Research Article

Pharmacodynamic Modeling of Sequence-Dependent Antitumor Activity of Insulin-like Growth Factor Blockade and Gemcitabine

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Abstract. Agents that block insulin-like growth factor (IGF) signaling are under investigation in clinical trials. Antitumor effects are likely to be enhanced when combined with other agents, but administration sequence effects on activity are not well-described. Three breast cancer cell lines (MCF-7, MDA-MB-231, and Hs-578T) were treated with Gemcitabine and small molecule receptor tyrosine kinase inhibitor cis-3-[3-(4-methyl-piperazin-1-yl)-cyclobutyl]1-(2-phenyl-quinolin-7-yl)-imidazo [1,5-a]pyrazin-8-ylamine (PQIP) as single agents and then in combination in the forward (Gemcitabine followed by PQIP) and reverse (PQIP followed by Gemcitabine) sequences. Antitumor effects were assessed longitudinally by Bayesian analysis using WinBUGS. The pharmacodynamic model adequately predicted the observed data. The differences in the cell-kill rate constants for the forward vs. reverse sequence ranged from 0.11 to 0.64 (day⁻¹), and statistical significance was generally dependent on cell line and PQIP concentration. These data indicate that treatment with Gemcitabine first, followed by PQIP is superior to the reverse sequence *in vitro*.

KEY WORDS: breast cancer; Gemcitabine; insulin-like growth factor; pharmacodynamic model; PQIP.

INTRODUCTION

A role for insulin-like growth factor (IGF) system in malignant transformation and stimulation of malignant phenotype has been established. Consequently, several small molecule inhibitors and antibodies targeting IGF system are being developed with clinical responses seen in phase I and II trials (1–3). The IGF system is composed of the circulating ligands, insulin-like growth factor-I (IGF-I), IGF-II, and insulin; multiple receptors, IGF-IR, IGF-IIR, insulin receptors (IR), and hybrid receptors composed of IGF-IR and IR; and binding proteins (4).

The insulin-like growth factor-I receptor (IGF-IR) is a tetrameric transmembrane receptor tyrosine kinase involved in embryonic development and postnatal growth with structural homology to insulin receptor. It is ubiquitously expressed by a wide variety of human tissues, and binds

IGF-I, IGF-II, and supraphysiological concentrations of insulin. Ligand activation of IGF-IR has been reported to stimulate proliferation, survival, transformation, metastasis, and angiogenesis. Ligand-binding activates the intrinsic tyrosine kinase activity of IGF-IR, which results in recruitment and phosphorylation of one of its two primary adaptor proteins, insulin receptor substrate 1 and sarcoma homology collagen-like docking protein. The phosphorylated adaptor proteins then serve as docking sites for other signaling molecules, resulting in the activation of the downstream pathways such as phosphatidylinositol 3'-kinase (PI3K) and the mitogen-activated protein kinase (MAPK). Both PI3K and MAPK influence key cell survival and proliferation events (4–7). IGF-1R has also been shown to mediate cell cycle reentry from a quiescent state through the induction of transforming growth factor α -mediated activation of epidermal growth factor receptor (5).

Cis-3-[3-(4-methyl-piperazin-1-yl)-cyclobutyl]1-(2-phenyl-quinolin-7-yl)-imidazo [1,5-a]pyrazin-8-ylamine (PQIP), is a potent and selective IGF-IR tyrosine kinase small molecule inhibitor. The molecule is an analogue to OSI-906, a compound under investigation in phase II clinical trials. PQIP is active against xenograft tumors grown in mouse models at concentrations that do not cause significant hyperglycemia (8).

