NHS76/PEP₂, a Fully Human Vasopermeability-Enhancing Agent to Increase The Uptake and Efficacy of Cancer Chemotherapy

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Abstract

Purpose: Previously, we have shown that the attachment of interleukin 2 (IL-2) to a tumor-targeting antibody can produce a 4-fold enhancement in the uptake of antibodies and drugs in tumors. More recently, we discovered that a 37-amino-acid linear sequence of IL-2 designated vasopermeability-enhancing peptide (PEP), contained the vasopermeability activity of IL-2, and could be used after linkage to tumor-targeting antibodies to produce the same enhancement of drugs and antibodies in tumors. We now describe the generation of a fully human antibody fusion protein, designated NHS76/PEP₂, which can be used in patients to enhance the therapeutic potential of chemotherapy.

Methods: NHS76/PEP₂ was expressed in NS0 cells using the glutamine synthetase gene amplification system. To show its clinical potential as a pretreatment to chemotherapy, NHS76/PEP₂ was given i.v. 2 hours before the injection of suboptimal doses of etoposide, doxorubicin, Taxol, Taxotere, 5-fluorouracil, or vinblastine in mice bearing established solid tumors. Results were recorded by measuring tumor volumes thrice per week.

Results: Compared with drug treatment alone, NHS76/PEP₂ pretreatment substantially improved the effectiveness of chemotherapeutic agents in solid tumor models. Tumor suppression was most pronounced in those groups of mice bearing tumors known to be sensitive to the specific drug under study. However, in certain instances, tumors previously known to be resistant to specific single chemotherapeutic agents were shown to respond by the addition of NHS76/PEP₂ pretreatment.

Conclusions: NHS76/PEP₂ seems an excellent candidate to improve the value of standard chemotherapy drug treatment by virtue of its ability to increase the uptake of drugs in solid tumors selectively.

Chemotherapy is dependent upon the delivery of cytotoxic drugs into the tumor parenchyma, where exposure to the cytotoxic activity of these reagents can affect the viability of tumor cells. Because tumors have a leaky periphery characterized by tissue invasion and neovascularization, blood-borne drugs and therapeutic antibodies preferentially enter the tumor at this site and penetrate deeper by diffusion and convection (1–4). To date, very few methods exist to enhance this process to improve significantly the therapeutic potential of cytotoxic drugs and antibodies in clinical oncology. To address this issue, our laboratory has developed a unique method of targeting solid tumors for the purpose of delivering vasopermeability-enhancing reagents to alter the leakiness of tumor vessels (5–8).

Proteins that induce vasopermeability such as interleukin 2 (IL-2), vascular endothelial growth factor, and tumor necrosis factor α are good candidates for the development of a clinically useful vasopermeability agent. Vasopermeability is a normal physiologic function of these molecules and naturally enhances the delivery of antibodies, growth factors, cytokines, coagulants, etc. to the site of tissue damage or inflammation. For IL-2, it is known that the induced vasopermeability is caused by the release of nitric oxide gas within the milieu of the tumor or inflammatory lesion resulting in the rounding up of the endothelial cell wall and the generation of microfenestrations that enable the leakage of fluid into affected tissues (8). For this to occur, we discovered that the localization of IL-2 in tumors need not be specific because after linkage to antibodies, IL-2 directed to the tumor cell surface, to necrotic regions, or to tumor vasculature, all cause increased vasopermeability in the tumor mass by approximately the same amount (9). IL-2, however, linked to a nontargeting control antibody, did not produce the same effect in tumor (5, 6, 9).

Human I is a globular protein consisting of 133 amino acids and is similar in structure to IL-4 and granulocyte macrophage colony-stimulating factor (10). Structural studies of IL-2 show that it is composed of four major amphipathic α helices arranged in an antiparallel fashion, with the hydrophobic faces making a stable, hydrophobic core (11, 12). In addition, a disulfide bond is important to the stability of the tertiary structure and is essential for the biological activity of IL-2 (13). Loss of this disulfide bond or even minor changes in the...
primary or secondary structure abrogate IL-2 cytokine activity, as shown by site-directed mutagenesis studies (14). Previously, we have shown that the vasopermeability activity of IL-2 is independent of its cytokine activity and receptor binding and can be localized to a 37-amino-acid stretch (amino acids 22-58) of the molecule (15). Moreover, this peptide fragment of IL-2, designated permeability-enhancing peptide (PEP), was found to retain its vasopermeability effect as a linear sequence, enabling it to be linked to antibodies and other targeting moieties. Devoid of IL-2’s cytokine activity, this peptide is an ideal vasopermeability-inducing reagent that can be used to enhance the delivery of drugs and antibodies into tumor or related lesions.

