Universitat Autònoma de Barcelona

Anticancer Targeted Agent Combination

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ABSTRACT

Cancer is a highly frequent disease associated to high mortality. Drug development in Oncology has shown to be inefficient, having one of the lowest success rate of drugs entering in phase I trials that finally achieves marketed authorization. The main reason for this high failure rate is lack of efficacy. Different strategies have been adopted to improved anti-cancer drug development with the aim of improving patient care. This strategies include the combinatorial use of agents, biomarker co-development, and optimization of clinical trial design with the use of pharmacokinetic-pharmacodynamic modeling.

This thesis is presented as compendium of work integrating two projects; the first project preclinically evaluates the combination of two PI3K-mTOR inhibitors and chemotherapy or the pan-HER inhibitor dacomitinib in patient derived xenografts. The second project evaluates de monoclonal antibody anti-CCL2 carlumab in patient derived xenografts.

Project 1: Three PDXs were selected for their lack of PTEN expression by immunohistochemistry: a triple-negative breast cancer (TNBC), a KRAS G12R low-grade serous ovarian cancer (LGSOC), and KRAS G12C and TP53 R181P lung adenocarcinoma (LADC). Two dual PI3K-mTOR inhibitors were evaluated—PF-04691502 and PF-05212384—in combination with cisplatin, paclitaxel, or dacomitinib.

The addition of PI3K-mTOR inhibitors to cisplatin or paclitaxel increased the activity of chemotherapy in the TNBC and LGSOC models; whereas no added activity was observed in the LADC model. Pharmacodynamic modulation of pS6 and pAKT was observed in the group treated with PI3K-mTOR inhibitor.

Our research suggests that the addition of a PI3K-mTOR inhibitor may enhance tumor growth inhibition when compared to chemotherapy alone in PTEN-deficient PDXs. However, this benefit was absent in the KRAS and TP53 mutant LADC model. The role of PTEN deficiency in the antitumor activity of these combinations should be further investigated in the clinic.

Project 2 is a first-in-human phase 1b study of carlumab with one of four chemotherapy regimens (docetaxel, gemcitabine, paclitaxel+carboplatin, and pegylated liposomal doxorubicin HCl [PLD]). Fifty-three patients with advanced solid tumors for which ≥1 of these regimens was considered standard of care or for whom no other treatment options existed

participated in the study: docetaxel (n=15), gemcitabine (n=12), paclitaxel or carboplatin (n=12), or PLD (n=14).

Dose-limiting toxicities included one grade 4 febrile neutropenia (docetaxel arm) and one grade 3 neutropenia (gemcitabine arm). The most common drug-related grade ≥3 adverse events were docetaxel arm—neutropenia (6/15) and febrile neutropenia (4/15); gemcitabine arm—neutropenia (2/12); paclitaxel+carboplatin arm—neutropenia, thrombocytopenia (4/12 each), and anemia (2/12); and PLD arm—anemia (3/14) and stomatitis (2/14). One partial response and 18 (38 %) stable disease responses were observed.

Combination treatment with carlumab had no clinically relevant pharmacokinetic effect on any of the chemotherapeutic agents tested. Free CCL2 declined immediately post-treatment with carlumab but increased with further chemotherapy administrations in all arms, suggesting that carlumab could sequester CCL2 for only a short time. Neither antibodies against carlumab nor consistent changes in circulating tumor cells (CTCs) or circulating endothelial cells (CECs) enumeration were observed. Three of 19 evaluable patients showed a 30 % decrease from baseline urinary cross-linked N-telopeptide of type I collagen (uNTx).

Carlumab could be safely administered at 10 or 15 mg/kg in combination with standard-of-care chemotherapy and was well-tolerated, although no long-term suppression of serumCCL2 or significant tumor responses were observed.

RESUMEN

Las toxicidades limitantes de dosis fuero una neutropenia febril grado 4 (en el brazo de docetaxel) y una neutropenia grado 3 en el brazo de gemcitabina. La combinación de carlumab no tuvo un impacto

El cáncer es una enfermedad altamente frecuente y con alta mortalidad. El desarrollo de fármacos contra el cáncer se ha caracterizado por su ineficiencia, con una de las tasas de aprobación de fármacos más baja entre las diferentes especialidades médicas. El principal motivo de esta baja tasa de éxito es la falta de eficacia de los nuevos fármacos que entran al desarrollo clínico. Se han planteado diferentes estrategias para mejorar la eficiencia del desarrollo de fármacos, incluyendo la combinación de fármacos antitumorales, el desarrollo en paralelo de biomarcadores y la optimización del diseño de los ensayos clínicos usando modelización basada en farmacocinética y farmacodinamia

Esta tesis es un compendio de dos artículos que evalúan estrategias para optimizar el desarrollo de fármacos mediante la combinación de agentes antitumorales. El primer proyecto es la evaluación preclínica en xenoinjertos derivados de pacientes (PDX) la combinación de inhibidores de PI3K-mTOR con diferentes agentes antitumorales y el segundo es el ensayo clínico fase I evaluando carlumab, un anticuerpo anti CCL2, en combinación con diferentes quimioterapias en pacientes con tumores sólidos avanzados.

Proyecto 1: se seleccionaron tres modelos de PDX con deficiencia en PTEN: un PDX de cáncer de mama triple negativo (TNBC), otro de carcinoma de ovario de bajo grado KRAS G12R mutado y otro de adenocarcinoma de pulmón con mutaciones en KRAS G12C y TP53 R181P. En estos modelos se evaluaron dos inhibidores de PI3K-mTOR—PF-04691502 and PF-05212384— en combinación con cisplatino, paclitaxel o dacomitinib.

La adición de los inhibidores de PI3K-mTOR a cisplatino o paclitaxel aumentó la actividad de la quimioterapia en los modelos de TNBC y LGSOC; sin embargo, no se objetivó este efecto en modelo de adenocarcinoma de pulmón con mutación de KRAS y TP53. Se objetivó modulación farmacodinámica de pAKT y pS6 en los grupos tratados con inhibidores de PI3K-mTOR.

Nuestra investigación sugiere que añadir un inhibidor de PI3K-mTOR puede aumentar el efecto inhibitorio sobre el crecimiento de la quimioterapia en modelos PDX con deficiencia en PTEN. Sin embargo, este beneficio no se observó en el modelo de adenocarcinoma

KRAS y TP53 mutado. En el futuro se deberá ahondar en el papel de la pérdida de PTEN en la actividad de estas combinaciones.

Proyecto 2: se trata de un ensayo clínico fase Ib evaluando carlumab, un anticuerpo monoclonal contra CCL-2, en combinación con cuatro regímenes de quimioterapia (docetaxel, gemcitabina, carboplatino + paclitaxel y doxorrubicina liposomal pegilda (PLD). En este estudio participaron 53 pacientes en los que o bien los agentes quimioterápicos eran parte del tratamiento convencional o no tenían otras opciones de tratamiento convencional: docetaxel (n=15), gemcitabina (n=12), carboplatino + paclitaxel (n=12) y PLD (n=14).

Las toxicidades limitantes de dosis incluyeron una neutropenia febril grado 4 (en el brazo de docetaxel) y una neutropenia grado 3 (en el brazo de gemcitabina). De acuerdo a los brazos de tratamiento, las toxicidades grado 3 o mayores más frecuentes fueron: neutropenia (6/15) y neutropenia febril (4/15) en el brazo de docetaxel, neutropenia (2/12) en el brazo de gemcitabina, neutropenia (4/12), trombocitopenia (4/12) y anemia (2/12) en el brazo de carboplatino-paclitaxel y anemia (3/14) y mucositis (2/14) en el brazo de PLD. Se objetivo una respuesta parcial y 18 estabilizaciones de la enfermedad (38%).

La adición de carlumab no tuvo cambios relevantes en el perfil farmacocinético de ninguna de las quimioterapias evaluadas. Los niveles de CCL2 libres descendieron inmediatamente tras el tratamiento con carlumab, pero aumentaron con las administraciones posteriores, sugiriendo que carlumab secuestraba CCL2 de manera temporal. No se objetivaron anticuerpos anti-droga que justificasen dicho efecto. No se objetivaron cambios en las células tumorales circulantes ni en las células circulantes endoteliales. En 3 de 19 pacientes evaluables se objetivó una reducción del 30% en los niveles de N-telopeptido de colágeno tipo I en orina (uNTx).

Carlumab es seguro administrado a dosis de 10 o 15 mg/kg en combinación con quimioterapia convencional y tiene buena tolerancia. Sin embargo, no se alcanza una inhibición sostenida de CCL2, ni se han objetivado un número de respuestas significativas.

TABLE OF CONTENTS

1. CONTENIDO

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DEDICATION

Para Emma

Para mis padres, Javier Manuel y María Antonia

Para mis hermanos, Manuel y Natalia

Para mi marido, Pedro

INTRODUCTION

1. INTRODUCTION

Cancer is a highly frequent disease with an estimated incidence of 14.1 million newly diagnosed cases worldwide (308.7 cases/100000 inhabitants/year) and the second leading cause of mortality after cardiovascular diseases causing 138 deaths per 100.000 inhabitants worldwide (1). Cancer has an enormous socioeconomic impact imposing an enormous burden of disability and morbidity for patients and their families and also an important cost to the Health System. According to a study evaluating the global burden of cancer in 2015, cancer caused 208.3 million disability-adjusted life years (DALYs) mainly due to years of life lost (YLLs) (2). A population-based cost analysis conducted in Europe revealed that cancer cost the European Union €126,000 million. Of those, €51,000 million were associated to Health Care and one of the main expenses was drug therapy (€13,000 million) (3). This expenditure on drugs is caused by both the high incidence of the disease and the elevated cost of anti-cancer treatments. The high cost of anti-cancer treatments reflects, at least in part, the high cost of drug development in Oncology. One of the causes associated to this high cost associated to anti-cancer Drug Development is the high attrition during the drug development process. Several studies evaluating the success rate of drug development during the 90s and early 2000s revealed that only 5%-18% of the anti-cancer drugs that entered phase I clinical trials was finally marketed (4, 5). This rate was one of the lowest across different specialties, highlighting the difficulties associated to drug development in Oncology.

The main pitfall preventing novel drugs in Oncology to be marketed is mainly their lack of efficacy. The improvement of drug development in Oncology requires changes at different levels including a better understanding of the tumor biology, better preclinical evaluation, better biomarker identification and development, and novel study designs. In the last decades there has been a better understanding of tumor biology including the description of different mechanisms of tumorigenesis (6, 7) and the genomic characterization of certain tumor types through international consortia (8). The efforts molecularly characterizing tumors have revealed that a minority of cancers are driven by a single genomic event, such as EGFR mutation or ALK translocation, leading to oncogene addiction which ultimately leads to the ability of inducing tumor regression by targeting that single mutant or translocated protein. However, in the vast majority of tumors there is not such oncogene addiction and tumor growth is led by the interaction of oncogenic pathways, which are activated by different genetic or epigenetic alterations, and also by the interaction between

tumor and its microenvironment. The better understanding of tumor biology has already translated into better results developing certain anti-cancer agents; the proportion of tyrosine kinase inhibitors entering phase I that got approval by the Food and Drug Administration was 47%, while in the same period, the approval rate of anti-cancer drugs in general has been 19% (5). However, further improvements are needed, as in most of cases the single agent activity of cancer drugs is limited and many times short-lived. Targeted agent combination represents an attractive strategy to circumvent resistance pathways or to simultaneously inhibit several oncogenic pathways. Likewise, some of the currently available targeted agents block pathways involved in chemoresistance, for that reason, the combination of these targeted agents with conventional chemotherapy might be a strategy to increase treatment efficacy.

Empiric evaluation of an extensive number of potential drug combinations in clinical trials would be neither scientifically sound nor feasible. For such reasons, rational preclinical research should be performed to select the most active combinations for further clinical development. Preclinical research may provide a better understanding of the mechanisms of action of the targeted agents and may identify biomarkers that can help confirming target modulation in subsequent clinical trials. Evaluating the antitumor effect of different drug combinations in preclinical models is essential to identify the most active combinations and to select the most appropriate schedule of administration. In addition, preclinical research is a key step to identify the molecular profiles or other biomarkers that could potentially predict the patient populations that most likely would benefit from the treatment. The selection of appropriate preclinical models is key for succeeding on selecting the most appropriate combinations and biomarkers to further test in early clinical trials. While the exclusive use of cell lines as the only preclinical model is discouraged due to concerns on their poorly reflection on patient tumors and their lack of stroma. Novel preclinical models, such as patient-derived xenografts (PDXs) or genetically-engineered mice models (GEMMs), offer an alternative to circumvent such problems. PDXs represent promising pre-clinical models as they seem to recapitulate some of the molecular characteristics of the primary tumor (9) as well as clinical tumor response (10-12). These models have the limitation of being generated on immunocompromised mice, which might limit their use to evaluate immunotherapeutic agents. On the contrary, GEMMs are generated in immunocompetent mice by inducing certain genomic alterations which finally leads to the generation of a tumor (13). The number of genes that can be induced or suppressed in GEMMs is limited and might not completely reflect the complexity of genomic alterations that patient tumors have,

but they have proved to be relevant models to understand tumor biology, to test anticancer therapies in models of oncogene addition and to understand mechanism of resistance to targeted therapies. In addition, the fact of being immunocompetent models make them ideal models to understand the interactions between tumor and its microenvironment, especially the interactions with the immune system.

In order to rationally develop anti-cancer agent combinations, scientifically driven preclinical evaluation needs to be coupled with efficient testing in clinical trials. The decisions to continue further development of anti-cancer drugs as single agents or in combinations have moved from late phases of development to earlier phases of development. Phase I clinical trials are no longer studies designed to define a recommended phase 2 dose (RP2D) based only on toxicity. Phase I have become the setting to evaluate target inhibition, safety, preliminary efficacy, and to further evaluate potential predictive biomarkers, if applicable, to enable patient selection. The drug development schema in which drugs should be subsequently tested into compartmentalized three-phase clinical trials has currently transitioned into seamless phase I/II clinical trials and phase I studies with large expansion cohorts in which to early evaluate biomarkers selection strategies and efficacy signals. Novel study designs, such as zone based design or Bayesian models, are helping the dose escalation process in phase I combination studies (14). Biomarker co-development in early phases of development would ensure target inhibition and the identification of a biological effective dose which needs to be at least reached by the recommended phase 2 dose in order to further develop the anticancer-drug (15).

This doctoral thesis is a compendium of work evaluating the combination of targeted agents with chemotherapy in the preclinical setting (Project 1) and in the early clinical setting (Project 2). In project 1 the PI3K-mTOR inhibitors PF-04691502 and PF-05212384 have been evaluated in combination with three different agents: Dacomitinib (PF-00299804)—a pan-HER inhibitor, cisplatin, and paclitaxel. These combinations have been evaluated in PDXs, aiming a better reflection of the molecular characteristics and behavior of the advanced tumors from which they were derived. The three PDXs selected in this project have been selected among a collection of genomically characterized PDXs by the presence of a common alteration in the PI3K pathway, namely PTEN deficiency. Project 2 is a phase I clinical trial investigating the combination of Carlumab—a monoclonal antibody against CCL-2—with four different chemotherapeutic agents or regimens (docetaxel, gemcitabine, pegylated liposomal doxorubicin and carboplatin-paclitaxel) in patients with advanced solid

tumors. In addition to evaluate safety of the combination, parallel pharmacokinetic (PK) and pharmacodynamics (PD) analysis has been integrated in order to confirm target modulation and select the optimal phase II recommended dose based on PK/PD modeling.

BACKGROUND

2. BACKGROUND

Cancer cells acquire different capabilities in order to become cancerous. These capabilities elegantly described by Hanahan and Weinberg as the hallmarks of cancer include sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, promoting tumor-induced inflammation, activating invasion and metastasis, inducing angiogenesis, suffering genomic instability and mutation, resisting cell death and deregulating cellular energetics (6, 7). In the last two decades most of the targeted agents have been directed to block proliferative signaling and angiogenesis. Recently, immunotherapy has become an additional main area on drug development leading to the approval of cancer therapies based on unprecedented improvement in overall survival in tumor types such as melanoma or non-small cell lung cancer.

Among the hallmarks of cancer, sustained growth signal autonomy was one of the firstly described oncogenic mechanisms. Tumors take advantage of the mechanisms by which proliferative signals are transmitted into the normal cells by transmembrane receptors in order to become growth-signal self-sufficient. This self-sufficiency can be acquired at three different levels: altering the extracellular growth signals, altering the transmembrane receptors or interfering in the intracellular transduction of the signal. Cancer cells can induce the production of the growth factors from the surrounding cells in the microenvironment or by the tumor cells themselves by an autocrine mechanism. Transmembrane receptors can be aberrantly activated in cancer cells by receptor overexpression or by alterations in the receptors, such as activating mutations, which constitutively activate the transmembrane receptors independently of the presence of ligand. Tumors can also become growth signal autonomous by activating intracellular pathways downstream the transmembrane. The mitogen-activated- p- kinase (MAPK) pathway and the Phosphatidylinositol 3-kinases (PI3K) pathways are the most relevant downstream signaling pathways activated in cancer.