Preclinical and clinical study results are mixed regarding the effects of IGF-1R blockade on response to chemotherapy (9, 10), although sequencing of IGF1R with the initiation of the cytotoxic insult may affect response (11). Since IGF-1R affects cell cycle progression, attention to the specific type of

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chemotherapy may be important. For instance, drugs that require active DNA synthesis for effect, *e.g.*, Gemcitabine, which exhibit cell cycle specificity by primarily killing cells undergoing DNA synthesis (S-phase) and also blocking the progression of cells through the G1/S-phase boundary, could be inhibited by IGF-1R signal inhibition due to inhibition of cell proliferation. In contrast, IGF-1R has been shown to signal to DNA repair mechanisms and it has been shown that inhibition of IGF-1R signaling may potentiate drugs that directly damage DNA. Hence, the potency of a particular drug combination may be, in part, associated with the sequence of administration for the IGF antagonist and a cytotoxic agent. The greatest anti-tumor effect of IGF-1R blockade is expected to be in combination regimens, but it is not clear how IGF-1R inhibitors should be combined with specific classes of chemotherapy. The purposes of these studies were to (1) evaluate the antitumor activity of PQIP and Gemcitabine as single agents *in vitro* and (2) to test the hypothesis that treatment with Gemcitabine first, followed by PQIP is associated with greater antitumor activity than the reverse sequence. Such information would ultimately inform the design of protocols for testing in clinical trials.

The methods of Chou and Talalay and use of Bliss independence/Lowe's additivity are widely accepted facile methods for determining combination indices (antagonism/additivity/synergy; 12). However, the estimations are reliant on cytotoxicity data collected at one time point, usually at the nadir of cell viability. Hence, information regarding rates of decline and recovery for cell viability are not included. A pharmacodynamic model that could account for these characteristics could inform decision-making regarding appropriate times for subsequent drug administration mimicking cycles of administration. Data analyses were performed by developing a pharmacodynamic model under Bayesian framework, which allows the incorporation of uncertainty in the parameter estimates in making inferences about the parameters or their functions.

MATERIALS AND METHODS

Reagents. Cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA) and fetal bovine serum (FBS), from Biosource (Rockville, MD, USA). Humulin R insulin was purchased from Lilly (Indianapolis, IN, USA) and IGF-1 was purchased from Novozymes. PQIP was kindly provided by OSI Pharmaceuticals and Gemcitabine was from Eli Lilly. Unless specified, all other reagents were purchased from Sigma (St. Louis, MO, USA).

Cell Lines and Culture. MCF-7, MDA-MB-231 and Hs-578T human breast cancer cell lines were used for the *in vitro* cell growth inhibition experiments and originally obtained from ATCC. MCF-7 were routinely maintained in Iscove's modified essential media with ZincOption (Richter's modification) with 10% FBS, and insulin to a final concentration of 0.068 ng/ml. MDA-MB-231 and Hs-578T cells were routinely maintained in Roswell Park Memorial Institute 1,640 media containing 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin.

In Vitro Cell Growth Inhibition Experiments. Cell lines were treated either with Gemcitabine or PQIP as single agents first. Upon completion of those experiments,

combination treatments were tested as described below. The exposure duration of Gemcitabine and PQIP, 1 and 24 h, respectively, was chosen based on the elimination half-lives of these drugs. Gemcitabine has very short elimination half-life in humans ranging from 10 to 30 min, hence in order to approximate the plasma exposure time observed in patients, 1-h exposure time was chosen for Gemcitabine. Ji *et al.* showed that PQIP concentrations of 2–4 µM were observed for approximately 16–24 h after oral administration in mice; hence, the exposure duration of PQIP was chosen to be 24 h (8). MCF-7 cells were plated in triplicate in 24-well tissue culture plates at a density of 30,000 cells per well and MDA-MB-231 and Hs-578T cells were plated at densities of 15,000 cells per well. Prior to and during treatment with PQIP, cells were switched to reduced serum media (0.5% FBS) containing 2 nM IGF. One day after plating, cells were treated with Gemcitabine for 1 h or PQIP for 24 h with concentrations listed in Table I. Immediately following drug exposure, the treatment media containing drug was replaced with drug-free growth media. The cell viability was measured using trypan blue staining in triplicate everyday following drug exposure until the wells became confluent. Cells were counted on a hemocytometer, and those excluding the blue dye were considered viable and those staining blue, nonviable. The experiments were terminated when the wells became confluent. Once sensitivity data were obtained following treatment with each single agent, concentrations were selected for testing in sequence of administration experiments. In these experiments, cells were either exposed to Gemcitabine first for 1 h followed 24 h later by PQIP for 24 h (GP), or in reverse sequence (PG). The concentration combinations of Gemcitabine and PQIP studied in sequence experiments are summarized in Table II. At each concentration combination, the GP and PG sequence was tested. Cell viability was measured following completion of treatment with both agents similar to the single agent experiments (described above). For each time point (*e.g.*, day), triplicate measures were taken and the mean number of independent triplicate measures per day was approximately three.

