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Synthesis of a covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic and its cytotoxic anti-neoplastic activity against chemotherapeutic-resistant SKBr-3 mammary carcinoma

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ABSTRACT

Gemcitabine is a potent chemotherapeutic that exerts cytotoxic activity against several leukemias and a wide spectrum of carcinomas. A brief plasma half-life in part due to rapid deamination and chemotherapeutic-resistance frequently limit the utility of gemcitabine in clinical oncology. Selective 'targeted' delivery of gemcitabine represents a potential molecular strategy for simultaneously prolonging its plasma half-life and minimizing exposure of innocent tissues and organ systems.

Materials and methods: Gemcitabine was combined in molar excess with *N*-[*p*-maleimidophenyl]-isocyanate (PMPI) so that the isocyanate moiety of PMPI which exclusively reacts with hydroxyl groups preferentially created a carbamate covalent bond at the terminal C_5-methylhydroxy group of gemcitabine. Monoclonal immunoglobulin with binding-avidity specifically for HER2/*neu* was thiolated with 2-iminothiolane at the terminal ε-*amine* group of lysine amino acid residues. The gemcitabine-(*carbamate*)-PMPI intermediate with a maleimide moiety that exclusively reacts with reduced sulfhydryl groups was then combined with thiolated anti-HER2/*neu* monoclonal immunoglobulin. Western-blot analysis was utilized to delineate the molecular weight profile for gemcitabine-(*carbamate*)-[anti-HER2/*neu*] while cell binding characteristics were determined by cell-ELISA utilizing SKBr-3 mammary carcinoma which highly over-expresses HER2/*neu* receptors. Cytotoxic anti-neoplastic potency of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] between the gemcitabine-equivalent concentrations of 10^{-12} and 10^{-6} M was determined utilizing vitality staining analysis of chemotherapeutic-resistant SKBr-3 mammary carcinoma.

Results: Gemcitabine-(*carbamate*)-[anti-HER2/*neu*] was synthesized at a molar incorporation index of 1:1.1 (110%) and had a molecular weight of 150 kDa that was indistinguishable from reference control immunoglobulin fractions. Cell-ELISA detected progressive increases in SKBr-3 mammary carcinoma associated immunoglobulin with corresponding increases in covalent gemcitabine immunochemotherapeutic concentrations. The in vitro cytotoxic anti-neoplastic potency of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] was approximately 20% and 32% at 10^{-7} and 10^{-6} M (gemcitabine-equivalent concentrations) after a 182-h incubation period.

Discussion: The investigations describes for the first time a methodology for synthesizing a gemcitabine anti-HER2/*neu* immunochemotherapeutic by creating a covalent bond structure between the C_5-*methylhydroxy* group of gemcitabine and thiolated lysine amino acid residues of monoclonal antibody or other biologically active protein fractions. Gemcitabine-(*carbamate*)-[anti-HER2/*neu*] possessed binding-avidity at HER2/*neu* receptors highly over-expressed by chemotherapeutic-resistant SKBr-3 mammary carcinoma. Alternatively, gemcitabine can be covalently linked at its C_5-*methylhydroxy* group to monoclonal immunoglobulin fractions that possess binding-avidity for other receptors and membrane complexes uniquely highly over-expressed by a variety of neoplastic cell types. Compared to chemotherapeutic-resistant SKBr-3 mammary carcinoma, gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic is anticipated to exert higher levels of cytotoxic anti-neoplastic potency against other neoplastic cell types like pancreatic carcinoma, small-cell lung carcinoma, neuroblastoma, glioblastoma, oral squamous cell carcinoma, cervical epithelioid carcinoma, or leukemia/lymphoid neoplastic cell types based on their reportedly greater sensitivity to gemcitabine and gemcitabine covalent conjugates.

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1. Introduction

Monoclonal immunoglobulin fractions with binding-avidity for over-expressed HER2/*neu* and EGFR receptors have demonstrated effectiveness for the treatment of HER2/*neu* and EGFR positive mammary carcinoma and other neoplastic conditions. Unfortunately, they reportedly have an inability to exert significant cytotoxic activity or completely resolve neoplastic disease states[1–7] unless they are applied in combination with chemotherapy or other forms of anti-cancer treatment.[8,9] Despite general familiarity with the influence of anti-HER2/*neu* immunoglobulin on the biology of cancer cells and it's application in clinical oncology, there is surprisingly little known about covalent gemcitabine-(anti-HER2/*neu*) immunochemotherapeutics and their potential to exert selectively 'targeted' cytotoxic anti-neoplastic activity against chemotherapeutic-resistant mammary carcinoma.[10]

Anthracyclines have traditionally been the class of chemotherapeutics utilized most extensively for the synthesis of covalent immunochemotherapeutics.[10–34] Several of these covalent anthracycline-immunoconjugates have utilized monoclonal immunoglobulin fractions that selectively recognize and bind to antigens highly over-expressed on the surface membrane of metastatic melanoma[35,36] and multiple myeloma.[37] Utilizing optimal semi-synthesis methodologies, anthracycline-immunoconjugates have been produced that possess higher[35,36,38–40] or relatively high (effective)[41] levels of potency compared to molar-equivalent concentrations of un-conjugated 'free' chemotherapeutic. Interestingly, some doxorubicin immunoconjugate that have low ex vivo levels of potency exert a surprisingly high level of in vivo anti-neoplastic activity compared to un-conjugated 'free' chemotherapeutic.[41]

Immunochemotherapeutics that selectively 'target' the delivery of doxorubicin[36,37,42–44] and to a lesser extent daunorubicin[38,45,46] and epirubicin[10,47,48] have been synthesized that exert high in vitro levels of cytotoxic anti-neoplastic potency against mammary carcinoma,[10] CD38 positive MC/CAR multiple myeloma,[37] B-lymphoma,[43] melanoma,[35,36,38] gastric carcinoma,[41] colon carcinoma,[47] and pulmonary carcinoma.[49] In direct accord with their level of in vitro efficacy, similar anthracycline-immunoconjugates are capable of reducing in vivo tumor burden and prolonging survival against human xenografts of gastric carcinoma,[41] breast cancer,[44] CD38 positive MC/CAR multiple myeloma,[37] B-lymphoma,[43] T-cell lymphoma,[50] colon carcinoma,[39,44,51,52] ovarian carcinoma,[39] pulmonary carcinoma,[44] metastatic melanoma,[35,36] hepatocellular carcinoma,[42] and intracerebral small-cell lung carcinoma.[53–55] Additionally, a number of clinical trails involved in evaluating the efficacy and potency of anthracycline-immunoconjugates continue to be conducted relevant to a small array of neoplastic disease states.[56,57]