The evasion from the immune system surveillance has been recently added as an emerging hallmark of cancer. The role of the immune system on recognizing and eliminating tumors at their initial phases was initially suggested based on population studies, in which increased incidence of cancer was observed in patients with acquired immunodeficiency such as transplant recipients or patients affected by the human immunodeficiency virus. Tumors have shown to be able to evade the response of immune system at two different levels: avoiding detection by the immune system and limiting the anti-tumor effect of the immune system. Tumors become less detectable by the immune system by altering the antigen

processing system; once detected by the immune system, tumors take advantage of physiological mechanisms developed in the healthy organisms to avoid autoimmunity and to protect normal tissues from the damage induced by the immune system if overactivated. These physiological mechanisms limit the capacity of the immune system to eradicate the tumors, by directly inhibiting cytotoxic lymphocytes ($CD8^+$ lymphocytes) through inhibitory immune-checkpoints, such as PD1-PDL1, or by enhancing inhibitory regulatory cells such as Tregs or M2-like macrophages. Hence, the balance between anti-tumor and tumor-promoting immune-cells is broken, leading to cancer immune-evasion and progression.

The current thesis is focused on evaluating anti-cancer agents targeting these two hallmarks of cancer and combining these agents with conventional treatment, such as chemotherapy. Two different PI3K-mTOR inhibitors have been evaluated targeting the PI3K-AKT-mTOR pathway, a key pathway involved on sustained growth signal autonomy; while carlumab, a monoclonal antibody against the chemokine CCL2, has been tested as a strategy to revert tumor immune evasion. The following subsections will summarize relevant aspects on the mechanism of action of each of these anticancer drugs. Additional subsections will provide strategies to improve the success of anticancer drug combinations development.

PI3K Pathway as a Paradigm of Autonomous Self Growth Pathway

Phosphatidylinositol 3-kinases (PI3Ks) represent a family of lipid kinases that plays a key role in signal transduction, cell metabolism and survival (16, 17). The PI3K family is divided into three classes, I, II and III, based on their substrate specificity and structure. Among them, class I PI3K seems to be the most relevant in cancer. Class I PI3K has a catalytic subunit (p110) and a regulatory subunit (p85) that stabilizes p110 and inactivates its kinase activity at basal state. Physiologically, PI3K transduces signals received from activated tyrosine kinase receptors (RTK), G protein-coupled receptors (GPCR) or from activated RAS. Upon receipt of such signals, the p85 regulatory subunit interacts with the phosphorylated tyrosine residues of activated RTKs. This engagement then causes release of the p85-mediated inhibition of p110, such that p110 can interact with the lipid membranes to phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). This reaction triggers a signaling cascade through the activation of AKT and its downstream effectors. The amount of PIP3 generated and resultant PI3K

pathway activation are tightly regulated by the tumor suppressor protein, phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PTEN can inactivate the PI3K pathway by converting PIP3 into PIP2 (Figure 1). The PI3K pathway can be activated not only via RTKs, but also by RAS and GPCR. RAS can activate the PI3K pathway by its direct interaction with p110α, p110γ, and p110δ subunits, while GPCRs can interact with p110β and p110γ subunits (17). The PI3K pathway is commonly deregulated in cancer, with the most common events being mutation or increased gene copy numbers of PIK3CA or other PI3K isoforms, loss of expression of the pathway suppressors (for example, PTEN), or hyperactivation of RTKs through receptor overexpression or activating mutations (Table 1).

Table 1. Common alterations in phosphatidylinositol 3-kinase p110α isoform gene (PIK3CA), PIK3CB and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) in cancer

Alteration	Occurrence (%)	References
PIK3CA mutations:		
Breast	26% (1,559/6,110)	(18)
Endometrium	24% (282/1,194)	(18)
Penis	29% (8/28)	(18, 19)
Urinary tract	20% (189/942)	(18)
Large intestine	12% (779/6,710)	(18)
Stomach	12% (96/824)	(18)
Ovary	10% (163/1,590)	(18)
Cervix	10% (25/256)	(18)
PIK3CA amplifications:		
Gastric	67% to 36%	(20, 21)
Papillary thyroid cancer	53% (265/499)	(22)
Head and neck	55% to 37%	(23, 24)
Non-small cell lung cancer	31%	(25)
Squamous cell carcinoma	59% (31/52)	(26)
Cervical	70% to 44%	(27, 28)
Ovarian	35% (54/152)	(29)
Prostate	28% (9/32)	(30)
Endometrial	12% to 15%	(31, 32)
Breast	8% (8/92)	(33)

Triple negative	31%	(34)
Chronic lymphocitic leukemia	5%	(35)
PIK3CB amplification:		
Breast	5%	(36)
Non-small cell lung cancer squamous cell carcinoma	56%	(26)
PTEN loss of heterozygosity:		
Glioblastoma	59%	(37)
Prostate	15% to 70%	(38-41)
Breast	11% to 38%	(42, 43)
Melanoma	33% (7/21)	(44)
Gastric	47% (14/30)	(21)
Uveal melanoma	76% to 39%	(45)
PTEN mutation:		
Endometrium	37% (690/1,860)	(46)
Vulva	62% (5/8)	(47)
Central nervous system	24% (491/2,055)	(48)
Prostate	14% (92/658)	(46)
Melanoma	16% (104/652)	(49)
Uveal melanoma	11% (4/35)	(45)

The PI3Ks are grouped into three classes (I, II and III) based on their structural characteristics and substrate specificity (17). Class I PI3Ks are further divided into class IA enzymes, which include p110α, p110β and p110δ, while p110γ constitutes class IB (17) (Figure 1). In mammals, p110α and p110β are ubiquitous while p110γ and p110δ are expressed preferentially in leukocytes (50, 51). This distribution justifies the most relevant role of p110γ and p110δ in inflammatory diseases and the implication of p110δ in hematological malignancies. Class II PI3Ks seem to be implicated in exocytosis, cell migration, smooth muscle cell contraction, glucose metabolism and apoptosis (52). Class III PI3Ks regulate cellular trafficking of vesicles and proteins (17). Class I PI3Ks are involved in cell growth, survival and metabolism, therefore represent one of the most sought after targets in cancer therapeutics. In addition to its effects on cell growth, proliferation and survival, class IA PI3K regulates glucose metabolism through insulin signaling (53-55). It is commonly deregulated in cancer through mutations or amplifications of the PIK3CA gene or

through alterations in the function of upstream tumor suppressors such as PTEN (Table 1). About 80% of the mutations of the PIK3CA gene are clustered in three hotspots in the p110α gene that encodes the catalytic subunit: two in the helical domain (E542K and E545K) and one in the kinase domain (H1047R) (56). PIK3CA mutations are oncogenic per se, as they can induce the generation of tumors in several preclinical models without other molecular aberrations (57-59). The signaling of the PI3Kβ isoform is mediated via GPCR (60-66) while the PI3Kα isoform preferentially mediates via RTK, however, platelet-derived growth factor receptor is able to sustain its signaling through the PI3Kβ isoform in the absence of the PI3Kα isoform (54).

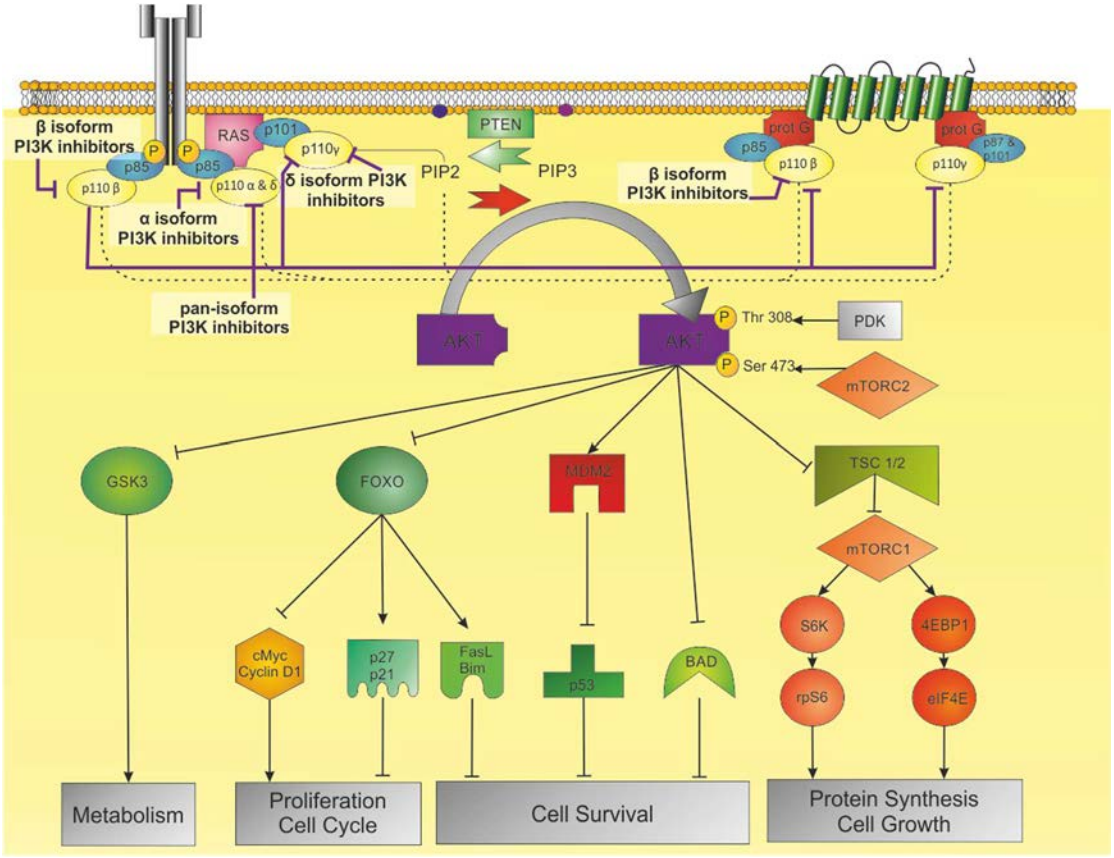

Figure 1. PI3K pathway: Class I PI3K isoforms and downstream pathways.

The PI3Kβ isoform is oncogenic when deregulated (61). *PIK3CB* occur rarely. The most common event that leads to PI3Kβ-isoform signaling deregulation is PTEN deficiency, although *PIK3CB* amplification has been described in breast cancer (36). PTEN is a lipid phosphatase that dephosphorylates the 3-phosphoinositide products of PI3K (67). PTEN deficiency is a frequent event in cancer (68) (Table 1), which can occur through several mechanisms including *PTEN* mutation, *PTEN* deletion, epigenetic changes (69), (70-73),

miRNA-mediated regulation (74-76) or post-translational modifications (77, 78). In preclinical models, it has been demonstrated that PTEN-deficient tumors depend on the PI3Kβ isoform for pathway activation, growth and survival (60, 79). The preclinical activity of several PI3Kβ-specific inhibitors in PTEN-deficient cell lines and xenograft models has been communicated (80-82). Additionally, some preclinical work suggests that the inhibition of both PI3Kβ and PI3Kα is necessary to avoid the appearance of resistance through PI3Kα.

In contrast to the ubiquitously expressed p110α and p110β isoforms, p110δ is mainly expressed in leukocytes (50, 83). Its overexpression has been observed in a wide range of lymphoproliferative disorders including chronic lymphocytic leukemia (CLL) (84), multiple myeloma (85), diffuse large B-cell lymphoma (86), B-cell acute lymphoblastic leukemia (86), follicular lymphoma (86), mantle cell lymphoma (87, 88), and Hodgkin's lymphoma (87). Both PI3Kδ-specific inhibitors and pan-isoform PI3K inhibitors have shown anticancer activity in hematological malignancies.

One of the major challenges in the clinical development of PI3K inhibitors is to identify the appropriate patient populations most likely to benefit from the treatment. In the current era where many drug targets are entering clinical evaluation and even more compounds are being developed to interrogate such targets, a rational approach is to intensify biomarker research in the preclinical setting and then incorporate them in early phase clinical trials. Both pharmacodynamic markers to prove biological effect and predictive biomarkers to identify sensitive or resistant populations are of interest, and their exploration in valid preclinical models would inform clinical development.

In preclinical models, cell lines harboring PIK3CA mutation, or amplification of PIK3CA or ERBB2 have shown sensitivity to different PI3K inhibitors, including pan-isoform PI3K inhibitors (89-91) or PI3Kα-specific inhibitors (92, 93). However, the role of PTEN loss as a predictor of responsiveness to PI3K inhibitors is less clear (94-97). In the clinical setting, the retrospective analysis of 217 patients referred to the MD Anderson Cancer Center revealed that those with PIK3CA mutant tumors treated with PI3K-AKT-mTOR axis inhibitors demonstrated a higher objective response rate than patients without such mutations (98, 99). However, the majority of these patients received combination therapies that included an mTOR inhibitor, and not a PI3K inhibitor. In addition, there are inherent biases to retrospective analyses, and these results should be considered exploratory and interpreted cautiously.

Phase I clinical trials with PI3K inhibitors have been initially developed in unselected patient populations. As preclinical data of sensitivity to pan-PI3K inhibitors in tumors harboring relevant molecular aberrations become available (89-91), different enrichment strategies have been adopted. These strategies range from the selection of patients with any PI3K pathway alterations in the expansion cohort of phase I trials, to the selection of patients since dose escalation, as performed in the development of isoform specific inhibitors. It is invalid to make a direct comparison between unselected versus selected approaches for patient recruitment, as other factors, such as the anticancer activity of each compound, the number of patients treated at suboptimal doses, pharmacokinetic issues, or the presence of different molecular events that can modify the sensitivity to PI3K inhibitors (such as KRAS mutations), can be confounding. However, preliminary experience from the phase I trial of BYL719 suggests that it is reasonable to select patients based on specific molecular aberrations which are justified by appropriate preclinical models. Importantly, this study has performed large scale screening in local institutions to identify patients with uncommon molecular characteristics without compromising timely enrollment, a finding that supports the feasibility of molecular prescreening already implemented by many large drug development programs (100, 101).

Role of Macrophages in Cancer

Tumors are integrated by cancer cells and stromal cells. The predominant stromal cells are lymphocytes (predominantly T-cells), macrophages, endothelial cells and fibroblasts. In addition, other stromal cells might be also relevant in some tumor types. These other stromal cells include eosinophils, granulocytes, and natural-killers (102). In recent years, new drugs blocking immune-checkpoints, which were suppressing the antitumor effect of cytotoxic T cells (103), have shown unprecedented antitumor activity. These activity has led to the approval of several agents targeting PD1 or PDL1, including nivolumab, pembrolizumab, atezolizumab and durvalumab. In parallel, new approaches are under development targeting other immune cells in the tumor microenvironment, such as tumor associated macrophages or natural killers. Tumor associated macrophages (TAMs) include a group of mononuclear cells with different ontogenic origin including circulating monocytes, circulating monocyte-related myeloid-derived suppressor cells (M-MDSC) and tissue resident macrophages (104). The ontogenic origin of TAMs does not seem to have an impact on the response the macrophages are performing in the tumor; this response is influenced however by different stimuli in the tissues. This stimuli lead to functional reprograming. Interferons produced by type 1 T-helper lymphocytes and bacterial products led to reprograming into classically activated or M1 macrophages, characterized by their anti-tumor effect. On the contrary, type 2 T-helper lymphocytes and type 2 innate immune cells differentiate TAMs into alternatively activated or M2 TAMs through IL-4 and IL13 production (104). M2 TAMs promote tumor progression and suppress the effect of adaptive immunity. Other stimuli can induce anti-tumor or pro-tumor TAM phenotypes, which are named M1-like and M2-like TAMs respectively by the similarities in their function with M1 and M2 TAMs. M2 and M2-like macrophages facilitates cancer cell proliferation, migration, intravasation and seeding at the pre-metastatic niche (104, 105). In most established tumors, M2 or M2-like TAMs are the predominantly phenotype. High TAM infiltration is associated to poor prognosis including breast, head and neck, pancreatic, bladder, thyroid, ovarian, endometrial and kidney cancer (104, 105). Based on the role of TAMs favoring cancer and their presence in a variety of tumors, targeting TAMs seems an attractive anti-cancer approach. One of the therapeutic approaches is blocking the migration of macrophages into the tumor which is orchestrated by chemokines.

Chemokines in Cancer – the Role of CCL2

Chemokines constitute a large family of small secreted proteins integrated by more than 50 members. Chemokines regulate immune cell trafficking and the development of lymphoid tissue. In contrast to other cytokines, chemokines are the only group that interact with G-protein-coupled receptors (CPCRs). Although there is little homology among their peptidic sequences, chemokines share a resembling tridimensional structure: 3 beta-strand with a Greek key configuration, one C-terminal alpha-helix and a flexible N-terminal region. Most chemokines have four cysteines which are linked through two bisulphide bridges. Chemokines are classified according to the position of the first two cysteines adjacent to the N-terminus into CC chemokines (the two cysteines are adjacent), CXC (two cysteines separated by one aminoacid), CX_3C (in which three aminoacids separate the cysteines) and C chemokines, which have only two cysteines in the whole molecule (106-108). Chemokines can be constitutive, which are involved in basal leukocyte trafficking and the development of lymphoid organs, and inducible, which can be produced at high levels as an inflammatory response. In tumors inducible chemokines can be produced by tumor cells, immune cells and stromal cells. These chemokines interact as a complex network that influence the phenotype and the amount of immune cells infiltrating the tumors. In cancer, chemokines induce the migration of different immune cell subsets towards areas with higher chemokine concentration. Based on the timing of chemokine secretion and the chemokine gradients, chemokines regulate immune responses in a spatio-temporal manner. In addition to module immune responses, chemokines can directly target tumor and vascular endothelial cells promoting proliferation, invasiveness and metastasis (108).

