

New Agents for Treatment of Recurrent Small-Cell Cancer: Tumor-Specific Abnormal Vasopressin V2 Receptor Antibodies

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ABSTRACT

We earlier demonstrated that small-cell lung cancer (SCLC) not only expresses a normal form of vasopressin V2 receptor but also an abnormal form, through alternate splicing, that lacks the seventh transmembrane region (named AbnR2). This tumor-specific abnormal receptor is expressed at the surface of cells and can be targeted by antibodies directed against a unique C-terminal region. Immunocytochemistry reveals AbnR2 is common to all or most small-cell lung tumors. Our generated mouse monoclonal antibody was found to decrease cell viability in vitro and promote apoptotic cascades. This mouse anti-AbnR2 antibody, and a humanized form derived from it, were each found to reduce the growth of SCLC xenografts representing recurrent disease in nu/nu mice, and to have synergistic, tumor anti-proliferative effects when used with cyclophosphamide, but not cisplatin, in completely inhibiting growth. Possible mechanisms might involve normal vasopressin V2 receptors.

Key Words: recurrent small-cell lung cancer, abnormal vasopressin V2 receptor, tumor xenografts, Abner humanized antibody, combination therapy

INTRODUCTION

There are about 40,000 new cases of SCLC per year in the US [ACS, Facts & Figure 2010] reflective of a high world-wide occurrence [Ferlay et al., 2015]. Present treatments of SCLC generally involve high-dose combination chemotherapy with or without radiation therapy [Wingo et al., 1995; Morabito et al., 2014; Morstyn et al., 1984; Grant et al., 1992; Ihde, 1995; Johnson et al., 1990; van Zandwijk, 1994; Wampler et al., 1991; Mor, 1994]. Although there is a high initial response rate to these treatments, and long-term survival in up to 10% of all cases [Morabito et al., 2014; Cook et al., 1994; Ciomber et al., 2006; Azim and Ganti, 2007; Hann and Rudin, 2008], average life expectancy is increased by only 8–15 months. While about 80% of these newly diagnosed SCLC patients respond to chemotherapy, remission generally lasts only 3 – 6 months. Unfortunately, there is no effective therapy to treat recurrent disease (*rSCLC*) because it is resistant to available approaches, including chemotherapies. All SCLC, including *rSCLC*, expresses vasopressin as an autocrine, growth-modulating agent [North et al., 1993; North, 2000; Woll and Rosengurt, 1989; North and Yu, 1993; Zachary et al., 1991; Bunn, 1994]. Additionally, all SCLC, including *rSCLC*, express all four known vasopressin receptors [North et al. 1993a; North et al. 1993b]. We were previously able to show that vasopressin acts through tumor vasopressin V1 receptors to promote growth and that the cascade associated with this promotion is disrupted in cell lines representing *rSCLC* [Piqueux et al., 2004; Keegan et al., 2002; North et al., 1997]. We were also able to show that vasopressin acts through vasopressin V2 receptors to inhibit tumor growth in primary and recurrent disease [North et al., 1998ab; Piqueux et al., 2004]. However, besides normal V2 receptors,

SCLC also express a tumor-specific abnormal V2 receptor through alternative splicing, that is referred to by us as AbnR2. This abnormal receptor lacks the seventh transmembrane region and has a unique C-terminal structure [North et al., 1998ab]. We are of the opinion that AbnR2 serves tumors as a null receptor. The current report describes the production of antibodies specific for AbnR2 and their use to examine the prevalence of AbnR2 in SCLC and to evaluate the use of anti-AbnR2 antibodies, alone or in combination, as potential therapeutic targeting agents for recurrent small-cell lung cancer.

METHODS

Tumor Cells

Human SCLC cell lines NCI H82, NCI H446, NCI 345, DMS-53, and NCI H146 were all obtained from ATCC (American Type Culture Collection, Rockville, MD) and maintained in DMEM medium (Mediatech, Inc., Herndon, Va.), containing 10% fetal bovine serum (Atlanta Biologicals, at 37°C, in an atmosphere of 5% CO₂ with medium changes every 3 and 4 days. These cultures were maintained in tissue culture flasks at densities from 1- 5 x 10⁵ cells/ml.

Antibodies

The rabbit polyclonal antibodies referred to as pAbner, and a mouse monoclonal antibody, mAbner, were generated against a 9-mer C-terminal segment of abnormal V2 receptor with added N-terminal tyrosine, H-YLEGGCSR₉-OH, coupled to thyroglobulin. Abner antibodies are the subject of a patent application by Woomera Therapeutics Inc. [US Patent Application:# 10/521,091]. mAbner is of the IgG1 subclass, and was used to generate first a human chimeric form (cAbner), and then a humanized form (hAbner). This procedure

will be described in detail elsewhere, but in summary is as follows:

The first step in this generation was the cloning and structural confirmation of the Vh and Vl regions of mAbner, a sub-class IgG1 molecule. Two expression vectors for the heavy and light chain of the antibody were then constructed that included a portion of the variable region of mouse Vh chain combined with a human IgG1 heavy chain, and a portion of the variable region of mouse Vl combined with a human kappa light chain constant region, respectively. The two expressions vectors were initially transiently transfected into human 293 cells, and later into CHO cells (Invitrogen) to form a cAbner-secreting stable cell line. The humanization process from cAbner was then facilitated by generating a homology-modeled antibody 3D structure and creating a profile of the parental antibody based on this structural modeling. Acceptor frameworks were identified based on the overall sequence identity across the framework, matching interface position, similarly classed CDR canonical positions, and the presence of N-glycosylation sites that would have to be removed. Based on these analyses, two light chain and two heavy chain frameworks were selected for the humanization design. The final designs of the humanized heavy and light chains were to have a maximum amount of human sequence in the final humanized antibodies while retaining the original antibody specificity. Accordingly, three humanized light chains and three humanized heavy chains were designed. Including the parental antibody sequences, a 4 x 4 matrix of 16 antibody combination were transiently expressed in HEK293 cells, and their expression levels and affinity to the antigen were evaluated by ELISA. Based on binding affinity and expression level, three antibodies were selected for 0.1 liter scale production and purification. Further analysis

of the three antibodies resulted in the selection of one antibody, now designated hAbner, for further study. Finally, both cAbner and hAbner were expressed through transfection as secretory products of stable CHO cell lines.

MOPC21 is a mouse monoclonal antibody also of the IgG1 sub-class and is a ubiquitous antibody produced by a mouse myeloma cell line [Schubert, 1968] and purchased from BioXcell Inc. of Lebanon, New Hampshire. For all studies, antibodies were purified from culture by Protein A affinity chromatography. Mouse monoclonal antibodies mAbner and MOPC21 were in some cases both modified by addition of a chelating agent. This was accomplished by reacting each at pH 8.3 overnight with CHXA⁷-DTPA reagent in 10-fold excess [Adams et al., 2004; Stein et al., 1997]. DTPA-modified antibodies were reacted with ⁹⁰Yttrium chloride, and ⁹⁰Yttrium-labeled products isolated by Sephadex G25 chromatography.

Biocore and RIA, and ELISA Antibody Evaluations

Biocore evaluation of chimeric and humanized Abner antibodies was performed on a G.E. Biacore X100. For these studies we employed Human Antibody Capture Kit (BR100839), an Amine Coupling Kit (BR100050), a Sensor Chip CM5 (BR100012), and HBS-EP+ buffer (BR100826), all from G.E. Anti-human antibody was attached to the chip with coupling agents following recommendations, and this was used to capture cAbner and hAbner at doses ranging from 5 to 20 µg/ml. The Analyte was the 9-mer peptide antigen used to generate mAbner and representing the C-terminus of the abnormal V2 receptor. Analysis was performed with up to seven concentrations of the peptide antigen from 0.05 to 10 µg/ml, and runs repeated a number of times.

Radioimmunoassay displacement curves were generated using mAbner and cAbner, ¹²⁵I-labelled peptide antigen, and concentrations of unlabeled peptide antigen ranging from 0.2 to 50 nM. Labelled peptide was produced as previously described using a lactoperoxidase procedure that yields a stable product of high specific activity, and counting of radioactivity pelleted with PEG was performed on a Cobra Auto-Gamma counter from Packard [North, 1991]. The binding coefficient in each case was assessed as the point of 50% displacement of label from antibody.

ELISA was performed for hAbner, but using decapeptide antigen bound covalently to BSA in the ratio of 10-fold peptide over protein through glutaraldehyde coupling [Friedmann et al., 1994]. Peptide complex was competed for binding to antibody with peptide covalently complexed with glutaraldehyde to biotinylated BSA. After equilibrating conditions, bound biotinylated complex was determined using peroxidase-labelled streptavidin and o-phenylenediamine dihydrochloride (OPD, Thermo Scientific) as a 1 mg/ml solution in 0.05 M citrate, pH5.0 with 0.003% peroxide. Absorbance was measured on a SYNERGY HT fluorescence plate reader at 450 nm or at 490 nm following a stop reaction with addition of strong acid (in our case 15µl 3M HCl to each 100 µl of reaction mixture).

Immunohistochemistry of Human Tumor Tissue:

Immunohistochemical (IHC) staining for human AbnR2 was performed, as previously described [34], on a SCLC microarray representing 40 patient cases of localized and metastatic disease (US Biomax LC10010), and on formalin-fixed preparations from 22 SCLC tumors, including 8 cases of recurrent disease, obtained from an archival tissue library at the Department of Pathology, Dartmouth-

Hitchcock Medical Center. Both monoclonal IgG1 mouse antibody (*mAbner*) and rabbit IgG2b polyclonal antibodies (*pAbner*) were used in IHC studies. Tissue sections (4µm) of formalin-treated material were steamed in citrate to recover antigen and then blocked by incubating them with 10% horse serum. They were then incubated for two hours at ambient temperature with *Abner* in PBS/ 5% bovine serum albumen alone, *Abner* in PBS/5% BSA containing an excess of peptide antigen as negative control, or PBS/5% BSA without antibody as a further negative control. Following washings (x3) in PBS/BSA, tissues were incubated for 1 hour at ambient temperature with biotinylated horse anti-rabbit IgG, washed (x3), and then incubated for 1 hour at ambient temperature with Streptavidin coupled to peroxidase. Tissues were again washed (x3) and finally reacted with a diaminobenzidine/peroxide mixture and contrasted with hemotoxylin.