Gemcitabine is a deoxycytidine nucleotide analog that functions as a chemotherapeutic when it is transformed into a triphosphate form which in turn can substitute for cytidine during DNA replication where it becomes incorporated into DNA strands and inhibits DNA polymerase. A second mechanism of action for gemcitabine involves its ability to inhibit and inactivate ribonucleotide reductase ultimately promoting suppressed deoxyribonucleotide synthesis in concert with diminished DNA repair and replication. Collectively these mechanisms of action ultimately contribute to the onset of induced apoptosis. In clinical oncology, gemcitabine is administered for the treatment of certain leukemias and potentially lymphoma conditions in addition to a spectrum of adenocarcinomas and carcinomas affecting the lung (e.g., non-small cell), pancreas, bladder, and esophogus. Gemcitabine has a brief plasma half-life because it is rapidly deaminated and the inactive metabolite is excreted into the urine.[58–60] Molecular design and synthesis of a covalent gemcitabine immunochemotherapeutic would provide several advantages due to the ability of such preparations to facilitate selective 'targeted' chemotherapeutic delivery.[10] In this form gemcitabine apparently becomes a poor substrate for both MDR-1 (multi-drug resistance efflux pump)[61] and presumably the rapid deaminating enzymes, cytidine deaminase, and (following gemcitabine phosphorylation), deoxycytidylate deaminase. In contrast to covalent anthracycline conjugates, a very limited number of published reports have described the molecular design, synthesis, and cytotoxic anti-neoplastic potency of gemcitabine covalent bound to selective 'targeting' ligands while an even fewer number of reports have described the production and potency of covalent gemcitabine immunochemotherapeutics.

2. Materials and methods

2.1. Synthesis methodology I: gemcitabine-(*carbamate*)-PMPI-immunoglobulin

2.1.1. Phase-I: immunoglobulin thiolation at lysine ε-amine groups

A purified fraction of monoclonal immunoglobulin with binding-avidity specifically for human HER2/*neu* (ErbB-2, CD 340) was utilized for the semi-synthesis of gemcitabine-(*carbamate*)-[anti-HER2/*neu*]. Desiccated anti-HER2/*neu* monoclonal immunoglobulin (1.5 mg) was combined with 2-iminothiolane (2-IT: 6.5 mM final concentration) in PBS (0.1 M, pH 8.0, 250 μl) and incubated at 25 °C for 1.5 h in combination with simultaneous constant gentle stirring.[19,62–64] Thiolated anti-HER2/*neu* monoclonal immunoglobulin was then buffer exchanged into PBS-EDTA (phosphate 0.1, NaCl 0.15 M, EDTA 10 mM, pH 7.3) using micro-scale column chromatography. Moles of reduced sulfhydryl groups covalently introduced into anti-HER2/*neu* monoclonal immunoglobulin was measured with a 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB reagent) based assay. The average number of thiolated lysine ε-*amine* groups introduced into anti-HER2/*neu* fractions (R-SH/IgG) was 3:1 using 2-IT reagent. Yield: 1.407 mg thiolated IgG or 9.38×10^{-6} mmol.

2.1.2. Phase-II: synthesis of gemcitabine-(carbamate)-PMPI sulfhydryl-reactive intermediate

Gemcitabine (10 mg/ml stock in DMSO) was combined with *N*-[*p*-maleimidophenyl]-isocyanate (PMPI)[65–67] at a 5:1 molar ratio in combination with constant gentle stirring at 25 °C for 3.5 h so that the isocyanate moiety of PMPI which exclusively reacts with hydroxyl (R-OH) groups preferentially created a carbamate covalent bond at the terminal C_5-methylhydroxy group of gemcitabine.[61,68–73] The highly selective reaction is reportedly complete within 2 h under the applied conditions. Gemcitabine was formulated at a large molar excess to deplete un-reacted PMPI and maximize synthesis of the sulfhydryl-reactive maleimide intermediate as validated by high-performance thin-layer chromatography analysis (HP-TLC).[74–79] Yield: 659 μg or 1.38 μM of the maleimide sulfhydryl-reactive gemcitabine-(*carbamate*)-PMPI intermediate.

2.1.3. Phase-III: covalent reaction of gemcitabine-(carbamate)-PMPI intermediate with thiolated immunoglobulin

The gemcitabine-(*carbamate*)-PMPI intermediate with a maleimide moiety that exclusively reacts with reduced sulfhydryl (R-SH) groups was combined at a 1.5:1 molar ratio with thiolated terminal lysine ε-*amines* in anti-HER2/*neu* monoclonal immunoglobulin fractions (PBS-EDTA: phosphate 0.1, NaCl 0.15 M, EDTA 10 mM, pH 7.3) and the formulation mixture incubated with constant stirring at 25 °C for 2 h.[10,12,13,17,34,37,42,65,80–84] Similar synthesis strategies in concept have previously been applied to generate covalent anthracycline immunochemotherapeutic

preparations.[10,19,62,63,85,86] Residual gemcitabine was removed from the final covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic preparation applying micro-scale 'desalting' column chromatography pre-equilibrated with PBS (phosphate 0.1, NaCl 0.15 M, pH 7.3). Yield: 710 μg of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic.

2.2. Synthesis methodology II: epirubicin-(C_{13}-*imino*)-EMCH-immunoglobulin

2.2.1. Phase-I: immunoglobulin thiolation at lysine ε-amine groups

Covalent introduction of reduced sulfhydryl groups into anti-HER2/*neu* immunoglobulin fractions was performed as described for the synthesis of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic.

2.2.2. Phase-II: synthesis of epirubicin-(C_{13}-imino)-EMCH sulfhydryl-reactive intermediate

The C_{13}-*keto* group of epirubicin (1.479×10^{-2} mg, 2.55×10^{-5} mm in methanol) was reacted with the hydrazide group of the heterobifunctional covalent cross-linking reagent, *N*-ε-maleimidocaproic acid hydrazide in the presence of trifluoroacetic acid (EMCH: 43.2 μg, 1.275×10^{-4} mmol in methanol) and then incubated at 25 °C for 96 h in concert with constant gentle stirring.[17,18] Crystalization of epirubicin-(C_{13}-*imino*)-EMCH was performed by the addition of acetonitrile until a slight opalescent appearance developed followed by incubation at −20 °C for 24 h. Recrystalized epirubicin-EMCH was harvested by centrifugation and rinsed in cold acetonitrile. The resulting recrystalization supernatant was partially evaporated under a stream of nitrogen (N_2) gas in order to maximize yield of total epirubicin-(C_{13}-*imino*)-EMCH product. Prior to Phase-II synthesis procedures, residual methanol in aliquots of recrystalized epirubicin-EMCH was removed under a gentle stream of nitrogen gas and then re-dissolved in dimethylsulfoxide (DMSO). Yield: 95.9 μg as epirubicin-(C_{13}-*imino*)-EMCH intermediate.

2.2.3. Phase-III: covalent incorporation of epirubicin-(C_{13}-imino)-EMCH into thiolated immunoglobulin at lysine ε-amine groups

The primary reduced sulfhydryl group (R-SH) of thiolated lysine ε-*amines* within HER2/*neu* monoclonal immunoglobulin contained in PBS-EDTA (phosphate 0.1, NaCl 0.15 M, EDTA 10 mM, pH 7.3) was combined with the sulfhydryl-reactive maleimido group of epirubicin-EMCH and allowed to react while incubating at 25 °C with continual gentle stirring for 2 h. Residual epirubicin was removed from epirubicin-(anti-HER2/*neu*) applying micro-scale 'desalting' column chromatography pre-equilibrated with PBS (phosphate 0.1, NaCl 0.15 M, pH 7.3). Yield: 723 μg of epirubicin-(C_{13}-*imino*)-EMCH immunochemotherapeutic.

