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### Preclinical efficacy evaluation of MLN2704: A chemotherapeutic-monoconal antibody conjugate targeting prostate-specific membrane antigen (PSMA)

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Targeted delivery of chemotherapeutic agents coupled to tumor-specific monoclonal antibodies may enhance anti-tumor efficacy and reduce systemic toxicity. We are developing MLN2704, a novel therapeutic combining DM1, a microtubule-inhibiting maytansinoid, with MLN591, a de-immunized Tumor targeted Monoclonal Antibody Vehicle (T-MAV) specific for PSMA. *In vitro* analysis of MLN2704 demonstrated rapid intracellular uptake and potent nanomolar, antigen-dependent cytotoxicity against LNCaP cells. Furthermore, *in vitro* pharmacodynamic analysis of MLN2704 indicates that exposure time is a relatively more important variable than concentration for cytotoxic effect on PSMA-positive LNCaP cells. We examined MLN2704 efficacy in the CWR22 and 22Rv1 prostate cancer xenograft models. By probing sections of CWR22 xenografts for anti-human IgG, we found that intravenously delivered MLN2704 distributed into the tumor through the vasculature, achieved maximal staining intensity by 24 hours and maximal distribution by 72 hours and human IgG was still detectable up to 21 days post-injection. In the CWR22 model, MLN2704 exhibited dose- and schedule-dependent efficacy that was distinct from either its antibody or DM1 constituents when administered individually. When delivered on a q3dX5 schedule, treatment of CWR22 xenografts with MLN2704 delayed tumor growth to 1000mm<sup>3</sup> by 46.4 days relative to PBS controls. On this regimen, MLN2704 treatment produced no overt toxicity as judged by body weight loss or mortality. By comparison, molar equivalent doses of DM1 and the MLN591 antibody delivered on the q3dX5 schedule produced tumor delays of 9.5 and 1.9 days, respectively. After the initial course of treatment with MLN2704, CWR22 xenografts eventually resumed growth, but tumors averaging ~1000mm<sup>3</sup> were sensitive to a second course of MLN2704 treatment, exhibiting 75-80% of the growth delays obtained in the initial treatment. Additionally, MLN2704 was effective against the growth of 22Rv1 xenograft models; however, these models exhibited a different dose interval dependency compared to the CWR22 model. Response of the CWR22 xenograft to MLN2704 was correlated with serum PSA levels. In summary, MLN2704 demonstrated efficacy against the growth of PSMA-expressing prostate cancer cells and xenograft tumors. Length of exposure to MLN2704 was a critical determinant of efficacy both *in vitro* and *in vivo*.

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### CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of non-hodgkin's lymphoma

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Antibody-targeted chemotherapeutic strategy relies on antibody-mediated preferential delivery of the cytotoxic agent to the tumor and reduces the exposure of normal tissues to the same cytotoxic agent. This has now been clinically validated in the form of Mylotarg (CMA-676) which remains the only US FDA-approved antibody-targeted chemotherapeutic to date and is indicated for the treatment of relapsed CD33+ AML. It is an immunoconjugate in which a derivative of Calicheamicin gamma (Calich.) is conjugated to a humanized IgG4 anti-CD33 mAb. Calicheamicin gamma binds DNA in its minor groove and causes double strand breaks leading to cellular apoptosis. Employing the same conjugation technology used in Mylotarg, we have developed a new immunoconjugate, CMC-544, targeted for B lymphoid malignancies. CMC-544 is an immunoconjugate in which Calich. is linked to a humanized IgG4 anti-CD22 antibody (G544). CD22, expressed by most B lymphoid malignancies, is internalized efficiently when bound by the antibody and thus, is ideally suited for the intracellular delivery of cytotoxic agents. CMC-544 binds CD22 with high affinity (KD = 120 pM) and is cytotoxic *in vitro* to a series of human CD22+ B lymphoma lines (IC<sub>50</sub> = 10 - 500 pM) being 20 fold more potent than a control conjugate CMA-676. In RL and RAMOS B lymphoma xenograft models in nude mice, CMC-544, when administered ip q4dx3 post-tumor-staging at 20 mcg of conjugated calich./kg, causes strong inhibition of B lymphoma growth (Therapeutic index = 12). CMC-544 also causes complete regression of both small and large established tumors at doses that are less than half of the LD10 of

