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Micellar Delivery of Cyclopamine and Gefitinib for Treating Pancreatic Cancer

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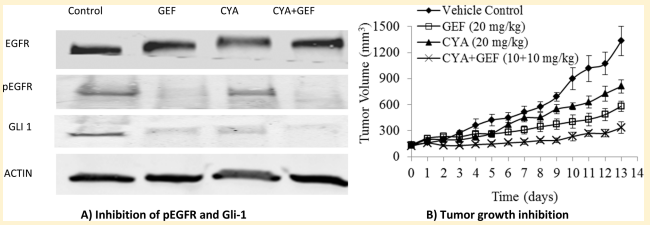
ABSTRACT: Hedgehog (Hh) and epidermal growth factor receptor (EGFR) signaling are involved in pancreatic cancer progression. Targeting these pathways simultaneously with cyclopamine (Hh inhibitor) and gefitinib (EGFR inhibitor) is a promising approach for treating pancreatic cancer. However, the major limitation for effective clinical translation of these molecules is their low aqueous solubility. We have previously demonstrated that methoxy polyethyleneglycol-b-poly-(carbonate-co-lactic acid) {mPEG-b-P(CB-co-LA)} copolymer solubilizes hydrophobic anticancer drugs and has the potential to deliver to tumors by an enhanced permeability and retention (EPR) effect. In this study, using the nanoprecipitation method, cyclopamine and gefitinib were efficiently loaded into mPEG-b-P(CB-co-LA) micelles with encapsulation efficiencies of 94.4 and 88.6%, respectively. These micelles had a narrow particle size distribution with a mean particle size of 54.3 nm and a PDI of 0.14. Combination therapy showed a synergistic effect against L3.6pl cells but an additive effect against MIA PaCa-2cells. Caspase 3/7 activity was also increased when this combination therapy was used, indicating apoptotic cell death. Gene and protein expression analysis indicated cross-talk between Hh and EGFR signaling. Furthermore, the combination decreased tumor growth rate in L3.6pl-derived xenograft mouse tumors. These data suggest the applicability of our micellar system to effectively load and deliver cyclopamine and gefitinib for combination chemotherapy.



KEYWORDS: *pancreatic cancer, cyclopamine, gefitinib, combination, micelles, drug delivery*

1. INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States with less than 5% 5 year survival.[1] The major concern in pancreatic cancer is metastasis to other organs due to late diagnosis.[2,3] At the time of diagnosis, 52% patients have distant disease, and 26% have regional spread, which decreases overall survival. Current treatment strategies for pancreatic cancer employ either surgery and/or chemotherapy using gemcitabine in combination with other anticancer agents. Chemotherapy has been shown to have only modest results due to the generation of multidrug resistance (MDR), and relapse is significant. Several lines of evidence have indicated that aberrant activation of Hedgehog (Hh)[4,5] and epidermal growth factor receptor (EGFR)[6] cascades plays a major role in pancreatic carcinogenesis, disease progression, metastasis, and their contribution to chemoresistance and subsequent relapse.

Binding of Hh ligands to patched (Ptch) receptor initiates a cascade of downstream signaling by relieving inhibition of smoothened (Smo) protein. Activated Smo in turn transduces downstream signals leading to translocation of active forms of glioma-associated oncogene (GLI) transcriptional effectors to the nucleus resulting in the increased expression of target genes.[7,8] In addition, EGFR and its ligands, EGF and transforming growth factor-α (TGF-α), can regulate key cellular events involved in cell proliferation and are normally overexpressed in pancreatic carcinomas. Ligand binding to EGFR induces dimerization of the receptor leading to autophosphorylation of intracellular tyrosine kinase, thereby resulting in activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-k) pathways.[9] Several studies have shown that there is a cross-talk between Hh and EGFR pathways,[10] which can increase chemoresistance and metastasis. Palma et al. have shown that sonic hedgehog (Shh) and EGFR cooperatively stimulated the cell proliferation in neocortical stem cells.[11,12] Furthermore, Shh overexpression in human keratinocyte cell line activates EGFR signaling.[13] Therefore, targeting these pathways simultaneously is a promising approach to improve the therapeutic outcome of pancreatic cancer treatment.