2.3. Analysis and property characteristics

2.3.1. General analysis

Determination of the IgG concentration within gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunoconjugates was determined by measuring absorbance at 280 nm and utilizing a 235 nm versus 280 nm standardized reference curve in order to accommodate for any potential absorption profile over-lap at 280 nm between gemcitabine and immunoglobulin. The gemcitabine molar incorporation index for the final synthetic covalent immunochemotherapeutic was determined by measuring difference in the moles of reduced sulfhydryl groups contained within thiolated anti-HER2/*neu* fractions and gemcitabine-(*carbamate*)-[anti-HER2/*neu*] preparations applying 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB) as a reagent for assaying reduced sulfhydryl groups (R-SH).[63,64] Quantitation of the amount of non-covalently bound gemcitabine contained within covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] preparations following separation by column chromatography was determined by measured absorbance at 265-267 nm[87,88] for the resulting supernatant after immunochemotherapeutic precipitation with methanol/acetonitrile (1:9 v/v).

Epirubicin concentrations were established by excitation at 485 nm and measurement of emission at 538 nm using known concentrations of epirubicin to generate a standard reference control curve sufficient to generate a linear equation for determination of epirubicin-equivalent concentrations between 10^{-9} and 10^{-5} M. Concentration of non-conjugated 'free' epirubicin contained in epirubicin-immunoconjugate preparations was determined by chloroform extraction,[81,89,90] with the organic phase collected by pipette, evaporated to dryness under a stream of nitrogen gas, and the resulting residue dissolved in Tris buffered saline (50 mM, pH 7.4) prior to further analysis. Adjusted epirubicin:immunoglobulin molar incorporation indexes were calculated by measuring absorbance at 485 nm and 280 nm, respectively and by correcting for absorbance from epirubicin at 280 nm.

2.3.2. Molecular mass-dependent separation of covalent immunochemotherapeutics by non-reducing SDS–PAGE

Covalent gemcitabine and anthracycline anti-HER2/*neu* immunochemotherapeutics in addition to the anti-HER2/*neu* immunoglobulin reference control fraction were adjusted to a standardized protein concentration of 60 μg/ml and then combined 50/50 v/v with conventional SDS–PAGE sample preparation buffer (Tris/glycerol/bromphenol blue/SDS) formulated without 2-mercaptoethanol or boiling. Each covalent immunochemotherapeutic, the reference control immunoglobulin fraction (0.9 μg/well) and a mixture of pre-stained reference control molecular weight markers were then developed by non-reducing SDS–PAGE (11% acrylamide) performed at 100 V constant voltage at 3 °C for 2.5 h.

2.3.3. Western-blot immunodetection analyses

Covalent gemcitabine and anthracycline anti-HER2/*neu* immunochemotherapeutics following mass-dependent separation by non-reducing SDS–PAGE were equilibrated in tank buffer devoid of methanol. Mass-separated gemcitabine and anthracycline anti-HER2/*neu* immunochemotherapeutics contained in acrylamide SDS–PAGE gels were then transferred laterally onto sheets of nitrocellulose membrane at 20 V (constant voltage) for 16 h at 2–3 °C with the transfer manifold packed in crushed ice.

Nitrocellulose membranes with laterally-transferred immunochemotherapeutics were then equilibrated in Tris buffered saline (TBS: Tris–HCl 0.1 M, NaCl 150 mM, pH 7.5, 40 ml) at 4 °C for 15 min followed by incubation in TBS blocking buffer solution (Tris 0.1 M, pH 7.4, 40 ml) containing bovine serum albumin (5%) for 16 h at 2–3 °C applied in combination with gentle horizontal agitation. Prior to further processing, nitrocellulose membranes were vigorously rinsed in Tris buffered saline (Tris 0.1 M, pH 7.4, 40 ml, $n = 3\times$).

Rinsed BSA-blocked nitrocellulose membranes developed for Western-blot (immunodetection) analyses were incubated with biotinylated goat anti-murine IgG (1:10,000 dilution) at 4 °C for 18 h applied in combination with gentle horizontal agitation. Nitrocellulose membranes were then vigorously rinsed in TBS (pH 7.4, 4 °C, 50 ml, $n = 3$) followed by incubation in blocking buffer (Tris 0.1 M, pH 7.4, with BSA 5%, 40 ml). Blocking buffer was decanted from nitrocellulose membrane blots which were then rinsed in TBS (pH 7.4, 4 °C, 50 ml, $n = 3$) before incubation with streptavidin–HRPO (1:100,000 dilution) at 4 °C for 2 h applied in combination with gentle horizontal agitation. Prior to chemiluminescent development nitrocellulose membranes were vigorously rinsed in

Tris buffered saline (Tris 0.1 M, pH 7.4, 40 ml, $n = 3$). Development of nitrocellulose membranes by chemiluminescent autoradiography following processing with conjugated HRPO-streptavidin required incubation in HRPO chemiluminescent substrate (25 °C; 5–10 min). Autoradiographic images were acquired by exposing radiographic film (Kodak BioMax XAR) to nitrocellulose membranes sealed in transparent ultraclear re-sealable plastic bags.

2.4. Mammary carcinoma tissue culture cell culture

The chemotherapeutic-resistant SKBr-3 human mammary carcinoma cell line was utilized as an ex vivo neoplasia model. Characteristically, SKBr-3 mammary carcinoma uniquely over-expresses epidermal growth factor receptor 1 (EGFR, ErbB-1, and HER1) and highly over-expresses epidermal growth factor receptor 2 (EGFR2, HER2/*neu*, ErbB-2, CD340, and p185) at 2.2×10^5/cell and 1×10^6/cell, respectively.

Populations of the SKBr-3 mammary carcinoma cell line were propagated in 150-cc^2 tissue culture flasks containing McCoy's 5a Modified Medium supplemented with fetal bovine serum (10% v/v) and penicillin–streptomycin at a temperature of 37 °C under a gas atmosphere of air (95%) and carbon dioxide (5% CO_2). Tissue culture media was not supplemented with growth factors, growth hormones or other growth stimulants of any type. Investigations were performed using SKBr-3 mammary carcinoma monolayer populations at a $\geqslant$85% level of confluency.