Because it was our aim to generate a universal vasopermeability agent that can be used to improve the selective uptake of chemotherapeutic drugs and antibodies in cancer patients, we chose to genetically engineer PEP to the human monoclonal antibody (mAb) NHS76, which targets necrosis and cellular degeneration commonly found in solid tumors. NHS76 is a third-generation tumor necrosis therapy (TNT) antibody that was produced by phage display as part of a collaboration with scientists at Cambridge Antibody Technologies (Cambridge, England; ref. 16). Like other TNT antibodies, it targets nuclear debris found in cell hosts or areas of cellular degeneration present in necrotic regions of tumors (17–21) and targets DNA/histone H1, a normal component of nucleosomes and chromatin. Because tumors generally have a high nuclear to cytoplasmic ratio, nucleosomes are an abundant component of tumors and become accessible in tumor cells that have lost their outer membrane integrity. Finally, although normal cells do undergo turnover, the reticuloendothelial system of the host actively removes dead and dying cells from normal tissues. This phenomenon, however, does not occur in necrotic regions of tumors (17). Described as “wounds that do not heal” (22) tumors uniquely display varying amounts of necrosis (range, 30–80% of the tumor mass), making necrosis a universal marker of solid tumors (23, 24).

As previously shown (5–9), vasopermeability-enhancing agents can induce a 2- to 4-fold increase in drug and antibody uptake selectively in tumors. In this report, we now describe a clinically relevant derivative, designated NHS76/PEP2, which consists of the human antibody NHS76 (16) and a dimer of the vasopermeability-enhancing peptide of IL-2 (PEP, ref. 15). This genetically engineered fusion protein was developed to deliver maximal but reversible vasopermeability into tumors as a method to enhance the tumor uptake of chemotherapeutic drugs and antibodies. Being fully human in design and devoid of predictable toxicity, NHS76/PEP2 should be compatible in man and safe for human clinical trials. As proof of its therapeutic potential, we now describe the benefit of NHS76/PEP2 pretreatment in solid tumor-bearing mice undergoing single-agent chemotherapy.

Materials and Methods

Reagents and analytic methods

All chemicals such as chloramine-T were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All solvents were of analytic grade and were used as purchased. 125I and 131I were obtained as sodium iodide in 0.1 N sodium hydroxide from Perkin-Elmer (North Billerica, MA). Radioactive samples were measured using either a 1282 Compugamma counter (LKB Instruments, Pleasant Hill, CA) or a CRC-7 dose calibrator (Capintec, Inc., Pittsburgh, PA).

Instant TLC (ITLC) was carried out on silica gel impregnated filters (Gelman Sciences, Ann Arbor, MI) and developed as specified below. Gel exclusion chromatography was done with Sephadex G-25 columns (Sigma-Aldrich Chemical). High-performance liquid chromatography was conducted on a Beckman System Gold Instrument (Beckman Instruments, Inc., Fullerton, CA) equipped with two 110B solvent pumps, a 210A injector valve, a 166 programmable absorbance detector, and a 406 analogue interface module. Fast protein liquid chromatography separations were done at room temperature using a Pharmacia ÄKTA system (Amersham Pharmacia Biotech, Piscataway, NJ). Buffers used in high-performance liquid chromatography and fast protein liquid chromatography procedures were filtered through 0.22-µm Nalgene disposable filter units before use.

Antibodies and cell lines

The fully human TNT antibody NHS76 was produced in our laboratory as previously published (16). The murine mAb B72.3 (IgG1), which recognizes the tumor-associated glycoprotein 72 (25), was a kind gift of Dr. Michiel Ultee (Cytogen Co., Princeton, NJ). The NS0 murine myeloma cell line was obtained from Lonza Biologics (Slough, United Kingdom). The Madison 109 (MAD109) murine lung carcinoma cell line and the LS174T human colon adenocarcinoma cell line were obtained from the National Cancer Institute (Frederick, MD). The Raji African Burkitt’s lymphoma cell line was obtained from the American Type Culture Collection (Manassas, VA).

Construction, expression, and purification of NHS76/PEP fusion protein

The human mAb, NHS76 (IgG1), was generated as previously described by our laboratory (16). For the construction of NHS76/PEP2, the cDNA of the variable regions of NHS76 were cloned into pee12 and pee6 vectors (Lonza Biologics) by standard molecular biology techniques and designated p6/NHS1 and p12/NHS6, respectively. The p6/NHS76T1, which contained the cDNA sequence for the NHS76 gamma chain under the control of the cytomegalovirus major intermediate early promoter, was used as our parent vector. Four primers (shown below) connected by a noncleavable linker (SSGGSSGGAS) were used to amplify the PEP gene from IL-2 cDNA for assembling the PEP dimer. The primers were as follows: pr1, 5'-CAGATGATTGATGGAATT-3'; pr2, 5'-GGCTAGCCAGCCCGCGCTGCGCAGCATTGAGCAG-3'; pr3, 5'-CTAGTGGTGCCGGGCTGAGCAGATGATTGGAATT-3'; and pr4, 5'-TCATGCGGCCGCTCAACACTGAAGATGTTTCAGTT-3'.

