molecular pharmaceutics

articles

Folate-Targeted Dinitrophenyl Hapten Immunotherapy: Effect of Linker Chemistry on Antitumor Activity and Allergic Potential

Yingjuan Lu,[†] Fei You,[†] Iontcho Vlahov,[†] Elaine Westrick,[†] Mingjin Fan,[†] Philip S. Low,[‡] and Christopher P. Leamon*,[†]

Endocyte, Inc., West Lafayette, Indiana 47906, and Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

Received April 27, 2007; Revised Manuscript Received July 27, 2007; Accepted August 7, 2007

Abstract: Targeting of malignancies with folate-linked therapeutics has proven to be a promising endeavor due to the preferential expression of folate receptors (FR) on human tumors. We have shown that folic acid (pteroyl-glutamate) can be used to deliver an antigenic hapten, fluorescein, to the surface of tumor cells to promote their opsonization within a fluorescein-immunized host. Here, we investigate structure–activity relationships among members of another class of folate–hapten conjugates (**EC57**, **EC63**, **EC0293**, and **EC0294**), namely, those containing the dinitrophenyl (DNP) group as the antigenic hapten. We report that despite exhibiting similar affinities for the FR, the antitumor activity and allergic potential of these DNP conjugates varied depending on their linker chemistries and abilities to bind anti-DNP IgG/IgE antibodies. Unlike **EC57** and **EC63**, both **EC0293** and **EC0294** (i) share the identical DNP bridging chemistry to that found in keyhole limpet hemocyanin (KLH)–DNP (i.e., the immunogen), (ii) efficiently recognize DNP-specific IgG, and (iii) mediate more pronounced antitumor responses. However, **EC0293** and **EC0294** were also found to recognize DNP-specific IgE, and they displayed a greater risk of allergy when evaluated in a passive cutaneous anaphylaxis assay. Nonetheless, upon co-stimulation with the appropriate cytokines (IL-2/IFN-α), the folate-targeted "haptenization" process allowed for tumor rejection and protective antitumor immunity without causing any visible allergy in immunized mice. Our data further support the concept that folate–hapten-targeted immunotherapy may offer an effective therapeutic option for treatment of FR-positive cancers, but such treatment should proceed with caution given the risk of a potential allergic reaction.

Keywords: Cancer immunotherapy; folate receptor; folate–DNP conjugates; anti-DNP antibody; cytokine co-stimulation; interleukin-2; interferon-α

Introduction

The folate receptor (FR) constitutes a useful target for tumor-specific drug delivery, primarily because it is up-regulated in many different types of human epithelial cancers, including those of the ovary, endometrium, lung, kidney, mesothelium, head and neck, and brain tissues.[1–6] Thus far, conjugation of folic acid to imaging and therapeutic agents has been shown to enhance their delivery to FR-expressing

* Author to whom correspondence should be addressed. Mailing address: Endocyte, Inc., 3000 Kent Avenue, Suite A1-100, West Lafayette, IN 47906. Tel: (765) 463-7175. Fax: (765) 463-9271. E-mail: chrisleamon@endocyte.com.

[†] Endocyte, Inc.

[‡] Purdue University.

(1) Toffoli, G.; Cernigoi, C.; Russo, A.; Gallo, A.; Bagnoli, M.; Boiocchi, M. Overexpression of folate binding protein in ovarian cancers. *Int. J. Cancer* **1997**, *74*, 193–198.

(2) Ross, J. F.; Chaudhuri, P. K.; Ratnam, M. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* **1994**, *73*, 2432–2443.

(3) Weitman, S. D.; Frazier, K. M.; Kamen, B. A. The folate receptor in central nervous system malignancies of childhood. *J. Neurooncol.* **1994**, *21*, 107–112.

10.1021/mp070050b CCC: $37.00
Published on Web 09/05/2007

cancer cells *in vitro* and in tumor-bearing animals.[7–11] In human clinical trials, folate-based radioimaging agents complexed with radionuclides (e.g., ^{111}In and ^{99m}Tc) have shown great promise in detecting FR-positive tumors in patients with malignant ovarian[12] and kidney cancers.[13]

Believing that the most effective way of controlling tumor growth must involve participation of the host's immune system, we have devised strategies to recruit immune effector cells to the tumor site by delivering an antigenic hapten linked to folic acid.[14] We have previously shown that folate targeting of a fluorescein (FITC) hapten in FITC-immunized mice can lead to the elimination of FR-positive tumor implants, especially in the milieu of proinflammatory cytokines [interleukin (IL)-2, interferon (IFN)-α].[9,14] Mechanistically, a folate–hapten conjugate is believed to form a bispecific molecular "bridge" between the tumor cell and the endogenous circulating anti-hapten antibody.[15,16] This "marking" step initiates an Fc (the constant region on an immunoglobulin molecule)-mediated immune response leading to the removal of the antibody-coated tumor cells via mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis.[15] Pharmacokinetic studies have revealed that the biodistribution of bispecific ligands depends on (i) how well the host is immunized against the hapten, and (ii) the ability of the ligand to recruit antibodies to FR-positive tumor, which in turn determines the antitumor activity.[16] More importantly, the development of a persistent tumor-specific cellular immunity was observed in cured animals, as evidenced by repeated rejection of new tumor challenges and the presence of memory $CD4^+$ and $CD8^+$ T cells.[14,15] At present, this targeted hapten therapy is under evaluation in early phase clinical trials of renal cell carcinoma, an immune responsive cancer with ~70% FR positivity (Endocyte, internal communications).

The purpose of this study was to enhance our understanding of this novel therapeutic approach by exploring the use of dinitrophenyl (DNP) as the targeted hapten in a DNP-immunized host. We chose DNP because it was synthetically simple to manipulate, and subtle alternations in the DNP linking chemistries could be evaluated. Interestingly, DNP has been used clinically to modify autologous tumor vaccines for the purpose of enhancing their immunological recognition.[17,18] Thus, a series of folate–DNP conjugates with different linker chemistries were synthesized, and their immunological and therapeutic activities were examined *in vitro* and in tumor-bearing mice that were co-stimulated with low doses of IL-2 and IFN-α. The conjugate series was ranked according to (i) binding affinity towards FR, (ii) ability to bind endogenously induced anti-DNP antibody, (iii) antitumor activity, and (iv) risk of producing allergic reaction. The conclusions from this investigation, in terms of safety and efficacy, may also provide guidance towards the ongoing clinical investigation of folate–fluorescein-targeted immunotherapy.[19]

Experimental Methods

Reagents. ^{3}H-Folic acid was purchased from Amersham (Arlington Heights, NY). Biotin-conjugated goat anti-mouse IgG, IgG1, IgG2a, and streptavidin-conjugated horseradish peroxidase were obtained from Caltag Laboratories (Burl-