2.5. Cell-ELISA IgG binding assay

Cell suspensions of SKBr-3 mammary carcinoma were seeded into 96-well microtiter plates in aliquots of 2×10^5 cells/well and allowed to form a confluence adherent monolayer over a 48 h period. The growth media contents of individual wells were then removed manually by pipette and serially rinsed ($n = 3$) with PBS followed by stabilization of adherent SKBr-3 cellular monolayers onto the plastic surface of 96-well plates with paraformaldehyde (4% in PBS, 15 min). Stabilized SKBr-3 monolayers were then incubated with gemcitabine-(carbamate)-[anti-HER2/neu] and epirubicin (C_{13}-imino)-[anti-HER2/neu] immunoconjugates formulated at gradient concentrations of 0.1, 0.25, 0.5, 1.0, 5.0, and 10 μg/ml in tissue culture growth media (200 μl/well). Direct contact incubation between SKBr-3 cellular monolayers and gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(*C*$_{13}$-*imino*)-[anti-HER2/*neu*] at 37 °C over an incubation period of 3 h using a gas atmosphere of air (95%) and carbon dioxide (5% CO_2). Following serial rinsings with PBS ($n = 3$), development of stabilized SKBr-3 mammary carcinoma monolayers entailed incubation with β-galactosidase conjugated goat anti-mouse IgG (1:500 dilution) for 2 h at 25 °C with residual unbound immunoglobulin removed by serial rinsing with PBS ($n = 3$). Final cell-ELISA development required serial rinsing ($n = 3$) of stabilized SKBr-3 monolayers with PBS followed by incubation with nitrophenyl-β-D-galactopyranoside substrate (100 μl/well of ONPG formulated fresh at 0.9 mg/ml in PBS pH 7.2 containing $MgCl_2$ 10 mM, and 2-mercaptoethanol 0.1 M). Absorbance within each individual well was measured at 410 nm (630 nm reference wavelength) after incubation at 37 °C for a period of 15 min.

2.6. Cell vitality assay for measuring immunoconjugate cytotoxicity

Individual preparations of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(*C*$_{13}$-*imino*)-[anti-HER2/*neu*] were formulated in growth media at standardized chemotherapeutic-equivalent concentrations of 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M (final concentration). Each chemotherapeutic-equivalent concentration of covalent immunochemotherapeutic was then transferred in triplicate into 96-well microtiter plates containing SKBr-3 mammary carcinoma monolayers (growth media 200 μl/well). Covalent immunochemotherapeutics where then allowed to directly contact-incubate with monolayer populations of SKBr-3 mammary carcinoma for a period of 182 h (gemcitabine-anti-HER2/*neu* with a replenishing of mediate at 96-h intervals) and 72 h (epirubicin-anti-HER2/*neu*) at 37 °C under a gas atmosphere of air (95%) and carbon dioxide (5% CO_2).

The contents of 96-well microtiter plates at 72 h was removed manually by pipette monolayers serially rinsed ($n = 3$) with PBS followed by incubation with MTT vitality stain reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide 5 mg/ml) formulated in RPMI-1640 growth media devoid of pH indicator or bovine fetal calf serum. During an incubation period of 3 h at 37 °C under a gas atmosphere of air (95%) and carbon dioxide (5% CO_2) the enzyme mitochondrial succinate dehydrogenase was allowed to convert MTT vitality stain to navy-blue formazone crystals. Contents of the 96-well microtiter plate was then removed, and serially rinsed with PBS ($n = 3$) followed by dissolving of the resulting blue intracellular formazone crystals with DMSO (300 μl/well). Spectrophotometric absorbance of the blue-colored supernatant was then measured at 570 nm using a computer integrated microtiter plate reader.

3. Results

The sequential order in combination with the type of selective organic chemistry reactions applied during the synthesis of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin- (*C*$_{13}$-*imino*)-[anti-HER2/*neu*] by design drastically minimizes the creation of side reactions and the formation of secondary products (Fig. 1). Molar incorporation indexes for gemcitabine and epirubicin in gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(*C*$_{13}$-*imino*)-[anti-HER2/*neu*] were 1.1:1 (110%) and 0.4:1 (40%), respectively. The percent of non-covalently bound gemcitabine or anthracycline contained in covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(*C*$_{13}$-*imino*)-[anti-HER2/*neu*] immunochemotherapeutics following the application of microscale desalting/buffer exchange column chromatography was consistently <4.0% (residual non-covalently bound chemotherapeutics generally can not be removed by performing serial/repeated column chromatography separations).[91] Higher molar incorporation indexes are possible to achieve with modifications in methodology but the harsher synthesis conditions required for such purposes are almost invariably accompanied by substantial reductions in the final yield of covalent immunochemotherapeutic,[16] and declines in antigen-immunoglobulin binding affinity.

Covalent gemcitabine and anthracycline (anti-HER2/*neu*) immunochemotherapeutics mass-separated by SDS–PAGE and developed by Western-blot immunodetection analysis in combination with chemiluminescent autoradiography detected a condensed 150-kDa band between a 5.0-kDa and 450-kDa molecular weight range (Fig. 2) The molecular weight for both gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epiribucin-(*C*$_{13}$-*imino*)-[anti-HER2/*neu*] of 150-kDa directly corresponded with the molecular weight/mass detected for the anti-HER2/*neu* immunoglobulin reference control fraction and the known molecular weight for immunoglobulin (Fig. 2). Analogous results have been reported for similar covalent immunochemotherapeutics.[10,12,49]

3.1. Cell-binding analysis

Utilizing standardized total immunoglobulin-equivalent concentrations formulated at 0.1, 0.25, 0.5, 1.0, 5.0, and 10.0 μg/ml analyses from cell-ELISA produced profiles that detected proportional