Immunocytochemistry and Confocal Microscopy

The coverslips were seeded with 10,000 NCI H82, or NCI H446 cells and grown to 100% confluence. The cells were fixed with formalin (Fisher Scientific), permeabilized with 0.5% NP40 (Sigma Aldrich), blocked with phosphate buffered gelatin and stained overnight at 4 °C with *mAbner* antibody. The coverslips were washed five times with 1% BSA in PBS and incubated with a 1:2000 (1 µg/ml) dilution of the Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes) for 60 minutes. The coverslips were then washed five times with PBS containing 1% Bovine serum albumen, post-fixed with 4% PFA and mounted in Vectashield (Vector Laboratories) on glass slides. The slides were visualized with an Olympus BX61WI fluorescent confocal microscope employing a Hamamatsu Orca-ER C4742-80 camera. In some cases cells were incubated with *mAbner* for four hours

at 37 °C prior to fixation, permeablizing, and addition of the secondary antibody.

MTT Cell Viability Assay

Trypsinized NCI H82 and NCI H345 cancer cells were seeded at approximately 20,000 per well into a 96 well plate, and allowed to recover from trypsinization for 24 hours. They were then treated with various dilutions of the *mAbner* or *cAbner* at concentration of 0.1 to 1 μ M for 24 hours. The MTT cell viability reagent was added to each well at a concentration of 10% and incubated at 37°C for overnight following which the substrate had changed to a pink color. The plate was read on a Synergy HT fluorescent plate reader at a florescent excitation wavelength of 570nm and emission wavelength of 590nm. The cell treatments were performed in quadruplicate and each experiment was performed three times. Cell viability was normalized to untreated cell values and expressed as a percentage of those values.

Generation of Small-Cell Tumor Xenografts

Male and female nu/nu mice 6-7 weeks of age were purchased from Harland. NCI H345, NCI H82, and NCI H446 cells were trypsinized, concentrated into growing medium by centrifugation ($4-5 \times 10^7$ cells/ml) and injected subcutaneously in the lower right flank quadrant ($1-2 \times 10^7$ cells per animal) using a 1 ml syringe and 22 gauge needle, as previously described [North et al., 2010]. Cells were allowed to generate tumor xenografts for 21 days before the initiation of the studies. At this time all of the mice receiving cells produced tumors that ranged in length from 0.5-0.75 cm. For the following four days tumors were evaluated for tumor growth by measuring length, width, and depth with a micrometer (Electronic Digital Caliper, Fisher Scientific Inc.), and size expressed as the product of all three parameters.

Treatment with Native and Yttrium-labelled Antibodies

Tumor-bearing mice were divided into three groups of eight animals in one short-term treatment study on the effects of native *mAbner* compared to treatment with saline vehicle and with the ubiquitous antibody MOPC2, and for one study comparing the effects of 190Yttrium-labeled antibodies. Animals were selected to provide a similar range in tumor size for each grouping. For each short-term study, group 1 comprised animals treated with four intra-peritoneal (i.p.) injections of 50 μ l of saline vehicle given on days 0, 2, 4 and 6. For one short-term study, animals of group 2 received four i.p. injections of 50 μ Ci (initially 20 mCi/mg protein) of 90Yttrium-labeled Abner made up to 100 μ g with naked Abner carrier (~3.3 mg/kg body weight) in 50 μ l saline, and animals of group 3 received four i.p. injections of 50 μ Ci of 90Yttrium-labeled MOPC21 with naked MOPC21 carrier, on days 0, 2, 4, and 6. The amount of radiolabel employed was based on earlier studies by others [Adams et al., 2004]. For the second short-term study, groups 2 and 3 comprised treatments on days 0, 2, 4, and 6, with 100 μ g/50 μ l of naked Abner (~3.3 mg/kg body weight) or naked MOPC21, respectively. Tumor volume was measured daily by micrometry, and the body weight of animals evaluated, for 16 days. For the long-term study, animals were injected i.p. daily for 16 days with, saline vehicle (group 1), naked *mAbner* (group2), or naked MOPC21 (group 3). Tumor size and animal body weight were assessed daily over this period, and daily measurements of tumor volume and body weight were extended for the *mAbner* treated group for an additional 20 days. Possible toxicity of treatment was measured by examining major organs (liver, kidneys) and tumors for necrotic changes at the completion of each study.