Human C-C chemokine ligand 2 (CCL2) binds preferentially CCR2 receptor. CCL2-CCR2 pathway is essential for TAM recruitment by attracting circulating monocytes, and M-MDSC mobilization from the bone marrow. High levels of CCL2 expression have been associated with tumor aggressiveness (109), poor prognosis, early relapse (110), and, in some cancers—including breast (109, 110), prostate (111), ovarian (112), and pancreatic (113)—advanced-stage disease and disease worsening or progression. In prostate cancer, CCL2 was shown to be highly expressed in bone marrow endothelial cells [16, 17], and in vitro studies have found that CCL2 stimulates cancer cell migration and growth in both breast [18] and prostate [16] cancers. These findings, along with CCL2 involvement in TAM-mediated actions, suggest that neutralizing CCL2 may provide a novel approach to controlling tumor growth.

Carlumab – a Monoclonal Antibody against Human CCL2

Carlumab (formerly CNTO 888) is a human immunoglobulin G1 kappa monoclonal antibody with high binding affinity and specificity for CCL2 (114). Carlumab has been previously evaluated as single agent in a phase I clinical trial in solid tumors (115) and in a phase II clinical trial in prostate cancer (116). Both studies has shown that Carlumab as single agent at a dose up to 15 mg/kg has been tolerable. In preclinical studies in prostate cancer, the murine anti-CCL2 antibody equivalent to carlumab suppressed tumor growth (117, 118) and formation of bone lesions (117) in murine models and attenuated cancer cell migration in in vitro human models (119). A murine melanoma model showed reduced tumor size and angiogenesis when treated with a CCL2 inhibitor (120). Chemokine CCL2 neutralization also modulated tumor inflammation by reducing CD68-positive macrophage/monocyte infiltration into tumor tissue in patients with pancreatic cancer (113). Preclinical studies suggest that carlumab may offer beneficial antitumor properties when used with standard-of-care chemotherapies (docetaxel (118, 121), gemcitabine, paclitaxel+carboplatin (122), or pegylated liposomal doxorubicin HCl [PLD]) for patients with solid tumors.

In the phase Ib study presented as part of this thesis, the primary objective was to evaluate the safety and tolerability of two dose regimens of carlumab in combination with four standard-of-care chemotherapies in patients with solid tumors. Major secondary objectives were to determine the effect of carlumab on the pharmacokinetics of docetaxel, gemcitabine, paclitaxel+carboplatin, and PLD and to assess the pharmacokinetics of carlumab in combination with these chemotherapies. Other secondary objectives were to evaluate pharmacodynamic biomarkers, carlumab immunogenicity, and rate of response to combination therapy.

PROJECT 1:

NOVEL COMBINATIONS OF PI3K-MTOR INHIBITORS WITH DACOMITINIB OR CHEMOTHERAPY IN PTEN-DEFICIENT PATIENT-DERIVED TUMOR XENOGRAFTS.

3. PROJECT 1: NOVEL COMBINATIONS OF PI3K-MTOR INHIBITORS WITH DACOMITINIB OR CHEMOTHERAPY IN PTEN-DEFICIENT PATIENT-DERIVED TUMOR XENOGRAFTS.

Hypothesis and Objectives

Hypothesis:

The addition of the PI3K-mTOR inhibitors PF-04691502 and PF-05212384 would increase the antitumor activity of chemotherapy (cisplatin and paclitaxel) and the pan-HER inhibitor dacomitinib.

Objectives

1) To evaluate the antitumor activity of each combination

2) To correlate the observed antitumor activity with the molecular alterations each model harbors

3) To evaluate the pharmacodynamic effects induced by the targeted agents.

Novel Combinations of PI3K-mTOR inhibitors with Dacomitinib or Chemotherapy in PTEN-deficient Patient-Derived Tumor Xenografts.

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Novel Combinations of PI3K-mTOR inhibitors with Dacomitinib or Chemotherapy in PTEN-deficient Patient-Derived Tumor Xenografts.

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ABSTRACT

PTEN inactivation occurs commonly in human cancers and putatively activates the PI3K/AKT/ mTOR pathway. Activation of this pathway has been involved in resistance to chemotherapy or anti-EGFR/HER2 therapies. We evaluated the combination of PI3K-mTOR inhibitors with chemotherapy or the pan-HER inhibitor dacomitinib in PTEN-deficient patient-derived tumor xenografts (PDX).

Three PDXs were selected for their lack of PTEN expression by immunohistochemistry: a triple-negative breast cancer (TNBC), a *KRAS* G12R low-grade serous ovarian cancer (LGSOC), and *KRAS* G12C and *TP53* R181P lung adenocarcinoma (LADC). Two dual PI3K-mTOR inhibitors were evaluated—PF-04691502 and PF-05212384—in combination with cisplatin, paclitaxel, or dacomitinib.

The addition of PI3K-mTOR inhibitors to cisplatin or paclitaxel increased the activity of chemotherapy in the TNBC and LGSOC models; whereas no added activity was observed in the LADC model. Pharmacodynamic modulation of pS6 and pAKT was observed in the group treated with PI3K-mTOR inhibitor.

Our research suggests that the addition of a PI3K-mTOR inhibitor may enhance tumor growth inhibition when compared to chemotherapy alone in certain PTEN-deficient PDXs. However, this benefit was absent in the *KRAS* and *TP53* mutant LADC model. The role of PTEN deficiency in the antitumor activity of these combinations should be further investigated in the clinic.

Word count: 199

INTRODUCTION

The phosphoinositide-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is commonly activated in cancer by several mechanism including activating mutations of *PIK3CA* or *AKT1* and or loss of phosphatase and tensin homolog (PTEN) [1] . This signaling pathway is critical in the regulation of cell growth, metabolism and survival, angiogenesis, tumor invasion, cell cycle regulation and DNA repair [2, 3]. The activity of different PI3K inhibitors in unselected population in the clinical setting has been limited [1]. Preclinical testing strategies to increase the antitumor activity of these compounds should address effectiveness of combination regimens with chemotherapy or other targeted agents and improvement of patient selection based on biomarkers predictive of an activated PI3K pathway.

Preclinical and clinical evidence supports a role of the PI3K pathway in chemoresistance in different tumor types including ovarian [4], breast [5], and non-small cell lung adenocarcinoma (LADC) [6]. Furthermore, inhibition of the PI3K pathway sensitizes preclinical models to chemotherapy [6-8]. Likewise, resistance to anti-EGFR and anti-HER2 therapies are associated with PI3K pathway activation by *PIK3CA* mutations in *EGFR* mutant LADC [9], and HER2-positive breast cancer [10] [11], or by loss of PTEN [12] or HER3 activation (which activates the PI3K pathway) in breast cancer models [13]. In cell lines, the addition of PI3K inhibitors overcomes resistance to anti-EGFR or anti-HER2 agents [10, 11, 14]. This evidence has supported the evaluation of the PI3K inhibitors in combination with anti-EGFR or anti-HER2 therapies in the clinical setting and several combinations are under investigation.

Patient derived tumor xenografts (PDXs) represent promising pre-clinical models as they seem to recapitulate some of the molecular characteristics of the primary tumor [15] as well as clinical tumor response [16-18]. To improve the clinical activity of the PI3K inhibitors, we tested several therapeutic strategies in three different tumor types using PDX selected for deficient PTEN expression as increased sensitivity

to PI3K and mTOR inhibitors has been previously described in cancer cell lines [19]. PTEN-deficient tumors seem to signal preferentially through the PI3K beta isoform (p110β) [20, 21] but it remains unclear whether single inhibition of the beta isoform would be sufficient to induce tumor growth inhibition, or whether dual PI3K alpha and beta isoform inhibition would be superior [22].

In the current study, we evaluated two PI3K-mTOR inhibitors in combination with a pan-HER inhibitor dacomitinib, cisplatin or paclitaxel. The two PI3K-mTOR inhibitors (PF-04691502 and PF-05212384), although different in route of administration and pharmacokinetics, both are potent inhibitors of all PI3K isoforms and mTOR [23, 24] and have shown clinical antitumor activity as monotherapies [25, 26]. Dacomitinib is an irreversible tyrosine kinase inhibitor targeting EGFR, HER2 and HER4 [27] with antitumor activity demonstrated pre-clinically in EGFR wild type and mutant LADC models [28], as well as clinically in head and neck squamous cell carcinoma [29] and LADC [30]. Cisplatin is commonly used as the backbone of chemotherapy regimens for many cancers, including LADC [31], ovarian [32], and triple negative breast cancers (TNBC) [33]. Likewise, paclitaxel is widely used in LADC [31], ovarian [32] , and breast cancers [34]. We hypothesize that in tumors deficient in PTEN protein expression, PI3K inhibition might increase the activity of cisplatin, paclitaxel or dacomitinib. We expect that the simultaneous evaluation of these compounds in PDX may expedite the translation of the most promising combinations into the clinical setting.

RESULTS

Molecular Characterization

Three PDX models were selected based on their deficient (null or low) PTEN expression. The three models display morphological and molecular characteristics from the original tumor (Supplementary Figure 1). The TNBC model lacks PTEN expression and did not harbor any of the mutations in the Oncocarta panel. PTEN staining was faint by immunohistochemistry in the low-grade serous ovarian cancer (LGSOC) and LADC PDX models. Furthermore, both of the latter models had *KRAS* mutations (G12R and G12C respectively in the LGSOC and LADC models). The LADC model also had a *TP53* co-mutation (R181P) detected by direct sequencing.

PI3K-mTOR inhibitor and Cisplatin

Treatment duration was up to 29 days when experiments were discontinued due to inaccessible tail veins for IV drug administration. Single agent PF-05212384 had no significant activity as single agent in any of the three models (Table 1 and Figure 1). As a single agent, cisplatin showed insignificant anti-tumor activity in the TNBC (Figure 1a) and LGSOC models (Figure 1b), but induced tumor growth inhibition (TGI) > 50% in the LADC model (Figure 1c). The combination of cisplatin and PF-05212384 induced TGI > 50% in all the three models (Table 1). While the combination was synergistic in the TNBC model ($p<0.05$), PF-05212384 did not enhance the TGI induced by cisplatin single agent in the LADC model ($p=0.113$), nor in the LGSOC model ($p=0.147$).

The initial dose selected for PF-05212384 (15 mg/kg) was found to be excessively toxic in the first model evaluated (LADC). Several cases of sudden death occurred after the first administration. Hence, a lower dose (10 mg/kg) was administered in subsequent experiments. After this dose reduction, the treatment was well-tolerated in the three models, with mean weight loss ranging 10 – 15% in the combination arm in the TNBC

model and in cisplatin-containing arm in the LGSOC model. No mean weight loss was observed in the LADC model.

PI3K-mTOR inhibitor and Paclitaxel

Although PF-05212384 and PF-04691502 showed no significant activity as single agents, paclitaxel induced TGI > 50% in the TNBC and LADC models (Table 1). The combination arm only induced TGI > 50% in the LGSOC and TNBC models (Figure 2a, b). Although the difference between the paclitaxel single agent and paclitaxel + PF-05212384 arms were not statistically significant, we observe signs of additive activity by adding PF-05212384 to paclitaxel in these two models; In the TNBC model, the combination arm induced tumor regression, which was not achieved in the paclitaxel single agent arm. In the LGSOC model, the combination arm achieved a TGI > 50%, while such TGI was not achieved by any of the single agent arm achieved (Table 1). The results of the LADC model (Figure 2c) should be evaluated with caution, as several mice in the paclitaxel arm suddenly died between weeks 1 and 2 of treatment. These deaths were directly attributed to dose initially selected of paclitaxel (15 mg/kg), as no new events were observed in any of the models once the paclitaxel dose was reduced to 10 mg/kg. No significant weight loss was observed in any of the treatment arms in the three models.

PI3K-mTOR inhibitor and Dacomitinib

As single agents, neither the PI3K-mTOR inhibitors nor dacomitinib achieved a TGI of 50%, (Table 1 and Figure 3). The combination arm only induced a mean TGI > 50% in the TNBC model (TGI = 55 %), but this did not achieve statistical significance according to the mixed effect model (p =0.09) (Table 1). No relevant weight loss or other toxicity was observed in any of the arms.

Evaluation of Downstream Effector Phosphoproteins

Despite the limited anti-tumor effect induced by the PI3K-mTOR inhibitors single agents or in combination with dacomitinib, downstream inhibition of the PI3K/AKT/mTOR pathway was observed in all of the arms containing a PI3K-mTOR inhibitor (Figure 4A-F): all models exhibited pS6 inhibition and the LGSOC and LADC models also exhibited pAKT inhibition. The inhibition of these phospho-proteins was, in general, greater in the combination arm, which might reflect the requirement for dual inhibition of the tyrosine kinase receptor and PI3K to completely abrogate the pathway. Substantial inhibition of AKT and S6 phosphorylation was observed 2 hours after treatment; while their phosphorylation levels partially recovered by 24 hours. The differences in phosphorylation patterns between the LADC, TNBC and LGSOC models might be partially due to the different molecular backgrounds, but also to the different pharmacokinetic profiles of PF-04691502 and PF-05212384.

Although the addition of dacomitinib was expected to induce some degree of inhibition in the MAPK signaling pathway, no effects on pEGFR, nor on pERK levels was observed in the three models (Figure 4A-F).

DISCUSSION

We simultaneously evaluated multiple combinations of novel therapeutic strategies using PDXs as an innovative platform for preclinical evaluation of these combinations. The selected models harbored molecular alterations commonly described in the matched tumor types, such as loss of PTEN expression present in around 30% in patients with TNBC [35] and LADC [36] or *KRAS* mutation described in around one third of LADC [37] and LGSOC [38]. The response to conventional chemotherapy in the three selected PDXs resembled the response in the matching tumor types reported in clinical trials and retrospective series, from a chemo-sensitive model represented by the LADC to a chemo-resistant model represented by the LGSOC [31, 33, 34, 39]. We observed that the addition of PF-05212384 to cisplatin enhanced the antitumor activity of cisplatin suggesting a chemo-sensitizing effect. There is preclinical evidence supporting the role of the PI3K-AKT-mTOR pathway in DNA repair. The beta isoform of PI3K (p110β) seems to have a role sensing DNA damage and facilitating the binding to DNA of DNA repair proteins from the ATM and ATR pathways [40]. Loss of p110β was found to induce genomic instability, whereas 110β inhibition increased sensitivity to DNA damaging agents [40]. The PI3K-mTOR pathway also regulates the expression of certain DNA repair proteins, such as BRCA1 [41] and FANCD2 [3], as decreased levels of these proteins are observed upon PI3K-mTOR inhibition. PTEN also is involved in chromosomal integrity and DNA repair, specifically the fraction of PTEN localized in the nucleus [42, 43]. In xenografts established from PTEN-deficient cell lines, the addition of a PI3K inhibitor increased the antitumor activity of cisplatin [43]. Our experiments did not assess DNA damage markers or DNA repair proteins, so the specific mechanism by which the addition of PF-05212384 might have induced cisplatin chemo-sensitization was not fully clarified. Furthermore, the use of a pan-isoform PI3K-mTOR inhibitor, does not allow to discern whether the chemo-sensitization induced by PF-05212384 depends on an individual PI3K isoform or whether simultaneous inhibition of different PI3K isoforms is needed. Experiments comparing the activity of pan-isoform PI3K inhibitors

and novel beta-isoform PI3K inhibitors combined with chemotherapy would help understanding the mechanism of chemo-sensitization observed in our experiments.

The effects of adding a PI3K-mTOR inhibitors to chemotherapy has varied among the three models tested: from a synergistic effect in the TNBC model to lack of effect in the LADC model; whereas an intermediate effect was achieved in the LGSOC model. These differences between these models may be probably related to their variable molecular backgrounds. The LADC had a *TP53* missense mutation causing a complete loss of function of p53 [44]. *TP53* mutations have been identified as potential mechanisms of resistance to PI3K inhibition in cell lines and in patients participating in the phase I clinical trial evaluating the alpha-specific PI3K inhibitor BYL719 [45]. Further research is warranted to characterize the role of *TP53* status on the activity of the PI3K inhibitors. The LADC and LGSOC models also harbored *KRAS* mutations (G12C and G12R respectively). Different *KRAS* mutations seemed to signal preferentially through different downstream pathways [46]; KRAS mutations signaling preferentially through the MAPK pathway, such as G12C, are a well-known resistance factor to PI3K inhibition [47]; whereas KRAS G12R signals through both the ERK and PI3K pathways, which might explain the moderate chemo-sensitization achieved in the LGSOC, disease characterized by its intrinsic relative chemo-resistance. Although the addition of a PI3K-mTOR inhibitor to chemotherapy might be a strategy to increase the antitumor activity of chemotherapy in LGSOC, the combination of MEK and PI3K inhibitors would probably be a more relevant combination based on the high response rate observed in this patient population in the early clinical trials evaluating these combinations [48, 49].

In the experiments evaluating the combination of PI3K-mTOR inhibitors and dacomitinib, no statistically significant difference was observed between the combination arm and the vehicle arm. This lack of activity contrasts with results from the western-blot, in which target inhibition was observed in the arms containing the PI3K-mTOR inhibitors. No significant pEGFR inhibition was observed in the dacomitinib containing arms which

might be related to the dose selected, lower than in prior *in vivo* experiments [27, 28, 50, 51]. The lack of MAPK pathway inhibition in the arm combining PIK3-mTOR inhibitor and dacomitinib may be related to an insufficient pEGFR inhibition or to the presence of activating events in the MAPK pathway, such as *KRAS* mutations, which may constitute potential mechanisms of resistance to this treatment combination.