CMC-544, which is 256 mcg of conjugated calich./kg. CMC-544 produces cures (tumor-free for >100 days) at 120 mcg of conjugated calich./kg in both these models (Curative index of >2). In contrast, the CD33-targeted conjugate, CMA-676, used as a negative control, fails to impact growth of CD22+ CD33- B lymphomas. Neither unconjugated calich. nor unconjugated anti-CD22 mAb G544 has any effect on the growth of B lymphoma xenografts in these models. In addition, in a model of systemically disseminated B lymphoma, CMC-544, but not the control conjugate CMA-676, significantly prolongs survival (>80 days) of mice with systemically disseminated CD22+ CD33- B lymphoma. Taken together, this demonstration of preclinical anti-tumor efficacy of CMC-544 strongly supports its clinical evaluation as a targeted chemotherapeutic for B lymphomas.

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### Enhanced antitumor activity of anti-epidermal growth factor receptor monoclonal antibody cetuximab (IMC-C225) in combination with irinotecan (CPT-11), 5-FU, and leucovorin against human colorectal carcinoma xenografts

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Colorectal carcinomas (CRC) frequently express the epidermal growth factor receptor (EGFR) and this expression correlates with more aggressive disease and poor prognosis. Specific inhibitors of EGFR activation such as the anti-EGFR monoclonal antibody ERBITUX<sup>TM</sup> (cetuximab) have been shown to inhibit the *in vitro* and *in vivo* growth of human CRC tumor cells in pre-clinical models. Cetuximab is currently undergoing clinical testing in a number of EGFR+ human cancers including CRC. In this study, we have evaluated the activity of cetuximab combined with a three-drug chemotherapy regimen of CPT-11/5-FU/leucovorin (LV) in both subcutaneous xenograft and intra-hepatic models of CRC. Athymic mice with established (0.2 cm<sup>3</sup>) DLD-1 or HT-29 tumors were treated with cetuximab (1 mg; q3d), CPT-11/5-FU/LV (42 mg-, 125 mg-, 6.7 mg/kg; q7dX4) or the combination. Treatment with cetuximab alone resulted in a modest inhibition of DLD-1 (48%) and HT-29 (29%) tumors. Combination therapy with cetuximab and CPT-11/5-FU/LV resulted in the greatest anti-tumor effect inhibiting both DLD-1 and HT-29 tumor growth 76% and 80%, respectively. Enhanced or synergistic activity between cetuximab and the three-drug regimen was seen from day 26 onward. Histological examination of tumors treated with combination therapy showed increased tumor cell apoptosis and extensive tumor necrosis. Cetuximab monotherapy inhibited the growth of intrahepatic DLD-1 and HT-29 tumors by 26% and 13%, respectively. The three-drug regimen of CPT-11/5-FU/LV resulted in >86% inhibition whereas combining cetuximab with the three-drug regimen resulted in >93% inhibition of intrahepatic tumor growth. Moreover, the majority of the animals given combination therapy showed no evidence of disease. No hepatic or metastatic masses were found in 56% of the DLD-1 or 67% of the HT-29 implanted livers. These results are consistent with previous reports showing the potentiation of anti-tumor activity when combining cetuximab with chemotherapeutic drugs. The present study suggests that combinations of cetuximab and CPT-11/5-FU/LV may be an effective therapy in the treatment of advanced colorectal carcinoma.

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### A calicheamicin conjugate that targets LewisY selectively destroys LewisY-positive human carcinoma cells and xenografts

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The low therapeutic index of most anticancer drugs prohibits administration of sufficient amounts to obtain a curative effect. Antibody-targeted chemotherapy can provide a solution to this problem. This strategy uses a cytotoxic agent bound to an antibody that recognizes a tumor-associated antigen. The antibody can deliver the cytotoxic agent specifically to the tumor and reduce systemic toxicity. We asked whether a conjugate of calicheamicin (CM) and an antibody that recognizes the LewisY (LeY) antigen would eliminate carcinomas that express this oligosaccharide antigen. CM is a potent cytotoxic antibiotic that causes double strand breaks of DNA. It has proven therapeutic value against acute myeloid leukemia when conjugated to an anti-CD33 antibody (Mylotarg<sup>TM</sup>). Expression of CD33 is limited to myeloid malignancies. In contrast, LeY is highly expressed on