(4) Ross, J. F.; Wang, H.; Behm, F. G.; Mathew, P.; Wu, M.; Booth, R.; Ratnam, M. Folate receptor type beta is a neutrophilic lineage marker and is differentially expressed in myeloid leukemia. *Cancer* **1999**, *85*, 348–357.
(5) Bueno, R.; Appasani, K.; Mercer, H.; Lester, S.; Sugarbaker, D. The alpha folate receptor is highly activated in malignant pleural mesothelioma. *J. Thorac. Cardiovasc. Surg.* **2001**, *121*, 225–233.
(6) Parker, N.; Turk, M. J.; Westrick, E.; Lewis, J. D.; Low, P. S.; Leamon, C. P. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal. Biochem.* **2005**, *338*, 284–293.
(7) Leamon, C. P.; Parker, M. A.; Vlahov, I. R.; Xu, L. C.; Reddy, J. A.; Vetzel, M.; Douglas, N. Synthesis and biological evaluation of EC20: A new folate-derived, (99m)Tc-based radiopharmaceutical. *Bioconjugate Chem.* **2002**, *13*, 1200–1210.
(8) Ke, C. Y.; Mathias, C. J.; Green, M. A. Folate-receptor-targeted radionuclide imaging agents. *Adv. Drug Delivery Rev.* **2004**, *56*, 1143–1160.
(9) Lu, Y.; Sega, E.; Leamon, C. P.; Low, P. S. Folate receptor-targeted immunotherapy of cancer: mechanism and therapeutic potential. *Adv. Drug Delivery Rev.* **2004**, *56*, 1161–1176.
(10) Leamon, C. P.; Reddy, J. A.; Vlahov, I. R.; Westrick, E.; Parker, N.; Nicoson, J. S.; Vetzel, M. Comparative preclinical activity of the folate-targeted vinca alkaloid conjugates EC140 and EC145. *Int. J. Cancer* **2007**, *121* (7), 1585–1592.
(11) Reddy, J. A.; Dorton, R.; Westrick, E.; Dawson, A.; Smith, T.; Xu, L. C.; Vetzel, M.; Kleindl, P. J.; Vlahov, I. R.; Leamon, C. P. Pre-clinical evaluation of EC145, a folate-vinca alkaloid conjugate. *Cancer Res.* **2007**, *67* (9), 4434–4442.
(12) Siegel, B. A.; Dehdashti, F.; Mutch, D. G.; Podoloff, D. A.; Wendt, R.; Sutton, G. P.; Burt, R. W.; Ellis, P. R.; Mathias, C. J.; Green, M. A.; Gershenson, D. M. Evaluation of 111In-DTPA-folate as a receptor-targeted diagnostic agent for ovarian cancer: initial clinical results. *J. Nucl. Med.* **2003**, *44*, 700–707.
(13) Naveed, F.; Fisher, R.; Engel, J. S.; Lu, J.; Low, P.; Amato, R. J. Folate-scan in subjects with suspected metastatic renal cell carcinoma. *J. Clin. Oncol., ASCO Annu. Meet. Proc.* **2004**, *22*, 4751.
(14) Lu, Y.; Low, P. S. Folate targeting of haptens to cancer cell surfaces mediates immunotherapy of syngeneic murine tumors. *Cancer Immunol. Immunother.* **2002**, *51*, 153–162.
(15) Lu, Y.; Sega, E.; Low, P. S. Folate receptor-targeted immunotherapy: Induction of humoral and cellular immunity against hapten-decorated cancer cells. *Int. J. Cancer* **2005**, *116*, 710–719.
(16) Lu, Y.; Xu, L. C.; Parker, N.; Westrick, E.; Reddy, J. A.; Vetzel, M.; Low, P. S.; Leamon, C. P. Preclinical pharmacokinetics, tissue distribution, and antitumor activity of a folate-hapten conjugate-targeted immunotherapy in hapten-immunized mice. *Mol. Cancer Ther.* **2006**, *5*, 3258–3267.
(17) Sato, T. Active specific immunotherapy with hapten-modified autologous melanoma cell vaccine. *Cancer Immunol. Immunother.* **1996**, *43*, 174–179.
(18) Berd, D. Autologous, hapten-modified vaccine as a treatment for human cancers. *Vaccine* **2001**, *19*, 2565–2570.
(19) Amato, R. J.; J., H.-M.; Conley, B.; Rogers, H.; Lu, J.; P.S., L.; Morgenstern, D.; Bever, B.; Messmann, R. A phase Ib study of FolateImmune (EC90 with GP1-0100 adjuvant followed by EC17) with low dose cytokines interleukin-2 (IL-2) and interferon-a (IFN-α) in patients with refractory or metastatic cancer. Presented at the 43rd ASCO Annual Meeting, **2007**.

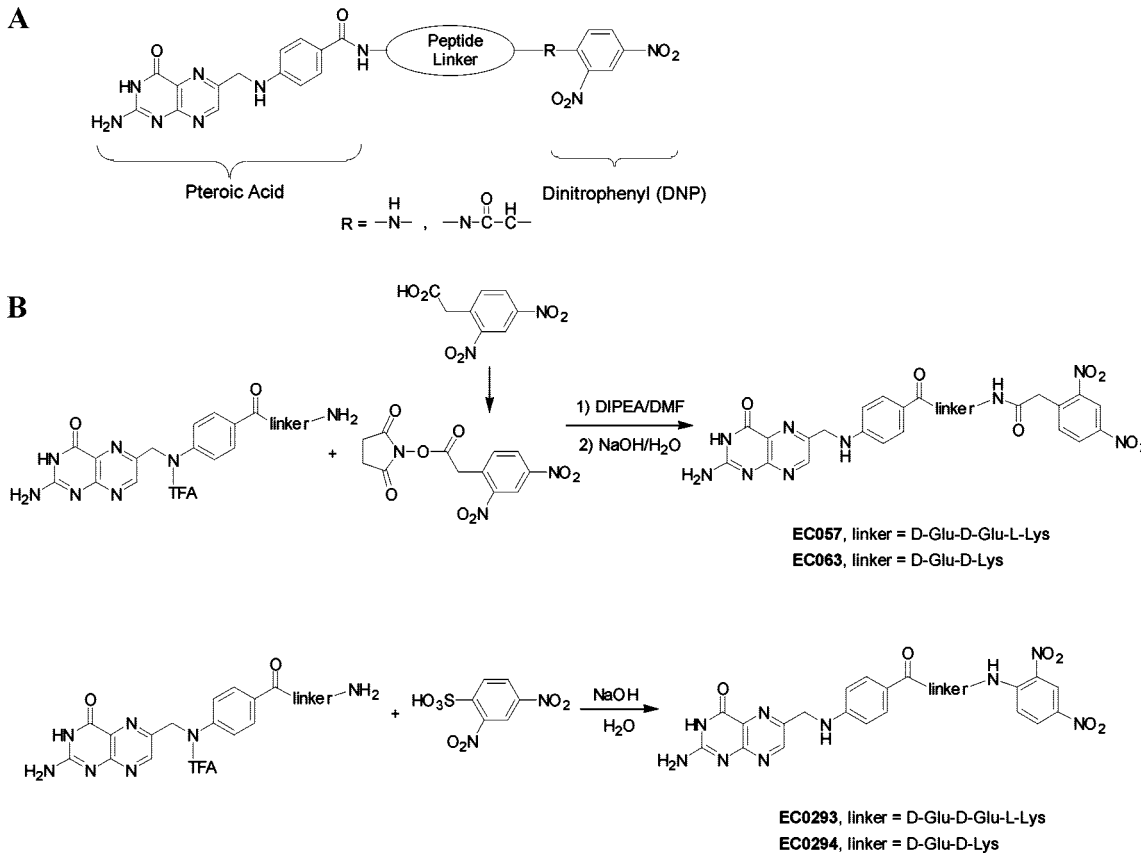

Figure 1. General structure (A) and synthetic scheme (B) of folate–DNP conjugates.

Table 1. Characteristics of Folate–DNP Conjugates

compound number	description[a]	net charge	approximate spatial distance from DNP to folate targeting moiety, (Å)[b]
41-AS-074	Pte-D-γGlu-eda-(DNP)	−1	6.04
EC57	Pte-D-γGlu-D-γGlu-Lys(DNP)	−3	9.72
EC63	Pte-D-γGlu-D-Lys(DNP)	−2	9.06
EC0293	Pte-D-γGlu-D-γGlu-Lys(DNP)	−3	8.89
EC0294	Pte-D-γGlu-D-Lys(DNP)	−2	7.47

[a] The folate moiety (D-enantiomer) consists of "Pte-D-γGlu". [b] Theoretical estimation calculated using the ArgusLab 4.0.1 program, M. A. Thompson, Planaria Software LLC, Seattle, WA. http://www.arguslab.com.

ingame, CA). 2,4-Dinitrobenzenesulfonic acid dihydrate was purchased from ICN Biomedical Research Products (Costa Mesa, CA). Keyhole limpet hemocyanin (KLH)–DNP (400 DNP groups per molecule) and bovine serum albumin (BSA)–DNP (>30 DNP groups per molecule of protein) conjugates were purchased from Calbiochem (San Diego, CA). Anti-DNP IgE mAb (Clone SPE-7) was purchased from Sigma Chemical Co. (St Louis, MO). TiterMax Gold adjuvant was obtained from CytRx Corporation (Norcross, GA). GPI-0100, a semisynthetic plant saponin adjuvant, was obtained from Galenica (Birmingham, AL). The alum adjuvant (Imject Alum) was purchased from Pierce Biotechnology (Rockford, IL). Bovine milk folate-binding protein (FBP) was purchased from Scripps Laboratories (San Diego, CA). Human recombinant IL-2 was purchased as a lyophilized powder either from CHEMICON International, Inc. (Temecula, CA) or PeproTech (Rocky Hill, NJ). Human recombinant IFN-α A/D was purchased from Research Diagnostics (Flanders, NJ) or PBL Biomedical Laboratories (Piscataway, NJ). All other biologic or chemical reagents were purchased from major suppliers.

Preparation of Folate–DNP Conjugates. The structures of the folate–DNP conjugates prepared for this study are shown in Figure 1 and Table 1.

Synthesis of 41-AS-074. Ethylenediaminefolic acid, γ-amide (i.e., Pte-γGlu-eda) was synthesized according to a procedure described by Fuchs et al.[20] This compound (48.4 mg, 0.1 mmol) was dissolved in 2 mL of dimethylsulfoxide after addition of 19.0 μL (0.15 mmol) 1,1,3,3-tetrameth-

(20) Luo, J.; Smith, M. D.; Lantrip, D. A.; Wang, S.; Fuchs, P. L. Efficient syntheses of pyrofolic acid and pteroyl azide, reagents for the production of carboxyl-differentiated derivatives of folic acid. *J. Am. Chem. Soc.* **1997**, *119*, 10004–10013.

ylguanidine and sonication for 1 h. To this solution was added 35 μL (0.2 mmol) of *N,N*-diisopropylethylamine and 50.0 mg (0.15 mmol) of the *N*-hydroxysuccinimidyl ester of 2,4-dinitrophenylacetic acid (NHS-DNP). Stirring was continued for 3 days, and the crude product was precipitated in acetonitrile to yield 47 mg of a yellow powder. The isolated product was unfortunately not soluble in water over a wide pH range (from 2.0 to 8.4).