Cyclopamine (CYA), a steroidal alkaloid derived from *Veratrum californicum*, is a selective Hh pathway inhibitor and binds to c-terminal of the Smo receptor leading to blockade of

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the downstream signaling cascade. A combination of CYA with EGF-EGFR pathway inhibitor, gefitinib (GEF), has been demonstrated to be effective for treating the solid organ tumors. Mimeault et al. have demonstrated that cytotoxic effects of this combination in prostate cancer resulted from a growth arrest and massive apoptotic cell death.[14] Combining CYA and GEF improved the outcome of docetaxel-based therapies in locally advanced prostate cancer.[15,16] Hu et al. have demonstrated the enhanced inhibitory effect and greater apoptotic death of pancreatic cancer cell lines using the combination therapy with CYA and GEF versus monotherapy.[17] However, systemic administration of these molecules is challenging due to their low water solubility. The current formulation strategies involve the use of either organic solvents and/or suspensions. For CYA, while β-cyclodextrin can effectively solubilize it, the pH of the resulting solution is extremely acidic, which decreases drug stability due to isomerization into less potent forms.[18] Polymeric micelles are an attractive alternative since they can enhance the solubility and stability of hydrophobic drugs and deliver them to the target tumors by enhanced permeability and retention (EPR) effect. We have previously used methoxypolyethyleneglycol-b-poly(carbonate-co-lactic acid) [mPEG-co-P(CB-co-LA)] for micellar drug delivery.[19] These micelles form nanosized (<100 nm) spherical assemblies with a hydrophobic core and PEG shell. Hydrophobic drugs can be efficiently loaded into the core, while the PEG shell provides the stealth effect and prevents recognition by the reticulo-endothelial system (RES), thereby further increasing the efficacy of the system.

The present work aims at developing multiple drug-loaded micelles and their in vitro and in vivo evaluation for treating pancreatic cancer. Drug-loaded micelles were characterized for particle size, drug loading, in vitro drug release, and antiproliferative effects in pancreatic cancer cell lines. The effect of combination therapy on gene and protein expression was assessed followed by evaluation in a xenograft nude mouse tumor model.

2. MATERIALS AND METHODS

2.1. Materials. CYA (free base, >99%) and GEF (free base, >99%) were purchased from LC Laboratories (Woburn, MA). Caspase GLO 3/7 assay kit was purchased from Promega (Madison, WI), and Gli1 (V812) antibody was from Cell Signaling Technology (Danvers, MA). EGFR antibody and Phospho-EGFR (pY1068) antibody were purchased from Epitomics, Inc. (Burlingame, CA), and ECM gel was from Sigma (St. Louis, MO). All other reagents were of analytical grade.

2.2. Cell Lines. The MIA PaCa-2 pancreatic cancer cell line was purchased from ATCC, while the L3.6pl cell line was a kind gift from Dr. Fazlul H. Sarkar of Wayne State University (Detroit, MI). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) in an incubator maintained at 37 °C/5%CO_2.

2.3. Synthesis and Characterization of mPEG-b-P(CB-co-LA). mPEG-b-P(CB-co-LA) was synthesized as reported by Danquah et al.[19] Briefly, benzyl 2,2-bis(methylol)propionate was first synthesized from 2,2-bis(hydroxymethyl)propionic acid by reaction with benzyl bromide at 100 °C for 15 h. The product formed was used to synthesize 5-methyl-5-benzyloxycarbonyl-1,3-dioxane-2-one by reacting with triphosgene at −78 °C in pyridine and dichloromethane. mPEG-poly(carbonate-co-lactide) copolymer was finally synthesized by ring-opening polymerization of mPEG, L-lactide, and 5-methyl-5-benzyloxycarbonyl-1,3-dioxane-2-one in the presence of stannous 2-ethylhexanoateas as a catalyst at 130 °C for 24 h. The copolymer was purified and characterized using ^{1}H NMR.

2.4. Preparation of Drug-Loaded Micelles. Drug-loaded micelles were prepared by nanoprecipitation using acetone as a water-miscible organic phase. Briefly, weighed amounts of mPEG-b-P(CB-co-LA) copolymer and drug(s) were dissolved in 1 mL of acetone. The drug solution was added to 5 mL of purified water while stirring at 1000 rpm. Nanoprecipitation occurred rapidly leading to self-assembly of copolymer to form drug-loaded micelles. Acetone was then evaporated under vacuum, and micelles were centrifuged at 5000 rpm for 5 min and filtered through 0.22 μm filter paper to remove the unentrapped drug followed by lyophilization using trehalose (2.5% w/v) as a lyoprotectant. Micelles were prepared at three different drug loadings of 2.5, 5, and 7.5% w/w for CYA and GEF to determine the effect of initial drug loading on particle size and encapsulation efficiency. Combination formulation was prepared by codissolving the drugs in acetone at a ratio of 1:1 and 1:4 of CYA:GEF with 5% total initial drug loading.

2.5. Characterization of Drug-Loaded Micelles. *2.5.1. Particle Size and Size Distribution.* The size and size distribution (PDI) of micelles was determined by dynamic light scattering using Zetasizer (Malvern Instruments, United Kingdom) at 2 mg/mL polymer concentration. All measurements were taken at a scattering angle of 173° at room temperature, and a *Z*-average size of 11 measurements was reported as the mean ± SD of triplicate samples.