Pharmacodynamic Model. The effect of single agent Gemcitabine, PQIP, and the combinations on cell kill were evaluated by Bayesian analysis using WinBUGS Differential Interface, WBDiff, in WinBUGS (13). WBDiff is an interface that allows the numerical solution of ordinary differential equations (ODEs) within WinBUGS models. The individual treatments with Gemcitabine and PQIP were co-modeled for each cell line. The pharmacodynamic model for the time

Table I. The Concentrations of Gemcitabine and PQIP Tested as Single Agents in the Treatment of Cell Lines

Cell line	Drug	Concentrations (µM)
MCF-7	Gemcitabine	0.1, 1, 10, 100
	PQIP	0.5, 5, 20, 50
MDA-MB-231	Gemcitabine	0.1, 1, 10, 100
	PQIP	0.5, 5, 20, 35
Hs-578T	Gemcitabine	0.1
	PQIP	5, 20

Table II. The Concentrations of Gemcitabine and PQIP Tested as Combinations in the Treatment of Cell Lines. Both the Forward and Reverse Sequences were Tested for Each Pair

Cell line	Dose combinations
MCF-7	1 μ M G and 5 μ M PQIP
	10 μ M G and 0.5 μ M PQIP
MDA-MB-231	1 μ M G and 5 μ M PQIP
	10 μ M G and 0.5 μ M PQIP
	10 μ M G and 5 μ M PQIP
HS-578T	0.1 μ M G and 5 μ M PQIP

course of effects of each drug in single agent experiments was described by the following equations:

$$\frac{dA}{dt} = Kg \times \left(1 - \left(\frac{A}{A_{\max}}\right)\right) \times A - Kd \times A \quad (1)$$

$$Kd = Kd_0 \times \exp(-K_0 \times t) \quad (2)$$

$$Kd_0 = \frac{E_{\max} \times C^H}{EC_{50}^H C^H} \quad (3)$$

where, A represents the number of viable cells; $A_{\max}$ is the maximum number of cells at which the wells become confluent; Kg (day^{-1}) is the first-order rate constant for net growth of cells; Kd is the time-dependent function allowing a change in the cell-kill rate constant as time increases; Kd_0 (day^{-1}) is the concentration-dependent cell-kill rate constant at time=0, which, depends on drug concentration C (μM), $E_{\max}$ (day^{-1}), the maximum cell-kill rate constant; EC_{50} (μM); and Hill coefficient (H).

According to Eq. 1, the rate of change of viable cell number was explained by a first-order ODE, where rate of change of viable cell number at any time point is equal to the difference between the rate of cell growth and the rate of cell death. The rate of cell growth and cell death are proportional to the number of viable cells. The rate of cell growth was given by the term $Kg \times A$ and rate of cell death by $Kd \times A$. As the cell number increases in each well over time, the cell growth slows down due to the limitation of the availability of nutrients as well as space to grow. This feature of cell growth was captured by incorporating $(1 - (A/A_{\max}))$ in Eq. 1 above, which causes Kg to decrease as the number of viable cells increases. The exposure time for Gemcitabine and PQIP was 1 and 24 h, respectively, but the viable cell count continues to drop up to day 3 following drug exposures. This delay in the time course of exposure and cell death was modeled by using exponential function, Kd , shown in Eq. 2. Kd allows the cell death parameters, Kd_0 (obtained from Eq. 3) to decay off the model gradually over time instead of abruptly at the end of drug exposure time. K_0 in Eq. 2 and K_{02} in Eq. 5 describes the decay of Kd and Kd_2 , respectively, over time; when Kd and Kd_2 becomes zero cells will grow at the same rate as they would in the absence of drugs.