Combination Treatments with Antibodies and Cyclophosphamide and Cisplatin

One six arm study compared the independent actions on variant NCI H82 tumor xenografts of native Abner antibody (daily treatment with 3.3 mg/kg bw, 14 days), cyclophosphamide (3x50 mg/kg bw given days 1, 2 and 3), cisplatin (10 mg/kg bw, single dose at Day1), and combination pretreatment with either cyclophosphamide followed by Abner treatment, or cisplatin treatment followed by Abner treatment (N=8 per 4 groups, N=4 for two groups). Control Group 1 received only saline vehicle as treatment. A second four arm study employed the same doses of mAbner and cyclophosphamide, but examined concomitant and instead of sequential combination treatment. A third four arm study substituted hAbner for mAbner. Tumor size was measured in semi-blinded fashion each day for two weeks. Body weights were measured daily throughout the studies to evaluate toxicity.

Statistical Analysis

To estimate the treatment effect in four groups of mice the longitudinal tumor growth data were fitted using exponential growth curves based on linear mixed effect model on the log scale [Demidenko, 2006; Demidenko, 2013]. Since tumor volume had considerable variation across mice the random intercept model has been used with the random effect associated with the animal heterogeneity of response to treatment [Demidenko, 2013. Computations were done in statistical language R (R Core Team, 2014). R: A language and environment for statistical computing (Vienna, Austria) using function 'lme' in the package 'nlme.' The synergy was tested using Z-test based on the difference in tumor growth rates compared with the control group.

RESULTS

Biocore, RIA, and ELISA Evaluation of Abner Antibodies

Biocore analysis typically gave Kds for mAbner from 84 to 210 nM with the corresponding on-off constants of k_a 3.7×10^5 M to 6.5×10^4 M and k_d 3.1×10^{-3} to 140×10^{-3} . cAbner had Kds of 94 to 141 nM with corresponding on-off constants 9.3×10^5 to 7.9×10^5 and k_d 8.8×10^{-2} to 11×10^{-2} ; while hAbner has Kds ranging from 70 to 285 nM with corresponding on-off constants k_a 2.7×10^5 to 5.3×10^3 and k_d 7.6×10^{-2} to 6.9×10^{-2} . This shows all forms of Abner bind to decapeptide antigen to a similar degree with KD ~100 nM and suitable on-off characteristics.

Radioimmunoassay analysis of binding gave smaller KDs of 27- 46 nM for mAbner and 27-36 nM for cAbner, with one evaluation of 22 nM for hAbner. These values are in reasonable agreement with the nevertheless higher values found through Biocore. Both Biocore and RIA assessments of binding were obtained with the free peptide antigen. While the dissociation constant for human Abner is therefore between 22 and 100 nM with the free antigen decapeptide, ELISA performed with the antigen attached to protein (as it would be as a component of AbnRV₂ receptor on cells) gave a much smaller KD of 6 nM, showing increased binding over that with free peptide (6-7-fold of RIA and >10-fold of Biocore). This finding, we believe, conveys an important message for others assessing binding constants for antibodies generated to any small peptide antigen representing a segment of tumor protein marker. This is because we expect binding assessed for antigen-protein complex should be more representative of that to marker on cells.

Immunohistochemistry and Confocal Microscopy

Immunohistochemistry with both *mAbner* and *pAbner forms* 66 cases of SCLC, including 8 with recurrent disease, revealed the presence of the abnormal vasopressin V2 receptor in seemingly all neoplastic cells of all tumor tissue microdots and tissue sections at a staining intensities of +3 and +4, as exemplified by Figure 1a. This staining was completely blocked by the presence of peptide antigen in antibody preparations, and no staining was found with sections of normal kidney, breast, liver and lung tissues, and with any of the cells in normal tissue microarray CHTN2002X from the Cooperative Human Tissue Network representing a combined total of 66 tissue types. Confocal evaluations on all five small-cell cultures showed the presence of the receptor on the surface of cancer cells (Figure 1b). Interaction of the receptor with Abner antibodies at 37 °C resulted in the complex becoming internalized.

Anti-proliferative Activity of Abner Antibody with and without Cyclophosphamide in Culture

mAbner in doses up to 100 nM antibody was shown to decrease cell viability over 24 hours of NCI H82 cells in culture by up to 30% compared to controls of antibody MOPC21. Cyclophosphamide had much more dramatic effects with reductions of greater than 50% viability produced by 50 nM concentrations of the agent. This anti-proliferative action was found to be additive to the inhibitory actions of *cAbner* on these cells, and was reflected by an increase in the level of apoptotic markers represented by the 89kD degradation product of PARP-1 (Poly ADP-ribose polymerase-1), a DNA repair enzyme. When NCI H82 cells were treated with *cAbner* at 67 nM, cyclophosphamide at 50 nM, or a combination of these agents, for 4, 8 16 and

24 hr at 37 °C the 89kd PARP-1 fragment was shown to increase as shown in Figure 2, with the highest concentration in cells treated with combination, indicating additive, and possibly synergistic, actions of these substances.