Synthesis of EC57. For **EC57**, the N^{10}-trifluoroacetyl-protected pteroyl (Pte)-containing peptidyl fragment, N^{10}-trifluoroacetyl-Pte-D-γGlu-D-γGlu-L-Lys-OH, was prepared using the Fmoc-strategy on the acid-sensitive Fmoc-Lys-(Boc)-Wang resin. PyBop was applied as the activating reagent to ensure efficient coupling using low equivalents of amino acids. Fmoc protecting groups were removed after every coupling step under standard conditions (20% piperidine in DMF, *N,N*-dimethylformamide). Fmoc-Glu-O*t*Bu and N^{10}-trifluoroacetyl-Pte-OH were used as protected amino acid building blocks. After the last assembly step, the peptide was cleaved from the polymeric support by treatment with trifluoroacetic acid (TFA), ethanedithiol and triisopropylsilane. This reaction also resulted in simultaneous removal of the *t*-Bu and Boc protecting groups. The crude peptide was purified by preparative HPLC to give N^{10}-trifluoroacetyl-Pte-D-γGlu-D-γGlu-L-Lys-OH as a TFA acid salt. The solution of 8.1 mg (0.01 mmol) of the prepared peptide in 0.5 mL of anhydrous DMF was treated with 28 μL (0.2 mmol) of triethylamine and 6.5 mg (0.02 mmol) of NHS-DNP. The reaction mixture was stirred for 48 h at room temperature, and the solvent was removed by freeze-drying. The trifluoroacetyl protective group was cleaved in aqueous ammonium hydroxide (pH = 10.0) and precipitated in acetonitrile. Finally, the crude conjugate was purified by preparative HPLC on NovaPak C18 (3.9 mm × 150 mm) using 10 mM NH_4HCO_3/CH_3CN as eluent. The fraction containing the conjugate was subjected to freeze-drying to yield 2.5 mg of a yellow amorphous material. The structure of the product was in agreement with its molecular mass spectrum (positive ESI, *m/z* 907.2 $[M + H]^+$; negative ESI, *m/z* 905.4 $[M - H]^+$).

Synthesis of EC63. EC63 is a folate–DNP conjugate containing a D-γGlu-D-Lys linker. This conjugate was prepared in similar manner as that described above for compound **EC57**. A solution of 66.5 mg (0.1 mmol) of peptide N^{10}-trifluoroacetyl-Pte-D-γGlu-D-Lys-OH in 5.0 mL of anhydrous DMF was treated with 52 μL (0.3 mmol) of diisopropylethylamine and 80 mg (0.248 mmol) of the NHS-DNP. The reaction mixture was stirred for 20 h at room temperature and then diluted with 50 mL of water. Some precipitate was formed and filtered off. The yellow filtrate was treated with aqueous ammonium hydroxide (pH = 10.0) for 15 min and then subjected to freeze-drying. Finally, the crude conjugate was purified by preparative HPLC on a NovaPak C18 (3.9 mm × 150 mm) column using 10 mM NH_4HCO_3/CH_3CN as the eluent. The fraction containing the conjugate was subjected to freeze-drying to yield 26.5 mg of a yellow amorphous material. The structure of the product was in agreement with its molecular mass spectrum (positive ESI, *m/z* 778.1 $[M + H]^+$; negative ESI, *m/z*: 776.3 $[M - H]^+$).

Synthesis of EC0293. EC0293 is a folate–DNP conjugate containing a D-γGlu-D-γGlu-L-Lys linker. N^{10}-trifluoroacetyl-Pte-D-γGlu-D-γGlu-L-Lys-OH, was prepared by a polymer-supported sequential approach using the Fmoc strategy on the acid-sensitive Fmoc-Lys(Boc)-Wang resin. After cleavage with TFA, ethanedithiol, and triisopropylsilane, the crude peptide was purified by preparative HPLC to give N^{10}-trifluoroacetyl-Pte-D-γGlu-D-γGlu-L-Lys-OH as a TFA acid salt. Peptide (69.8 mg, 0.1 mmol) and 33.5 mg of 2,4-dinitrobenzene sulfonic acid were dissolved in 2.0 mL of 0.1 N NaOH aqueous solution. The pH of the mixture was adjusted to 10.5 by 1.0 N NaOH. The reaction mixture was stirred for 1 day under argon. HPLC purification with 0.1% TFA/acetonitrile as eluent gave 46 mg of desired product as yellow amorphous material after lyophilization. The structure of the product was in agreement with its molecular mass spectrum (positive ESI, *m/z* 865.4 $[M + H]^+$).

Synthesis of EC0294. EC0294 is a folate–DNP conjugate containing a D-γGlu-D-Lys linker, and it was prepared in a similar manner as that described above for **EC0293**. Briefly, the N^{10}-trifluoroacetyl-protected Pte-containing peptidyl fragment N^{10}-trifluoroacetyl-Pte-D-γGlu-D-Lys-OH was prepared by a polymer-supported sequential approach using the Fmoc strategy. The crude peptide was purified by preparative HPLC to give N^{10}-trifluoroacetyl-Pte-D-γGlu-D-Lys-OH as a TFA salt. To the solution of 114.0 mg (0.2 mmol) of peptide in 3.0 mL of 0.1 N NaOH aqueous solution, 45.0 mg of 2,4-dinitrobenzene sulfonic acid was added. The pH of the mixture was adjusted to 10.5 by 1.0 N NaOH. The reaction mixture was stirred under argon, after 1 day 25 mg more of 2,4-dinitrobenzene sulfonic acid was added, and the mixture was stirred for one more day. HPLC purification with 0.1% TFA/acetonitrile as eluent yielded 85 mg of desired product as yellow amorphous material after lyophilization. The structure of the product was in agreement with its molecular mass spectrum (positive ESI, *m/z* 736.2 $[M + H]^+$).

Cell Culture, Animals, and Tumor Models. The KB cell line is derived from a human nasopharyngeal epidermal carcinoma, and it is well known to overexpress cell surface FRs.[7] M109 cells are a high FR-expressing subclone of the Madison lung carcinoma cell line of Balb/c mouse origin.[21] 4T1 parent and 4T1c2 constitute a matched pair of mouse mammary carcinoma cell lines with the latter transfected with a murine folate-binding protein (M. Vetzel, unpublished data). The levels of functional FR on M109 and 4T1c2 tumors were comparable based on flow cytometric analyses using a folate-linked fluorescent dye (data not shown). All

(21) Gabizon, A.; Horowitz, A. T.; Goren, D.; Tzemach, D.; Mandelbaum-Shavit, F.; Qazen, M. M.; Zalipsky, S. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. *Bioconjugate Chem.* **1999**, *10*, 289–298.

cell lines were cultured in folate-free RPMI 1640 medium (Gibco BRL) supplemented with 10% v/v heat-inactivated fetal calf serum.

Female Balb/c mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN) and used when they reached 6–8 weeks of age. M109 tumors were maintained in Balb/c mice and regenerated as described previously.[14] For intraperitoneal tumor implants, 5×10^5 viable M109 tumor cells at an early passage (P_0 or P_1) were suspended in 400 μL of folate-free RPMI-1640 (FFRPMI) supplemented with 1% syngeneic mouse serum (antibiotic-free) and then injected into the peritoneal cavity. For subcutaneous tumor challenges, 1×10^6 cells per 100 μL of M109 or 4T1c2 were injected into the shoulder or dorsal caudal region, respectively. Female Lewis rats (~3.5 months of age, 202–218 g) were purchased from Harlan and used for allergy studies.

Relative Affinity Assay. The relative affinities of folate–DNP conjugates (**EC57**, **EC63**, **EC0293**, and **EC0294**) were determined according to a previously established method[7] with the exception that the test articles were incubated with the cells for 1 h at 37 °C. Notably, relative affinities were defined as the inverse molar ratio of compound required to displace 50% of ^{3}H-folic acid bound to FR on KB cells, and the relative affinity of folic acid for the FR was set to 1.