A sequential modeling approach was used to estimate the rate of cell kill with sequence of administration experi-

ments. Concentrations for the combinations were selected based initially upon data from single agent experiments. To observe for concentration effects, we tested combinations of relatively high Gemcitabine+low PQIP (e.g., 10 μM Gemcitabine+0.5 μM PQIP) and low Gemcitabine+high PQIP (e.g., 1 μM Gemcitabine+5 μM PQIP). The rate of cell growth was fixed to the posterior of the cell growth parameters, Kg and $A_{\max}$, determined from the single-agent data by using the “cut” logical function in WinBUGS. The rate of cell kill following administration sequence experiments was estimated by Eq. 4, where Kd_2 represents the rate constant for the total effect of the sequence.

$$\frac{dA}{dt} = Kg \times \left(1 - \left(\frac{A}{A_{\max}}\right)\right) \times A - Kd_2 \times A \quad (4)$$

$$Kd_2 = \theta \times \exp(-K_{02} \times t) \quad (5)$$

In Eq. 5, θ represents the total effect of a sequence at time zero. In developing the model, the assumption is that drugs interact at their sites of action immediately upon initiating treatment, and illicit their effects. Likewise, when drug is removed, further drug interaction with the pharmacologic site stops. Both Kd_0 and θ are therefore modeled to have a non-zero value at time=0, which is estimated by model fitting. The comparison of sequence effects was based on the posterior of the difference in the total effect of GP (θ_{GP}) and PG (θ_{PG}) sequences at each dose combination as shown in Eq. 6. In Bayesian analyses, the inferences concerning parameters are based on posterior distribution of the parameters, which represents all of the likely values of the estimated parameters, given the data and prior information.

$$\text{Diff} = \theta_{GP} - \theta_{PG} \quad (6)$$

If the posterior of “Diff” in Eq. 6 is positive than GP sequence is superior to the PG sequence at a particular dose combination of Gemcitabine and PQIP, otherwise PG sequence is superior. The P value was calculated as how often the estimate of the difference in total effect in GP and PG sequence at each dose combination was less than zero.

The data were log transformed for the analysis and the mean of log-number of viable cells (Y) was used as the response variable, which was assumed to follow a normal distribution (Eq. 7)

$$Y \sim N(A, \sigma^2/n) \quad (7)$$

where, A is the solution to the differential equation (model prediction), σ^2 is the residual variance, and n is the number of observations used to calculate Y .

Standardized residuals, r , were calculated as shown in Eq. 8

$$r = \left(\frac{Y - A}{\sqrt{\sigma^2/n}}\right) \quad (8)$$

The Gibbs sampler, which is the algorithm in WinBUGS for estimating model parameters, was run for a total of 100,000 iterations, of which 4,000 were burn-in, for obtaining

the summary of posterior distributions of the model parameters. Model comparisons were done by deviance information criterion (DIC). A difference in DIC of more than five was considered important to rule out the model with higher DIC. Goodness-of-fit diagnostics included plots of observed vs. model predictions and standardized residuals. Informative but uniform priors were used for all of the parameters except $A_{\max}$, which used a log normal prior, while a vague gamma prior was used for the precision ($1/\sigma^2$) of the response variable.

MODEL EVALUATION

Model evaluation was performed by doing posterior predictive check (PPC) using the posterior predictive distribution as defined by Eq. 9.

$$p(y^{\text{rep}}|y) = \int p(y^{\text{rep}}|\theta) \times p(\theta|y) d\theta \quad (9)$$

where, y^{rep} denotes replicated or future values of y , $p(\theta|y)$ is the posterior distribution of the parameters estimated with the observed data y , and $p(y^{\text{rep}}|\theta)$ is the sampling distribution (or likelihood) of the data, given the model parameters.

The PPC P value was calculated as per Eq. 10:

$$\text{PPC } P \text{ value} = \Pr(T(y^{\text{rep}}) \geq T(y)|y) \quad (10)$$

where, $T(y)$ is the test statistic based on the observed data and $T(y^{\text{rep}})$ is the same test statistic based on the replicated data. We used each observed data value, y , as the test statistic. PPC P values less than 0.025 and greater than 0.975 were considered extreme values indicating lack of fit of the model to the data.