Similar Inhibitory Effects Produced by Native and 90-Yttrium-labelled Antibody

There were significant and almost identical effects of *mAbner*, *cAbner*, and *hAbner* on tumors with native *mAbner* treatment (shown) reducing growth to about half of the rate of MOPC21 treated tumors ($p < 0.03$), with an almost 3-fold increase in doubling time (Figure 3A). An initial reduction in tumor volume to ~80% occurred with *mAbner* treatment. For the second study (Figure 3B), ⁹⁰Yttrium-labelled MOPC21 treatment gave a growth curve indistinguishable from saline control, while ⁹⁰Yttrium-labelled *mAbner* treatment reduced the growth rate to about one-fourth of controls for the dosing period ($p < 0.007$). Following this time, growth rates increased to parallel the ⁹⁰ Yttrium-MOPC21 and control group. No differences were seen between the body weights of control and treated groups shown for the ⁹⁰Yttrium study in Figure 2C. The dip in body weight at day 10 was caused by an unintended interruption of water supply to animals.

In Growth Tumor Inhibitory Effects Abner Synergizes with Cyclophosphamide but not Cisplatin

As can be seen from Figure 4A and 4B, tumors treated with native *mAbner* had their volumes reduced to ~80% and their growth interrupted so initial doubling time was increased ~5-fold compared with saline controls ($p < 0.001$). Nevertheless, with this concentration of 3.3 mg/kg, as seen before, tumors eventually overcame inhibitory effects of antibody and recommenced growing at approximately the same rate as

controls. Cyclophosphamide treatment increased tumor initial doubling time ~3-fold, but tumors then recommenced growing at the rate shown by controls. Cisplatin treatment (Fig. 3B), like antibody, reduced tumor volume by ~20%, increased initial doubling time ~5-fold, and then allowed normal growth to recommence. Combination use of cisplatin and antibody did not seem to alter effects produced with each of them as single agents. However, when cyclophosphamide treatment preceded treatment with mAbner, tumor growth was almost prevented for the entire time of observation (Fig.4A). This observation time covered 8 days beyond the day of the last treatment. There were no recognizable pathological changes in normal tissues, and no body weight differences between control and treatment groups (Fig. 4C). A similar outcome was found when the 3-dose regimen of cyclophosphamide was given concomitantly with mAbner. Figure 5 illustrates that treatments with hAbner and cyclophosphamide, alone and in combination, gave very similar outcomes. Analyzing for synergy for data in Figure 5 through tumor overall growth rates in individual animals shows doubling times of 6 days for controls, 8 days for *hAbner* treatment, 10 days for cyclophosphamide, and 48 days for the combination and a probability for synergy of $P < 0.00001$.

DISCUSSION

Recurrent small-cell lung cancer is a deadly disease with no effective treatment. We believe the studies outline in this manuscript clearly demonstrate a potential effective treatment modality of antibody and cyclophosphamide combination that inhibits or limits tumor growth in animal studies, and could be adapted for patients. Our antibody called Abner, now humanized, is directed against an apparently tumor-specific abnormal vasopressin V2 receptor, AbnV2R, because it does not give positive

staining of normal tissues. This would seem to provide it with a distinct advantage over other tumor-targeting antibodies, like Herceptin, which are tumor-selective rather than tumor-specific [Romond et al. 2005]. It has been proposed that a truncated receptor such as AbnV2R would not be inserted into the plasma membrane of cells [Sedeghi et al., 1997]. Nevertheless, Zhu and Weiss [1998] found that a similar, but inherited, truncated vasopressin receptor molecule referred to the Utah mutation when co-expressed with wild-type V2 receptor dimerized with this form and effectively inhibited all downstream activity. Since we, and others [North, 2000; North et al. 1998ab; Taylor et al., 1990; Ripoll et al. 1990; Garona et al., 2015], have demonstrated that vasopressin action through V2 receptors is inhibitory to the growth of solid tumors, we have concluded that tumors probably manufacture AbnV2R as a null receptor to impair such growth inhibition. Since Alonso and coworkers [Ripoll et al., 2013; Garona et al., 2015] have shown that activation of tumor V2 receptors seems to impair angiogenesis and tumor metastasis as well as retard growth of breast cancer, it is conceivable that all these mechanisms could also play some role in the inhibitory actions of Abner if the antibody internalizes and removes the null receptor and allows influences by tumor-generated vasopressin to be increased through normal V2 receptors. Treatments with Abner, instead of administering V2 receptor agonist proposed by Garona et al. [2015] for breast cancer, would have the advantage of not exacerbating the condition of SIADH present in many cases of small-cell lung cancer. While such reasoning about inhibiting a null receptor could well apply to primary forms of small-cell lung cancer that are known to express both normal and abnormal forms of the vasopressin V2 receptor, we were previously unable to demonstrate that NCI H82 cells,

representing recurrent disease, expressed the wild-type V2 receptor [North et al., 1998; North, 2000]. It is however possible that, as AbnR2 is a product of post-transcriptional regulation, NCI H82 cells revert to producing normal V2 receptors as a consequence of the actions of Abner antibodies. While such a possibility should be readily testable, an entirely separate mechanism of action cannot be ruled out.