Immunizations. For induction of anti-DNP antibodies in mice, KLH–DNP (either prepared according to a published procedure[22] or purchased from Calbiochem) was used at a DNP-to-protein molar ratio of ~400. For therapy studies, mice were first immunized subcutaneously at the base of the tail with 50 μg of KLH–DNP formulated with either 100 μL of TiterMax Gold adjuvant (emulsions prepared according to manufacturer's instructions) or 100 μg of GPI-0100. The KLH–DNP/TiterMax Gold immunized mice were boosted once (2 weeks later) with 80% of the primary antigen dose injected on the back of the neck, and their serum samples were collected 11 days later. The KLH–DNP/GPI-0100 immunized mice were boosted twice at 2-week intervals with the same primary antigen doses, and the serum samples were collected 1 week later. For allergy studies, mice were immunized ip against 10 μg of KLH–DNP and 3 mg of alum and boosted 1 month later with 2 μg of KLH–DNP and 2 mg of alum (ip). Sera pooled from these mice 1 week later were used as a source of "allergic serum" for passive cutaneous anaphylaxis assay in rats (see below). All mouse serum samples were stored at −20 °C until further use.

ELISA Analyses of Anti-DNP Antibody Responses. Unless otherwise specified, preimmune serum was used as controls, and all incubation steps were done at room temperature on a horizontal shaker. For the detection of anti-DNP IgG and IgG isotypes in mouse sera, BSA–DNP-coated ELISA plates were saturated with 0.2% gelatin in phosphate-buffered saline (PBS; 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) containing 0.05% Tween-20 for 1 h. After washing with buffer consisting of 0.05% Tween-20 in PBS, serial dilutions of pooled DNP antiserum were added to the plates and incubated for 1 h. The plates were washed and incubated further with biotin-conjugated goat anti-mouse IgG, IgG1, or IgG2a as the primary antibody and streptavidin-conjugated horseradish peroxidase (Caltag) as the secondary reagent. For the detection of anti-DNP IgE levels in mouse sera, a capture ELISA assay was developed employing a biotin–BSA–DNP reagent made by reacting BSA–DNP with EZ-Link sulfo-NHS–LC–biotin following manufacturer's instructions (Pierce). Briefly, plates coated with anti-mouse IgE capture mAb (BD Biosciences) were blocked with PBS, pH 7.4, containing 1% BSA for 30 min. After washing, serial dilutions of pooled DNP antiserum were added to the plates and incubated for 1 h. The plates were washed and incubated further with biotin–BSA–DNP as the primary reagent and streptavidin-conjugated horseradish peroxidase as the secondary reagent. Finally, the presence of mouse anti-DNP antibodies was revealed by adding 150 μL/well of *o*-phenylenediamine dihydrochloride in substrate buffer (Sigma Fast *o*-phenylenediamine dihydrochloride tablet sets). The enzymatic reaction was stopped by addition of 37 μL of 3 N HCl, and the optical density was read at 490 nm. The average absorbance values at 490 nm were plotted against log serum dilution factors (e.g., 1:100 dilution = $\log_{10}$ 100 = 2) to generate titration curves. A titer is defined as the serum dilution that yields 50% of the maximal binding on the BSA–DNP-coated plate.

Evaluation of Binding of Anti-DNP IgG to FBP-Immobilized Folate–DNP Conjugates. Bovine milk FBP-coated plates were preblocked with SuperBlock blocking buffer (Pierce) before exposure to **EC57**, **EC63**, **EC0293**, and **EC0294** (0–26 nM) in PBS buffer containing 0.05% Tween-20 and 10% SuperBlock blocking buffer. After 1 h incubation, the plates were washed and incubated with 1:1000 dilution of DNP antiserum obtained from KLH–DNP/GPI-0100 immunized mice. After stringent washing, a biotin-conjugated goat anti-mouse IgG secondary antibody (Caltag) was added to the plates followed by streptavidin-conjugated horseradish peroxidase (Caltag). Following 1 h incubation at each step and intermittent washes, the presence of folate–DNP/anti-DNP IgG immune complexes was revealed by adding 150 μL/well of *o*-phenylenediamine dihydrochloride in substrate buffer (Sigma Fast *o*-phenylenediamine dihydrochloride tablet sets). The average OD values at 490 nm were fitted against concentrations of folate–DNP conjugates, and the concentration of each conjugate that achieved 50% of the maximal binding was calculated from the curves.

Survival Studies. For *in vivo* use, folate–DNP conjugates were prepared in sterile PBS. IL-2 and IFN-α were mixed together in sterile PBS containing 1% syngeneic mouse serum and stored at −80 °C in small aliquots. All compounds were administered ip except that folate–DNP conjugates were given in separate syringes from IL-2/IFN-α. In study I,

(22) Wu, Y.; Zhou, Q.; Zheng, P.; Liu, Y. CD28-independent induction of T helper cells and immunoglobulin class switches requires costimulation by the heat-stable antigen. *J. Exp. Med.* **1998**, *187*, 1151–1156.

KLH–DNP/TiterMax Gold immunized mice ($n = 4$) bearing ip M109 tumors (implanted 18 days after the secondary immunization) were treated with PBS alone or 1500 nmol/kg of **EC63** or DNP (nontargeted hapten) in combination with 5000 units/dose of IL-2 and 25 000 units/dose of IFN-α on days 7, 8, 9, 11, and 14 post-tumor cell implantation. The DNP dosed in mice was deactivated by neutralizing 2,4-dinitrobenzenesulfonic acid (ICN Biomedical Research Products) with NaOH before diluting into PBS. In study II, KLH–DNP/GPI-0100 immunized mice ($n = 5$) bearing ip M109 tumors (implanted 2 days after the third immunization) were treated with 1500 nmol/kg of **EC57**, **EC63**, **EC0293**, or **EC0294** in combination with 5000 units/dose of IL-2 and 25 000 units/dose of IFN-α on days 7, 8, 9, 11, and 14 post-tumor cell implantation. To reduce mouse serum folate levels to values characteristic of humans (~20 nM), mice were maintained on a folate-deficient diet (Harlan Teklad, Inc.) in both studies starting ~3 weeks before the first day of treatment. Animals were monitored daily thereafter, and those found dead or sacrificed in moribund conditions were necropsied for visual examination of tumor load within the peritoneal cavity. For tumor rechallenge studies, five long-term survivors (>100 days) that had been treated with **EC0293** and **EC0294** in study II were rechallenged sc with 1×10^6 of both M109 and 4T1c2 cells in the same animal on the respective shoulder and caudal regions. Five naïve mice were given the same tumor implants as controls. The tumor dimensions were measured 2–3 times a week, and tumor volumes were calculated by the following formula: $V = ½ab^2$, where a is the longest axis across the tumor and b is the shorter axis perpendicular to a. Since 4T1c2 tumors grow more slowly than M109 tumors in Balb/c mice, animals were euthanized if the M109 tumor had reached ~1000 mm^3.

Passive Cutaneous Anaphylaxis Assay. Female Lewis rats ($n = 2$) were sensitized intradermally in duplicates on the shaved dorsal surface with 0.1 mL of a 1:1000 dilution of anti-DNP IgE mAb (Clone SPE-7) or 1:2 serum dilution of DNP antiserum obtained from KLH–DNP/alum immunized mice. The injection spots were circled with a marker. Forty-eight hours later the animals were challenged by injecting intravenously 0.25 mL of a 1% Evans Blue dye solution containing either BSA–DNP (4 mg/mL), **EC57** (0.56 mg/mL), **EC63** (0.48 mg/mL), **EC0293** (0.59 mg/mL), or **EC0294** (0.45 mg/mL). Thirty minutes after challenge, rats were euthanized by CO_2 asphyxiation, and the skin spots were excised and extracted with 1 mL of 1 N KOH overnight at 37 °C (shaking). Nine milliliters of 0.6 N H_3PO_4/acetone (5:13) was added to the tissue extracts on the next day, and the mixture was shaken vigorously for a few seconds. After centrifuging at 3000 rpm for 15 min, the supernatants were collected, and Evans Blue content was measured at 620 nm.

Statistical Analysis. Statistical analyses were performed using the computer program GraphPad Prism (GraphPad Software Inc., San Diego, CA). Survival data were analyzed using the log rank test. Comparisons of tumor growth curves in the rechallenge studies were made by two-way ANOVA. Differences in survival or tumor growth were considered significant when $P \leq 0.05$.

Results

Syntheses of Folate–DNP Conjugates. The structures and brief synthetic schemes of all folate–DNP conjugates used in this investigation are shown in Figure 1. The folate moiety (D-enantiomer) on each conjugate consisted of "Pte-D-γGlu", and the specific linkages placed between folate and the DNP hapten are listed in Table 1. Since it was known that the γGlu-eda linker was long enough to effectively present a fluorescein hapten to the immune system,[14] Pte-γGlu-eda–DNP (**41-AS-074**; an analogue of folate–FITC or Pte-γGlu-eda–FITC) was synthesized to begin our studies. Unfortunately, this compound was never biologically evaluated due to its poor water solubility. However, four other folate–DNP conjugates (**EC57**, **EC63**, **EC0293**, or **EC0294**), each constructed with a different peptidic linker, were found to be sufficiently water soluble to evaluate. Table 1 summarizes the characteristics of these conjugates with respect to peptide configuration, molecular charge, and relative spatial distance between the hapten and folate-targeting moiety. **EC57** and **EC0293** shared the same "D-γGlu-D-γGlu-Lys" peptide linker. The linkers in **EC63** and **EC0294** were also identical to one another, but they differed from **EC57** and **EC0293** in that they contained only one D-Glu residue and had a D-Lys residue instead of the natural L amino acid isomer. Besides obvious differences in the linker length, more subtle structural variations were also designed into the DNP conjugates to (i) keep the DNP linking motif in **EC0293/EC0294** consistent with the linkage found in KLH–DNP (the immunogen), (ii) allow the DNP linking motif in **EC57/EC63** to vary slightly from that of **EC0293/EC0294**, (iii) permit **EC63/EC0294** to possess the biologically stable all D-residue linker, and (iv) allow **EC57/EC0293** to share the natural L configuration in their terminal lysine residue.