RESULTS

The cell lines were chosen for these studies, based on prior knowledge regarding IGF responsiveness. MCF-7 cells show both proliferative and anti-apoptotic response to IGF, while MDA-MB-231 cells exhibit only an anti-apoptotic response (14–16). In contrast, Hs-578T cells have low levels of IGF-1R and do not show either proliferative or anti-apoptotic responses to IGF stimulation; hence, considered mostly resistant to IGF-1R inhibitors (17).

Linear, $E_{\max}$, and Sigmoid $E_{\max}$ structural models were tested during model building. The posterior densities of the parameters were smooth after 100,000 Gibbs samples. The history plots of the Gibbs sampler revealed that it converged to the stationary distribution within 4,000 burn-in iterations.

SINGLE AGENT EXPERIMENTS WITH GEMCITABINE AND PQIP

The time course of MCF7, MDA-MB-231, and Hs-578T cell growth for single-agent Gemcitabine and PQIP treatment are shown in Fig. 1. In the absence of drug treatment, all three cell lines grew exponentially, which appears as linear growth on the log scale; however, their

rate of growth appears to decrease over time. Cell growth was fastest for Hs-578T followed by MDA-MB-231 then MCF-7 cells. $E_{\max}$ and sigmoid $E_{\max}$ models adequately described the drug effects in MCF-7 and MDA-MB-231 cell lines, respectively. The parameter estimates for MCF-7 and MDA-231 cell lines from single agent experiments with Gemcitabine and PQIP are presented in Table III. The maximum number of MCF-7, MDA-MB-231, and Hs-578T cells at which the wells became confluent ($A_{\max}$) were estimated to be 2.029, 0.579, and 0.459 million cells, respectively. Cell kill (MCF-7 and MDA-MB-231) was concentration-dependent for both single agents. The maximum effect ($E_{\max}$) of PQIP was more than that of Gemcitabine for both MCF-7 and MDA-MB-231 cells; however, the EC_{50} estimate of Gemcitabine was less than that of PQIP. The estimated effects of single agents PQIP at 5 and 20 μM and Gemcitabine 0.1 μM treatments of Hs-578T cells were not significantly different than control. The standard deviation (σ) of the log viable cells was estimated to be 0.95, 0.74, and 0.87 for MCF-7, MDA-MB-231, and Hs-578T, respectively.

SEQUENCE OF ADMINISTRATION EXPERIMENTS

The estimates of the difference in cell-kill rate constant in GP and PG sequences, Diff, at each of the Gemcitabine and PQIP dose combinations tested in the study are shown in Table IV. The time course of MCF7, MDA-MB-231 and Hs-578T cell growth for sequence of administration experiments are shown in Figs. 2, 3, and 4, respectively.

For MCF-7 cell line, the GP sequence was better than PG sequence at the two dose combinations, 1 μM Gemcitabine/5 μM PQIP and 10 μM Gemcitabine/0.5 μM PQIP, evaluated in the study with estimated difference of 0.48 and 0.14 in cell-kill rate constant (P value, <0.05). For MDA-MB-231 cell line, the GP sequence was better than PG sequence at two dose combinations, 1 μM Gemcitabine/5 μM PQIP and 10 μM Gemcitabine/5 μM PQIP, with estimated difference of 0.26 and 0.46 in cell-kill rate constant (P value, ≤ 0.05). A statistically significant difference was not observed in the difference of cell-kill rate constant between GP and PG sequence at 10 μM Gemcitabine/0.5 μM PQIP dose combination; however, there was a trend for the superiority of GP sequence over PG sequence with estimated difference of 0.11 (P value = 0.13). For Hs-578T cell line, a trend towards superiority of the GP sequence over PG sequence was observed at the only dose combination, 0.1 μM Gemcitabine/5 μM PQIP, evaluated in the study with estimated difference of 0.64 in cell-kill rate constant (P value = 0.1). Plots of standardized residuals *versus* time and standardized residuals *versus* model prediction, for both single-agent experiments and sequence of administration experiments, did not show any trend to suspect model misspecification (plots not shown).

