tumor-suppressing effect, their side effects were simultaneous and unavoidable due to nontargeted delivery and various biological functions (42). This problem is a significant bottleneck that blocks their future use. There is an urgent need for a targeted drug delivery system to address these clinical limitations.

Historically, solid tumors are treated mostly through chemotherapy or radiotherapy. However, these nontargeting therapeutic options are usually associated with many adverse effects. In recent years, monoclonal antibody drugs have been developed in the field of cancer therapy. The therapeutic antibody is representative of a novel targeted drug with high target recognition, affinity, and internalization rate. However, many monoclonal antibodies face the problem of limited targeting ability and limited therapeutic efficacy. To better focus the targeted function of antibodies, we use antibodies as biological missiles specifically to deliver cytotoxins to tumor cells with lower toxicity and a higher therapeutic window. Antibody drug conjugates are representative of novel targeted drug delivery systems that use an antibody as a vehicle, and they have become one of the best alternatives in cancer immunotherapy (43, 44). CD24 is a membrane protein that is localized in lipid membrane raft domains (24, 45, 46). It is also a receptor that can mediate antibody internalization (46, 47). CD24 is known as a hepatocellular carcinoma stem cell marker and is upregulated in chemoresistant residual HCC (24). Previously, we developed the anti–CD24 antibody G7mAb, which selectively targeted HCC in vitro and in vivo (25, 48). What's more, previous studies showed that NO sensitized HCC to chemotherapeutic compounds by nitrosylation of critical thiolos in DNA repair enzymes. The increased expression of p53 gene families members played an important role in the induction of extrinsic and intrinsic pathways to cell death induced by chemotherapy in HCC (49).

Combining the antitumor effects of NO donors and G7mAb on hepatic carcinoma, we generated the antibody-nitric oxide conjugate HN-01 using a thioether bond to combine NO-donating HL-2 to G7mAb. By the specific targeting of G7mAb, the toxin molecules were enriched on the surface of tumor cells, which improved the therapeutic index of toxic molecules and reduced the toxic and side effects on normal tissues.

One hundred milligram of G7mAb was prepared through mice celiac inoculation. We conjugated the NO-donating HL-2 that contained a disulfide bond and maleimide with G7mAb to generate HN-01, and we obtained 16.8 mg ADC with 56.0% recoveries of G7mAb. An average DAR 3.327 was determined by HIC, which was consistent with the expected coupling of two to four toxins per antibody (Fig. 3B). The specific binding of HN-01 to CD24+ Huh7 and BEL-7402 cells was analyzed by flow cytometry (Fig. 3C). Except the in vitro targeting, our results also showed that HN-01 specifically targeted to HCC xenografts in vivo. Moreover, the binding of HN-01 to tumor was blocked by free HN-01. That suggested that HN-01 has specificity to CD24+ HCC, which might result in higher antitumor efficacy with fewer side effects (Supplementary Fig. S6).

The endocytosis and endocytic activity of HN-01 to target cells were examined using flow cytometry and a LSCM. HN-01 had equivalent endocytosis with its parental G7mAb, and both of them were internalized into target cells effectively. The effector cell-mediated cytotoxicity that was induced by the Fc fragment was detected using human PBMCs as effector cells and Huh7 or BEL-7402 cells as target cells. HN-01 had similar specificity and an ADCC effect similar to that of naked G7mAb (Fig. 5A). Moreover, the MIT assay suggested that HN-01 exhibited significantly higher antiproliferative effects in a dose-dependent manner on target cells compared with G7mAb or HL-2 alone (Fig. 5B). Furthermore, the FCM-based apoptotic assay suggested that the cytotoxicity was partially due to proapoptotic activity of the NO donor (Fig. 5C and D). In vitro studies showed that HN-01 agreed with the essential requirements of ADC, and the antitumor activity of HN-01 increased significantly, which was consistent with the expected results.

Finally, BEL-7402-bearing and Huh7-bearing nude mice models were established to evaluate the antitumor activity of HN-01 in vivo, and tumor volume of HCC-bearing nude mice was measured after treatment with HN-01. As anticipated, HN-01 inhibited the growth of xenografted tumors significantly and durable and significantly prolonged the survival of tumor-bearing mice (Fig. 6B). In addition, the decrease of Ki67 and increase of CC3 (Supplementary Fig. S5) indicated that HN-01-mediated tumor suppression was associated with inhibition of tumor cell proliferation and increased cell apoptosis. NO inhibited cell mitochondrial respiration and caused Cyt c release into the cytosol. The caspase activation cascade was triggered when Cyt c was released into the cytosol and led to cell death. Following
HYN treatment, the expression of Cyt c in tumor tissues was monitored by Western blot analysis. The results demonstrated that HYN-01 increased the amount of Cyt c in the cytoplasm of tumor cells (Fig. 6C), which proved that NO played a role in the inhibition of the mitochondrial respiratory chain and induced the release of Cyt c into cytoplasm. What’s more, the appropriate half-life of HYN-01 (Supplementary Fig. S6B) and low toxicity (Supplementary Fig. S7) laid the foundation for further development of the drug.

In this study, we created the first generation of ANC, which is a new tumor-targeted NO delivery system, which we named HYN-01. Basically, HYN-01 maintained specific binding ability, the ADC effect, and endocytosis efficiency. In addition, HYN-01 showed more effective antitumor activity than either component, G7mAb or HL-2, in vivo. In conclusion, HYN-01 combined fully targeted affinity of antibodies with a NO donor to achieve superior efficiency, which provides a new approach for the treatment of CD24+ malignant tumors.

Finally, the antibody-nitric oxide conjugate design, which used G7mAb coupled with HL-2, expands the concept of ADCs to the point that they are no longer limited to highly toxic, small-molecule drugs. Instead, gas drugs such as NO can be targeted and delivered accurately and, therefore, can be used for therapeutic purposes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Antibody-Nitric Oxide Conjugate Suppresses Hepatic Carcinoma


Anti-CD24 Antibody–Nitric Oxide Conjugate Selectively and Potently Suppresses Hepatic Carcinoma

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