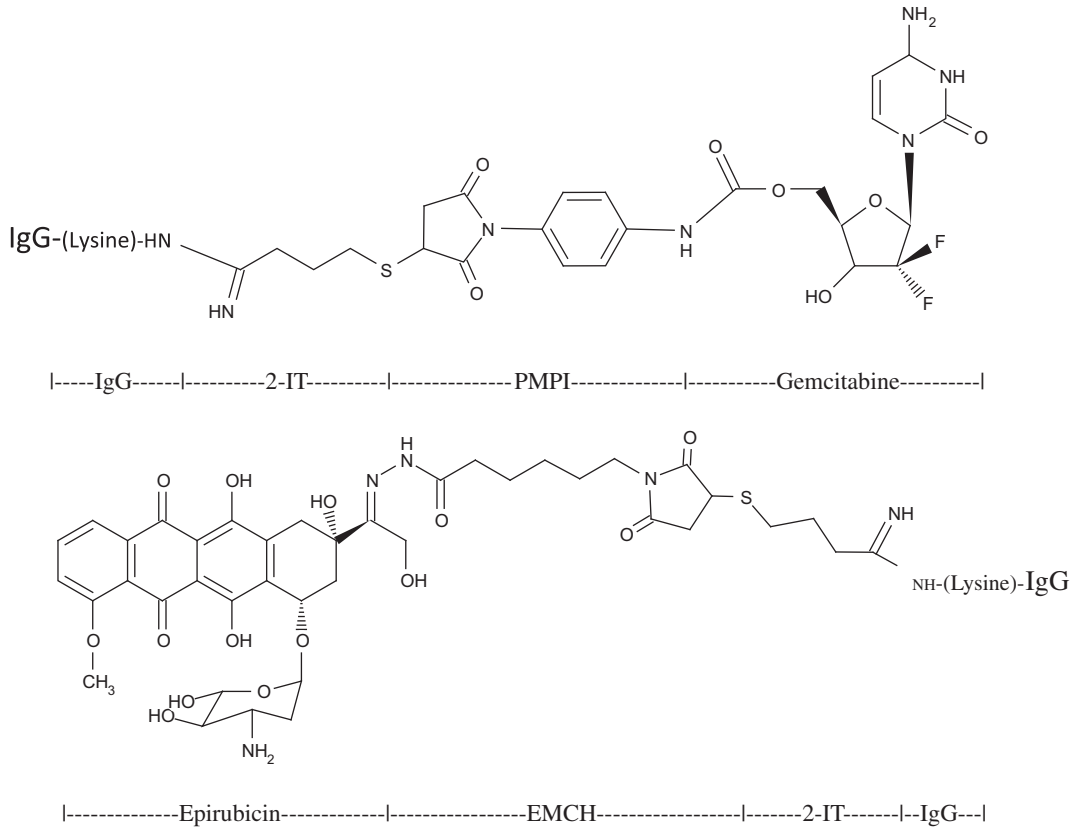

Figure 1. Top panel: gemcitabine-(*carbamate*)-[anti-HER2/*neu*] covalent biopharmaceutical created using a 2-stage synthesis scheme. Stage-I: a carbamate is created at the C_5-methylhydroxy group of gemcitabine through the selectively hydroxyl-reactive isocyanate group of *N*-[*p*-maleimidophenyl]-isocyanate (PMPI). Stage-II: the selectively sulfhydryl-reactive maleimide group of the gemcitabine-(*carbamate*)-PMPI intermediate is covalently linked at/to thiolated lysine α-*amine* groups residing within anti-HER2/*neu* monoclonal immunoglobulin fractions. Bottom panel: epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] covalent biopharmaceutical created using a 2-stage synthesis scheme. Stage-I: a maleimidocaproyl)hydrazone is created at the C_{13}-*keto* group of the anthracycline through the selectively carbonyl-reactive hydrazide group of *N*-ε-maleimidocaproic acid hydrazide (EMCH). Stage-II: the selectively sulfhydryl-reactive maleimide group of the epirubicin-(C_{13}-*imino*) maleimodocaproyl)-hydrazone intermediate is covalently linked at/to thiolated lysine α-*amine* groups residing within anti-HER2/*neu* monoclonal immunoglobulin fractions.

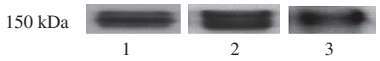

Figure 2. Western-blot autoradiography of anti-HER2/*neu* reference control, compared to gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] covalent immunochemotherapeutics. Lane-1: murine anti-human HER2/*neu* immunoglobulin; Lane-2: gemcitabine-(*carbamate*)-[anti-HER2/*neu*]; Lane-3: epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*]. Immunoglobulin preparations were mass-separated by SDS–PAGE followed by lateral transfer onto sheets of nitrocellulose membrane and detected with biotinylated goat anti-mouse IgG. Subsequent analysis entailed incubation of membranes with conjugated streptavidin-HRPO in combination with the use of an HRPO chemiluminescent substrate to facilitate the acquisition of autoradiography images.

increases in SKBr-3 membrane binding for gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] in a manner that validated retained selective binding of the covalent immunochemotherapeutics at membrane HER2/*neu* receptor sites (Fig. 3). Differences in total membrane-bound immunoglobulin detected between anti-HER2/*neu* compared to anti-EGFR reflect the greater membrane expression densities for HER2/*neu* (2.2×10^5/cell) compared to EGFR (1×10^6/cell) in populations of SKBr-3 mammary carcinoma (Fig. 3).[10]

3.2. Cytotoxicity analysis

Gemcitabine-(*carbamate*)-[anti-HER2/*neu*] exerted a 32% level of selectively 'targeted' cytotoxic anti-neoplastic activity against chemotherapeutic-resistant SKBr-3 mammary carcinoma (Figs. 4 and 5). The cytotoxic anti-neoplastic potency of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] was not greater than gemcitabine at chemotherapeutic-equivalent concentrations particularly at molar concentrations of 10^{-8}, 10^{-7} and 10^{-6} M (Fig. 4). However, the cytotoxic anti-neoplastic potency profile for covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic at 182-h was very similar to the potency profile for gemcitabine at 72-h but was slightly lower at gemcitabine-equivalent concentrations of 10^{-7} and 10^{-6} M (Fig. 4). Gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic also exerted levels of cytotoxic anti-neoplastic activity against chemotherapeutic SKBr-3 mammary carcinoma that were similar to epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] but was lower at the chemotherapeutic-equivalent concentration of 10^{-7} M while maximal levels of anti-neoplastic potency for the two preparations were 32% (10^{-6} M at 182-h) and 52% (10^{-7} M at 72-h), respectively (Fig. 5). The cytotoxic anti-neoplastic potency of gemcitabine against chemotherapeutic-resistant SKBr-3 mammary carcinoma was nearly equivalent to epirubicin at 72-h but was much less than levels detected for gemcitabine at 182-h where differences were most prominent at 10^{-8} and 10^{-7} M when compared at chemotherapeutic-equivalent concentrations (Fig. 6). Individual anti-HER2/*neu* and anti-EGFR monoclonal immunoglobulin fractions alone did not exert any detectable anti-neoplastic activity against SKBr-3 mammary carcinoma (Fig. 7) which is in accord with previous investigations.[10,35–38,49]

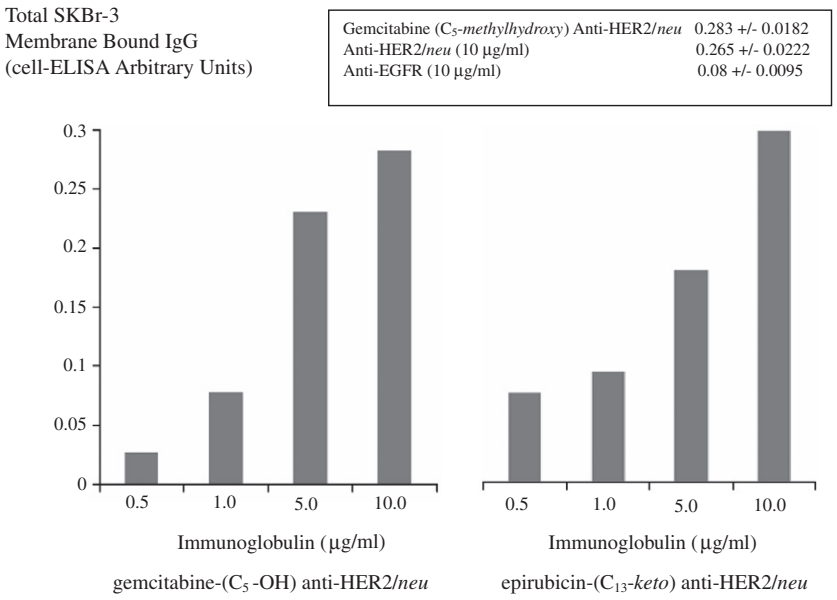

Figure 3. Immunoglobulin cell binding analysis for populations of chemotherapeutic-resistant SKBr-3 mammary carcinoma. Legend left panel: gemcitabine-(*carbamate*)-[anti-HER2/*neu*]; right panel: epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*]. Monolayer populations of SKBr-3 mammary carcinoma were incubated with covalent immunochemotherapeutics over a 4-h period and total immunoglobulin bound on the exterior surface membrane was measured by cell-ELISA.