MODEL EVALUATION

The goodness-of-fit plots for MCF-7, MDA-MB-231, and Hs-578T cell line models shows the combined data from both single agent and sequence of administration experiments and are shown in Fig. 5. The PPC indicates that the model describes the data well. The PPC P values for each of the

Fig. 1. Time course of cell growth for single agent Gemcitabine (a, c, and e) and PQIP (b, d, and e). The plots (a and b) are data for MCF-7, the plots (c and d) are data for MDA-MB-231, and plot (e) are data for Hs-578 cells. Solid circles mean of $\ln$ viable cells, error bars SEM, and solid lines model prediction

three cell lines are shown in [Supplementary Figures](#). Only three PPC P values for MCF-7 cell line, two PPC P values for MDA-MB-231 cell line and none of the PPC P values for Hs-587T cell line were outside the 0.025–0.975 range.

DISCUSSION

The antitumor activity following treatment with combinations of selected cytotoxic agents and targeted therapies

Table III. Parameter Estimates (Median Values from the Posterior Density of the Parameters) for MCF-7 and MDA-231 Cell Lines Following Treatment with Either Gemcitabine or PQIP as Single Agents

Cell line	Drug	$A_{\max}$ ($\times 10^6$ cells)	K_g (day^{-1})	$E_{\max}$ (day^{-1})	EC_{50} (μM)	H	K_0 (day^{-1})	K_{02} (day^{-1})
MCF-7	Gemcitabine	2.029	0.518	1.71	0.19	1	0.90	0.29
	PQIP	3.89	9.53					
MDA-MB-231	Gemcitabine	0.579	0.773	1.03	0.157	0.3	0.116	0.373
	PQIP	1.41	20.4	6.2	0.324			
Hs-578T		0.459	2.38					

$A_{\max}$ maximum number of cells at which the cells become confluent, K_g rate constant for the net growth of cells, $E_{\max}$ maximum cell-kill rate constant, EC_{50} Michaelis–Menton constant, H hill coefficient, K_0 describes the decay of K_d over time

may be sequence dependent. A large cooperative group trial showed an estimated disease-free survival advantage of 7% for sequential rather than concurrent chemotherapy with tamoxifen in an adjuvant setting (18). Treatment with doxorubicin first, followed by anti-IGF1R antibodies (scFv-Fc or EM164), was superior over the reverse order of treatment with these agents (11). We extend this knowledge by showing that treatment with Gemcitabine first, followed by treatment with a small molecule inhibitor for IGF-1R is superior to the reverse sequence. This effect was most readily apparent with breast tumor cells (MCF-7), which are responsive to both the proliferative and anti-apoptotic effects of IGF. The longitudinal effects on both cell death and cell recovery were characterized following drug treatment.

Several issues arose during the modeling process. First, the experimental design resembles a serial sacrifice design in which the cells in each well provide information about viable cells (or observations for modeling) at only one time point. Three replicates were done to increase the precision or certainty. Means of those three replicates was used in the modeling instead of naïve pooling as conceptually both approaches will provide same results after accounting for the sampling distribution of mean, which we did by Eq. 7. This provides weighting for the number of observations used to calculate mean at each time point. We cannot model individual curves in this case as we do not have multiple measurements on the same experimental unit (wells) over time. Second, we tested individual kill rate constants for each drug and an interaction term for the combinations with concentrations, but this approach fit the data poorly, and it was inferior to our proposed model. The time course of effect differs between single-agent treatments and the combinations. The interaction term was different for different regimens and we could not relate it to the concentrations of two drugs. We would need to test more drug combinations to estimate the interaction as currently we have data at just two drug-combination levels for both MCF-7 and MDA-MB-231 cell

lines (for MDA-MB-231 cell line we have data at three dose levels, but the observed effect was apparent at only two combinations using 10 μM Gemcitabine), which were chosen based on the clinically relevant concentrations of both drugs. As our aim was to demonstrate the effects of the sequences at the clinically relevant (or anticipated) concentrations of these drugs, we did not explore more combinations to make the model more mechanistic for drug effects. Third, the apparent effect of the drugs (2–5 days) exceeded the duration of exposure. Therefore, we incorporated an exponential decline of the effect over time rather than abruptly allowing the effect to vanish at the end of drug exposure.