All four conjugates were evaluated biologically using an *in vitro* relative affinity assay that measured a conjugate's ability to directly compete with ^{3}H-folic acid for binding to cell surface FRs. As shown in Figure 2, each water-soluble folate–DNP conjugate was found to efficiently bind cell surface FRs with similar binding affinities (within experimental error). This outcome was anticipated, since these conjugates were less than 1000 in molecular weight and shared the same folate-binding moiety.

Anti-DNP Antibody Responses and Subtypes in DNP-Immunized Mice. Since the basis of the hapten-mediated therapy is to redirect the immune system to the tumor, it is important to build a strong anti-hapten antibody response that can guide effector immune cells to the malignant tissue. To assure that the host developed a potent immune response to the DNP hapten, the targeted immunotherapy began with serial inoculations of KLH–DNP admixed with an immunologically effective adjuvant. For the studies described below, we used either (i) TiterMax Gold, which

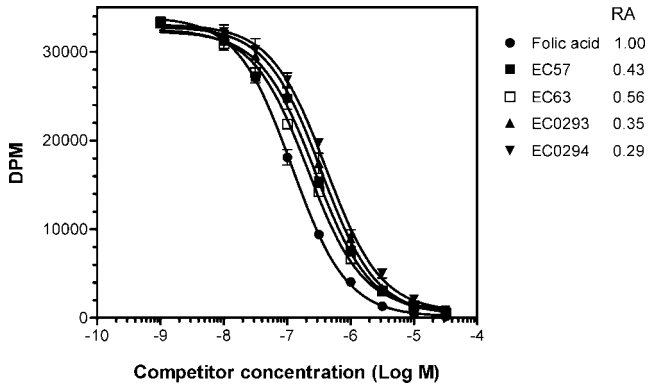

Figure 2. Relative affinity (RA) of folate–DNP conjugates for binding to human FR. FR-positive KB cells were incubated for 1 h at 37 °C with 100 nM of ^{3}H-folic acid in the absence and presence of increasing concentrations of **EC57** (■), **EC63** (□), **EC0293** (▲), and **EC0294** (▼). Relative affinities (RA) were defined as the inverse molar ratio of compound required to displace 50% of ^{3}H-folic acid bound to FR on KB cells, and RA of folic acid (●) for the FR was set to 1. Each data point was averaged from three independent measurements.

is an emulsifier-based adjuvant known to generate a persistent mixed Th1 and Th2 response,[23] or (ii) GPI-0100, which is a Th1-biased adjuvant and a potent inducer of IgG2a in mice.[24] Alum, which skewed the immune system towards an allergic Th2-type response,[25] was only used to generate DNP antiserum for passive cutaneous anaphylaxis evaluations (*vide infra*).

The data in Figure 3 depict the antibody responses in mice that were immunized against DNP according to the aforementioned vaccination regimens. According to the literature, an increased antigen-specific IgG2a to IgG1 ratio in mice is often associated with increased tumor cell killing and long-term tumor-protective immunity.[26] KLH–DNP/TiterMax Gold-immunized mice produced a good anti-DNP IgG response with a DNP-specific IgG2a to IgG1 ratio of 0.39

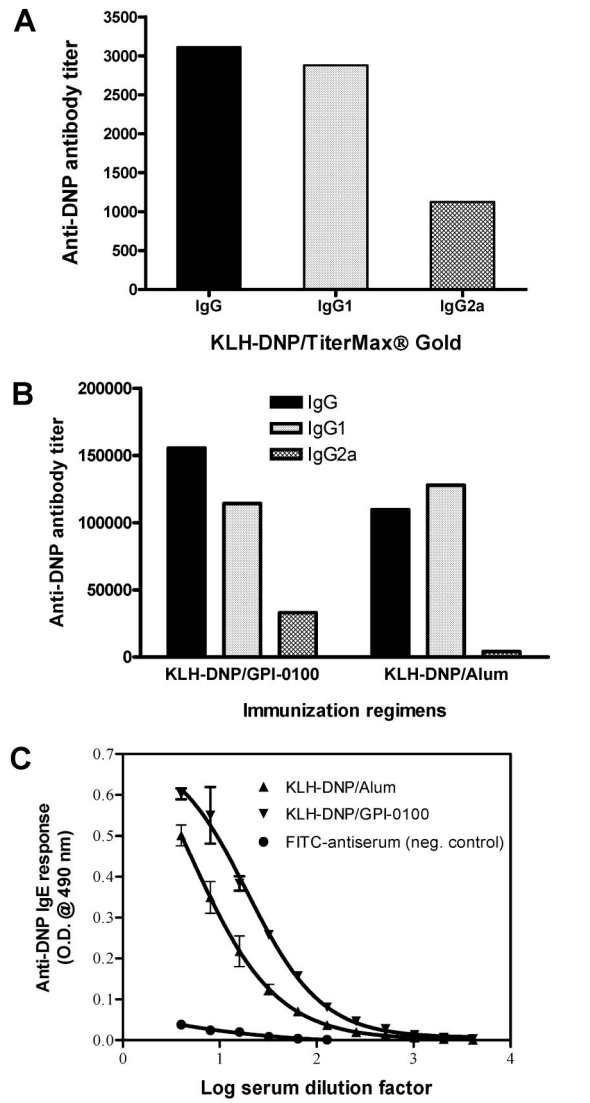

Figure 3. Anti-DNP antibody responses in DNP-immunized mice. Female Balb/c mice were immunized against KLH–DNP formulated in TiterMax Gold (A), GPI-0100 (B, C), or alum (B, C), as described in Experimental Methods. Equal volumes of antisera pooled from individual mice given the same immunization regimen were analyzed for DNP-specific IgGs (A, B) or IgE (C) responses. The average OD (absorbance) values shown in panels A and C are the mean of three independent measurements. FITC antiserum was used as negative control as an irrelevant hapten in panel C. The *Y*-axis in panel B represents anti-DNP antibody titer derived from corresponding titration curves (not shown).

(Figure 3A). Both KLH–DNP/GPI-0100- and KLH–DNP/alum-immunized mice produced very strong anti-DNP IgG and IgG1 responses; however, the former generated a greater anti-DNP IgG2a response (Figure 3B). Consequently, the ratios for DNP-specific IgG2a to IgG1 responses were 0.29 and 0.03 for KLH–DNP/GPI-0100- and KLH–DNP/alum-immunized mice, respectively.

(23) Bennett, B.; Check, I. J.; Olsen, M. R.; Hunter, R. L. A comparison of commercially available adjuvants for use in research. *J. Immunol. Methods* **1992**, *153*, 31–40.

(24) Marciani, D. J.; Press, J. B.; Reynolds, R. C.; Pathak, A. K.; Pathak, V.; Gundy, L. E.; Farmer, J. T.; Koratich, M. S.; May, R. D. Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. *Vaccine* **2000**, *18*, 3141–3151.

(25) Adel-Patient, K.; Creminon, C.; Bernard, H.; Clement, G.; Negroni, L.; Frobert, Y.; Grassi, J.; Wal, J. M.; Chatel, J. M. Evaluation of a high IgE-responder mouse model of allergy to bovine beta-lactoglobulin (BLG): development of sandwich immunoassays for total and allergen-specific IgE, IgG1 and IgG2a in BLG-sensitized mice. *J. Immunol. Methods* **2000**, *235*, 21–32.

(26) Weiner, G. J.; Liu, H. M.; Wooldridge, J. E.; Dahle, C. E.; Krieg, A. M. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10833–10837.