Our reported studies show we were clearly able to retain both binding characteristics and biological activities of Abner when transforming our mouse monoclonal antibody to a humanized form. Binding to peptide antigen was actually marginally enhanced through this process. Of particular interest was our finding that binding is increased 10-fold when the antigen is covalently linked to protein. The latter binding seems more appropriate since use will be directed towards targeting a protein surface marker of which the antigen is part. A similar reasoning could apply when evaluating antibodies from any small peptide antigen because such antibodies may represent much better targeting agents than assessed by binding to non-protein linked antigen.

All forms of Abner, in most cases when used alone on a daily basis, had their maximal effects on growth for the first 2-3 days and then rapidly lost any ability to inhibit small-cell tumor growth. The tumors either shrank or stopped growing within the first 24 hours of treatment, but thereafter became resistant so that growth gradually approached that of saline or MOPC21 antibody controls. It is uncertain whether this might be due to a down-grading of abnormal receptor surface expression or to a decreased capacity to internalize complexed receptor. In a similar fashion, growth inhibition produced by three days of treatment with cyclophosphamide was generally soon afterwards followed by a

gradual return of growth to parallel that of control treatment. However, a combination treatment of Abner with three days of cyclophosphamide, given either concomitantly or sequentially, dramatically increased efficacy by producing a synergistic prolonged growth inhibition that extended beyond the time of treatment. This effect of combination treatment on tumors in animals was evidenced *in vitro* by the additive effects these agents were shown to have on PARP-1 degradation, reflective of increased cell apoptosis. Such an increased treatment efficacy was not found when Abner was combined with the chemotherapeutic agent, cisplatin.

It is hoped the synergy exhibited in our studies between Abner antibody and cyclophosphamide will apply to combinations with additional chemotherapeutic agents, such as the topoisomerase I inhibitor Topotecan, that is a preferred treatment for recurrent small-cell lung cancer [Garst, 2007]. Nevertheless, current findings provide the promise that the humanized form of Abner antibody can be added to chemotherapy to produce more effective outcomes of patients with this fatal condition.

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AUTHOR CONTRIBUTIONS

WGN drafted the manuscript and was the chief architect of all of the research conducted. FL was involved in performing confocal, cell culture, RT-PCR and binding studies. VM selected tumor cases and reviewed IHC, and ED performed statistical evaluations. RP and RT actively participated in performing and planning animal studies.

CONFLICT OF INTEREST STATEMENT

Concerning COI, all authors except for William G. North and Roy H.L. Pang have no commercial interest relating to the material in the manuscript. However, as explained in the referenced patent application, and by institutional designation, Drs. North and Pang do have a commercial interest in Woomera Therapeutics Inc., the patent applicant. Dr. North is a full time faculty member of Geisel Medical School at Dartmouth, but is also President of Woomera Therapeutics Inc. and holds >40% interest in the company. Dr. Pang, until recently, was CEO and CSO of the company.