carcinomas of colon, breast, lung, ovary and prostate while its low expression on normal tissue is restricted to the gastric mucosa, small intestine and pancreas. A calicheamicin conjugate that targets LeY could therefore provide a treatment option for a variety of cancers. The humanized IgG1 antibody, Hu3S193, that recognizes LeY was conjugated to a CM analog. FACS analysis showed that this conjugate (Hu3S193-CM) bound LeY-positive cells with similar selectivity and avidity as Hu3S193 indicating that conjugation did not influence these parameters. When tested on monolayer cultures of various human LeY-positive carcinoma cells, Hu3S193-CM was consistently more cytotoxic than a control conjugate. This difference in efficacy was not noted on LeY-negative cells. The efficacy of Hu3S193-CM depended qualitatively on the expression of LeY and on the sensitivity to CM. Quantitatively, the efficacy was neither directly proportional to the CM-sensitivity of tumor cells nor to their amount of LeY. Hence, other factors such as the nature of the surface proteins that carry LeY can co-determine the sensitivity to Hu3S193-CM. *In vivo*, Hu3S193-CM inhibited tumor growth of xenografted human gastric (N87), colon (LOVO) and prostate carcinomas (LNCaP) more effectively than control conjugates. When used against the N87, Hu3S193-CM (160 ug/kg, q4dx3) was curative. Our data show that Hu3S193-CM can specifically eliminate tumors that express LeY and therefore encourage development of this conjugate as an option for the treatment of carcinomas.

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### Inhibition of tumor metastasis by ING-1(heMab), a human-engineered(TM) monoclonal antibody targeting the epithelial cell adhesion molecule

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Tumor metastasis is one of the hallmarks of malignancy and remains the main cause of death in cancer patients. Despite this, very few treatment strategies are effective in preventing metastasis from primary tumors. Therapeutic monoclonal antibodies, either alone or conjugated with cytotoxic agents, have shown promise in inhibiting tumor invasion and metastasis. Here we demonstrate that a Human-Engineered(TM) monoclonal antibody, ING-1(heMab), which recognizes a 40-KDa glycoprotein, Epithelial Cell Adhesion Molecule (Ep-CAM), significantly inhibits tumor metastasis in a murine model of human cancer. To investigate the efficacy of ING-1(heMab), we established an *in vivo* adenocarcinoma-lung metastasis model, in which athymic nude mice received an intravenous (IV) injection of human HT-29 colon adenocarcinoma cells via their tail veins. All mice were pre-treated with human interleukin-1beta to augment tumor metastasis. In all studies, mice were observed for 8 weeks to allow for lung metastasis development. Mice (10 per group) received IV dosing of 1 mg/kg ING-1(heMab) twice weekly for 3 weeks starting on day 2 or day 5. A negative control group received human IgG with the same dosing schedule starting on day 2. In the positive control group, 100 mg/kg 5-FU/leucovorin was injected intraperitoneally once weekly for 4 weeks. At the end of the study, the mice were sacrificed for gross necropsy and tissue collection. Three different methods were used for data analysis. First, visible tumor nodules (>3 mm) were counted throughout animal body cavities. Second, tumor nodules on lung surfaces were counted under a dissecting microscope. Last, micrometastatic foci in sections of lung tissues were counted after detection with hematoxylin/eosin staining. ING-1(heMab) treatment starting on day 2 significantly reduced the number of tumor nodules visible during necropsy ( $P < 0.01$ , vs IgG group). When nodules on lung surfaces were examined, a significant reduction was again observed in the ING-1(heMab)/day 2-treated group ( $P < 0.005$ , vs IgG group). Lastly, ING-1(heMab) treatment starting on day 2 resulted in 91% reduction of micrometastases in lung tissues ( $P < 0.005$ , vs IgG group). Delaying ING-1 treatment until day 5 still led to 54% reduction in micrometastases ( $P < 0.005$ , vs IgG group). The above results have been confirmed in another independent study. In conclusion, ING-1(heMab) effectively reduced tumor metastasis in a murine model of human cancer. While ING-1(heMab) was particularly effective when administered early in the study on day 2, day 5 treatment was also effective in reducing micrometastases. These data suggest that ING-1(heMab) may show promise in treating metastatic diseases in humans.