The preclinical results generated by our study have supported the translation of these combinations into the clinic and helped define the patient populations for each combination. The combination of cisplatin and PF-05212384 in patients with TNBC is currently under evaluation in a phase I clinical trial (NCT01920061), which has three parallel arms evaluating PF-05212384 in combination with cisplatin, docetaxel, and dacomitinib respectively. Although no molecular selection is required, paired biopsies are being collected for biomarker analysis. This trial should provide further insights into the role of PI3K-mTOR inhibitors as chemosensitizing agents and could help to clarify the effect of PF-05212384 on DNA damage and repair and the role of different molecular biomarkers, such as PTEN deficiency, on such effect. The results provide some evidence that preclinical studies of novel targeted drugs in PDX may reveal the treatment response heterogeneity, which could be informative in refining the design of early stage clinical trials for these drugs.

MATERIALS AND METHODS

Drugs

The two PI3K-mTOR inhibitors evaluated, PF-05212384 and PF-04691502, were provided by Pfizer Inc. (New York City, NY), as well as the pan-HER inhibitor dacomitinib; cisplatin (Teva Parenteral Medicines, Inc., Irvine, CA) and paclitaxel (Sigma-Aldrich Corporation, St. Louis, MO) were purchased from the hospital pharmacy.

PDX Models

The three PDX models were selected from a repository at the University Health Network (UHN) that has been established with patient consent and in accordance to the guidelines of the UHN Human Research Ethics Board. Animal experiments were performed at the Toronto Centre for Phenogenomics in compliance with regulatory guidelines and a protocol approved by the UHN Animal Care Committee. Three models were selected based on their deficient PTEN expression as assessed by immunohistochemistry: a TNBC model established from a liver metastasis biopsy, a LGSOC model established from an ascites sample, and a lung adenocarcinoma (LADC) model established from a surgical specimen. Breeders were purchased from Jackson Laboratories (Bar Harbor, ME) and were used for up to four generations to avoid genetic drift. Cryobanked tumor fragments were revived and expanded as donors in 2-3 serial mouse generations at the subcutaneous flank site of non-obese diabetic severe combined immunodeficiency (NOD-SCID) NOD.CB17-Prkdc^{scid}/J mice.

PDX Therapeutic Studies

In the experimental phase, groups of 12 tumor-bearing mice were randomized to treatment or vehicle once tumors reached an average volume of 250 mm^3 by caliper measurements.

Treatment doses were selected according to previously performed maximum tolerated dose experiments and were refined further based on the observed toxicity profile. Three

different drug combinations were evaluated with their matching single agent and vehicle arms. Treatment arm details are summarized in Table 2. In the LADC model, both PI3K-mTOR inhibitors—PF-05212384 and PF-04691502—were tested. However, in the subsequent models, only PF-05212384 was tested as PF-05212384 would be the compound that would be further evaluated in clinical trials based on additional clinical data that became available before starting the TNBC and LGSOC models. Tumor size and mouse weight were evaluated twice per week. Tumor volume was calculated using the formula = length^2 x width x 0.52. Mice were sacrificed once tumors reached 1500 mm^3 or when other human endpoints were observed in compliance with regulatory guidelines of the Institutional Animal Care Committee. Pieces of extracted tumors were snap-frozen in liquid nitrogen and formalin-fixed and paraffin-embedded for further analysis.

A parallel acute dose experiment was performed to evaluate the pharmacodynamic effect of PF-05212384, PF-04691502, and dacomitinib on downstream effectors in the treatment groups. Three mice replicates were harvested at 2 and 24 hours post-dosing.

Genomic Characterization of Patient-Derived Tumor Xenografts

PDXs in the repository were characterized using the OncoCarta Panel v 1.0 (238 mutations in 19 key oncogenes) on the MassARRAY System (Agena Bioscience, San Diego, CA) to determine their tumor mutational profile.

Immunoblots of Downstream Effector Proteins

Aliquots of approximately 50 mg of tissue were mixed with 1 ml of lysis RIPA buffer (R 0278, Sigma, Saint Louis, MO) buffer containing a protease inhibitor cocktail (11 836 170 001, Roche Diagnostics GmbH, Mannheim, Germany), 8 mM sodium orthovanadate, and 0.2 mM phenylmethanesulfonyl fluoride. Protein extracts were collected after tissues were homogenized (1 min) and centrifuged (20000 × g, 20 min). Aliquots of 30 µg of protein were mixed with an equal volume of 2 X SDS-PAGE sample loading buffer, and

resolved by SDS-PAGE. Resolved samples were transferred to polyvinylidene fluoride (PVDF) membranes by using Trans-Blot® Turbo™ Midi PVDF Transfer Packs, 170-4157 BioRad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in Tris-Buffered Saline with Tween 20 (1X TBST). Blots were incubated overnight at 4ºC with primary antibodies (EGFR Tyr 1068, ERK, pERK1/2 Thr202/Tyr204, AKT, pAKT Ser 473, pS6 Ser235/236, and β-actin) at 1:1000 dilutions, followed by incubation with a 1: 4000 dilution of HRP-linked anti-rabbit IgG secondary antibodies for one hour . All antibodies used for blotting were from Cell Signaling Technology, Danvers, MA. Immunoreactive protein bands were detected by ECL-Prime Western blotting detection reagent (RPN2236, GE Healthcare, Little Chalfont, UK).

Immunohistochemistry

PTEN immunohistochemistry was performed using the Ventana Benchmark XT autostainer for anti-PTEN (138G6, Cell Signaling Technology, Danvers, MA) with the iVIEW DAB detection system (Ventana Medical Systems, Inc., Tucson, AZ). Absence of staining of tumor cell cytoplasm was classified as loss of expression whereas faint staining was considered as low PTEN expression.

Statistical Analysis

Mixed effect model was used to test the differences in tumor growth rates overtime between treatment and control groups within each PDX model. SAS 9.2 (SAS Institute Inc., Cary, NC) was used for the analysis.

Relative mean tumor growth was calculated with the formula

$$\frac{\Delta \text{ mean tumor volume treatment arm} - \Delta \text{ mean tumor volume control arm}}{\Delta \text{ mean tumor volume treatment arm}} \text{ x } 100$$

Tumor volume were plotted as mean +/- standard deviation with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA)

TABLES

Table 1. Patient derived xenograft molecular profile and tumor growth inhibition per treatment arm

PDX tumor type	TNBC		LGSOC		LADC	
Molecular profile	PTEN null		*KRAS* G12R PTEN low		*KRAS* G12C *TP53* R181P PTEN low	
	TGI%	ΔV p-value	TGI%	ΔV p-value	TGI%	ΔV p-value
Experiment 1						
PF-05212384	32%	0.07	39%	0.19	-1%	0.78
Cisplatin	42%	<0.05**	43%	0.03	97%	<0.05**
PF-05212384 + Cisplatin	96%	<0.05**	68%	<0.05**	92%	<0.05**
Experiment 2						
PF-05212384	22%	0.64	40%	0.21		
PF-04691502					33%	<0.05**
Paclitaxel	84%	<0.05**	38%	0.20	79%	<0.05**
PF-05212384 + Paclitaxel	110%^	<0.05**	56%	<0.05**		
PF-04691502 + Paclitaxel					45%	<0.05**
Experiment 3						
PF-05212384	22%	0.61	40%	0.26		
PF-04691502					33%	<0.05**
Dacomitinib	15%	0.99	8%	0.53	23%	0.35
PF-05212384 + Dacomitinib	55%	0.09	45%	0.47		
PF-04691502 + Dacomitinib					32%	0.055

TNBC = triple negative breast cancer; LGSOC = low grade serous ovarian cancer; LADC = non-small cell lung cancer; TGI%: percentages of tumor growth inhibition; ΔV: differences between daily tumor volume change of each treatment arm and the control arm; ^ Tumor regression; ** statistically significant

Table 2. Treatment arms, dose, frequency and administration route

Cisplatin + PI3K-mTOR inhibitor experiment				
Model	Drug	Dose	Frequency	Route
LADC [1] TNBC LGSOC	Cisplatin	3 mg / kg	Weekly	IP
	PF-05212384	10 mg/ Kg	2 times / week	IV
	Cisplatin PF-05212384	3 mg / kg 10 mg/ Kg	weekly 2 times / week	IP IV
	Vehicle		weekly 2 times / week	IP + IV
Paclitaxel + PI3K-mTOR inhibitor experiment				
Model	Drug	Dose	Frequency	Route
LADC	Paclitaxel	15 mg / kg [2]	2 times / week	IP
	PF-04691502	5 mg / kg	daily	PO
	Paclitaxel PF-04691502	15 mg / kg 5 mg / kg	2 times / week daily	IP PO
	Vehicle		daily 2 times / week	PO + IP
TNBC LGSOC	Paclitaxel	10 mg / kg	2 times / week	IP
	PF-05212384	10 mg / kg	2 times / week	IV
	Paclitaxel + PF-05212384	10 mg / kg 10 mg / Kg	2 times / week 2 times / week	IP IV
	Vehicle		2 times / week	IP + IV
Dacomitinib + PI3K-mTOR inhibitor				
Model	Drug	Dose	Frequency	Route
LADC	Dacomitinib	3 mg / kg	daily	PO
	PF-04691502	5 mg / kg	daily	PO
	Dacomitinib PF-04691502	3 mg / kg 5 mg / kg	daily daily	PO PO
	Vehicle		daily	PO
TNBC LGSOC	Dacomitinib	3 mg / Kg	daily	PO
	PF-05212384	10 mg/ Kg	2 times / week	IV
	Dacomitinib PF-05212384	3 mg / Kg 10 mg/ Kg	daily 2 times / week	PO IV
	Vehicle		daily 2 times / week	PO IV

LADC: non-small cell lung cancer; TNBC: triple negative breast cancer; LGSOC: low-grade serous ovarian cancer; IP: intraperitoneal; IV: intravenous; PO: oral gavage; [1] In the LADC model, PF-05212384 at a dose of 15 mg / kg was initially tested. Several sudden deaths occur after the first drug administration; thus, PF-05212384 was reduced to 10 mg / kg in subsequent administrations. The models evaluated afterwards (TNBC and LGOSC) received a dose of 10 mg / kg. [2] Paclitaxel was initially evaluated at a dose of 15 mg / kg in the LADC and subsequently changed to 10 mg/kg based on several unexpected deaths in the paclitaxel-containing arms. Paclitaxel was tested at a dose of 10 mg / kg in the remaining models.

FIGURE LEGENDS

Figure 1: Combined cisplatin and PI3K-mTOR inhibitor in PTEN-deficient gPDX. Tumor growth of A) triple-negative breast cancer (TNBC), B) KRAS mutant (G12R) low-grade ovarian cancer (LGSOC) and C) TP53 (R181P) and KRAS (G12C) mutant lung adenocarcinoma (LADC). The three models were treated with vehicle, PF-05212384 (10 mg/kg, twice weekly, intravenously, cisplatin (3 mg/kg, once weekly, intraperitoneally) or the combination of both agents. Relative tumor volumes are displayed as mean +/- SE. p: p value for daily tumor volume change for each arm in comparison to vehicle arm. TGI%: percentages of tumor growth inhibition in comparison to vehicle arm.

Figure 2: Combined Paclitaxel and PI3K-mTOR inhibitor in PTEN-deficient PDX. Tumor growth of A) triple-negative breast cancer (TNBC); B) KRAS mutant (G12R) low-grade ovarian cancer (LGSOC) and C) TP53 (R181P) and KRAS (G12C) mutant lung adenocarcinoma (LADC). The TNBC and LGSOC models were treated with vehicle, PF-05212384 (10 mg/kg, twice weekly, intravenously, paclitaxel (10 mg/kg, twice weekly, intraperitoneally) or the combination of both agents. The LADC was treated with vehicle, PF-04691502 (5 mg/kg, daily, oral gavage), paclitaxel (10 mg/kg, twice weekly, intraperitoneally) or the combination of both agents. Relative tumor volumes are displayed as mean +/- SE. p: p value for daily tumor volume change for each arm in comparison to vehicle arm. TGI%: percentages of tumor growth inhibition in comparison to vehicle arm.

Figure 3: Combined dacomitinib and PI3K-mTOR inhibitor in PTEN-deficient PDX. Tumor growth of A) triple-negative breast cancer PDX (TNBC), KRAS mutant (G12R) low-grade ovarian cancer (LGSOC) and TP53 (R181P) and KRAS (G12C) mutant lung adenocarcinoma (LADC). The TNBC and LGSOC models were treated with vehicle, PF-05212384 (10 mg/kg, twice weekly, intravenously, dacomitinib (3 mg/kg daily, oral gavage) or the combination of both agents. The LADC was treated with vehicle, PF-04691502 (5 mg/kg, daily, oral gavage), dacomitinib (3 mg/kg daily, oral gavage) or the combination of both agents. Relative tumor volumes are displayed as mean +/- SE. p: p value for daily tumor volume change for each arm in comparison to vehicle arm. TGI%: percentages of tumor growth inhibition in comparison to vehicle arm.

Figure 4: Western blot analysis of the indicated proteins in three independent tumors of the triple-negative breast cancer (TNBC), KRAS mutant (G12R) low-grade ovarian cancer (LGSOC) and TP53 (R181P) and KRAS (G12C) mutant lung adenocarcinoma (LADC). The TNBC (A, B) and LGSOC (C, D) models were treated with vehicle, PF-05212384 (10 mg/kg, twice weekly, intravenously, dacomitinib (3 mg/kg daily, oral gavage) or the combination of both agents. The LADC model (E, F) was treated with vehicle, PF-04691502 (5 mg/kg, daily, oral gavage), dacomitinib (3 mg/kg daily, oral

gavage) or the combination of both agents. Tumors were collected 1 hour after treatment (A, C, E) or 24 hours after treatment (B, D, F).

AUTHOR CONTRIBUTIONS

Experiment design: Irene Brana, Nhu-An Pham, Rafael Sierra, Lillian L Siu and Ming-Sound Tsao.

Model generation: Nhu-An Pham, Ming Li, Rafael Sierra, Benjamin G Neel, Lillian L Siu and Ming-Sound Tsao.

In vivo experiments: Ming Li, Christine Ng, Yuhui Wang, Peter Loparco, Rafael Sierra, Irene Brana, Nhu-An Pham, Ming-Sound Tsao.

Western-blot: Christine Ng, Irene Brana, Nhu-An Pham.

Pathology review and sample quality assessment: Lucia Kim, Shingo Sakashita, Blaise A. Clarke, Ming-Sound Tsao.

Statistical analysis: Lisa Wang.

Data interpretation: Irene Brana, Nhu-An Pham, Benjamin G. Neel, Lillian L Siu, Ming-Sound Tsao.

Manuscript preparation: Irene Brana, Nhu-An Pham, Lillian L Siu, Ming-Sound Tsao.

Manuscript review: all authors.