Initial clinical studies suggest that inhibition of IGF-1R with the antibody, figitumumab enhances response to chemotherapy (9); however, phase III trials were discontinued due to potential futility (19). Given the long half-life of monoclonal antibodies *in vivo*, it would be difficult to study the sequencing of anti-IGF-1R antibody with chemotherapy. After the first administration of antibody, IGF-1R inhibition is essentially complete and not easily reversed. Hence, it would be difficult to shut off the inhibition of IGF-1R after treatment *in vivo*. However, tyrosine kinase inhibitors have a much shorter half-life, and can be used to study the short-term effects of short term IGF-1R inhibition on chemotherapeutic benefit.

Our study shows the sequence-dependent effects of combining IGF-1R inhibitor with cytotoxic chemotherapy. Exposure with Gemcitabine first, followed by PQIP, was found to be either superior or equivalent to the reverse sequence in all of the three breast cancer cell lines used in the study. The sequence-dependent effect of Gemcitabine with PQIP is consistent with their mechanisms of action. Gemcitabine is S-phase dependant, primarily killing cells undergoing DNA synthesis, while IGF-1R inhibition would slow proliferation. Hence, one could hypothesize that IGF-1R inhibition first followed by Gemcitabine could theoretically render a lower fraction of cells in the S-phase; consequently, a

Table IV. Estimates (Median Values from the Posterior Density of the Parameters) of the Difference in Cell-Kill Rate Constant in GP and PG Sequences

Cell-line	Treatment combination	Estimate (Diff) (day^{-1}) [95% credible interval]	P value
MCF-7	1 μM G/5 μM PQIP	0.48 [0.31, 0.67]	0.000
	10 μM G/0.5 μM PQIP	0.14 [−0.02, 0.32]	0.039
MDA-MB-231	1 μM G/5 μM PQIP	0.26 [−0.05, 0.57]	0.051
	10 μM G/0.5 μM PQIP	0.11 [−0.08, 0.30]	0.13
	10 μM G/5 μM PQIP	0.46 [0.26, 0.68]	0.00005
Hs-578T	0.1 μM Gem/5 μM PQIP	0.64 [−0.66, 1.97]	0.10

Fig. 2. Time course of MCF-7 cell growth for sequence of administration experiments **a** 1 μ M Gemcitabine/5 μ M PQIP in GP and PG sequences and **b** 10 μ M Gemcitabine/0.5 μ M PQIP in GP and PG sequences

lower fraction of cells would respond to Gemcitabine treatment. The largest sequence effects were observed for MCF-7 cells. This is consistent with the proliferative response of MCF-7 cells to IGF, but not MDA-MB-231 cells. We did observe sequence effects for MDA-MB-231 cells treated at the highest concentrations of both agents, suggesting the role of other mechanisms or possibly the cross-reactivity of PQIP at other sites at higher concentrations. The Hs-578T cells were extremely sensitive to Gemcitabine and have very fast doubling time. We also collected data for 1 μ M Gemcitabine treatment of Hs-578T in single-agent experiments, but do not present it. The cells did not recover out to 9 days; therefore, we discontinued measurement. Hs-578T cells have very low level of expression of IGF-1R, and even after we treated with 5 and 20 μ M PQIP, cell survival did not differ from control. Hence, several different concentrations of PQIP and Gemcitabine were not tested in Hs-578T to develop the $E_{\max}$ model for this cell line, as we did for the other two. This cell line only served the purpose of confirming the sequence effect in a cell line which has low IGF-1R expression.