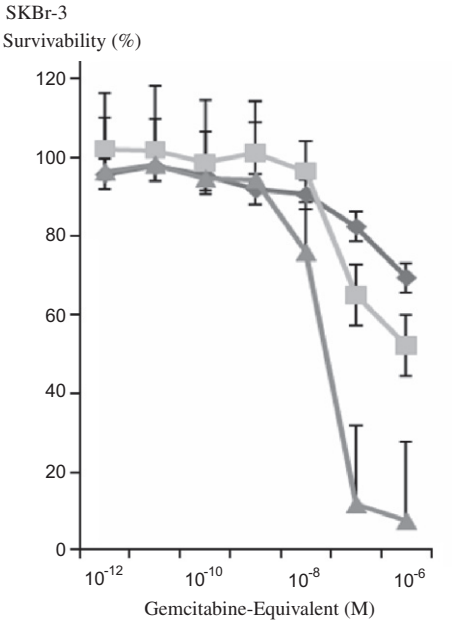

Figure 4. Influence of immunoconjugation on the potency of epirubicin anti-neoplastic activity against chemotherapeutic-resistant SKBr-3 mammary carcinoma. Legend: (♦) gemcitabine-(*carbamate*)-[anti-HER2/*neu*] (182-h incubation period); (■) gemcitabine chemotherapeutic (72-h incubation period); and (▲) gemcitabine chemotherapeutic (182-h incubation period). SKBr-3 monolayers were incubated with the covalent gemcitabine immunochemotherapeutic, gemcitabine or epirubicin and cytotoxicity measured using a MTT vitality assay relative to matched negative reference controls.

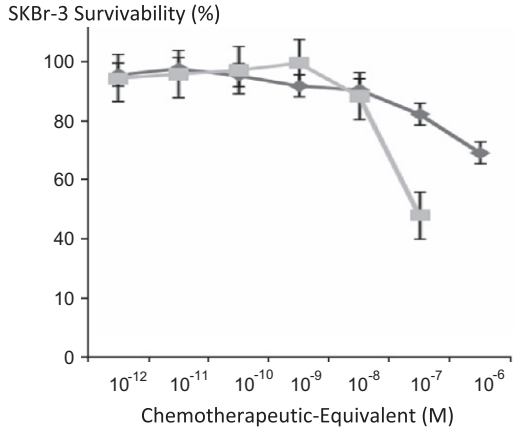

Figure 5. Relative cytotoxic anti-neoplastic potency of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] compared to epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] against chemotherapeutic-resistant SKBr-3 mammary carcinoma. Legend: (♦) gemcitabine-(*carbamate*)-[anti-HER2/*neu*]; and (■) epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*]. SKBr-3 monolayers were incubated with gemcitabine and epirubicin covalent immunochemotherapeutics for 182 and 72 h, respectively and cytotoxicity measured using a MTT vitality assay relative to matched negative reference controls.

4. Discussion

4.1. General

A small collection of semi-synthetic heterobifunctional organic chemistry reactions can potentially be used to covalently link gemcitabine to monoclonal immunoglobulin or other biologically active protein fractions. One methodology involves creation of a covalent bond at the cytosine *amine* group of gemcitabine either as a direct link to a 'targeting' ligand or for the purpose of creating a gemcitabine reactive intermediate.[30,71,73,92,93] Similar molecular strategies have been employed to synthesize covalent anthracycline immunochemotherapeutics through the formation of a covalent bond with the α-monoamine (C_3-*amine*) group on the carbohydrate moiety of doxorubicin, daunorubicin, or epirubicin.[10,15,17,20–25,27,28,32,19] Generation of a covalent bond at the C_5-*methylhydroxy* group of gemcitabine represents a second alternative molecular strategy for synthesizing covalent gemcitabine–ligand conjugates.[61,68–73]

Gemcitabine has been covalently bound to a number of biologically relevant ligands. Most prominent in this regard have been poly-L-glutamic acid (polypeptide configuration),[70] cardiolipin,[68,69] 1-dodecylthio-2-decyloxypropyl-3-phophatidic acid,[61,72] lipid–nucleosides,[94] *N*-(2-hydroxypropyl)methacrylamide polymer

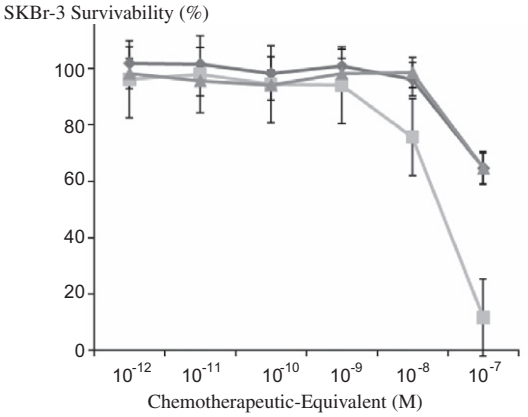

Figure 6. Influence of immunoconjugation on the potency of epirubicin anti-neoplastic activity against chemotherapeutic-resistant SKBr-3 mammary carcinoma. Legend: (♦) gemcitabine chemotherapeutics (72-h incubation period); (■) gemcitabine chemotherapeutic (182-h incubation period); and (▲) epirubicin chemotherapeutic (72-h incubation period). SKBr-3 monolayers were incubated with the covalent gemcitabine immunochemotherapeutic, gemcitabine, or epirubicin and cytotoxicity measured using a MTT vitality assay relative to matched negative reference controls.

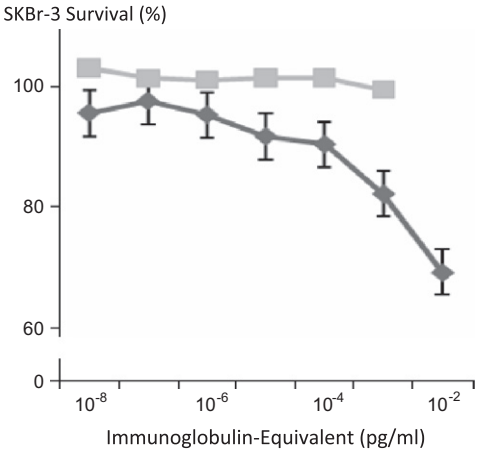

Figure 7. Relative anti-neoplastic potency of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] compared to monoclonal immunoglobulin anti-HER2/*neu* and anti-EGFR fractions against chemotherapeutic-resistant SKBr-3 mammary carcinoma. Legend: (♦) gemcitabine-(*carbamate*)-[anti-HER2/*neu*]; and (■) anti-HER2/*neu* monoclonal antibody. SKBr-3 monolayers were incubated with immunoconjugates cytotoxicity measured at 72 h using the MTT vitality assay relative to matched negative reference controls.