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CONFLICT OF INTEREST

Lillian L. Siu: funding from Pfizer to support clinical trials and to conduct of this preclinical study
Ming S. Tsao: funding from Pfizer to conduct this preclinical study
Rest of the authors: no conflict of interest to disclose

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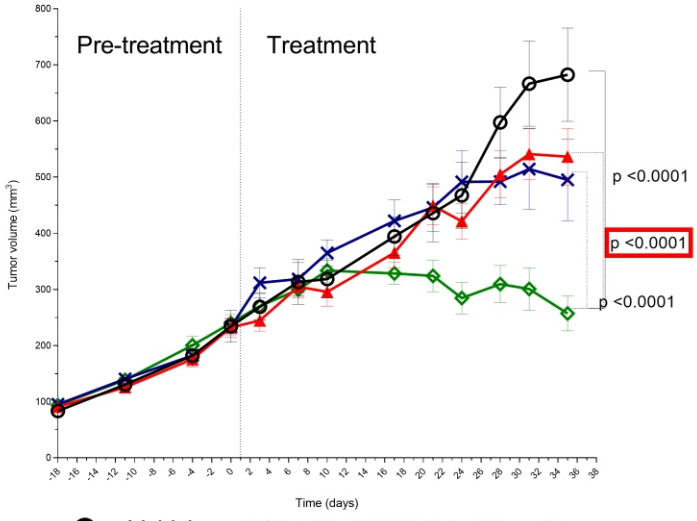
Figure 1

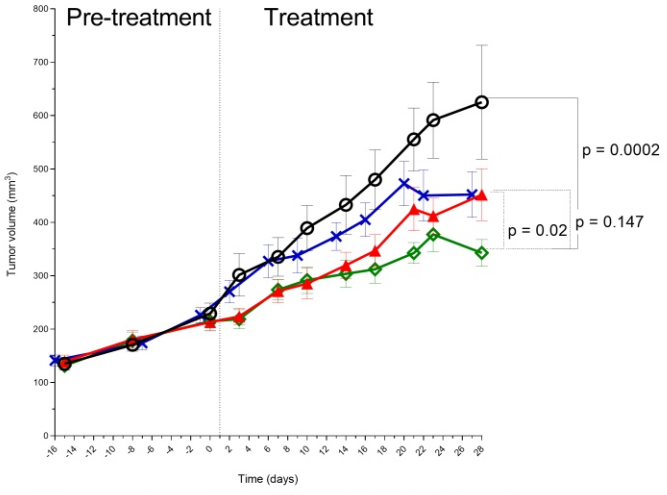
A TNBC



Vehicle
PF-05212384 (10mg/Kg)
Cisplatin (3mg/Kg)
PF-05212384 (10mg/Kg) +Cisplatin (3mg/Kg)

	TGI%
PF-05212384	32%
Cisplatin	42%
PF-05212384 + Cis	96%

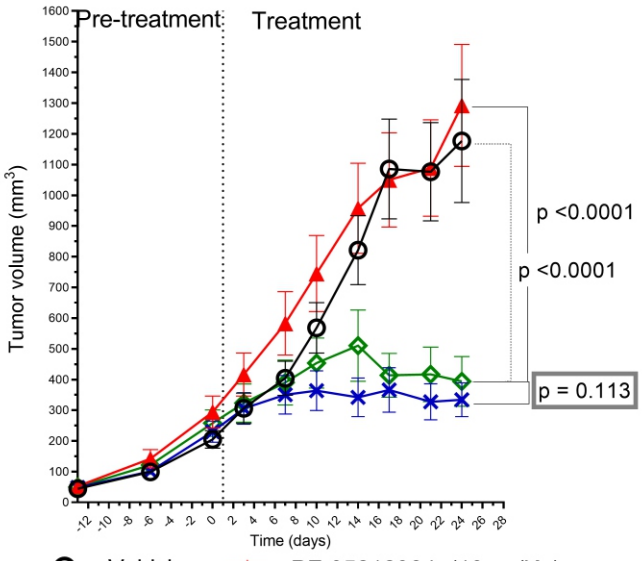
B LGSOC
***KRAS* G12R mutant**



Vehicle
PF-05212384 (10mg/Kg)
Cisplatin (3mg/Kg)
PF-05212384 (10mg/Kg) +Cisplatin (3mg/Kg)

	TGI%
PF-05212384	39%
Cisplatin	43%
PF-05212384 + Cis	68%

C LADC
***KRAS* G12C, *TP53* R181P mutant**

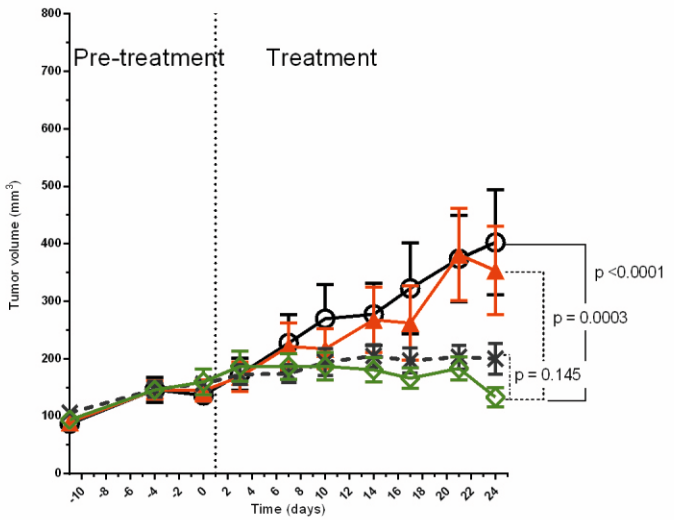


Vehicle
PF-05212384 (10mg/Kg)
Cisplatin (3mg/Kg)
PF-05212384 (10mg/Kg) +Cisplatin (3mg/Kg)

	TGI%
PF-05212384	-1%
Cisplatin	97%
PF-05212384 + Cis	92%

Figure 2

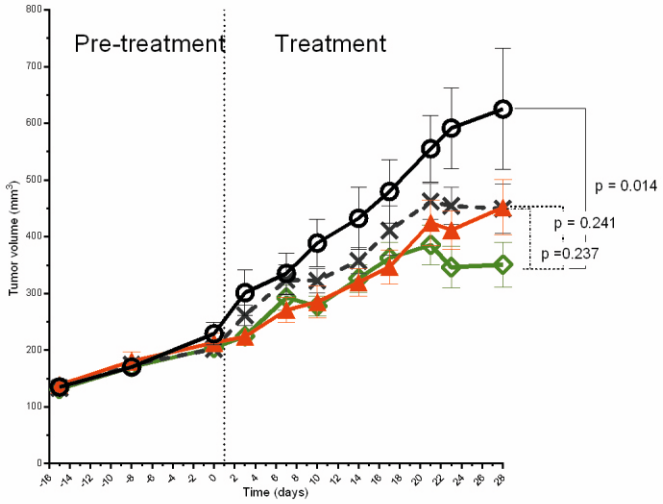
A TNBC



Vehicle
PF-05212384 (10 mg/kg)
Paclitaxel (10 mg/kg)
PF-05212384 (10 mg/kg) + paclitaxel (10 mg/kg)

	TGI %
PF-05212384	22%
Paclitaxel	84%
PF-05212384 + Paclitaxel	110%

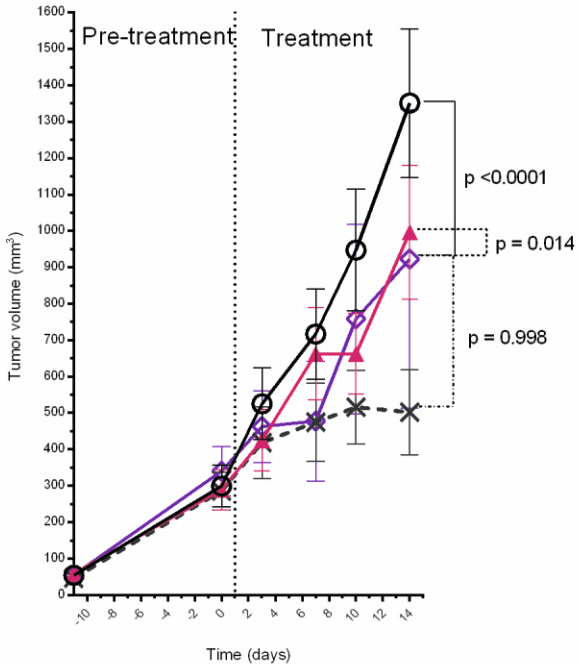
B LGSOC
KRAS G12R mutant



Vehicle
PF-05212384 (10 mg/kg)
Paclitaxel (10 mg/kg)
PF-05212384 (10 mg/kg) + paclitaxel (10 mg/kg)

	TGI %
PF-05212384	40%
Paclitaxel	38%
PF-05212384 + Paclitaxel	56%

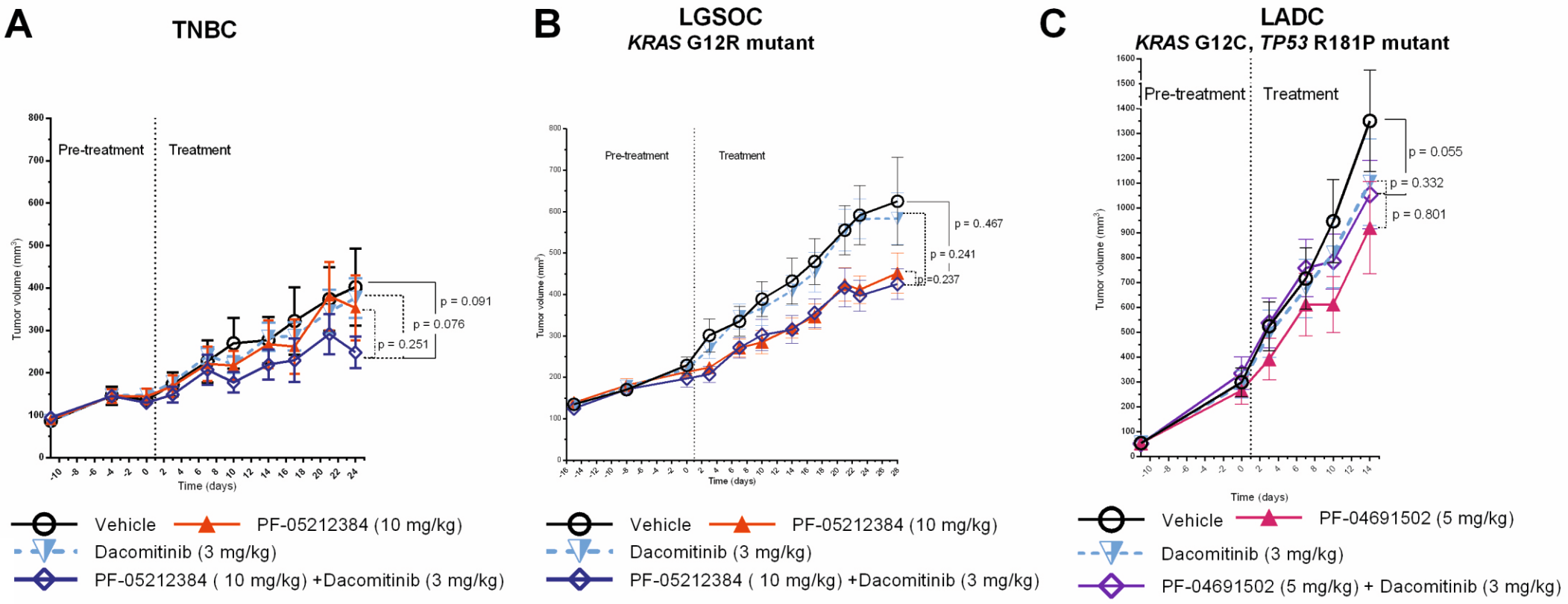
C LADC
KRAS G12C, *TP53* R181P mutant



Vehicle
PF-04691502 (5 mg/kg)
Paclitaxel (10 mg/kg)
PF-04691502 (5 mg/kg) + Paclitaxel (10 mg/kg)

	TGI %
PF-04691502	33%
Paclitaxel	79%
PF-04691502 + Paclitaxel	45%

Figure 3

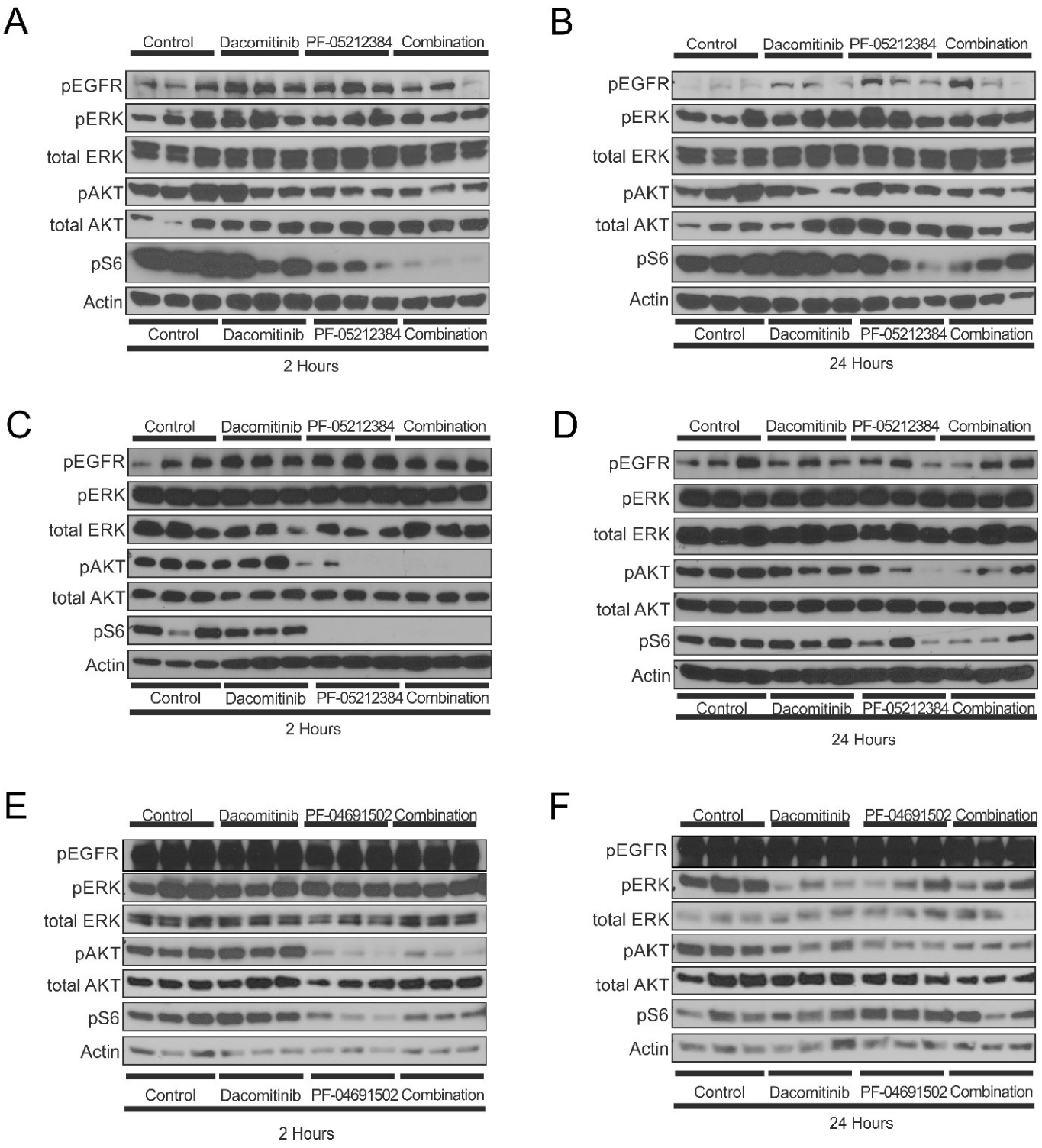


	TGI %
PF-05212384	22%
Daccomitinib	15%
PF-05212384 + Dac omitinib	55%

	TGI %
PF-05212384	40%
Dacomitinib	8%
PF-05212384 + Dac omitinib	45%

	TGI %
PF-04691502	33%
Dacomitinib	23%
PF-04691502 + Dac omitinib	32%

Figure 4


A
Control
Dacomitinib
PF-05212384
Combination
pEGFR
pERK
total ERK
pAKT
total AKT
pS6
Actin
Control
Dacomitinib
PF-05212384
Combination
2 Hours
B
Control
Dacomitinib
PF-05212384
Combination
pEGFR
pERK
total ERK
pAKT
total AKT
pS6
Actin
Control
Dacomitinib
PF-05212384
Combination
24 Hours
C
Control
Dacomitinib
PF-05212384
Combination
pEGFR
pERK
total ERK
pAKT
total AKT
pS6
Actin
Control
Dacomitinib
PF-05212384
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2 Hours
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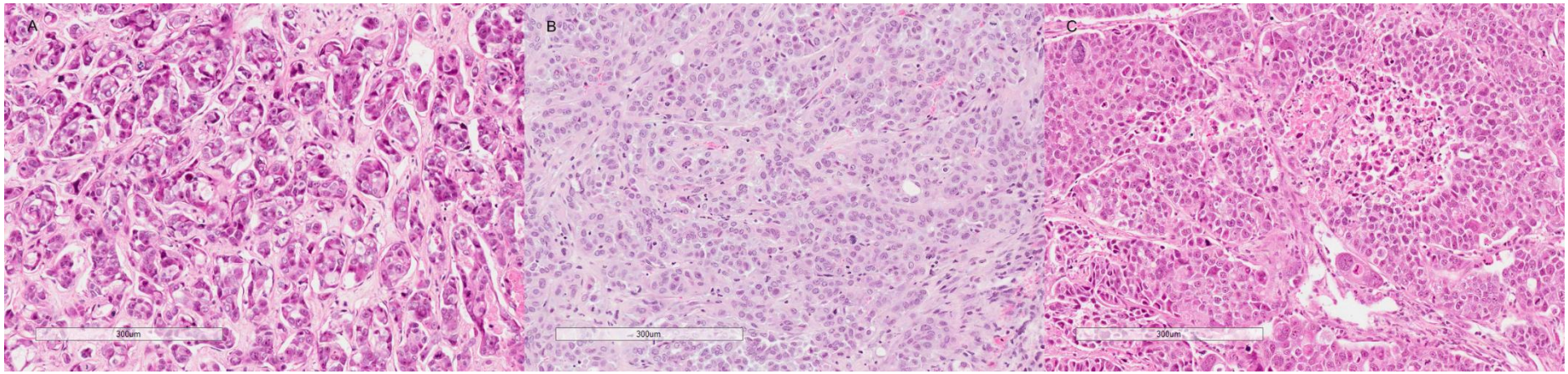
Dacomitinib
PF-05212384
Combination
pEGFR
pERK
total ERK
pAKT
total AKT
pS6
Actin
Control
Dacomitinib
PF-05212384
Combination
24 Hours
E
Control
Dacomitinib
PF-04691502
Combination
pEGFR
pERK
total ERK
pAKT
total AKT
pS6
Actin
Control
Dacomitinib
PF-04691502
Combination
2 Hours
F
Control
Dacomitinib
PF-04691502
Combination
pEGFR
pERK
total ERK
pAKT
total AKT
pS6
Actin
Control
Dacomitinib
PF-04691502
Combination
24 Hours

SUPPLEMENTARY MATERIAL

Supplementary Figure 1

Hematoxylin and eosin stain of patient derived xenografts: A) Triple Negative Breast Cancer model; B) Low Grade Serous Ovaria Cancer model; C) Lung Adenocarcinoma model

Supplementary Figure 1



PROJECT 2: CARLUMAB, AN ANTI-C-C CHEMOKINE LIGAND 2 MONOCLONAL ANTIBODY, IN COMBINATION WITH FOUR CHEMOTHERAPY REGIMENS FOR THE TREATMENT OF PATIENTS WITH SOLID TUMORS: AN OPEN-LABEL, MULTICENTER PHASE 1B STUDY

4. PROJECT 2: CARLUMAB, AN ANTI-C-C CHEMOKINE LIGAND 2 MONOCLONAL ANTIBODY, IN COMBINATION WITH FOUR CHEMOTHERAPY REGIMENS FOR THE TREATMENT OF PATIENTS WITH SOLID TUMORS: AN OPEN-LABEL, MULTICENTER PHASE 1B STUDY

4.1 Hypothesis and Objectives

Hypothesis

The addition of carlumab to standard chemotherapy would be tolerable and would inhibit free CCL2 levels.