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### Development of monoclonal antibodies targeting the uPA system for diagnosis and therapy

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The urokinase plasminogen activator system, comprised of the serine protease urokinase (uPA), its cell surface receptor uPAR and plasminogen activator inhibitor-1 (PAI-1), has been implicated in the neo-vascularization, invasion and metastasis of many solid tumors. uPAR plays an essential role in the regulated degradation and remodeling of the extracellular matrix by tumor cells and angiogenic endothelial cells. uPA-uPAR dependent cascades also result in the activation of proMMP-9 and the activation and release of growth factors and angiogenic factors including HGF, VEGF and TGFbeta. The important role of uPA-uPAR in tumor growth and its abundant expression within tumor, but not normal tissue, makes it an attractive diagnostic and therapeutic target. We previously described the production and characterization of a panel of monoclonal antibodies against the amino terminal fragment (ATF) of uPA. We have tested the ability of two of these antibodies, ATN-291 and ATN-292, to inhibit tumor growth *in vivo*. Briefly, 7x105 MDA MB 231 cells were injected into the mammary fat pad of Balb/c nu/nu mice. Tumors were staged to 35 mm3 before the start of treatment with anti-ATF or isotype matched control antibodies (10 mg/kg, 3 x week, IP). Treatment with ATN-291 or ATN-292 significantly inhibited tumor growth in this model. In addition, we have generated a panel of monoclonal antibodies against domains 2 and 3 (D2D3) of uPAR conjugated to KLH. Initial characterization of the antibodies indicates that they bind suPAR with affinities of 3-80 nM in direct binding assays and recognize suPAR, D2D3 but not domain 1 (D1) of uPAR, as determined by western blot. In functional assays a D2D3 antibody, as well as a polyclonal antibody against D2D3, effectively inhibited adhesion of integrin alpha5beta1 expressing CHO cells to suPAR. Furthermore, the antibody also inhibited uPA induced cell migration. Preliminary experiments indicate that the antibodies can be efficiently labeled with I-125 with no significant change in the binding characteristics of the antibodies. We will present results of internalization studies using these labeled antibodies and assess their potential to deliver cytotoxic agents to tumor cells.

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### Characterization of the intracellular mechanisms behind the cytotoxic effect of the immunotoxin, 425.3-PE in two breast cancer cell lines

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The aim of the study was to investigate the mechanisms behind immunotoxin (425.3-PE)-induced cell death. In addition, the combinational effect of 425.3-PE and cycloheximide (CHX) in cell culture was evaluated. Our immunotoxin consists of antibody 425.3, recognizing the EGF receptor, chemically conjugated to the bacterial toxin *Pseudomonas* exotoxin (PE). The two different breast cancer cell lines used, MA11 and MT1, respond differently to 425.3-PE treatment in several aspects, thereby giving us opportunity to evaluate mechanistic events in 425.3-PE induced cell death. The immunotoxin treatment of these two cell lines do not only inhibit protein synthesis (PS) ( $IC_{50} = 0.5-2$  ng/ml) by ADP-ribosylation of EF-2, it also induces caspase activation, cleavage of poly(ADP)-ribose polymerase (PARP) and DNA fragmentation, characteristic events of apoptosis. Western blot demonstrated immunotoxin-induced PARP cleavage, more rapidly induced in MA11 cells (maximal 5h) contra MT1 cells (maximal 10h). The broad-spectrum caspase inhibitor (z-VADfmk) totally inhibited PARP cleavage and improved the cell viability (approx. 3-fold) without altering inhibition of PS in both MA11 and MT1 cells treated with 425.3-PE. The specific caspase-9 inhibitor (z-LEDHfmk) had no effect on 425.3-PE induced cell death in MT1 cells. However, in MA11 cells this specific inhibitor made them 1.6 times more resistant to 425.3-PE, suggesting activation of caspase-3 by caspase-9, mediated by mitochondria alterations. By combination of two PS inhibitors, CHX and 425.3-PE, the cell viability decreased in both MA11 and MT1 cells to a level corresponding to that of a 10 times higher concentration of 425.3-PE alone. The CHX concentration used gave by itself minimal effects on PS inhibition and cell viability. For MA11 cells the combination of CHX+425.3-PE increased the PS inhibition effect further, whereas MT1 cells were insensitive to the combination. Notably, 425.3-PE also decreased the p53 protein level in MA11 cells to 30% of control. The increased activity of 425.3-PE when combined with CHX will be further examined. In conclusion, the apoptotic program which 425.3-PE starts in MA11 and in MT1