Briefly, the primer pair pr1/pr2 was used to amplify the first PEP sequence from IL-2 cDNA and primer pair pr3/pr4 was used to amplify the second PEP sequence. Finally, the primer pair pr1/pr4 was used to assemble the complete PEP dimer sequence, which was inserted into Smal and NotI sites of p6/NHS761 to produce an expression vector encoding the NHS76 gamma chain with the PEP dimer at its COOH terminus. The fusion protein was expressed in NS0 murine myeloma cells according to the protocol of the manufacturer (Lonza Biologics). Briefly, linearized plasmids were electroporated into NS0 cells, which were plated in nonselective Hybridoma-SEM medium (Life Technologies, San Diego, CA) containing 10% characterized FCS (HyClone, Logan, UT), 1% l-glutamine/penicillin streptomycin solution (Gemini BioProducts, Woodward, CA), and 1% nonessential amino acids solution (Sigma Chemical Co., St. Louis, MO) at a concentration of 2 x 105 cells per well for 24 hours, at which time selective glutamine-free medium consisting of Hybridoma-SEM medium containing 10% dialyzed FCS (HyClone), G418 supplement (Sigma Chemical), 1% nonessential amino acids solution, and 1% penicillin/streptomycin solution (Sigma Chemical) when transfectants appeared ~3 weeks later, supernatants were tested for the presence of fusion protein by indirect ELISA. The highest-producing clones were identified by...
24-hour rate of production assays. After subcloning by limiting dilution, the highest-producing clones were expanded in selective medium shown above but with 5% heat-inactivated dialyzed FCS to prevent the induction of endogenous proteolytic enzyme which can cleave the fusion protein. Incubation was done in 8-liter aerated stir flasks for ~10 to 12 days. The fusion protein was purified stepwise from cell culture medium by fast protein liquid chromatography using tandem Protein A affinity and ion-exchange chromatography, as described previously (21). The purity of the NHS76/PEP2 fusion protein was examined by SDS-PAGE in a reducing gel according to the method of Laemmli (26) and by high-performance liquid chromatography as previously described (21). Antibody concentrations were estimated by optical spectroscopy at 280 nm.

**Radioiodination and in vitro stability**

The murine mAb B72.3 and NHS76/PEP2 fusion protein were radiolabeled with radioiodine using a modified chloramine-T method (27, 28). Typically, all antibodies were radioiodinated with 1 mCi of carrier-free Na\(^{125}\text{I}/\text{I}\) in the presence of chloramine-T/antibody ratio, 0.4:1, w/w. The radioiodinated derivatives were analyzed using an analytic ITLC system as described previously (21). In vitro serum stability was also evaluated for 5 days in mouse serum at 37°C as described previously (28). The immunoreactivities for each radiolabeled antibody preparation were monitored by RIA as previously described (29).

**Determination of avidity constant**

The avidity constant of radiolabeled NHS76/PEP2 was determined by a fixed-cell RIA developed in our laboratory (29), using the method of Frankel and Gerhard (30). Scatchard analysis was done to obtain the slope. The equilibrium or avidity constant \(K_s\) was calculated by the equation \(K = -(\text{slope} / n)\), where \(n\) is the valence of the antibody \((n = 2)\).

**Pharmacokinetic and biodistribution studies of NHS76/PEP2**

It has previously been shown that half-life values of immunglobulin G clearance from BALB/c mice determined by whole-body radioactivity are statistically indistinguishable from those calculated by blood sampling (31). Therefore, whole-body radioactivity was done for these pharmacokinetic studies. Six-week-old female BALB/c mice were used to determine the pharmacokinetic clearance of the radiolabeled fusion protein. Groups of mice \((n = 5-8)\) previously fed potassium iodide in the drinking water for 1 week to block thyroid uptake of radioiodine, were given i.v. injections of \(^{125}\text{I}/\text{I}\) labeled NHS76/PEP2 \((1.0-1.5\text{ MBq per 10}\mu\text{g per mouse})\) in PBS solution. The whole-body activity immediately after injection and at selected times thereafter was measured with a CTC-7 microdosimeter (Capintec). The data were analyzed to calculate the whole-body half-lives as described previously (32). Significance levels \((P)\) in whole-body radioactivity of \(^{125}\text{I}/\text{I}\) NHS76 and \(^{125}\text{I}/\text{I}\) NHS76/PEP2 were determined using the Wilcoxon rank sum test (33).

To quantitate the tissue biodistribution, groups \((n = 5-8)\) of 6-week-old female athymic nude mice were injected s.c. in the left flank with a 0.2-mL inoculum containing \(5 \times 10^6\) LS174T human colon adenocarcinoma cells using a University Animal Care Committee–approved protocol. The tumors were grown for 7 to 10 days until they reached ~0.5 to 1 cm in diameter. Within each group, individual mice were injected i.v. via the tail vein with 3 to 4 MBq/10 \(\mu\text{g}\) of \(^{125}\text{I}/\text{I}\) labeled mAb in PBS. All mice were sacrificed by sodium pentobarbital overdose at 3 days post-injection, and tissues were removed, lightly blotted to remove excess blood, weighed, and their radioactivity measured in a LKB gamma counter. For each mouse tissue or organ, the data were expressed as a mean of percentage of injected dose per gram of tissue (%ID/g) and mean tumor-to-organ ratios (cpm per gram/cpm per gram organ) as described previously (32). From these data, the mean and SD were calculated for each group. Statistical significance \((P)\) of differences in radiolabel tracer uptake of \(^{125}\text{I}/\text{I}\) NHS76 and \(^{125}\text{I}/\text{I}\) NHS76/PEP2 in tumor-bearing mice at 3 days post-injection was determined using the Wilcoxon rank sum test as described above.