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Figure 1

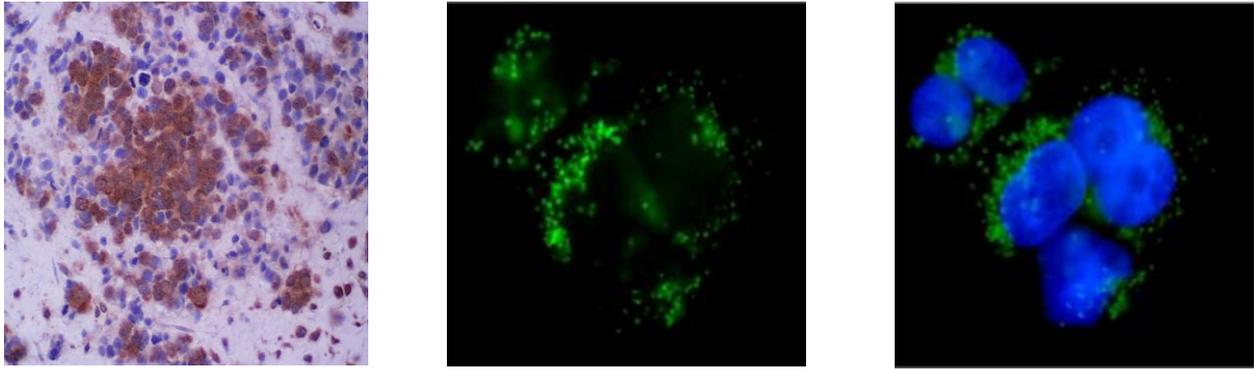


Figure 2

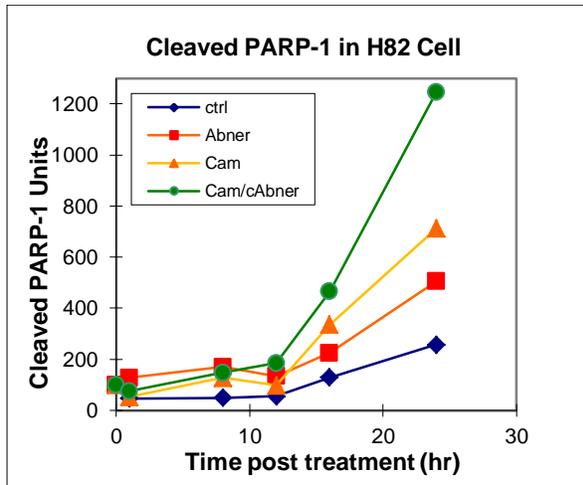


Figure 3

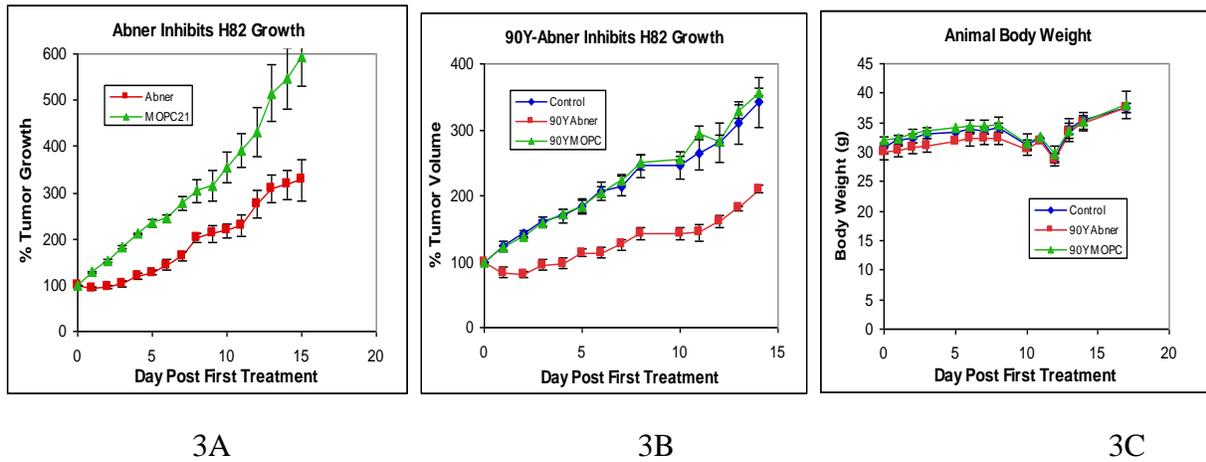


Figure 4

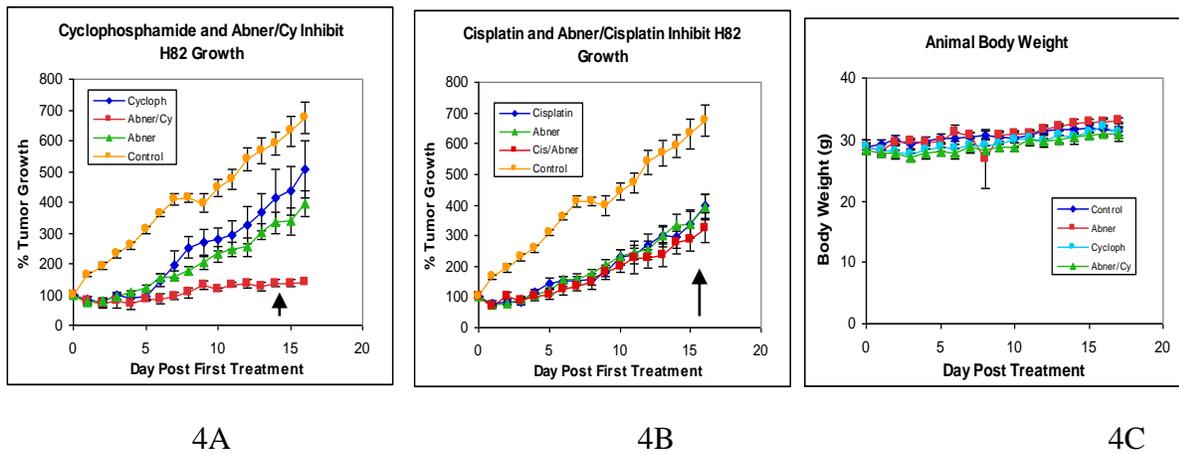
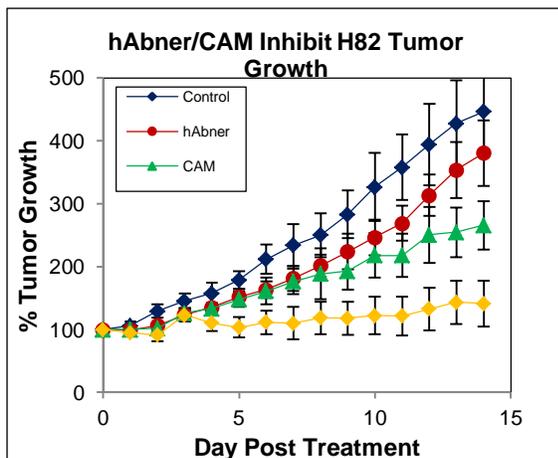


Figure 5



Legends

Figure 1. (a). Representative immunohistochemical staining of SCLC with Abner antibody. Staining was completely blocked by adding 20 $\mu\text{g}/\text{ml}$ peptide antigen to antibody preparations (b) Confocal Imaging of NCI H82 SCLC cells with Abner antibody.