In single-agent experiments, the rate constant for cell growth, K_g , was highest for Hs-578T cell line followed by MDA-MB-231 and then MCF-7 cell line, which would explain

Fig. 3. Time course of MDA-MB-231 cell growth for sequence of administration experiments **a** 1 μ M Gemcitabine/5 μ M PQIP in GP and PG sequences, **b** 10 μ M Gemcitabine/0.5 μ M PQIP in GP and PG sequences, and **c** 10 μ M Gemcitabine/5 μ M PQIP in GP and PG sequences

the fastest doubling time for Hs-578T followed by MDA-MB-231 and MCF-7 in the absence of drugs. MCF-7 cells are smaller in size than MDA-MB-231 and Hs-578T, consequently the estimate of the maximum number of cells at which the wells become confluent, $A_{\max}$, was highest for MCF-7. For MCF-7 cell line, PQIP was found to be more effective in causing cell-kill than Gemcitabine. $E_{\max}$ of PQIP was higher

Fig. 4. Time course of Hs-578T cell growth for sequence of administration experiment with 0.1 μM Gemcitabine/5 μM PQIP in GP and PG sequences

for MCF-7 compared to the MDA-MB-231 cell line, which could be due to the fact that MCF-7 cells are more sensitive to IGF stimulation as they show both proliferative and anti-apoptotic responses. On the other hand, MDA-MB-231 cells only show anti-apoptotic response. The estimate of the hill coefficient, H , for PQIP against MDA-MB-231 cell line was 6.2, and indicates a steep exposure–response relationship, which we suspect could be due to cross-reactivity of PQIP with other receptor types. A more thorough analysis of the effects of PQIP on other receptor tyrosine kinases (*i.e.*, phosphorylation) has not been reported, though a similar IGF-1R antagonist, OSI-906 is reported to inhibit insulin receptor (20). Positive uniform priors, which assign equal probabilities to all possibilities in the range specified in the prior distribution, were used for all parameters except $A_{\max}$ for the analysis of single-agent experiments based on our prior experience with these cell lines in our laboratory. Uniform priors were used for the cell-kill rate constant in the sequence of administration experiments based up on the results of the single agent experiments. A vague gamma prior was used for the precision ($1/\sigma^2$) of the response variable. Gamma distribution, applies to the unknown quantities that take values between 0 and ∞ (such as the precision parameter), serve as a conjugate prior for the precision ($1/\sigma^2$) of the response. Such conjugate priors allow the posteriors to emerge without numerical integration, which makes the analysis less computationally burdensome.

In summary, our study has important implications in designing the combination therapy of IGF-1R inhibitors with other cytotoxic drugs, which require DNA synthesis for their antitumor activity. We found that exposure with Gemcitabine first followed by IGF-1R inhibitor, PQIP, was either superior or equivalent to the reverse sequence. This observation could be critical as several IGF-1R inhibitors are currently under development and being evaluated in combination with other cytotoxic drugs in clinical trials. Gemcitabine is commonly given on a days 1 and 8 schedule, and our data suggest that small molecule inhibitors such as PQIP are more effective when given for a brief period (*e.g.*, 2–3 days) after Gemcitabine. Longer duration of PQIP may have an interfering effect on Gemcitabine that is administered on day 8. Furthermore, long-term inhibition of host IGF-1R or insulin receptor signaling is associated with hyperglycemia (21). Therefore,

Fig. 5. Goodness-of-fit plots depicting observed cell viability data vs. model prediction for **a** MCF-7, **b** MDA-MB-231, and **c** Hs-578T cell lines. Blue lines Lowess smooth throughout the data

consideration should be given to stopping the PQIP approximately 3 days prior to Gemcitabine doses given on day 8. Current phase I studies of the PQIP analog OSI-906 are testing these intermittent *versus* continuous schedules. Future studies will be aimed at the evaluation of sequence effects and tolerability of IGF-1R inhibitors with Gemcitabine in mouse models of breast cancer *in vivo*, which could help in the development of treatment protocols which can be tested clinically.

CONCLUSION

Differences in cell-kill rate constants were estimated using a pharmacodynamic model and Bayesian analysis in WinBUGS. Based on our *in vitro* data, this pharmacodynamic model has the potential to enable prediction of the timing of subsequent drug administration (mimicking treatment cycles for patients) that would enhance overall antitumor effects. The sequence of Gemcitabine first, followed by PQIP was superior to or equivalent to the reverse sequence, depending on the drug concentrations and cell line tested. These results suggest that consideration of administration sequencing for Gemcitabine and OSI-906 combination is warranted if tested in a phase I trial.

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