**In vivo toxicity studies**

To determine the maximum tolerated dose of the fusion protein, groups of BALB/c mice \((n = 5-8)\) received a single dose of NHS76/PEP2 or five daily consecutive doses by i.v. injection \((0.1\text{ mL})\) starting with 100 \(\mu\text{g/dose}\) and increasing up to and including 400 \(\mu\text{g}\). Toxicity was measured by documenting mortality and secondarily, by observing the daily activity levels of the mice, the absence or presence of ruffled fur, and the loss of weight (measured thrice a week). Survival time was used as an end point of these protocols. Twenty days after NHS76/PEP2 initial injection, all the survived mice were sacrificed by sodium pentobarbital overdose.

**Vasopermeability studies**

**Tumor models.** All animal studies were conducted under University Animal Care Committee–approved protocols. The murine MAD109 lung carcinoma and the human LS174T colon carcinoma cell lines were transplanted in the left flank of 6-week-old female BALB/c and athymic nude mice, respectively, as described above.

**Biodistribution and imaging of tracers after NHS76/PEP2 pretreatment.** The purified NHS76/PEP2 fusion protein was evaluated for biological activity by performing in vivo biodistribution analysis. In a dose-dependence study, groups of LS174T-bearing nude mice \((n = 5-8)\) were injected i.v. \((0.1\text{ mL})\) with saline or 15, 30, and 45 \(\mu\text{g of NHS76/PEP2}\) 2 hours before the i.v. injection of \(^{125}\text{I}/\text{I}\) labeled B72.3 \((3-34\text{ MBq per 10}\mu\text{g per 0.1}\text{ mL})\) tracer. Because we previously showed that the highest tumor accretion of antibodies occurred when vasconjugates were injected 2 hours before administration of radiolabeled antibodies \((8, 15)\), this pretreatment time was used in these studies. All mice were sacrificed 3 days after administration of the tracer dose for biodistribution analyses as described above. For each mouse tissue or organ, the data were expressed as a mean of %ID/g and mean tumor-to-organ ratios as described above. Significance levels \((P)\) were determined using the Wilcoxon rank sum test (33).

For the imaging studies, groups of LS174T-bearing nude mice were pretreated with 30 \(\mu\text{g of NHS76/PEP2}\) 2 hours before the administration of \(^{125}\text{I}/\text{I}\) labeled B72.3 \((0.1\text{ mL inoculum containing 7-8 MBq/10}\mu\text{g})\). At 3 days post-injection, the mice were anesthetized with a s.c. injection of ketamine \((50 \mu\text{g/g})/\text{xylazine (10}\mu\text{g/g})\) cocktail (Sigma-Aldrich Chemical). The immobilized mice were imaged in a posterior position with a Spectrum 91 gamma camera equipped with a pinhole collimator (Raytheon Medical Systems, Melrose Park, IL) set to record 10,000 counts using the Nuclear MAX Plus image analysis software package (MEDX, Inc., Wood Dale, IL).

**Chemotheraphy studies with NHS76/PEP2 pretreatment.** To test the therapeutic efficacy of NHS76/PEP2 pretreatment, groups of MAD109-bearing BALB/c mice \((34, 35)\) and LS174T-bearing nude mice \((36)\) were used. Five to 8 days after tumor implantation, groups of mice \((n = 8)\) were treated either daily or every 3 or 4 days for two to four treatments of doxorubicin \((\text{Sigma-Aldrich Chemical}; 4\text{ mg/kg})\), Taxol \((\text{Mead Johnson, Princeton, NJ}; 10/\text{mg/kg})\), vinblastine \((\text{Bedford Laboratories, Bedford, OH}; 1.4\text{ mg/kg})\), etoposide \((\text{Bedford Laboratories; 15 mg/kg})\), vinblastine \((\text{Bedford Laboratories, Bedford, OH}; 1.4\text{ mg/kg})\), etoposide \((\text{Bedford Laboratories; 15 mg/kg})\), 5-fluorouracil \((\text{5-FU, Sigma-Aldrich Chemical; 35 mg/kg})\), or Taxotere \((\text{Aventis Pharmaceutical Products, Bridgewater, NJ}; 5-30\text{ mg/kg})\) i.p. using a 1-mL injectate diluted in sterile PBS. Other groups received an i.v. injection of a 0.1-mL inoculum containing 30 \(\mu\text{g of NHS76/PEP2}\) 2 hours before the administration of the chemotherapeutic drug. Drug doses were chosen to produce low or suboptimal effects on tumor growth so that the action of NHS76/PEP2 pretreatment could be measured. In all treatment studies, other control groups of mice were injected with either PBS or NHS76/PEP2 \((30\mu\text{g alone})\). Tumor growth was monitored every other day by caliper measurement in three dimensions. Tumor volumes were calculated using the formula:
length × width × height. The results were expressed as the mean of tumor size (cm³). The statistical significance (P’s) of differences in tumor size in control and treated tumor-bearing mice was determined using the Wilcoxon rank sum test (33). Eighteen days after tumor implantation, all the mice were sacrificed by sodium pentobarbital overdose.