Objectives

1) To evaluate the safety and tolerability of two dose regimens of carlumab in combination with docetaxel, pegylated-liposomal doxorubicin (PLD), gemcitabine and carboplatin-paclitaxel

2) To assess the levels of CCL2 after the administration of carlumab

3) To evaluate the effect of carlumab on the pharmacokinetics of docetaxel, gemcitabine, PLD, and carboplatin-paclitaxel.

4) To evaluate potential pharmacodynamics markers such as urinary cross-linked N-telopeptide of type I collagen.

5) To evaluate carlumab immunogenicity.

6) To evaluate the preliminary response rate of carlumab in combination with docetaxel, gemcitabine, PLD, and carboplatin-paclitaxel in patients with advanced solid-tumors.

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ORIGINAL RESEARCH

Carlumab, an anti-C-C chemokine ligand 2 monoclonal antibody, in combination with four chemotherapy regimens for the treatment of patients with solid tumors: an open-label, multicenter phase 1b study

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Abstract C-C chemokine ligand 2 (CCL2) stimulates tumor growth, metastasis, and angiogenesis. Carlumab, a human $IgG_1\kappa$ anti-CCL2 mAb, has shown antitumor activity in preclinical and clinical trials. We conducted a first-in-human phase 1b study of carlumab with one of four chemotherapy regimens (docetaxel, gemcitabine, paclitaxel+carboplatin, and pegylated liposomal doxorubicin HCl [PLD]). Patients had advanced solid tumors for which ≥1 of these regimens was considered standard of care or for whom no other treatment options existed. Dose-limiting toxicities included one grade 4 febrile neutropenia (docetaxel arm) and one grade 3 neutropenia (gemcitabine arm). Combination treatment with carlumab had no clinically relevant pharmacokinetic effect on docetaxel (n=15), gemcitabine (n=12), paclitaxel or carboplatin (n=12), or PLD (n=14). Total serum CCL2 concentrations increased post-treatment with carlumab alone, consistent with carlumab-CCL2 binding, and continued increase in the presence of all chemotherapy regimens. Free CCL2 declined immediately post-treatment with carlumab but increased with further chemotherapy administrations in all arms, suggesting that carlumab could sequester CCL2 for only a short time. Neither antibodies against carlumab nor consistent changes in circulating tumor cells (CTCs) or circulating endothelial cells (CECs) enumeration were observed. Three of 19 evaluable patients showed a 30 % decrease from baseline urinary cross-linked N-telopeptide of type I collagen (uNTx). One partial response and 18 (38 %) stable disease responses were observed. The most common drug-related grade ≥3 adverse events were docetaxel arm—neutropenia (6/15) and febrile neutropenia (4/15); gemcitabine arm—neutropenia

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(2/12); paclitaxel+carboplatin arm—neutropenia, thrombocytopenia (4/12 each), and anemia (2/12); and PLD arm—anemia (3/14) and stomatitis (2/14). Carlumab could be safely administered at 10 or 15 mg/kg in combination with standard-of-care chemotherapy and was well-tolerated, although no long-term suppression of serum CCL2 or significant tumor responses were observed.

Keywords Carlumab · Chemokine CCL2 · Combination chemotherapy · Clinical trial · Phase 1 · Solid tumors

Introduction

Human C-C chemokine ligand 2 (CCL2) is one of the most commonly observed chemokines in malignant solid tumors [1]. A chemoattractant for T-cells, monocytes [2], fibrocytes [3, 4], and natural killer cells [5], CCL2 mediates its actions by binding to C-C chemokine receptor 2 (CCR2), a G-protein-coupled, 7-transmembrane-spanning receptor [6]. By drawing monocytes—in which CCR2 is highly expressed [7]—to tumors, CCL2 leads monocytes to differentiate and become tumor-associated macrophages (TAMs) [7, 8]. Tumor-associated macrophages alter the surrounding tumor microenvironment [9] and promote tumor progression through metastasis, angiogenesis, and immunosuppression [10].

High levels of CCL2 expression have been associated with tumor aggressiveness [11], poor prognosis, early relapse [12], and, in some cancers—including breast [11, 12], prostate [13], ovarian [14], and pancreatic [15]—advanced-stage disease and disease worsening or progression. In prostate cancer, CCL2 was shown to be highly expressed in bone marrow endothelial cells [16, 17], and in vitro studies have found that CCL2 stimulates cancer cell migration and growth in both breast [18] and prostate [16] cancers. These findings, along with CCL2 involvement in TAM-mediated actions, suggest that neutralizing CCL2 may provide a novel approach to controlling tumor growth. In preclinical studies in prostate cancer, anti-CCL2 antibodies suppressed tumor growth [17, 19] and formation of bone lesions [17] in murine models and attenuated cancer cell migration in in vitro human models [16]. A murine melanoma model showed reduced tumor size and angiogenesis when treated with a CCL2 inhibitor [20]. Chemokine CCL2 neutralization also modulated tumor inflammation by reducing CD68-positive macrophage/monocyte infiltration into tumor tissue in patients with pancreatic cancer [15].

Carlumab (formerly CNTO 888) is a human immunoglobulin G_1 kappa monoclonal antibody [21] with high binding affinity and specificity for CCL2 [22]. Preclinical studies suggest that carlumab may offer beneficial antitumor properties when used with standard-of-care chemotherapies (docetaxel [19, 23], gemcitabine, paclitaxel+carboplatin [24], or pegylated liposomal doxorubicin HCl [PLD]) for patients with solid tumors. Additionally, carlumab was demonstrated to have an acceptable safety profile in previous phase 1 [21] and phase 2 [25] clinical studies when administered at up to 15 mg/kg.

The primary objective of this phase 1b study was to evaluate the safety and tolerability of two dose regimens of carlumab in combination with four standard-of-care chemotherapies in patients with solid tumors. Major secondary objectives were to determine the effect of carlumab on the pharmacokinetics of docetaxel, gemcitabine, paclitaxel+carboplatin, and PLD and to assess the pharmacokinetics of carlumab in combination with these chemotherapies. Other secondary objectives were to evaluate pharmacodynamic biomarkers, carlumab immunogenicity, and rate of response to combination therapy.

Patients and methods

Patient selection

Eligible patients were ≥18 years old and had a histologically or cytologically confirmed advanced or refractory solid tumor considered measurable or evaluable as defined by Response Evaluation Criteria in Solid Tumors (RECIST) v1.1, Eastern Cooperative Oncology Group (ECOG) performance status score ≤2, and adequate liver, renal, and bone marrow function. Additionally, patients receiving carlumab with either docetaxel, gemcitabine, or paclitaxel+carboplatin had to have ≤2 previous anticancer therapies. Previous therapy with the same chemotherapy was allowed. Patients receiving PLD had no limitations on the number of prior anticancer therapies, though it was recommended not to enroll any patient with a body surface area of >2.0 m^2.

Patients receiving carlumab with docetaxel, paclitaxel+carboplatin, or PLD were prohibited from any concomitant medication use that could significantly modulate hepatic drug metabolism via enzyme induction or inhibition from ≤2 weeks before the first study-agent dose through the treatment period. Patients receiving carlumab with paclitaxel+carboplatin or gemcitabine were prohibited from any medication use that could markedly affect renal function from ≤2 weeks before study entry through the treatment period.

Study design and drug administration

This study was an open-label, nonrandomized, multicenter, phase 1b clinical trial. The study was approved by the institutional review board or independent ethics committee at each site and was conducted in accordance with the ethical standards of the Declaration of Helsinki. All patients provided

written informed consent. Patients were assigned in a nonrandomized manner based on investigator judgment to one of four treatment arms, in which they received 15 mg/kg carlumab (Janssen Research & Development, LLC, Spring House, PA, USA) as a 90-min intravenous infusion every 3 weeks (q3w) with either (1) 75 mg/m^2 docetaxel q3w, (2) 1,000 mg/m^2 gemcitabine q3w, or (3) 175 mg/m^2 paclitaxel+ carboplatin dosed to area under serum concentration versus time curve (AUC) 6 q3w or received (4) 10 mg/kg carlumab every 2 weeks (q2w) with 50 mg/m^2 PLD (Doxil/Caelyx) every 4 weeks. Patients were treated until disease progression, treatment delay of ≥2 weeks or ≥2 episodes of treatment delay, discontinuation of any part of the combination therapy, or any relevant toxicity. Toxicities warranting discontinuation of study treatment included arterial thromboembolism, gastrointestinal perforation or fistula, grade ≥3 wound dehiscence or symptomatic bleeding, severe infusion reactions, or any toxicity considered relevant by the investigator.

The two carlumab dose regimens were based on preliminary pharmacokinetic data from a previous phase 1 study [21] and the compatibility of these treatment regimens with those of the chemotherapeutic agents. For cycle 1, chemotherapy and carlumab were administered on days 1 and 3, respectively, allowing for a 2-day period to evaluate pharmacokinetics of chemotherapy alone. For all subsequent cycles, both chemotherapy and carlumab—in that order—were administered on day 1, though gemcitabine was additionally given on day 8 and, in the PLD arm, carlumab was additionally given on day 15. The previous study also showed that 10 or 15 mg/kg q2w were safe and well-tolerated dose regimens for carlumab, and clinical biomarker and tumor penetration data did not support using a lower dose [21]. Additionally, these dose regimens were predicted to achieve steady-state trough serum concentrations above the preclinical target of 10 μg/mL following the first dose, and no preclinical evidence suggested that chemotherapy could affect the pharmacokinetic profile for carlumab. Because no evidence suggested that carlumab could significantly affect renal or hepatic elimination pathways involved in metabolism or excretion, the most commonly used clinical doses and schedules of the selected chemotherapies were evaluated [26].

Patients were required to meet the following retreatment criteria before study administration on day 1 of each cycle: absolute neutrophil count ≥1.5×10^9/L (1,500/mm^3); platelet count ≥100,000/mm^3; hemoglobin ≥9.0 g/dL; total bilirubin ≤1.5×upper limit of normal (ULN); aspartate aminotransferase or alanine aminotransferase ≤3.0×ULN; serum creatinine ≤1.5×ULN or calculated glomerular filtration rate >60 mL/min/1.73 mm^2; alkaline phosphatase <2.5×ULN for patients in the docetaxel arm; and any other clinically significant toxicity must have recovered to grade ≤2 or pretreatment grade. All patients were followed at 4 and 8 weeks after the last carlumab dose to assess safety, efficacy, and immunogenicity.

Safety and dose-limiting toxicity evaluations

Dose-limiting toxicity was defined as any toxicity-related delay in the start of cycle 2 treatment for ≥2 weeks or any grades 3−4 hematologic/nonhematologic toxicity during cycle 1. The following known chemotherapy-related toxicities were excluded from defining dose-limiting toxicity (DLT): afebrile neutropenia of grade 3 (any duration) or 4 (≤5 days); grade 3 anemia; grade 3 clinically nonsignificant transient lab abnormalities resolved before next drug administration; grade 3 nausea, vomiting, or diarrhea responsive to symptomatic treatment; and grade 3 fatigue that was grade 1−2 before dose administrations.

Safety data were evaluated according to a cohort expansion scheme in which six patients were enrolled in each treatment arm. If ≤1 patient experienced a DLT during the first full cycle, six more patients were enrolled. If ≥2 patients experienced a DLT in this cycle, six more patients were enrolled but treated with the same dose of carlumab and a reduced chemotherapy dose. Among these additional patients, if ≤1 patient experienced a DLT, six more patients were enrolled and treated at this same dose. If ≥2 patients experienced a DLT, six more patients were enrolled and treated at reduced doses of carlumab and chemotherapy; if no further DLTs were experienced, then six more patients were to be enrolled and treated at both reduced doses.

Carlumab-treated patients who completed the safety follow-up from start of the first carlumab dose through the first full dosing cycle of combination therapy were evaluable for DLT. Any carlumab-treated patient who experienced a DLT, however, was considered evaluable for DLT and considered in the Study Evaluation Team's overall decisions on safety regardless of follow-up period.

Safety evaluations were based on adverse events (AEs), including DLTs, signs of clinical sequelae from lack of efficacy, allergic reactions, hypersensitivity, cytokine release syndrome, or acute infusion reactions. Adverse events were graded according to the National Cancer Institute—Common Terminology Criteria for Adverse Events, V4.0.

Pharmacokinetic evaluations

Validated, specific, and sensitive immunoassay methods (data on file) were used to analyze blood samples to determine serum concentrations of carlumab and plasma concentrations of docetaxel, gemcitabine, paclitaxel, carboplatin, and PLD. Reversed-phase high-performance liquid chromatography with mass spectrometry/mass spectrometry detection were used to determine concentrations of docetaxel, gemcitabine, paclitaxel, and PLD in human plasma. The lower limit of quantification (LLOQ) was 2.0 ng/mL for docetaxel samples, 0.50 ng/mL for gemcitabine samples, 2.0 ng/mL for paclitaxel samples, and 1.0 ng/mL for PLD samples. A validated assay

method using inductively coupled plasma mass spectrometry (data on file) was used to determine platinum concentration from carboplatin samples in human plasma (carboplatin was quantified as total platinum). The LLOQ for total platinum concentration was 2.0 ng/mL. A validated assay method based on an enzyme-linked immunosorbent assay format (data on file) was used to determine serum concentrations of carlumab, the lowest quantifiable concentration of which was 0.07810 μg/mL.

Blood samples to determine plasma concentration of chemotherapy alone were collected after the first chemotherapy dose at selected time points, while those to determine concentration of chemotherapy in combination with carlumab were collected after the second chemotherapy dose. Additional samples were collected before and after chemotherapy infusion in cycles 3 and 4. Serum samples to determine serum concentration of carlumab were collected before and at the end of infusion for cycles 2 (and selected time points after cycle 2), 3, and 4.

Pharmacokinetic data analysis methods are described in supplementary material (Online Resource 1). A statistical analysis of maximum observed serum concentration (C_{max}) and $AUC_{(0–48\ h)}$ was conducted for patients with paired data in cycles 1 and 2 to show the effect of carlumab on each chemotherapy pharmacokinetic profile.

Immunogenicity evaluations

A validated immunoassay (Janssen Research & Development, LLC) was used to detect antibodies against carlumab. Serum samples were collected from all enrolled patients before the first carlumab dose, at 4 and 8 weeks after the last carlumab dose, and at any time when an infusion reaction was observed or reported during the study.

Pharmacodynamic assessment

Blood samples were analyzed for target inhibition by carlumab with combination therapy by measuring total and free CCL2 concentrations in serum. Total serum CCL2 was measured using a bead-based, multiplex method (Myriad RBM). The Meso Scale Discovery plate-based method was used to measure free CCL2. Bone remodeling markers in urine were evaluated for osteoclastic and osteoblastic activity (urinary cross-linked *N*-telopeptide of type I collagen [uNTx]), whereby uNTx response was defined as a ≥30 % reduction from baseline, confirmed by a second uNTx value ≥3 weeks later. Circulating tumor cells (CTCs) and circulating endothelial cells (CECs) were analyzed from whole blood pre- and post-treatment using the CellSearch assay (Veridex, LLC).

Efficacy evaluations

Radiological disease assessments (computed tomography [CT]/magnetic resonance imaging) were conducted every 8 weeks for patients in the PLD arm and every 9 weeks for patients in the docetaxel, gemcitabine, and paclitaxel+carboplatin arms after the first dose. Best response was assessed by the local investigator according to RECIST guidelines v1.1 [27]. Patients with known bone metastasis at screening had radionuclide bone scans performed. Repeat assessments were performed ≥4 weeks from the initial observation to confirm a complete response (CR) or partial response (PR). For a response to qualify as stable disease, follow-up measurements had to meet the stable disease criteria at least once at a minimum interval of ≥8 weeks.

Statistical analysis

A sample size of 12 patients per treatment arm was chosen to allow for sufficient data to be collected for DLT evaluation of each arm. No formal statistical analyses were planned. Descriptive statistics were used to summarize study data.

Results

Baseline patient characteristics

Of the 76 patients enrolled between May 2010 and November 2010 in this multicenter (two US and two Spain) study, 53 patients were treated. Twenty-three patients were not treated due to screening failure (n=19), consent withdrawal (n=3), or financial coverage issues (n=1).