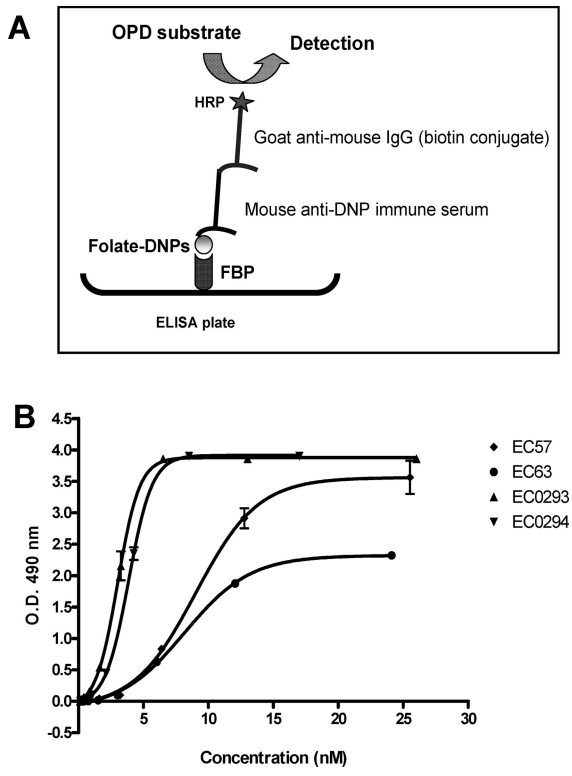

Figure 4. Recognition of DNP-specific IgG antibody by folate–DNP conjugates *ex vivo*. Serial dilutions of folate–DNP conjugates (**EC57**, **EC63**, **EC0293**, or **EC0294**) were added to FBP-coated 96-well plates followed by a fixed dilution of DNP antiserum. The formation of anti-DNP IgG/folate–DNP immune complex was detected as described in Experimental Methods. The basic "sandwich ELISA assay" schema is shown in panel A. Representative titration curves are shown in panel B. Results were confirmed by three separate analyses.

Since IgE is a good marker for induction of allergic responses in mice, the DNP-specific IgE response was also assessed by a capture ELISA assay (see Experimental Methods section). Both KLH–DNP/GPI-0100- and KLH–DNP/alum-immunized mice generated significant quantities of anti-DNP IgE as compared with the negative control with an irrelevant FITC hapten (Figure 3C). This result indicated that allergic responses were possible if immunized mice were treated with hapten-based formulations (*vide infra*).

Differential Binding of Folate–DNP Conjugates by anti-DNP Immune Serum. Based on previous experience with folate–FITC,[16] we anticipated that systemically administered folate–DNP conjugates would form immune complexes with anti-DNP antibodies in DNP-immunized mice. Since **EC57**, **EC63**, **EC0293**, and **EC0294** all displayed similar binding affinities towards FR, we developed an *ex vivo* ELISA assay in an effort to distinguish the abilities of these folate–DNP conjugates to recruit anti-DNP antibody. As cartooned in Figure 4A, bovine milk FBP was adsorbed onto high-protein-binding polystyrene plates, which were then used to immobilize the folate–DNP conjugates. A 1:1000 dilution of polyclonal DNP antiserum, obtained from KLH–DNP/GPI-0100-immunized mice, was added to allow anti-DNP antibodies to bind to the FBP-immobilized folate–DNP conjugates. The presence of folate–DNP/anti-DNP IgG antibody immune complexes on the plates was then detected following a routine procedure (see Experimental Methods). As shown in Figure 4B, all four of the evaluated folate–DNP conjugates were found to bind/trap anti-DNP antibody in this assay; however, **EC0293** and **EC0294** displayed the greatest binding efficiency. When the total antibody binding capacity is considered, as indicated by the maximal signal on the titration curves, the rank order among these compounds was **EC0293/EC0294** > **EC57** > **EC63**.

Antitumor Effect of Folate–DNP Conjugates Co-stimulated with IL-2 and IFN-α We have previously shown that the administration of folate–FITC supplemented with a low dose of IL-2 or IL-2 plus IFN-α can significantly extend the lifespan of FITC-immunized, M109 tumor-bearing mice.[14,15] In the present study, the same tumor model was used to evaluate the antitumor activity of our folate–DNP conjugates in KLH–DNP immunized mice. First, **EC63** was compared with unconjugated DNP in a preliminary study to demonstrate the requirement for FR targeting. As shown in Figure 5A, the unconjugated DNP with IL-2/IFN-α treatment produced only a minor improvement in the lifespan of the tumor-bearing mice (median survival time extended only 5 days longer than control animals). This was expected, since an untargeted FITC hapten administered with or without low levels of IL-2 and IFN-α was also shown to not extend the lives of FITC-immunized M109 tumor-bearing mice.[14] In contrast, animals treated with **EC63** plus the cytokines survived nearly 3 times longer than control mice (Figure 5A), thereby confirming the importance of folate targeting.

In a second study, we evaluated the antitumor activities of all four folate–DNP conjugates and directly compared their performances against cytokine-only or **EC63**-only control cohorts. As shown in Figure 5B and detailed in Table 2, **EC63** therapy alone was found to be completely ineffective against 7-day-old ip M109 tumors, a finding that was similar to previous results with folate–FITC alone.[14] Cytokine treatment alone also produced only a minimal (30%) increase in survival compared with untreated animals ($P \approx 0.003$). However, when the cytokine therapy was combined with **EC57**, **EC63**, **EC0293**, or **EC0294** treatment, the median survival times of the treated mice were increased to 30, 40, 46, and >100 days, corresponding to a 50%, 100%, 130%, and >400% increase in lifespan, respectively. The **EC63**, **EC0293**, and **EC0294** conjugates were found to significantly improve the therapeutic efficacy beyond that of cytokine treatment alone ($P \approx 0.0018$ for all). However, only **EC0293** and **EC0294** were observed to generate cures in these mice at rates of 40% and 60%, respectively, although the effectiveness of **EC0293** and **EC0294** was largely comparable ($P > 0.3$). This latter observation was interesting because the chemical linkage of DNP to the folate moiety in these two compounds was similar to the expected linkage produced in KLH–DNP (i.e., DNP anchored to the primary amine on

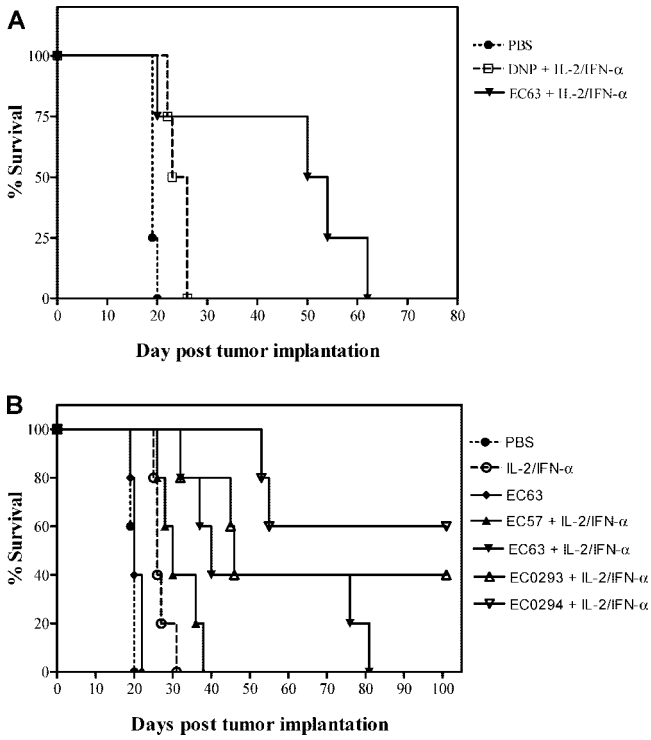

Figure 5. Survival of M109 tumor-bearing mice following immunotherapy with folate–DNP conjugates supplemented with IL-2/IFN-α. Mice were immunized against KLH–DNP formulated in TiterMax Gold (A, $n = 4$) or GPI-0100 (B, $n = 5$) prior to tumor challenge on day 0 (5×10^5 cells) and immunotherapy on days 7, 8, 9, 11, and 14. As specified in the figure legends, folate–DNP conjugates (**EC57**, **EC63**, **EC0293**, or **EC0294**) or a nontargeted DNP (1500 nmol/kg) were administered together with IL-2 (5000 units/dose) and IFN-α (25 000 units/dose). Control mice were given diluents only.

Table 2. Summary of Therapeutic Efficacy

treatment	median survival (days)[a]	% increase in survival[b]	% cured
PBS	20		0
EC63	20	0	0
IL-2/IFN-α	26	30	0
EC57 + IL-2/IFN-α	30	50	0
EC63 + IL-2/IFN-α	40	100	0
EC0293 + IL-2/IFN-α	46	130	40
EC0294 + IL-2/IFN-α	>100	>400	60

[a] Obtained from the survival profiles shown in Figure *4*. Note that all mice except the PBS group received low dose cytokines, accounting for the extension in survival of the group treated with nontargeted DNP. [b] %Increase in survival = 100 × (Treatment group - PBS control)/PBS control.

a lysine side chain of KLH). This data confirmed that folate–DNP conjugates with the greatest binding efficiency to DNP-specific IgG antibody (Figure 4) were also more effective in mediating an antitumor response (Figure 5B). Notably, no allergy or gross toxicities (weight loss, rough fur coat, etc.) were observed throughout the entire study, suggesting that this form of therapy may be well tolerated.