Results

Generation and testing of the vasopermeability-enhancing agent NHS76/PEP₂

The fusion protein NHS76/PEP₂ was expressed in NS0 murine myeloma cells using the glutamine synthetase gene amplification system. The highest-expressing clone producing the fusion protein was selected for large-scale cell culture. A 24-hour production assay showed that this clone generated between 8 and 12 μg/mL of product. Cell culture was done in selective medium using 5% dialyzed FCS which was previously heat-inactivated at 68°C for 1 hour to prevent the induction of proteolytic enzymes in the NS0 cells. Under these conditions, an intact fusion protein with the expected molecular weight was obtained as shown by reducing SDS-PAGE electrophoresis (Fig. 1). Two bands were resolved for NHS76/PEP₂ at ~Mₐ 26,000 and 60,000, corresponding to the molecular weights of the immunoglobulin light chain and heavy chain plus PEP₂, compared with NHS76 and NHS76/IL-2, the heavy chain of which exhibited molecular weights of ~Mₐ 52,000 and 68,000, respectively. After purification, the product was aliquoted and stored frozen at −20°C until use. The purity of the NHS76/PEP₂ was confirmed by high-performance liquid chromatography and revealed one sharp peak with a retention time of ~550 seconds.

Radiolabeling efficiency and stability

All mAb preparations showed a radiolabeling efficiency of 80% to 85%. ITLC analysis of all 125I/131I-labeled NHS76/PEP₂ revealed an Rₜ value of 0 (mAb bound) and a radiochemical purity of ≥99%. In addition, the fusion protein was examined for deiodination in mouse serum over a 5-day incubation period at 37°C. Approximately 95% of the activity was trichloroacetic acid precipitable for all the derivatives, and virtually no release of free radioiodine was detected over this period at 37°C for deiodination in mouse serum over a 5-day incubation period. Using fixed Raji Burkitt’s lymphoma cells, the enhancement of proteolytic enzymes in the NS0 cells. Under these conditions, an intact fusion protein with the expected molecular weight was obtained as shown by reducing SDS-PAGE electrophoresis (Fig. 1). Two bands were resolved for NHS76/PEP₂ at ~Mₐ 26,000 and 60,000, corresponding to the molecular weights of the immunoglobulin light chain and heavy chain plus PEP₂, compared with NHS76 and NHS76/IL-2, the heavy chain of which exhibited molecular weights of ~Mₐ 52,000 and 68,000, respectively. After purification, the product was aliquoted and stored frozen at −20°C until use. The purity of the NHS76/PEP₂ was confirmed by high-performance liquid chromatography and revealed one sharp peak with a retention time of ~550 seconds.

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Avidity determination of NHS76/PEP₂

A binding study was conducted in which 125I-labeled NHS76/PEP₂ was incubated with fixed Raji cells, and the bound radioactivity was used to calculate the avidity constant (Kₐ) by Scatchard analysis. As expected, the genetic linkage of PEP₂ to the Fc region had no effect on binding. The NHS76/PEP₂ was found to have an avidity constant of 1.06 × 10⁹ (mol/L)⁻¹, compared with 1.65 × 10⁹ (mol/L)⁻¹ for NHS76 (16). This study indicates that the NHS76/PEP₂ fusion protein has the same binding ability towards its target antigen and hence should have the same targeting capabilities as NHS76.

Pharmacokinetic and biodistribution studies of NHS76/PEP₂

Whole-body radioactivity studies in BALB/c mice were done to establish differences in pharmacokinetic characteristics between the 125I-labeled NHS76 and the 125I-labeled NHS76/PEP₂. It has previously been shown that NHS76 clears slowly with a whole-body half-life of 212 hours in BALB/c mice (16). As depicted in Fig. 2, NHS76/PEP₂ showed a more rapid total whole-body clearance than that of NHS76 (a half-life of 96 versus 212 hours; P ≤ 0.01). This is an expected result because most fusion proteins tend to have a shorter half-life in mice than their parent antibodies.

Relative tumor uptake of the NHS76/PEP₂ and NHS76 was determined in LS174T human colon adenocarcinoma tumor-bearing nude mice. For these studies, biodistribution was done 3 days after i.v. injection of 125I-labeled derivatives to insure low background of the slower clearing NHS76. 125I-NHS76/PEP₂ showed a tumor uptake of 2.33% ID/g (P ≤ 0.01) at 3 days post-injection compared with a 3.94% ID/g for 125I-NHS76 (P ≤ 0.01; Fig 3A). The rapid clearance of NHS76/PEP₂ showed a decrease in radioactivity levels in blood and all of the other normal tissues (P ≤ 0.01), resulting in approximately similar tumor-to-organ ratios compared with NHS76 (Fig 3B). These data illustrate the specificity of tumor targeting with NHS76/PEP₂ and its rapid elimination from the blood and normal organs.

In vivo toxicity studies

In all four groups of BALB/c mice, there were no mortalities or secondary signs of morbidity seen during the observation period of 3 weeks post-injection. Our conclusion from these studies is that at the doses studied, NHS76/PEP₂ is nontoxic in normal mice.

Vasopermeability studies

Biodistribution and imaging with NHS76/PEP₂ pretreatment. Vasopermeability studies were done to show the activity of the NHS76/PEP₂ fusion protein and to determine the optimal dose required to obtain maximum vasopermeability. Because previous studies by our laboratory have shown that pretreatment with antibody alone does not show any vasopermeability effect (5–9), this control was not used in the present studies. The relationship between the dose of NHS76/PEP₂ and tumor uptake of 125I-labeled B72.3 was examined in LS174T-bearing nude mice using a 2-hour pretreatment.