2.5.2. Drug Loading and Encapsulation Efficiency. To determine the drug loading and encapsulation efficiency, 9 mL of acetonitrile was added to 1 mL of micellar formulation, and drug concentrations were analyzed by HPLC-UV for GEF and LC-MS for CYA. For GEF, chromatography was performed using Inertsil ODS 3 column (5 μm, 4.6 mm × 250 mm) with acetonitrile:sodium acetate buffer (pH 4.5, 20 mM) (60:40) as the mobile phase run at a flow rate of 1 mL/min and analytical wavelength of 330 nm. CYA was analyzed using LC-MS with acetonitrile:water (containing 0.1% formic acid) (80:20) as the mobile phase run at a flow rate of 0.35 mL/min. Chromatographic separation was performed on XterraMS C18 column (2.5 μm, 4.6 mm × 50 mm). A mass transition of 412.3 → 394.2 was taken for analysis. Drug loading and encapsulation efficiency were calculated using the following formulas:

$$\text{encapsulation efficiency (\%)} = \frac{\text{weight of drug encapsulated}}{\text{initial weight of drug taken}} \times 100$$

$$\text{drug loading (\% w/w)} = \frac{\text{weight of drug encapsulated}}{\text{total weight of formulation}} \times 100$$

2.5.3. Drug Release Study. Drug-loaded micelles containing 0.5 mg each of CYA and GEF were placed in a dialysis bag with a molecular weight cutoff of 2000 Da (Spectrum Laboratories Inc., Rancho Dominguez, CA) and suspended in 50 mL of drug release media consisting of PBS (pH 7.4) containing 50% ethanol as a cosolvent to maintain the sink conditions. A drug release study was performed in a thermocontrolled shaker maintained at 37 °C with a stirring speed of 100 rpm. Samples (1 mL) were taken at regular time intervals and replaced with the fresh media. The drug content in the samples was analyzed

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Table 1. Particle Size and Encapsulation Efficiency of CYA and GEF in Micelles[a]

Single Drug-Loaded Micelles						
	CYA-loaded micelles			GEF-loaded micelles		
initial loading (% w/w)	particle size (PDI) (nm)	EE[c] (%)	drug loading (% w/w)	particle size (PDI) (nm)	EE[c] (%)	drug loading (% w/w)
2.5	56.7 ± 1.01 (0.13)	94.7 ± 0.99	2.45 ± 0.05	56.8 ± 7.22 (0.14)	96.7 ± 0.98	2.41 ± 0.10
5	54.3 ± 1.97 (0.15)	92.9 ± 4.67	4.71 ± 0.22	59.8 ± 2.82 (0.18)	87.1 ± 5.0	4.36 ± 0.25
7.5	57.6 ± 4.11 (0.12)	85.2 ± 3.42	6.35 ± 0.45	57.0 ± 8.92 (0.15)	81.2 ± 3.62	5.79 ± 0.63
Simultaneously Loaded Micelles[b]						
ratio	particle size (PDI) (nm)	EE[c] (%)		drug loading (% w/w)		
1:1	54.3 ± 2.08 (0.14)	CYA: 94.4 ± 3.13 GEF: 88.6 ± 3.09		CYA: 2.33 ± 0.08 GEF: 2.22 ± 0.15		
1:4	55.03 ± 4.05 (0.17)	CYA: 92.3 ± 3.7 GEF: 85.9 ± 4.3		CYA: 0.99 ± 0.08 GEF: 3.82 ± 0.18		

[a]Data are means ± SDs (n = 3). [b]Micelles were prepared at total initial loading of 5% w/w. [c]EE, encapsulation efficiency.

by HPLC-UV for GEF and LC-MS for CYA as described above. The cumulative drug released into the media was determined after correction for the volume and drug losses during sampling and plotted against time. Release profiles were fitted into different kinetic models such as zero order, first order, Higuchi, Weibull, Baker Londsdale, and Hixon-Crowell, and regression analysis was performed to determine the best fit.