Among treated patients, the median age was 62 (range, 40–81) years, and 31 of 53 patients were male. Most (51 of 53) patients were Caucasian, and 44 were of Hispanic or Latino ethnicity. Of the 53 patients, 47 had prior chemotherapy, 25 had prior radiotherapy, and 41 had previous cancer-related surgery. Two patients in the docetaxel arm, three patients in the gemcitabine arm, and one patient in the PLD arm were treated with the same respective chemotherapy prior to the study. Eleven patients in the paclitaxel+carboplatin arm received prior platinum-based chemotherapy (data on file). Twenty-four and 26 of 53 patients reported an ECOG performance status score of 0 or 1, respectively, at study entry (Table 1).

The most common reasons for discontinuation from study treatment or participation were disease progression (n=37), AEs (n=7), and physician decision (n=5), while two patients discontinued for other reasons (one death and one refusal of further treatment).

Table 1 Patient demographics and disease characteristics at baseline

	Carlumab+docetaxel	Carlumab+gemcitabine	Carlumab+paclitaxel+carboplatin	Carlumab+PLD
Patients treated	15	12	12	14
Age, years				
Median (range)	61.0 (46, 70)	66.0 (43, 81)	62.0 (40, 70)	61.5 (46, 76)
Gender (men:women)	13:2	5:7	5:7	8:6
Race				
Caucasian	14	11	12	14
Black	1	1	0	0
Hispanic/Latino	12	9	11	12
Tumor type				
Pancreas	7	3	1	1
NSCLC	0	2	0	1
Prostate	1	0	0	0
Other[a]	7	7	11	12
Patients with prior chemotherapy	14	8	11	14
Patients with prior radiotherapy	9	3	6	7
ECOG performance scale				
0	6	4	7	7
1	8	7	4	7
2	1	1	1	0

All values are presented as *n* unless otherwise specified

ECOG Eastern Cooperative Oncology Group, *NSCLC* non-small cell lung cancer, *PLD* pegylated liposomal doxorubicin HCl

[a] Includes the following primary tumor types: anal (*n*=1), breast (*n*=1), cervix (*n*=2), cholangiocarcinoma (*n*=6), colorectal (*n*=12), cutaneous or other melanoma (*n*=2), gastric (*n*=2), maxilar sinus carcinoma (*n*=1), ocular melanoma (*n*=3), small cell lung cancer (*n*=2), squamous cell carcinoma (*n*=1), squamous cell carcinoma of the head and neck (*n*=2, including one supraglottic larynx carcinoma), thyroid (*n*=1), and uterine cancer (*n*=1)

DLTs

One DLT was observed in the docetaxel arm, which involved a grade 4 febrile neutropenia episode that occurred 6 days after the first chemotherapy dose and 4 days after the first carlumab dose. The episode lasted 2 days and evolved into a grade 4 neutropenia without fever, which lasted 5 days. The investigator determined the event to be probably related to both carlumab and docetaxel, and the patient continued the study at the same carlumab dose and a reduced docetaxel dose.

One DLT was also observed in the gemcitabine arm, which involved a grade 3 neutropenia episode that developed 6 days after the first chemotherapy dose and 4 days after the first carlumab dose and lasted 8 days. Notwithstanding the per-protocol exclusion of grade 3 neutropenia as a DLT, the episode was determined to be a DLT because of its recurrent and persistent nature despite dose reduction. The investigator determined the episode to be unrelated to carlumab and probably related to gemcitabine. The patient continued the study at the same carlumab dose and a reduced gemcitabine dose.

Since these were the only DLTs observed in both treatment arms, no expansions with dose adjustments were made, no additional dose levels were explored, and all four treatment arms enrolled another minimum of six patients.

Safety

Patient exposure to chemotherapy and carlumab is described in ESM Table 1. The number of chemotherapy administrations ranged from 1 to 29 (median range, 2–6), while the median durations ranged from 29.0 to 71.5 days. The median duration for carlumab exposure was 43.0 (range, 3–308) days.

Adverse events (any grade) considered by the investigator to be reasonably related to carlumab and/or chemotherapy treatment (i.e., drug-related) were observed in 13 of 15 patients in the docetaxel arm, 10 of 12 patients in the gemcitabine arm, 12 of 12 patients in the paclitaxel+carboplatin arm, and 13 of 14 patients in the PLD arm. The most frequently reported drug-related AEs for the docetaxel arm were neutropenia (6 of 15); stomatitis (5 of 15); and fatigue, febrile neutropenia, and alopecia (4 of 15 each). Those for the gemcitabine arm were anemia (5 of 12) and neutropenia, nausea, asthenia, decreased appetite, and arthralgia (2 of 12 each). Those for the paclitaxel+carboplatin arm were thrombocytopenia (8 of 12); alopecia (6 of 12); neutropenia, asthenia, and fatigue (5 of 12 each); anemia and arthralgia (4 of 12); and nausea, vomiting, paraesthesia, and peripheral sensory neuropathy (3 of 12 each). Those for the PLD arm were

stomatitis and rash (6 of 14); fatigue (4 of 14); and nausea, anemia, and neutropenia (3 of 14 each; Table 2).

Adverse events of grades 3−4 considered by the investigator to be reasonably related to carlumab and/or chemotherapy treatment (i.e. drug-related) were observed in 8 of 15 patients in the docetaxel arm; the most frequently reported were neutropenia (6 of 15) and febrile neutropenia (4 of 15). Drug-related AEs of grade 3 were observed in 5 of 12 patients in the gemcitabine arm, the most frequent of which was neutropenia (2 of 12). Drug-related AEs of grades 3−4 were observed in 8 of 12 patients in the paclitaxel+carboplatin arm; the most frequently reported were neutropenia and thrombocytopenia (4 of 12 each) and anemia (2 of 12). Drug-related AEs of grade 3 were observed in 6 of 14 patients in the PLD arm, the most frequent of which were anemia (3 of 14) and stomatitis (2 of 14; Table 2). No grade 4 AEs were observed in the gemcitabine or PLD arms.

In the gemcitabine arm, 2 of 12 patients discontinued treatment due to the following drug-related AEs: grade 2 splenic infarction (n=1) and grade 2 asthenia (n=1). In the paclitaxel+carboplatin arm, 2 of 12 patients discontinued treatment due to drug-related grade 4 thrombocytopenia (n=1) and grade 3 neutropenia (n=1).

Infusion reactions were observed in 1 of 15 patients in the docetaxel arm (grade 1 hyperhidrosis; very likely related to carlumab but not docetaxel); 2 of 12 patients in the paclitaxel+carboplatin arm (grade 3 back pain and grade 1 phlebitis; very likely related to paclitaxel and to carlumab, respectively); and 2 of 14 patients in the PLD arm (grade 2 back pain and grade 1 cough and flushing; very likely related and possibly related to PLD, respectively, but not to carlumab). For the phlebitis and hyperhidrosis cases, neither carlumab nor chemotherapy dose was changed or interrupted. For the grade 2 back pain and cough and flushing cases, chemotherapy administration was interrupted. For the grade 3 back pain case, paclitaxel but not carboplatin administration was interrupted. All events resolved without sequelae following standard medical care and administration of concomitant standard treatments.

Forty-one of 53 patients (12 docetaxel, 8 gemcitabine, 9 paclitaxel+carboplatin, and 12 PLD) had progressive disease (PD) or died. Of these, seven patients (four docetaxel, one

Table 2 Drug-related adverse events

	Carlumab+docetaxel		Carlumab+gemcitabine		Carlumab+paclitaxel+carboplatin		Carlumab+PLD	
Patients treated, *n*	15		12		12		14	
Patients experiencing ≥1 drug-related AE, *n*	13		10		12		13	
	All Grades	Grade ≥3	All Grades	Grade ≥3	All Grades	Grade ≥3	All Grades	Grade ≥3
Adverse events, *n*[a]								
Hematological								
Anemia	3	0	5	1	4	2	3	3
Neutropenia	6	6	2	2[b]	5	4	3	1
Febrile neutropenia	4	4[b]	0	0	1	1	0	0
Thrombocytopenia	2	1	0	0	8	4	2	0
Nonhematological								
Fatigue	4	1	0	0	5	1	4	1
Asthenia	2	0	2	1	5	0	2	0
Stomatitis	5	0	0	0	2	0	6	2
Nausea	2	0	2	0	3	0	3	0
Vomiting	2	0	1	0	3	0	0	0
Alopecia	4	0	0	0	6	0	1	0
Arthralgia	3	0	2	0	4	0	2	0
Peripheral sensory neuropathy	3	0	1	1	3	0	0	0
Decreased appetite	0	0	2	0	2	0	2	0
Rash	0	0	0	0	1	0	6	1

PLD pegylated liposomal doxorubicin HCl

[a] Reported in ≥5 patients

[b] One grade 4 febrile neutropenia observed in the docetaxel arm and one grade 3 neutropenia observed in the gemcitabine arm were each considered dose-limiting toxicities (DLTs). Since these were the only DLTs observed in each treatment arm, no expansions with dose adjustments were made and no additional dose levels were explored

gemcitabine, and two PLD) died, four of whom died due to PD that was unrelated to the study agents. Two patients had AEs with an outcome of death—one suicide (docetaxel arm) and one pneumonia resulting in respiratory septic shock (PLD arm). One patient (docetaxel arm) had an intracranial bleed, which occurred after study drug discontinuation and resulted in death. All deaths occurred >30 days after the last dose and were determined by investigators to be unrelated to carlumab or chemotherapy. Across all treatment arms, median progression-free survival (PFS) was 65.0 days (95 % confidence interval [CI] 63.0, 92.0); PFS data were censored for 12 of 53 patients. For overall survival, data were censored for 46 of 53 patients. Due to limited week-8 follow-up assessments, median overall survival was not estimable.

Pharmacokinetics

Median concentration values for each chemotherapeutic agent are presented in ESM Fig. 1. Pharmacokinetic parameter estimates for carlumab are shown in Table 3. Following the second carlumab dose, serum samples were collected at numerous specified time points prior to the third dose. The C_{max} was evaluable for all treated patients across all treatment arms. Due to patients stopping therapy prior to the third carlumab dose, the $AUC_{(0–21\ days)}$ was only evaluable in 29 of 53 patients across the treatment arms.

The intersubject variability, defined as the SD divided by the respective mean value times 100, was 17 % for C_{max} and 28 % for $AUC_{(0–21\ days)}$ in the docetaxel arm, 18 % for C_{max} and 20 % for $AUC_{(0–21\ days)}$ in the gemcitabine arm, 29 % for C_{max} and 32 % for $AUC_{(0–21\ days)}$ in the paclitaxel+carboplatin arm, and 27 % for C_{max} in the PLD arm. The mean C_{max} and $AUC_{(0–21\ days)}$ values were similar across treatment arms.

A statistical analysis of C_{max} and $AUC_{(0–48\ h)}$ in patients with paired data in cycles 1 and 2 is presented in Table 4. As the ratio of geometric means (cycle 2/cycle 1) was between 80 and 125 %, carlumab did not substantially affect C_{max} and $AUC_{(0–48\ h)}$ for docetaxel, paclitaxel, carboplatin, or PLD. Additionally, while the 90 % CI measurements for C_{max} and $AUC_{(0–48\ h)}$ for carboplatin and PLD were within 80 to 125 %, the 90 % CIs for docetaxel and paclitaxel C_{max} and $AUC_{(0–48\ h)}$ were outside this range, likely due to the small number of patients with paired data in each treatment arm (n=13, docetaxel; n=10, paclitaxel+carboplatin). A high degree of intersubject variability for C_{max} and $AUC_{(0–48\ h)}$, expressed as coefficient of variation, was also observed, ranging from 43 to 56 % for docetaxel and from 30 to 55 % for paclitaxel (Table 4).

For gemcitabine (n=8 for patients with paired data), C_{max} was 71 % higher in cycle 2 versus cycle 1, and the 90 % CI was outside the no-effect boundary of 100 %, ranging from 111 to 263 %. The $AUC_{(0–48\ h)}$, however, was 23 % higher in cycle 2 versus cycle 1, and the 90 % CI ranged from 82 to 186 %. Consistent with the wide-ranging 90 % CI, intersubject variability for gemcitabine C_{max} and $AUC_{(0–48\ h)}$ ranged from 55 to 117 % (Table 4).

Immunogenicity

Of the 38 patients across the four treatment arms who were evaluable for immunogenicity, none tested positive for antibodies against carlumab.

Pharmacodynamics

Total serum CCL2 concentrations did not increase with chemotherapy treatment alone. A large increase in total serum

Table 3 Carlumab pharmacokinetic parameter estimates, cycle 2

	Carlumab+docetaxel	Carlumab+gemcitabine	Carlumab+paclitaxel+carboplatin	Carlumab+PLD
Evaluable patients, *n*	15	12	12	14
$AUC_{(0–21\ days)}$, μg day/mL				
n	11	7	11	0
Mean±SD	1,747.32±494.814	1,597.64±316.148	1,964.06±653.293	NA±NA
Median	1,875.28	1,611.88	2,029.28	NA
Range	(908.4, 2,613.5)	(1,071.7, 2,003.4)	(1,083.6, 3,115.4)	(NA, NA)
C_{max}, μg/mL				
n	13	10	11	9
Mean±SD	283.81±47.620	250.66±46.215	306.16±87.281	233.57±63.756
Median	291.44	251.80	304.55	214.18
Range	(193.3, 344.2)	(174.7, 312.5)	(163.9, 446.6)	(139.9, 361.2)

AUC area under serum concentration versus time curve, C_{max} maximum observed serum concentration, *NA* not available, *PLD* pegylated liposomal doxorubicin HCl

Table 4 Carlumab effect on chemotherapy pharmacokinetic parameters

	Carlumab+docetaxel		Carlumab+gemcitabine		Carlumab+paclitaxel+carboplatin				Carlumab+PLD	
					Paclitaxel		Carboplatin			
	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1
Evaluable patients, *n*	15	15	12	12	12	12	12	12	14	14
C_{max}, ng/mL										
n	13	15	8	12	10	12	11	12	9	14
Mean±SD	2,134.17± 1,064.594	2,369.87± 1,008.528	14,621.20± 9,997.649	11,076.75± 12,988.75	3,541.45± 1,312.894	3,745.77± 1,393.894	24,390.02± 7,535.947	24,268.82± 7,924.711	41,978.21± 6,751.073	42,815.49± 8,475.470
Median	2,289.95	2,328.68	11,922.16	8,229.86	3,670.02	3,882.69	20,924.00	22,892.43	41,302.29	45,702.93
Range	588.5, 4,703.4	1,250.9, 5,209.8	7,441.7, 37,961.5	2,082.3, 51,033.4	661.8, 5,361.6	1,524.2, 6,752.5	14,943.4, 35,513.7	13,416.5, 41,033.2	33,728.9, 53,005.6	25,088.5, 54,048.6
Coefficient of variation, %	50	43	68	117	37	37	31	33	16	20
Geometric mean	1,786.8	2,215.0	13,453.4	7,858.8	3,372.1	3,484.7	23,207.8	23,189.5	41,942.2	41,935.0
Ratio of geometric means[a]		80.7		171.2		96.8		100.1		100.0
90 % CI		64.28, 101.24		111.39, 263.10		68.13, 137.44		88.38, 113.33		97.18, 102.94
$AUC_{(0–48\ h)}$, ng h/mL										
n	13	15	7	11	10	12	10	12	9	14
Mean±SD	2,598.70± 1,460.615	2,804.95± 1,283.905	6,589.09± 3,652.138	5,719.84± 4,892.217	6,212.67± 1,872.089	6,983.86± 3,835.222	109,822.5± 22,313.74	105,318.6± 22,528.40	1,538,776± 397,449.5	1,517,369± 497,230.3
Median	2,299.96	2,550.28	6,113.22	5,000.78	6,931.53	6,154.96	111,249.46	97,692.05	1,483,809.3	1,709,483.5
Range	1,048.6, 6,821.1	1,288.3, 6,406.6	2,723.0, 13,944.7	1,597.9, 19,812.2	2,360.7, 8,167.7	2,882.7, 17,312.8	81,680.1, 146,872.5	75,756.8, 151,890.0	989,212.6, 2,136,200.5	477,091.2, 2,198,708.3
Coefficient of variation, %	56	46	55	86	30	55	20	21	26	33
Geometric mean	2,281.4	2,581.7	5,725.6	4,642.1	6,645.0	6,212.5	108,401.8	103,290.2	1,476,847.1	1,417,687.6
Ratio of geometric means[a]		88.4		123.3		107.0		104.9		104.2
90 % CI		72.22, 108.12		81.85, 185.85		87.12, 131.33		97.94, 112.46		98.50, 110.17

PLD pegylated liposomal doxorubicin HCl

[a] Ratio is the respective pharmacokinetic parameter of cycle 2 over cycle 1, corresponding to the chemotherapy given in combination with carlumab over the chemotherapy alone

CCL2 concentration was, however, observed at 2 h post-treatment with carlumab alone, consistent with the binding of carlumab to CCL2 (i.e., carlumab–CCL2 complex). Total CCL2 concentrations continued to increase with further doses of carlumab in combination with chemotherapy in all treatment arms (Fig. 1a–d). On the other hand, free serum CCL2 concentrations increased approximately twofold at 48 h after treatment with chemotherapy alone in each treatment arm; however, free CCL2 concentrations declined rapidly to the level of detection at 2 h post-treatment with carlumab alone. Median free CCL2 concentrations continued to increase with administrations of carlumab in combination with chemotherapy in all the treatment arms, suggesting that carlumab was able to sequester binding to CCL2 only for a short time period. Despite some variability, an overall trend of a rapid reduction in free CCL2 concentrations at 2 h after carlumab dose and subsequent three- to fivefold increase over baseline concentrations with further carlumab and chemotherapy doses remained consistent for all treatment arms (Fig. 2a–d).