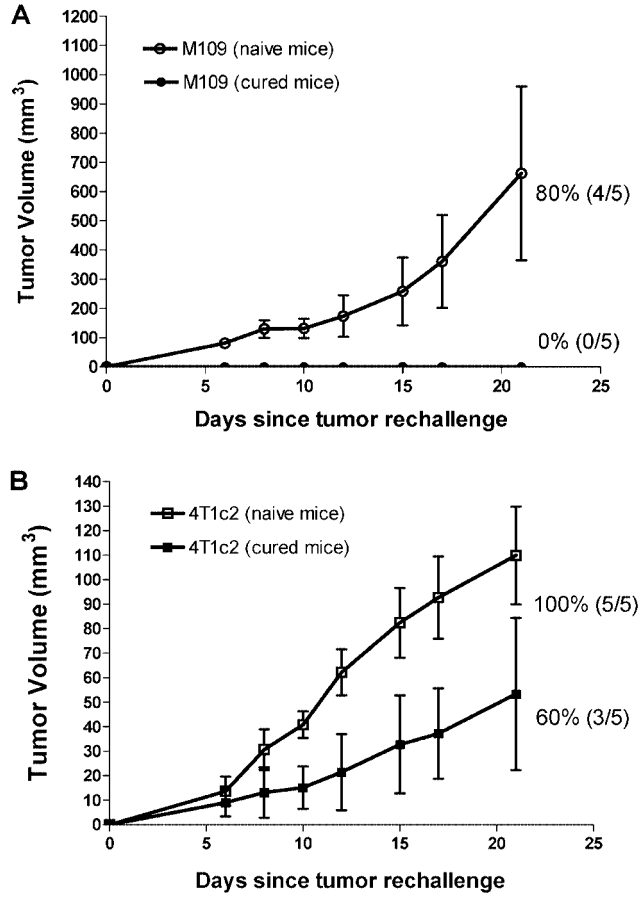

Figure 6. Evaluation of tumor-specific memory in long-term survivors after DNP-mediated immunotherapy. Five animals in groups treated with **EC0293** and **EC0294** (see Figure 5) were still alive 126 days after the initial M109 tumor challenge. These cured mice were rechallenged subcutaneously on day 0 with 1×10^6 viable M109 (A) and 4T1c2 tumor cells (B). Five naïve mice (no previous exposure to folate–DNP immunotherapy) were also inoculated with the same tumor cells to serve as controls. Points represent mean tumor volumes of five animals; bars indicate SEM. Next to each tumor growth curve is the percentage of animals that were euthanized due to excessive tumor burden.

Tumor-Specific Protective Immunity in Mice Treated with DNP Immunotherapy. Since untreated control mice "died" of peritoneal M109 tumors around ~20 days postinoculation, five long-term survivors from the previous study (Figure 5B) in **EC0293** and **EC0294** treated groups were considered cured by day 126. To determine whether any of these mice had developed an independent protective immunity against hapten-free M109 tumor cells, we rechallenged the cured mice (along with five naïve mice) with freshly prepared M109 or 4T1c2 cells (1×10^6 cells each) at distinct subcutaneous injection sites. Without any further treatment with a folate–DNP conjugate or cytokine, these mice were found to reject 100% of the freshly inoculated

M109 lung cancer cells (Figure 6A). A similar potent tumor rejection response was also seen in mice after a folate–FITC-mediated immunotherapy, as published previously.[14,15] Interestingly, as shown in Figure 6B, two of the re-inoculated mice (40%) also rejected unrelated 4T1c2 tumor cells, which like the M109 cells were FR-positive but derived from a breast rather than lung cancer of a Balb/c mouse. Regardless, the difference in tumor growth cures between M109 and 4T1c2 cells in previously cured mice was statistically significant ($P = 0.0004$; two-way ANOVA). In addition, the differences in the growth of M109 or 4T1c2 tumors in naïve versus cured mice were statistically significant with regards to prior therapy ($P < 0.0001$ for both tumors; two-way ANOVA).

Based on the above findings, we wondered whether the long-term survivors had acquired an immunity against the FR, which was expressed at similar levels on both M109 (30 ± 12 pmol/mg) and 4T1c2 (22 ± 9 pmol/mg) tumor cells (N. Parker, Endocyte internal communications). To test that hypothesis, we inoculated the remaining two mice (which had rejected the growth of both M109 and 4T1c2 tumors) with FR-negative 4T1-parent cells (on the opposite flank of the previous 4T1c2 implantation site) and observed the growth of the derived tumors. Importantly, these mice did not reject the 4T1 parent tumor cells, but eventually had to be euthanized due to excess tumor burden (data not shown).

Allergic Responses Induced by Folate–Hapten Conjugates. Vaccine strategies for cancer immunotherapy rarely involve systemic administration of an antigenic compound in an actively immunized host. Although no allergic reactions to any folate–hapten conjugate were observed in our immunotherapy studies (Figure 5), there was the potential for DNP-immunized hosts to produce an allergic response, especially when the host developed an allergic Th2-type response (see Figure 3C). To test this hypothesis, **EC57**, **EC63**, **EC0293**, and **EC0294**, along with BSA–DNP as a positive multihaptenated control, were evaluated in a passive cutaneous anaphylaxis assay that was analogous to the skin test used to predict drug allergies in humans. Here, rats were sensitized intradermally with anti-DNP IgE mAb or polyclonal DNP antiserum and then intravenously challenged 48 h later with the test article in the presence of Evans Blue dye solution. In cases where a localized allergic reaction occurs, the Evans Blue dye would escape the more permeable vasculature and accumulate in the inflamed tissue. In other words, where no allergic reaction is obtained, no blue coloration would be observed. As shown in Figure 7A, rats presensitized with anti-DNP IgE mAb generated a strong allergic response when injected with BSA–DNP. Neither **EC57** nor **EC63** produced any detectable dye extravasation, but **EC0293** and **EC0294** unfortunately did produce low but notable allergic responses. Considering the epitope specificity of anti-DNP IgE mAb, a second test was conducted in rats presensitized with polyclonal DNP antiserum generated from KLH–DNP/alum ip immunized mice (see Experimental Methods). As shown in Figure 7B, the allergic responses induced by **EC0293** and **EC0294** were amplified in the DNP-antiserum-sensitized rats,

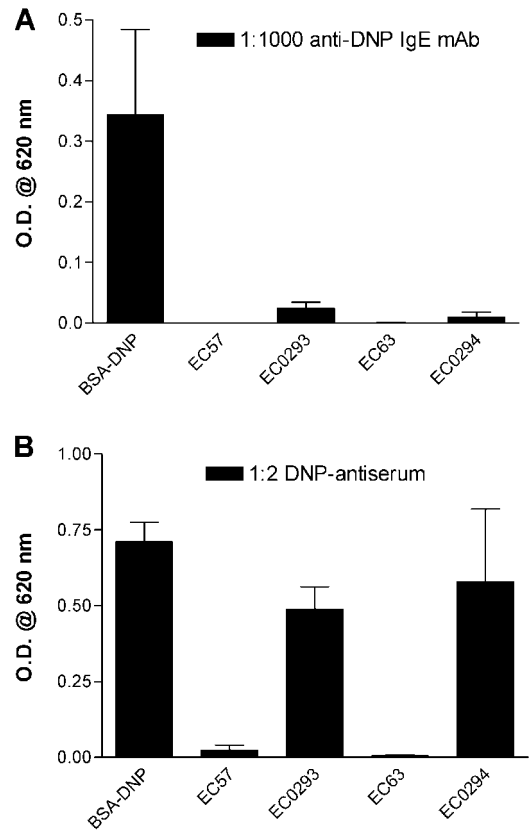

Figure 7. Investigation of hypersensitive potential of folate–DNP conjugates in rats presensitized with anti-DNP IgE mAb or DNP antiserum. Female Lewis rats ($n = 2$) were intradermally injected in duplicate with a 1:1000 dilution of anti-DNP IgE mAb (Clone SPE-7) or 1:2 dilution of DNP antiserum (from KLH–DNP/alum-immunized mice). Forty-eight hours later, rats were intravenously challenged with BSA–DNP (positive control), **EC57**, **EC63**, **EC0293**, or **EC0294** in 1% Evans Blue dye. Thirty minutes after the challenge, skin spots were excised, and dye was extracted and measured at 620 nm.

while the responses to **EC57** and **EC63** remained mild or negligible. Collectively, these results suggest that the risk of allergy is somewhat dependent on the hapten's structural motif in the folate–DNP conjugate.