Fig. 1. SDS-PAGE identification of recombinant fusion proteins. Coomassie blue – stained 10% acrylamide reducing gel of purified NHS76/IL-2 fusion protein (lane 1), NHS76/PEP₂ fusion protein (lane 2), and NHS76 human mAb (lane 3).
protocol. Biodistribution analyses were done 3 days after 125I-B72.3 tracer injections. In these studies, a dose of 30 μg resulted in the greatest increase in tumor uptake (Fig. 4A). With higher doses, the magnitude of tumor uptake began to diminish as previously seen when chTNT-3/IL-2 was used as the vasoconjugate reagent (8). This may be due to the underlying physiologic cause of vasopermeability in which nitric oxide release becomes potentially toxic in the tumor microenvironment when over produced. In the current studies, a 30-μg i.v. pretreatment dose gave optimal results and produced a 4.4-fold increase in antibody uptake in tumor (from a median of 4.1-18.1%ID/g after this pretreatment dose; P < 0.02), without altering the biodistribution of antibody in normal tissues. Finally, biodistribution analyses of the pretreated groups revealed markedly higher tumor-to-normal organ ratios for all normal tissues (Fig. 4B).

Imaging studies were also done to show the significance of the observed improved uptake in tumor produced by NHS76/PEP2 pretreatment. As shown in Fig. 5, 3-day images of 131I-labeled B72.3 mAb in LS174T-bearing nude mice showed faster and better uptake in tumor with 30-μg pretreatment compared with control animals receiving antibody alone. Like the biodistribution results shown in Fig. 4, images of 131I-B72.3 in NHS76/PEP2 pretreated mice were better than the non-treated mice and showed little evidence of radioactivity in normal tissues. Additionally, enhanced uptake was only seen in the tumor, which showed excellent localization of the radio-labeled antibody in this tumor model. Thus, pretreatment with NHS76/PEP2 improved the immunoscintigraphy of the colon adenocarcinoma xenograft. In all the above studies, consistent results were obtained within the groups of mice.

**Therapeutic efficacy of NHS76/PEP2 pretreatment on the chemotherapy of transplanted solid tumors.** For these studies, two different solid tumor models (LS174T and MAD109) were used to show the efficacy of NHS76/PEP2 pretreatment on chemotherapy. Two models were chosen to provide sensitive tumors to various chemotherapy drugs. As shown in Figs. 6 and 7, single-agent chemotherapy regimens using doxorubicin, Taxol, vinblastine, etoposide, 5-FU, and taxotere, at doses that by themselves displayed little or no clinical benefit in different solid tumor models, could be made therapeutically effective at these same reduced doses by pretreatment with 30 μg of NHS76/PEP2. As described previously, treatment did not commence until day 5 to 8 when the tumors were palpable. In those groups of mice receiving NHS76/PEP2 alone, tumor growth was either unaffected, slightly faster, or slightly slower than that of the untreated mice. These differences, however, did not change the slope of the growth curves indicating that NHS76/PEP2 alone was ineffective in altering the overall outcome of tumor progression. By contrast, the combination of NHS76/PEP2 pretreatment and doxorubicin in the LS174T human tumor model dramatically altered the slope of the growth curve because of marked tumor suppression (Fig. 6A). The results show that the average tumor volumes of the NHS76/PEP2 + doxorubicin–treated groups on the 12th day after the first injection were <30% of the tumor volume in the doxorubicin alone–treated control group. However, the NHS76/PEP2- and control PBS-treated mice, which received four consecutive doses, showed no significant inhibition of tumor growth (Fig. 6A).

Similar treatment results with each of the other chemotherapeutic drugs such as Taxol, vinblastine, etoposide, and 5-FU were observed in the MAD109 lung tumor model (Fig. 6B-D). The results show that the average tumor volumes of the NHS76/PEP2 + chemotherapeutic drug–treated groups were
45% to 60% of those in the chemotherapy drug–treated control groups. In all of the above experiments, NHS76/PEP2 pretreatment showed improved results compared with drug treatment alone and showed the clinical potential and versatility of this method in the treatment of relatively chemo-resistant solid tumors. In some instances such as shown in Fig. 6B, the chemotherapeutic drug (Taxol) at the chosen dosage showed no clinical effect when used alone. The results showing a significant tumor regression with the addition of NHS76/PEP2 pretreatment in this setting is particularly exciting because it proves the concept that critical dosing is necessary in chemotherapy-resistant tumors. This point is further explored in subsequent studies in which broader ranges of chemotherapeutic drug doses were used with and without NHS76/PEP2 pretreatment. As shown in Fig. 7A, treatment with a combination of the NHS76/PEP2 (30 μg) and a low Taxotere dose (5 mg/kg) dramatically decreased the tumor growth, with the average tumor volumes on the 10th day after the first injection being <30% of the Taxotere-treated control group. However, the combination of NHS76/PEP2 (30 μg) and a higher dose of Taxotere (30 mg/kg) did not cause a significant reduction of tumor compared with the same dose level of Taxotere given alone (Fig. 7B). These results may reflect the chemotherapy sensitivity levels of tumors and point to the possibility that tumors with characteristic steep dose response curves like breast cancer might respond best to NHS76/PEP2 pretreatment. It should be noted that some of these studies (see Fig. 6C, D, and E) did...
not include vasoconjugate treatment alone as a control group because other studies showed no significant effects in these tumor models (except slight increases or decreases) over untreated mice.