Fifty-one of the 53 patients had evaluable CTCs at baseline. Forty-six of these patients had ≤5 CTCs at baseline, while 28 and 31 patients had ≤5 CTCs after two treatment cycles and at the week-4 follow-up assessment, respectively. No consistent changes in CEC enumeration were observed after two treatment cycles or at the week-4 follow-up assessment for any treatment arm.

Nineteen patients (five docetaxel, five gemcitabine, three paclitaxel+carboplatin, and six PLD) had evaluable uNTx values, three of whom showed a uNTx response. One patient from each of the docetaxel, paclitaxel+carboplatin, and PLD arms showed a 30 % decrease in uNTx from baseline.

Efficacy

A best overall response of stable disease was seen in 18 (4/13 docetaxel, 4/11 gemcitabine, 7/11 paclitaxel+carboplatin, and 3/13 PLD) of 48 evaluable patients, the duration (median [range]) of which was 6.3 (2.0,[1] 10.8) months—6.7 (3.5, 6.7) months for the docetaxel arm, 10.8 (2.0,[1] 10.8) months for the gemcitabine arm, 5.8 (2.1,[1] 6.5) months for the paclitaxel+carboplatin arm, and 3.7 (3.6, 7.0)months for the PLD arm. One PR by RECIST v1.1 criteria was observed in a patient with treatment-naive pancreatic carcinoma in the gemcitabine arm after seven cycles (4.2 months). Following PR (duration, 5.8 months), the investigator resected the tumor, and the patient discontinued the study after achieving CR from the surgery. An overall best response of PD was observed in 28 of 48 evaluable patients.

Four of the 48 patients had best overall responses of PR that were not confirmed by RECIST v1.1 and thus not counted as PRs. Of the four patients, one showed a PR lasting for 1.8 months that progressed to PD and led to discontinuation from the study, while two patients showed PRs after three and six cycles that lasted for nearly 2.5 and 2 months, respectively, which likewise led to PD and discontinuation from study. The fourth patient showed PR after approximately 4 months of treatment, which was confirmed via CT scan at two subsequent disease assessments and lasted for nearly 4 months.

[1] Censored

Discussion

This open-label, phase 1b clinical study evaluated the potential for anti-CCL2 treatment with carlumab in combination with four standard-of-care chemotherapy regimens (docetaxel, gemcitabine, paclitaxel+carboplatin, and PLD) in patients with advanced or refractory solid tumors. While the primary objective was to analyze the safety and tolerability of these treatment regimens, additional pharmacokinetic, pharmacodynamic, and tumor response assessments were also performed.

Safety assessment for carlumab was confounded by chemotherapy administration, and the contribution of carlumab to AEs could not be discerned from the effect of the co-administered chemotherapies. The safety profile was as expected for patients with late-stage disease treated with standard-of-care chemotherapies and for AEs commonly associated with the co-administered chemotherapy regimens. Although two DLTs occurred, neither resulted in changes in the carlumab dose regimen or treatment schedule at the time of treatment arm expansion.

Carlumab did not affect the pharmacokinetic parameters of C_{max} or $AUC_{(0–48\ h)}$ for docetaxel, paclitaxel, carboplatin, or PLD. Based on the ratio of geometric means, gemcitabine C_{max} did increase by approximately 71 % in cycle 2 compared with cycle 1, a difference indicated by the 90 % CI to be statistically significant. The $AUC_{(0–48\ h)}$, however, was only 23 % higher in cycle 2 than in cycle 1, a difference that was not statistically significant. Also, because only 8 of the 12 gemcitabine patients had paired samples in cycles 1 and 2, these findings should be viewed with caution.

Additionally, the intersubject variability for gemcitabine C_{max} and $AUC_{(0–48\ h)}$, respectively, ranged from 68 to 117 % and from 55 to 86 %. The high variability in C_{max} may be attributed to blood sample processing. Specifically, to prevent gemcitabine from being catabolized by cytidine deaminase before sample bioanalysis, tetrahydrouridine (THU)—a potent cytidine deaminase inhibitor [28]—had to be added to the sample. Although THU was added to all samples, the timing relative to sample processing varied. Despite an increased C_{max} in the presence of carlumab, no additional toxicities were observed when gemcitabine was administered in combination with carlumab. Therefore,

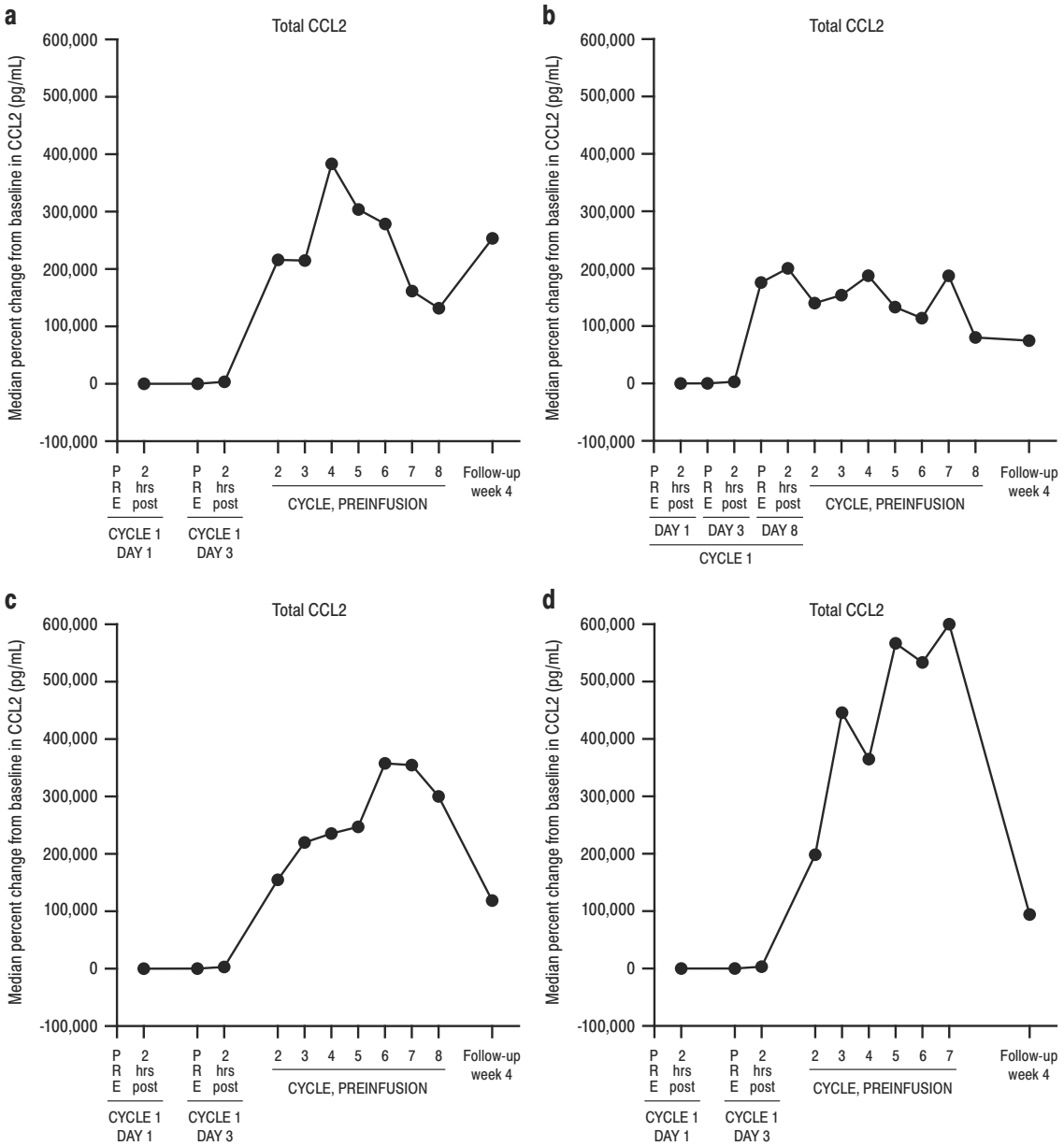

Fig. 1 Median percent change from baseline in total C-C chemokine ligand 2 (CCL2) serum concentration for patients in the **a** docetaxel arm, **b** gemcitabine arm, **c** paclitaxel+carboplatin arm, and **d** pegylated liposomal doxorubicin HCl arm

carlumab does not appear to have a clinically meaningful effect on gemcitabine pharmacokinetics.

Carlumab was administered at 15 mg/kg q3w in the docetaxel, gemcitabine, and paclitaxel+carboplatin treatment arms. The C_{max} and $AUC_{(0–21\ days)}$ values were within the ranges of previously reported single-agent pharmacokinetic data at the same dose [21, 25]. The minimum steady-state trough concentrations were approximately 50 % lower than the previously reported values (data not shown). In the previous studies, however, carlumab was administered once q2w and, with an observed terminal-phase half-life of approximately 7 days, these steady-state minimum concentrations are expected. Moreover, since carlumab was administered once q2w in the PLD arm, the minimum steady-state concentration can be directly compared with previous single-agent pharmacokinetic data. The minimum steady-state concentration for this arm, 52.15 μg/mL, was within the range of previously reported concentration data in the initial phase 1 study (data not shown). These data suggest that co-administering carlumab with standard-of-care chemotherapy regimens does not affect the pharmacokinetic profile of carlumab.

Pharmacodynamic and biomarker results showed that total serum CCL2 concentrations indicative of carlumab-CCL2

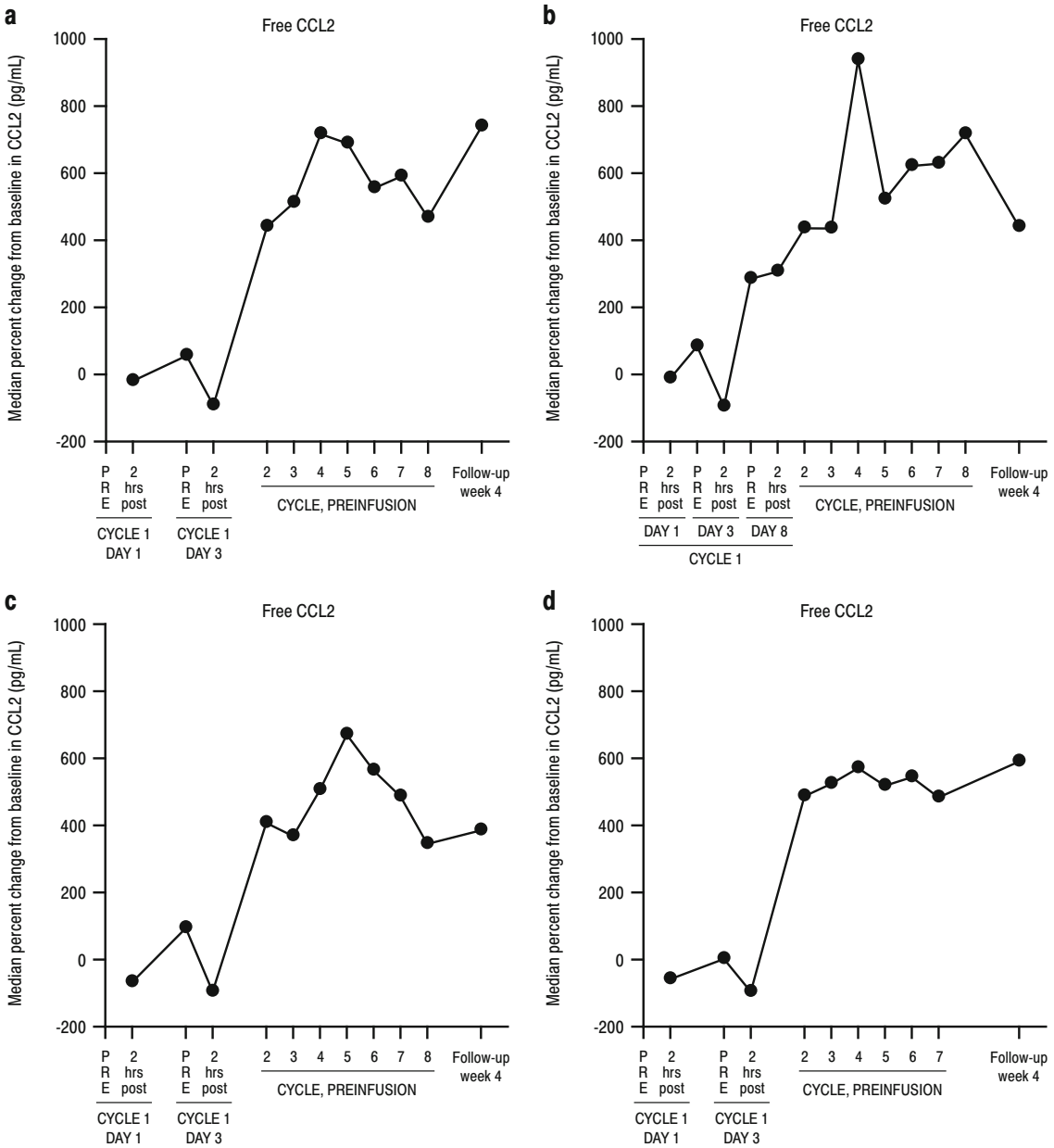

Fig. 2 Median percent change from baseline in free C-C chemokine ligand 2 (CCL2) serum concentration for patients in the **a** docetaxel arm, **b** gemcitabine arm, **c** paclitaxel+carboplatin arm, and **d** pegylated liposomal doxorubicin HCl arm

complex increased in combination with chemotherapy and that free serum CCL2 concentrations were only transiently suppressed after carlumab treatment, regardless of treatment arm. Taken together, these data suggest that carlumab was able to bind CCL2 but was ineffective at suppressing CCL2 for a clinically meaningful time period. The reason for this is unknown; however, the weak binding affinity of the antibody, resulting in a large amount of free CCL2 being dissociated from the carlumab–CCL2 complex, has been proposed as a potential explanation for increased free CCL2 concentrations in serum [25]. Furthermore, consistent with an inability to impact CCL2 signaling as demonstrated by previous biomarker data [21, 25], carlumab did not affect other biomarkers including uNTx or CTC enumeration. No AEs were determined to be associated with increased serum CCL2 concentrations.

The lack of sustained suppression of CCL2 was independent of the chemotherapy regimen and was similar to the patterns of transient CCL2 suppression found with carlumab treatment in a phase 1 study in a similar patient population [21, 29] and in a phase 2 study in patients with metastatic castration-resistant prostate cancer [25]. Additionally, the high baseline endogenous production of CCL2 coupled with the low-binding affinity of carlumab is sufficient to explain the

lack of clinical efficacy. Future studies to assess alternative dosing schedules of carlumab and assessment of duration of CCL2 suppression may be indicated before any further clinical investigation.

Evaluation of tumor response according to RECIST v1.1 showed no clinically significant responses aside from one patient with pancreatic carcinoma who had a PR after seven cycles of carlumab and gemcitabine and was able to undergo tumor resection following treatment. Additionally, four patients had PRs that were not confirmed by RECIST v1.1 and thus not counted as PRs but which lasted for as briefly as 56 days to as long as nearly 4 months. Efficacy evaluations, however, were limited by the small sizes of the study population and individual treatment arms, limited follow-up assessments, presence of different tumor types, and lack of a placebo or comparator arm to assess whether the combination treatment proved more effective. Therefore, no meaningful conclusions regarding efficacy (i.e., response and PFS) could be formed.

Although the study is limited by its lack of a placebo or an active comparator arm, this phase 1b design was unique in that it evaluated carlumab in combination with four separate standard-of-care chemotherapies using treatment arms run in parallel. Several advantages have been suggested for this design [30], such as running one multiarm trial under one protocol rather than four separate trials, thus minimizing costs, time, and efforts associated with study conduct [31]. This design can also expedite patient enrollment, particularly when a patient is already receiving or is a candidate for the standard-of-care chemotherapy [31]. The present study had an approximately 6-month recruitment period. The design also provides a potentially larger sampling of patients from whom to collect data pertaining to the study drug than might individual phase 1b studies [30, 31]. The present trial enrolled 53 patients (76 patients if including screening failures) and evaluated efficacy as well as safety and pharmacokinetics, whereas four separate phase 1 studies of carlumab in combination with docetaxel, gemcitabine, paclitaxel+carboplatin, and PLD followed by subsequent phase 2 studies for each would have likely required substantially higher patient enrollment and lengthier study durations.

In conclusion, the safety and pharmacokinetic results of this study showed that carlumab administered at 10 or 15 mg/kg with standard-of-care chemotherapies at commonly used dose regimens was safely administered and generally well tolerated in patients with solid tumors. This combination therapy, however, was ineffective in yielding durable, sustained suppression of serum CCL2 concentrations, leading to a steady rise in free CCL2 during treatment. Based on these findings and on evidence of minimal clinical activity, the combination of carlumab—on this dosing regimen—in combination with docetaxel, gemcitabine, paclitaxel+carboplatin, or PLD is not recommended for further clinical assessment.

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Conflict of interest Drs. Puchalski, Seetharam, Zhong, and de Boer are employees of Janssen, own stock in Johnson & Johnson, and/or are currently conducting research sponsored by Janssen. Antonio Calles is a Rio Hortega fellowship grant recipient from the Instituto de Salud Carlos III (CM09/00283). All remaining authors have declared no conflicts of interest.

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