(HPMA),[30] benzodiazepine receptor ligand,[71,73] 4-(*N*)-valeroyl, 4-(*N*)-lauroyl, 4-(*N*)-stearoyl,[93] 1,1′,2-tris-nor-aqualenecarboxylic acid,[95] and the 4-fluoro[^{18}F]-benzaldehyde derviative[92] for application as a positron emitting radionuclide. Few if any reports have described the molecular design and efficacy evaluation of a covalent immunochemotherapeutic synthesized through the generation of a covalent bond structure at either the cytosine *amine* or C_5-*methylhydroxy* groups of gemcitabine.

A common reaction scheme and identical conditions were applied for the thiolation of anti-HER2/*neu* monoclonal immunoglobulin fractions utilized to synthesize both gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] immunochemotherapeutics. Similarly, the synthetic gemcitabine-PMPI and epirubicin-EMCH intermediates both contained sulfhydryl-reactive maleimide groups. Despite these similarities, higher molar incorporation indexes were attained during the synthesis of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] (Gem-IgG of 1.1-to-1 or 110%) relative to epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] (0.4-to-1 or 40%). Similar results have been observed for the synthesis of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] (0.275-to-1 or 27.5%),[10] and epirubicin-(C_3-*amide*)- [anti-EGFR] (0.407-to-1 or 40.7%).[10] Conservative speculation suggests that one reason for the higher molar incorporation index observed in the final gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic was due to the implementation of a synthesis scheme that involved a distinctly different organic chemistry reaction for production of the gemcitabine-PMPI reactive intermediated. A second likely reason was because PMPI is able to form a covalent bond at the C_5-*methylhydroxy* (R-OH) group of gemcitabine with a lower degree of steric hindrance compared to the synthesis of the epirubicin-(C_3-*amide*)-SMCC intermediate resulting from covalent bond formation at the carbohydrate moiety *amine* group,[10] and epirubicin-(C_{13}-*imino*)-EMCH intermediate at the side-chain ketone group of epirubicin (Fig. 1).

4.2. Cell binding

Total membrane-bound IgG profiles in SKBr-3 mammary carcinoma populations for gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] revealed increases that were proportional to elevations in total immunoglobulin (Fig. 3). Previous investigations have noted that seemingly modest alterations in synthetic chemistry and elevations in the chemotherapeutic molar incorporation index can profoundly influence immunoglobulin binding properties.[36] The relatively mild conditions employed during synthesis procedures and the modest molar incorporation index of 1.1 contributed to the high biological integrity of covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] immunochemotherapeutics based on results from SDS–PAGE/immunodetection and cell-ELISA analyses. Relatively higher chemotherapeutic-immunoglobulin molar incorporation indexes could possibly be attained through implementation of harsher synthesis conditions. Such modifications can result in only modest declines in immunoreactivity (e.g., 86% for a 73:1 ratio) but also be accompanied by disproportionate declines in cytotoxic anti-neoplastic activity down to potency levels substantially lower than those found with non-conjugated 'free' chemotherapeutic.[41] Internalization of covalent immunochemotherapeutics following binding to membrane HER2/*neu* and EGFR receptors expressed by SKBr-3 mammary carcinoma is assumed to occur through mechanisms of receptor-mediated endocytosis.[96] Although specific data for SKBr-3 HER2/*neu* and EGFR receptor complexes is limited,[10] other neoplastic cell lines like metastatic multiple myeloma are known to internalize and metabolize approximately 8×10^6 molecules of anti-CD74 monoclonal antibody per day.[97] Receptor-mediated endocytosis at membrane HER2/*neu* complexes in this fashion can lead to intracellular chemotherapeutic levels that approach and exceed 8.5[51] to >100-fold greater[98] concentrations than is possible by passive chemotherapeutic diffusion.

4.3. Cytotoxicity

Progressive increases in gemcitabine-(*carbamate*)-[anti-HER2/*neu*] concentration resulted in proportional incremental declines in the survival (%) of chemotherapeutic-resistant SKBr-3 mammary carcinoma (Figs. 4 and 5). In contrast to epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] which possesses greater cytotoxic potency than epirubicin, the covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic was not more potent than gemcitabine during a 182-h incubation period (Fig. 4). Despite this difference, the cytotoxic anti-neoplastic potency of covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] at 182-h was almost identical to

levels for gemcitabine after a 72-h incubation period.[99] Levels of covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] potency at 182-h were also very close to levels of survivability detected for chemotherapeutic-resistant SKBr-3 mammary carcinoma incubated with epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] for a 72-h period where the most profound difference was observed at 10^{-7} M (chemotherapeutic-equivalent concentration).[100] Individual cytotoxic anti-neoplastic potencies of gemcitabine and epirubicin against chemotherapeutic-resistant SKBr-3 mammary carcinoma at the end of a 72-h incubation period were nearly identical.(Fig. 6) Covalent gemcitabine conjugates have been synthesized that exert greater cytotoxic anti-neoplastic potency than gemcitabine but these preparations were in the form of gemcitabine-(oxyether phospholipid)[61,72] or dual gemcitabine/doxorubicin-HPMA (*N*-(2-hydroxypropyl)methacrylamide polymer).[30] Cytotoxic anti-neoplastic activity of these covalent gemcitabine conjugates were evaluated against populations of human mammary carcinoma (MCF7/WT-2′)[72] and mammary adenocarcinoma (BG-1)[72] promyelocytic leukemia[61,72] a T-4 lymphoblastoid clone,[72] glioblastoma,[61,72] cervical epithelioid carcinoma,[72] colon adenocarcinoma,[72] pancreatic adenocarcinoma,[72] pulmonary adenocarcinoma,[72] oral squamous cell carcinoma,[72] and prostatic carcinoma.[30]