Discussion

In this study, a recombinant fusion protein containing the human mAb NHS76 and a dimer of the vasopermeability-enhancing agent PEP was generated for use as a universal pretreatment to enhance the delivery of therapeutic molecules to solid tumors. The NHS76/PEP$_2$ fusion protein was expressed in mammalian cells using the glutamine synthetase gene amplification system so that large-scale production will yield sufficient recombinant product for clinical use (37). Acrylamide gel electrophoresis showed that the human heavy chain fusion protein remained intact after purification (Fig. 1). As described previously by our laboratory for other antibody/cytokine fusion proteins (8), the PEP cDNA was inserted downstream of the terminal codon of the heavy chain. This fusion protein retains the immunoreactivity of the parent antibody, as evidenced by binding of $^{125}$I-labeled NHS76/PEP$_2$ to fixed Raji Burkitt's lymphoma cells. Moreover, NHS76/PEP$_2$ maintains the high avidity constant of NHS76. Despite its rapid clearance profile (Fig. 2), the NHS76/PEP$_2$ retains the ability to localize to tumor effectively (2.33%ID/g), whereas normal organ uptake remains relatively low (Fig. 3). The rapid clearance of the fusion protein may prove beneficial in the clinical setting because exposure of normal organs and tissues will be minimized.

Our laboratory was the first to use targeted vasoconjugates containing cytokines to increase tumor vascular permeability (5–8). As shown by us (5) and by others (38), pretreatment with free vasoactive cytokines increases vascular permeability systemically as well as in the tumor. Furthermore, attached to an irrelevant antibody, the vasoactive immunoconjugate shows no vasopermeability effect in tumor compared with results obtained when tumor targeting antibodies are used (5, 9). The fusion protein described in the present study was designed to enhance the tumor uptake of therapeutic molecules in a wide variety of human cancers due to the ability of NHS76, which recognizes a universal nuclear antigen exposed in the degenerating and necrotic cells present in all solid tumors (16).

The ability of vasoconjugates to increase tumor uptake of both a mAb and a drug (IUdR) was previously reported in the LS174T colon adenocarcinoma xenograft model (7, 8). These studies showed that increased tumor uptake after pretreatment with vasoconjugates was both time- and dose-dependent and showed that the highest tumor accretion of radiolabeled antibodies and radiolabeled IUdR occurred when vasoconjugates were injected 1.5 to 2.5 hours before administration of radiolabeled tracers. For this reason, a 2-hour interval between NHS76/PEP$_2$ pretreatment and radiolabeled B72.3 injection was used in the present studies. In addition, dosing studies shown in Fig. 4 show that at the optimal dose of 30 $\mu$g/mouse, pretreatment with NHS76/PEP$_2$ results in a 4.4-fold increase in tumor accretion of $^{125}$I-labeled B72.3 with no effect on normal tissues. Hence, it is clear that NHS76/PEP$_2$ is as effective at enhancing tumor vasopermeability as chemically conjugated mAb/PEP reagents used in earlier studies (15). Furthermore, the imaging results verify the ability of NHS76/PEP$_2$ pretreatment to enhance the tumor uptake of $^{131}$I-B72.3 mAb in LS174T colon tumor–bearing nude mice shown (Fig. 5). These data are consistent with previously published findings (39) in which the clarity and intensity of the tumor images were enhanced by the pretreatment approach compared with antibody alone.

To test the therapeutic potential of NHS76/PEP$_2$, a series of experiments were done using frequently used single-agent chemotherapeutic drugs. In these studies, groups of mice were treated with chemotherapeutic drugs using suboptimal therapeutic doses with and without NHS76/PEP$_2$ pretreatment to show that increased vasopermeability at the tumor site could translate into better therapy as a result of increased drug uptake in tumor. As shown in Fig. 6A–E, five such studies are presented that clearly show the value of NHS76/PEP$_2$ pretreatment. In general, those tumors that were sensitive to specific drugs had
the most profound effects (Fig. 6A), but a response could now be generated in tumors normally resistant to a specific drug such as the MAD109 murine lung carcinoma (34, 35) treated with Taxol (Fig. 6B), vinblastine (Fig. 6C), or 5-FU (Fig. 6E). These results highlight the efficacy of vasopermeability enhancement at improving the therapeutic value of chemotherapeutic drugs and lay the foundation for using NHS76/PEP2, a completely human fusion protein, for the therapy of patients with cancer and related diseases.

Although the literature is scarce in describing methods for improving the delivery of drugs to tumors, there are a number of areas being actively investigated. Mechanical methods such as the use of microinfusional delivery as described by Morrison et al. (40) are currently being employed for intratumoral drug delivery in brain cancer lesions. Clinical trials using this method with 131I-chTNT are currently underway in the treatment of refractory brain tumors and advanced lung cancer.