Figure 2. Induced cellular apoptosis of NCI H82 cells assessed by the increase in PARP-1 89 kd breakdown product over time of treatment with humanized Abner antibody, cyclophosphamide, and a combination of both agents. Combination treatment results in increased apoptosis. Cyclophosphamide (CAM) concentration, 20 mM, Abner concentration, 300 μM .

Figure 3. (A). Immunofluorescence and Confocal Imaging of NCI H82 cells with MAG-1 and MOPC21 (center insert); the blue staining of the confocal image is DAPI; (B) In vivo imaging of NCI H345 tumor in nu/nu mouse with ^{99}Tc -DTPA-CHX-A''-Fab MAG-1 at 20 hours after administration of label; (C) Treatment of NCI H345 tumor in nu/nu mice with 50 μCi ^{90}Y -DTPA-CHX-A''-MAG-1 (50 $\mu\text{g}/30$ g body weight total antibody) given alternate days (x4). Values expressed as % change in mean tumor size ($\pm\text{SEM}$) from day 0 [n=4]; (D) Treatment of NCI H345 cells tumors in nu/nu mice with native MAG-1 (50 $\mu\text{g}/\text{gram}$ body weight) given alternate days (x4). Values expressed as % volume change ($\pm\text{SEM}$) from day 0 (n=4); (E) Treatment of NCI H345 cells tumors in nu/nu mice with native MAG-1 (100 $\mu\text{g}/30$ gram body weight) given each day for 14 days. Values expressed as % change ($\pm\text{SEM}$) from day 0 (n=8); (F) Histology of control H345 tumor (G) and MAG-1 treated NCI H345 tumor (H) Treatment of NCI H82 tumor in nu/nu mice with native MAG-1, MOPC21 isotype antibody control, or saline vehicle. Antibody (100 $\mu\text{g}/30$ gm body weight) was given daily for 14 days. Values expressed as % volume change for 14 days (n=8). (: end of treatment)

Figure 3: Effect of mAbner antibody and ^{99}Y trium-labelled Abner antibody on growth of recurrent SCLC tumors in female nu/nu mice. Tumors were derived from NCI H82 cells. (A) Tumor growth assessed by micrometer as LxBxH in response to treatments for 11 days with daily intraperitoneal

dosing of 3 mg/kg body weight of either mouse Abner, or of MOPC21 antibody control. Abner treatment decreased growth to approximately half that of controls ($p<0.01$);(B) Tumor growth assessed by micrometer as LxBxH in response to treatments with daily intraperitoneal dosing of 1.5 mCi/kg body weight of either mouse ^{99}Y trium-labelled Abner, or of ^{99}Y trium-labelled MOPC21 antibody control. For each dosing the total amount of antibody was adjusted to 3 mg/kg body weight. ^{99}Y trium-labelled Abner treatment decreased growth rate to approximately one-fourth that of controls by the end of the treatment period ($p<0.01$);(C) Daily body weight assessment during and beyond treatments with ^{99}Y trium-labelled antibody. The dip in body weight at day 10 was due to an unplanned failure to provide water on the previous day.

Figure 4. Effects of mAbner antibody combinations with cyclophosphamide or cisplatin on growth of recurrent SCLC tumors in female nu/nu mice. Six arm study on tumors derived from NCI H82 cells. (A) Treatments with saline daily (yellow), 3 mg/kg body weight Abner daily (green), 3x50 mg/kg body weight CAM over six days (blue), and Abner daily with CAM pretreatment (red); (B) Treatments with saline daily (yellow), 3 mg/kg body weight Abner daily (green), a single dose of 10 mg/kg body weight cisplatin (blue), and Abner with cisplatin pretreatment (red). All treatments delayed growth compared to saline controls ($p<0.02$), but only the Abner and CAM in combination seemed to synergize to prevent any tumor growth ($p<0.001$). (C) Body weights of animals for Study. Mean \pm SEM (Arrow indicates end of treatment)

Figure 5: Effects of hAbner antibody combination with cyclophosphamide on growth of recurrent SCLC tumors in female nu/nu mice. Treatments with saline daily (blue), 3 mg/kg body weight Abner daily (red), 3x50 mg/kg body weight CAM over six days (blue), and Abner daily with CAM pretreatment (yellow). hAbner and CAM in combination synergize to prevent any tumor growth ($p<0.01$)