Discussion and Conclusions

In this current study, we provide additional evidence that a potent antitumor immune response can be directed against a FR-positive tumor when a hapten-vaccinated host is treated with a folate–hapten conjugate. More importantly, we show that the potency of such conjugates can vary depending upon how the hapten is linked to the folate-targeting moiety. Four water-soluble folate–DNP conjugates (**EC57**, **EC63**, **EC0293**, **EC0294**) were constructed with various peptide-based linkers, and all of them maintained their abilities to bind to FR-positive cells yet differed significantly in their immunological responses. To assess the bispecific binding

properties of these conjugates, we developed an ELISA assay that measures the formation of immune complex between anti-DNP IgG and FBP-immobilized folate–DNPs (Figure 4A). Both **EC0293** and **EC0294** were found to bind anti-DNP antibodies efficiently, and they exhibited higher binding capacities compared with **EC57** and **EC63** (Figure 4B). This result was somewhat expected, since the DNP in **EC0293** and **EC0294** is linked via an identical bridging chemistry to that in KLH–DNP, that is, the immunogen used to generate the DNP antiserum. The same polyclonal anti-DNP antibody did not recognize **EC57** and **EC63** as well, likely due to the slight mismatch in bridging chemistry (see structures in Figure 1).

Although **EC63** appeared to be the weakest bispecific ligand *ex vivo*, **EC63** combined with low doses of IL-2/IFN-α was effective in extending the lifespan of M109 tumor-bearing mice, whereas a nontargeted DNP was found to be ineffective in combination with the same cytokine regimen (Figure 5A). This result suggests that targeting of the DNP hapten to the cell surface FR was critical for effective immunotherapy and that **EC63** was sufficiently active *in vivo*. When all four folate–DNP conjugates were directly compared in a subsequent study, the two strongest bispecific ligands (**EC0293** and **EC0294**) yielded the best efficacy against 7-day-old ip M109 tumor implants, where 40–60% cures occurred in the presence of IL-2/IFN-α (Figure 5B). It is noteworthy that no cures have been produced in the same ip tumor model by folate–FITC in FITC-immunized mice,[14] suggesting that in the presence of cytokine co-stimulation, **EC0293** and **EC0294** may be more effective than the folate–FITC conjugate tested previously. While **EC63** alone was completely ineffective in this tumor model, **EC63** supplemented with cytokines again produced statistically significant antitumor activity beyond that of cytokine treatment alone. Surprisingly, despite the favorable binding characteristics predicted by the *ex vivo* ELISA assay, **EC57** only generated a 50% increase in the lifespan, a result that was not statistically different from the 30% increase generated by cytokines alone. One possible explanation for this discrepancy is that the aforementioned *ex vivo* ELISA analysis would not have been sensitive to differences in the stability of folate–DNP conjugates *in vivo*. Thus, we intentionally replaced the stable D-lysine residue in **EC63/EC0294** with a cleavable L-lysine in **EC57/EC0293** to explore the importance of conjugate stability. The fact that **EC0293** was found to be highly active *in vivo* (despite having an L-lysine residue) could be attributed to the fact that it is a much stronger antibody binder than **EC57**. Although we did not test the antitumor activity of **EC0293** and **EC0294** without cytokines, our experience with folate–FITC (which consequently has a thiourea linking motif identical to that found in KLH–FITC) suggested that a folate–hapten conjugate dosed alone is not very effective against the established 7-day-old ip M109 tumor. Nonetheless, given the strong bispecific binding potential of **EC0293** and **EC0294**, we would imagine that these two conjugates might perform better than **EC63** if they are given to mice with more freshly implanted ip tumors.

As we have reported previously using FITC as the hapten,[14,15] a tumor-specific protective immunity was developed in mice after folate–DNP targeted immunotherapy. In the current study, we rechallenged mice that had been cured of peritoneal M109 tumors after **EC0293/EC0294** immunotherapy (Figure 5B) with fresh inoculations of both M109 and 4T1c2 tumor cells on distant subcutaneous sites. As expected, we saw 100% rejection of the M109 tumor, but two of the five animals also developed immunity against the 4T1c2 tumor (Figure 6). This was not anticipated, since these two cell lines were not related except for expression of FR, with the former being syngeneic and the latter being artificially transfected with FR. Out of curiosity, these two surviving mice were rechallenged 30 days later with 4T1 cells, the FR-negative parent of 4T1c2,[6] and no immunity against the 4T1 parent cells was observed. These intriguing observations might suggest that a cancer cell-specific protective immunity had developed during the initial course of the DNP-mediated immunotherapy and that such antitumor immunity might have been (in part) directed against the FR. Further studies with additional animals are necessary to confirm this hypothesis; however it should be noted that the FR is a recognized tumor-associated antigen[27] and that anti-FR antibodies are naturally generated in patients with ovarian cancers.[28]

A risk of administering a folate–hapten conjugate to a hapten-immunized host is the potential for the host to develop hypersensitivity to the folate–hapten conjugate. In our therapy studies (Figure 5), we did not observe an allergic reaction in any of the treated mice. However, ELISA analyses for anti-DNP antibody isotypes (Figure 3) did show that the KLH–DNP immunized mice could conceivably develop an allergic Th2-type response due to low IgG2a to IgG1 ratio and the observed induction of IgE antibody, especially when alum was used as adjuvant. To further explore this issue, we used a commercially available anti-DNP IgE mAb (Clone SPE-7) as well as the induced DNP polyclonal antiserum from KLH–DNP/alum-immunized mice in a rat passive cutaneous anaphylaxis assay (Figure 7). Despite the fact that our folate–hapten conjugates are monovalent in nature (1:1 ligand to hapten), **EC0293** and **EC0294** were found to produce weak allergic responses in rats presensitized with the anti-DNP IgE mAb and strong allergic responses in rats presensitized with DNP polyclonal antiserum. On the other hand, **EC57** and **EC63** appeared to be either nonallergic or

(27) Coney, L. R.; Tomassetti, A.; Carayannopoulos, L.; Frasca, V.; Kamen, B. A.; Colnaghi, M. I.; Zurawski, V. R., Jr. Cloning of a tumor-associated antigen: MOv18 and MOv19 antibodies recognize a folate-binding protein. *Cancer Res.* **1991**, *51*, 6125–6132.

(28) Knutson, K. L.; Krco, C. J.; Erskine, C. L.; Goodman, K.; Kelemen, L. E.; Wettstein, P. J.; Low, P. S.; Hartmann, L. C.; Kalli, K. R. T-cell immunity to the folate receptor alpha is prevalent in women with breast or ovarian cancer. *J. Clin. Oncol.* **2006**, *24*, 4254–4261.

only mildly allergic, suggesting that a slight variation in the hapten chemistry (adjacent to the DNP moiety) rendered them safer than **EC0293** and **EC0294**. This was encouraging, since structurally modified allergens have been used to develop vaccines for the treatment of allergies.[29] The fact that none of the **EC0293-** and **EC0294**-treated mice ever displayed any symptoms of allergy (e.g., itching/scratching, redness, labored breathing, decreased body temperature, etc.) is now puzzling, but this effect may depend on the extent to which the immune system was biased towards a Th2 response.[25,30,31]

Immunotherapy strategies for treating FR-positive cancers are still in an early stage of development, and they can involve anti-FR mAbs (MOv18, MOv19), radioimmunotherapy, bispecific antibodies, and tumor vaccines (reviewed in ref 32). We believe that our ligand-targeted immunotherapy may offer a unique mechanistic approach where (i) FR-positive tumors are noninvasively marked with a small folate–hapten conjugate, (ii) endogenous anti-hapten antibodies are recruited to opsonize the tumor cells, and (iii) Fc receptor-bearing immune effector cells recognize and kill the opsonized tumor cells. More importantly, a long-term antitumor immunity may result from phagocytosis and antigen presentation of the marked/dying tumor cells by local antigen-presenting cells, such as dendritic cells and macrophages. Ultimately, such tumor antigen presentation may then lead to the development of cellular immunity against the tumor-associated antigens, including the FR, as suggested by the current study (Figure 6). Taken together, our results suggest that a folate–hapten conjugate may be designed to maximize its ability to bind to endogenous anti-hapten antibody, but one should also carefully consider its potential for allergy in a hapten-sensitized host before consideration is given for development.

Acknowledgment. The authors acknowledge Rich Messmann, Apparao Satyam, Nikki Parker, Marilynn Vetzel, and Sheri Wilson for their technical assistance.

MP070050B

(29) Akdis, C. A.; Blaser, K. Regulation of specific immune responses by chemical and structural modifications of allergens. *Int. Arch. Allergy Immunol.* **2000**, *121*, 261–269.

(30) Blaser, K. Allergen dose dependent cytokine production regulates specific IgE and IgG antibody production. *Adv. Exp. Med. Biol.* **1996**, *409*, 295–303.

(31) Riedl, M. A.; Landaw, E. M.; Saxon, A.; Diaz-Sanchez, D. Initial high-dose nasal allergen exposure prevents allergic sensitization to a neoantigen. *J. Immunol.* **2005**, *174*, 7440–7445.

(32) Lu, Y.; Low, P. S. Immunotherapy of folate receptor-expressing tumors: Review of recent advances and future prospects. *J. Controlled Release* **2003**, *91*, 17–29.