These trials showed excellent uptake and therapy in patients and in June 2003, this reagent was approved in China for the treatment of lung carcinoma, only the second of three existing approved radiolabeled antibodies for the treatment of cancer worldwide (41). In addition, external beam radiation has been shown to cause modulation of tumor vasculature function. Studies by Sonveaux et al. (42) have shown that after a local 6 Gy irradiation, the tumor microvasculature exhibits a 4-fold increase in iNOS abundance and a simultaneous decrease in caveolin-1 expression. These investigators also observed that focal irradiation significantly increases tumor blood flow and tumor oxygenation as measured 24 hours after treatment, a response attributable to nitric oxide release (43, 44). In studies by de Lange Davies et al. (45), radiation was likewise shown to improve both the distribution and the uptake of liposomal doxorubicin in human osteosarcoma xenografts. In these studies, radiotherapy increased the tumor uptake of
doxorubicin by 2- to 4-fold and showed localization of the drug in more central areas of the tumor compared with nonirradiated controls. However, colocalization of doxorubicin and hypoxic cells revealed the absence of drug distribution into hypoxic areas. Similarly, studies by Schwichtert et al. (46) using contrast-enhanced MRI imaging showed that single 5 to 15 Gy doses of X-rays in mammary adenocarcinoma implanted rats showed increased capillary permeability 1 and 3 days post-irradiation, which ultimately resulted in higher concentrations of cis-platinum in tumors compared with nonirradiated controls.

From the pioneering work of Tannock (4) and Jain (1–3), the limited penetration of drugs in tumors has been ascribed to structural properties of tumors, which produce increased intratumoral pressures and poor diffusion of small molecules from the tumor microvasculature. Noting that blood flow in tumors is often irregular and the intracapillary distances larger than that seen in normal tissues, these investigators speculate that drug delivery is hampered in tumors. To determine if elevated interstitial fluid pressures produced by physiologic barriers found in tumors can be modified in vivo, the authors chose to enhance the delivery of drugs, Tong et al. (47) used the anti–vascular endothelial growth factor antibody

![Chemotherapy treatment of human LS174T colon carcinoma in nude mice](image)

Fig. 7. Chemotherapy treatment of human LS174T colon carcinoma in nude mice with and without NHS76/PEP2 pretreatment using different Taxotere dose levels. A, 5 mg/kg (suboptimal treatment level); B, 30 mg/kg (optimal treatment level).

DC101 to decrease the interstitial fluid pressure of tumors. After 3-day treatment with this antibody, it was observed that the architecture of tumor vasculature was normalized with respect to vascular density, vessel diameter, and basement membrane structure. Structural normalization of tumor vessels was shown to translate into functional normalization as determined by vascular permeability, plasma and interstitial oncotic pressures, and interstitial fluid pressure. By contrast, the plasma oncotic pressure remained unchanged, causing an inward flux of fluids into the tumor. This mechanism of enhanced drug uptake is different in nature from that seen with NHS76/PEP2 pretreatment. Unlike DC101 treatment, which requires several days to normalize the structure of tumor vessels, NHS76/PEP2 causes improved drug uptake in 1 to 2 hours. Because the oncotic pressures before and after NHS76/PEP2 pretreatment have not been measured, it would of interest to study these variables to determine the biophysical nature of the phenomenon caused by NHS76/PEP2.

Using yet another approach, Salnikov et al. (48) used prostaglandin E1 treatment to lower the tumor interstitial fluid pressure and found that chemotherapeutic treatment of experimental tumors with 5-FU was enhanced. In these studies, prostaglandin E1 treatment produced edema formation in the tumor interstitium, thereby increasing capillary-to-interstitium transport of 5-FU by convection. In tumor spheroid studies by Croix et al. (49) and clinical studies by Klocker et al. (50) and Pillwein et al. (51), the addition of hyaluronidase to standard chemotherapy was found to improve the clinical outcome of therapy in patients. These investigators contend that by degrading hyaluronic acid, a major component of tumor stroma and vessels, hyaluronidase loosens the intercellular substance of tumors, thereby increasing the permeability of vessel membranes. Known as “spreading factor,” hyaluronidase facilitates the diffusion of chemotherapeutic drugs. Finally, using a similar approach, Eikenes et al. (52) used collagenase treatment to increase transcapillary pressure gradient and improve the uptake and distribution of mAbs in tumor xenografts. Taken in concert, the above studies show that it is possible to improve drug uptake in tumor by altering the existing physiologic variables found in tumors and their vessels. Further studies such as those done by Netti et al. (53) are warranted to clarify the mechanism of action of each of these methods in order for us to better understand their potential uses.

In summary, NHS76/PEP2 seems a clinically useful reagent that can be given in concert with chemotherapy in the treatment of solid tumors. NHS76/PEP2 is an ideal tumor permeability–enhancing agent because it is compatible in humans, has a low toxicity profile, has a single and defined mechanism of action, is applicable to the majority of patients with solid tumors, has a rapid and reversible onset of activity, and is able to improve the effects of different chemotherapeutic drugs. Further studies are warranted to determine if NHS76/PEP2 pretreatment can change the therapeutic index of drugs in those tumors, which show a steep dose response curve.

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NHS76/PEP$_2$, a Fully Human Vasopermeability-Enhancing Agent to Increase The Uptake and Efficacy of Cancer Chemotherapy

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