In contrast to most covalent anthracycline immunochemotherapeutics described to date, a longer 182-h incubation period was applied to evaluate the cytotoxic anti-neoplastic activity of gemcitabine-(*carbamate*)-[anti-HER2/*neu*]. Longer incubation periods were used based on results observed during in vitro efficacy evaluations performed at the end of 72-h incubation periods which directly correlated with previously published reports for related covalent gemcitabine-ligand preparations.[30,61,71] Several explanations may account for the requirement to use longer incubation periods for the in vitro evaluation of gemcitabine compared to anthracycline covalent conjugates. Since gemcitabine-(*carbamate*)-[anti-HER2/*neu*] and epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] covalent immunochemotherapeutics were both selectively 'targeted' for delivery at the same membrane HER2/*neu* receptor highly over-expressed in chemotherapeutic-resistant SKBr-3 mammary carcinoma populations, it is possible if not probable that differences in their cytotoxic anti-neoplastic activity can be attributable to; (i) differences in the vulnerability of synthesized covalent bond structures to enzymatic degradation or acid-mediated hydrolysis within the endosome/lysosome micro-environment; (ii) differences in neoplastic cell type expression profiles for enzyme fractions necessary to liberate gemcitabine from covalent immunochemotherapeutics; (iii) better capacity of anthracycline moieties in intact covalent epirubicin immunochemotherapeutics to exert one of their multiple mechanisms of action compared to gemcitabine in covalent gemcitabine immunochemotherapeutics; or (iv) greater vulnerability of the gemcitabine moiety in fractions of membrane-bound gemcitabine immunochemotherapeutic to inactivation by deamination compared to 'free' gemcitabine which can more readily diffuse across intact cancer cell membranes. The fact that gemcitabine-(*carbamate*)-[anti-HER2/*neu*] at the end of a 182-h incubation had a cytotoxic anti-neoplastic potency profile that was very similar to gemcitabine after a 72-h incubation period tends to diminish the validity of the latter possible mechanism.

Several modifications in analytical methodology could have been implemented to substantially increase the level of anti-neoplastic activity exerted by the covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic. The time frame that chemotherapeutic-resistant SKBr-3 mammary carcinoma were exposed to gemcitabine-(*carbamate*)-[anti-HER2/*neu*] could simply have been extended beyond 182 h since there was no indication that the level of cytotoxic activity achieved against chemotherapeutic-resistant SKBr-3 mammary carcinoma had reached a plateau by the end of this incubation period (Figs. 4 and 5). Further support for this consideration in based on the observation that chemotherapeutic-resistant SKBr-3 mammary carcinoma survivability was very similar when challenged with gemcitabine (*carbamate*)-[anti-HER2/*neu*] (182 h) compared to gemcitabine (72 h), but increased dramatically for gemcitabine when the incubation period was extended (72–182 h). In addition, the cytotoxic activity of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] could have instead been assessed with a breast cancer cell type that was not chemotherapeutic-resistant similar to the populations utilized to evaluate majority of the covalent biochemotherapeutics reported in the literature to date. The rare exceptions in this regard have been the evaluation of anti-chondroitin sulfate proteoglycan 9.2.27 surface marker daunorubicin conjugates against chemotherapeutic-resistant M21 metastatic melanoma;[36,38,101] anthracycline conjugates of epidermal growth factor (EGF) or an EGF fragment evaluated for their anti-neoplastic potency against chemotherapeutic-resistant MCF-7AdrR mammary carcinoma;[102] and epirubicin-(anti-HER2/neu) or epirubicin-(anti-EGFR) potency assessed against chemotherapeutic-resistant SKBr-3 mammary carcinoma.[10] In this context, measurement of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] cytotoxic anti-neoplastic potency could also have been determined in an entirely different neoplastic cell type such as pancreatic carcinoma,[103] small-cell lung carcinoma,[104] neuroblastoma,[105] or leukemia/lymphoid[72,106] populations due to their relatively high gemcitabine sensitivity. Similarly, human promyelocytic leukemia,[61,72] T-4 lymphoblastoid clones,[72] glioblastoma;[61,72] cervical epithelioid carcinoma,[72] colon adenocarcinoma,[72] pancreatic adenocarcinoma,[72] pulmonary adenocarcinoma,[72] oral squamous cell carcinoma,[72] and prostatic carcinoma[30] have been found to be sensitive to gemcitabine and gemcitabine-(oxyether phospholipid) covalent chemotherapeutic conjugates. Within this array of neoplastic cell types, however, human mammary carcinoma (MCF7/WT-2′)[72] and mammary adenocarcinoma (BG-1)[72] are known to be relatively more resistant to gemcitabine and gemcitabine-(oxyether phospholipid) chemotherapeutic conjugate. Presumably this pattern of gemcitabine sensitivity is directly relevant to the cytotoxic anti-neoplastic potency detected for gemcitabine-(*carbamate*)-[anti-HER2/*neu*] in chemotherapeutic-resistant SKBr-3 mammary carcinoma (Figs. 4 and 5). Similarly, the cytotoxic anti-neoplastic potency of the covalent gemcitabine-(*carbamate*)-[anti-HER2/*neu*] immunochemotherapeutic would likely have been higher if it had been accessed in vivo since other related anti-cancer immunochemotherapeutics frequently produce much greater cytotoxic activity in vivo compared to their in vitro level of efficacy.[39,107] The added level of cytotoxic anti-neoplastic activity for gemcitabine-(*carbamate*)-[anti-HER2/*neu*] is presumably evoked at least in part through the host's immune system by mechanisms of antibody/complement initiated cytolysis and/or antibody induced cell-mediated immune responses. Lastly, analytical methodologies could have been modified to detect cytotoxic anti-neoplastic potency in chemotherapeutic-resistant SKBr-3 mammary carcinoma in a more sensitive manner than is possible with MTT vitality/proliferation stain assay systems. Instead, declines in viability could have been measured by employing either the [H^3]-thymidine cell proliferation assay, or ATP-based assay since they reportedly are $\geqslant$10-fold more sensitive than MTT vitality stain assays.[108,109] Despite this consideration, measurement of cell vitality utilizing MTT reagent based assays have, and continue to be extensively employed for the routine assessment of chemotherapeutic anti-neoplastic activity.[110–114]

In conclusion, gemcitabine-(*carbamate*)-[anti-HER2/*neu*] was synthesized that possessed properties that included a 1.1:1 gemcitabine/IgG molar incorporation index, an overall molecular weight of 150 kDa analogous to immunoglobulin reference controls, and retained HER2/*neu* binding-avidity based on results from

cell-ELISA analyses. Cytotoxic anti-neoplastic potency of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] was similar to gemcitabine at the end of 182 h and 72 h incubation periods respectively. Cytotoxic anti-neoplastic potency of gemcitabine-(*carbamate*)-[anti-HER2/*neu*] would likely have been greater if it have been evaluated using populations of human promyelocytic leukemia, T-4 lymphoblastoid clones, glioblastoma; cervical epithelioid carcinoma, colon adenocarcinoma, pancreatic adenocarcinoma, pulmonary adenocarcinoma, oral squamous cell carcinoma, or prostatic carcinoma. Research investigations in the immediate future plan to enhance the in vitro potency of covalent gemcitabine immunochemotherapeutic within a 72–96-h incubation period relative to non-selectively 'targeted' gemcitabine. Among the parameters to be delineated include; (i) assessment of cleavable covalent gemcitabine immunochemotherapeutic synthesis schemes; (ii) design of synthesis regimens that will increase the gemcitabine molar incorporation index; (iii) dual simultaneous and selective 'targeted' delivery at over-expressed HER2/*neu* and EGFR membrane receptors; and (iv) utilization of covalent bond forming chemical reagents that present less steric hindrance to endogenous enzymes capable of liberating the chemotherapeutic moiety of covalent gemcitabine immunochemotherapeutics.

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