2.5.4. In Vitro Cytotoxicity Assay. For cytotoxicity assays, MIA PaCa-2 and metastatic L3.6pl cell lines (5000 cells/well) were seeded in a 96-well cell culture plate and incubated at 37 °C/5%CO_2 for 24 h. Cells were then treated with different concentrations of CYA, GEF, and their combination for 72 h. Because DMSO was used to solubilize free drugs, DMSO controls were also included in the study. The media were replaced with the fresh one containing 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT;100 μL, 0.5 mg/mL) with further incubation for 4 h at 37 °C/5%CO_2. Media were removed, formed formazan crystals were dissolved in DMSO, and the absorbance was measured at 560 nm after subtraction for cell debris at 655 nm. The cell viability was calculated using the following equation:

$$\text{cell viability (\%)} = \frac{\text{absorbance of test sample}}{\text{absorbance of control}} \times 100$$

Mean inhibitory concentration (IC_{50}) values (i.e., drug concentrations required to reduce the cell viability to 50%) were calculated graphically from the plots of cell viability versus drug concentration. To assess the synergistic, additive, or antagonistic interaction between CYA and GEF, isobologram analysis was used.[20,21] Combination indexes (CIs) were determined using the following formula:

$$\text{CI} = \frac{d1}{D_{50}1} + \frac{d2}{D_{50}2}$$

where $d1$ and $d2$ are doses of drug 1 and drug 2 required to produce a 50% effect when used in combination and $D_{50}1$ and $D_{50}2$ are doses of drug 1 and drug 2 required to produce a 50% effect when used alone. CI values <0.9, =0.9−1.1, and >1.1 indicate synergistic, additive, and antagonistic effects, respectively.

2.5.5. Caspase GLO 3/7 Assay. Cells (5000 cells/well) were seeded in a 96-well cell culture plates for 24 h at 37 °C/5%CO_2. The media were replaced with the fresh media containing drug (CYA, GEF, or their combination)-loaded micelles, and the cells were incubated for 24 h at 37 °C/5%CO_2. Caspase GLO 3/7 assay was then performed as per the manufacturer's instructions, and luminescence was measured by a Luminometer (Berthold Detection Systems, Huntsville, AL).

2.5.6. mRNA Levels of Gli1 and EGFR by Real-Time Reverse-Transcribed Polymerase Chain Reaction (RT-PCR). The total RNA was extracted from cultured pancreatic cancer cells using a RNeasy RNA isolation kit (Qiagen, MD), and its quality was determined using Nanodrop 2000 instrument and reverse transcribed into cDNA template as previously described.[20] A total of 100 ng of cDNA was then amplified by real-time PCR using SYBR Green dye universal master mix on a Light Cycler 480 instrument (Roche, Indianapolis) using the primers for Gli-1 (5′-AGTTTCCAGCCTGGACCACG and 5′-GAGGTCCGGATTACGGTTT) and EGFR (5′-GGCACTTTTGAAGATCATTTTCTC and 5′-CTGTGTTGAGGGCAATGAG) for 40 cycles. Crossing point (Cp) values were used for calculating the relative amount of mRNA as compared to the S19 (housekeeping gene) level and scaled relative to control samples set at a value of 1. Results for gene expression in experimental samples were plotted as compared with the control.

2.5.7. Protein Expression of Gli1, EGFR, and Phospho-EGFR (pEGFR) by Western Blot. L3.6pl cancer cells were seeded into six-well cell culture plates (3×10^5 cells/well) and treated with drug-loaded micelles for 48 h. Cells were then lysed with RIPA buffer containing a protease inhibitor cocktail and phosphatase inhibitor cocktail 2 and 3 (Sigma, St. Louis, MO). The total protein concentration was determined using the bicinchoninic acid (BCA) method. Proteins were resolved on sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred to an Immobilonpolyvinylidene fluoride (PVDF) membrane using an iBlot dry blotting system (Invitrogen, Carlsbad, CA) followed by blocking with Odyssey blocking buffer for 1 h at room temperature with shaking. Membranes were then incubated with an appropriate dilution of the primary antibody for 16 h at 4 °C. β-Actin was used as the loading control, and target proteins (Gli1, EGFR, and pEGFR) were detected by LI-COR secondary antibodies using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

2.5.8. In Vivo Efficacy of Drug-Loaded Micelles in Xenograft Model. All animal experiments were performed in accordance with the NIH animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center (UTHSC, Memphis, TN). Heterotopic xenograft flank tumors were established in 6 week old male athymic nude mice

(Jackson Laboratory, Bar Harbor, ME) by subcutaneous injection of 1 million L3.6pl pancreatic cancer cells suspended in 1:1 serum-free media and ECM gel. When tumors reached ~150 mm^3, mice were randomized and assigned to different groups (N = 5). These tumor-bearing mice were treated with drug-loaded micelles containing CYA (20 mg/kg), GEF (20 mg/kg), or their combination (10 mg/kg each) given intratumorally for 5 days a week for 2 weeks. Tumors were measured with a caliper before each injection, and their volumes were calculated using the formula (length × $width^2$)/2.

3. STATISTICS

Statistical analyses were done using Student's t test with a p value <0.05 considered to be a statistically significant difference.

4. RESULTS

4.1. Preparation and Characterization of Drug-Loaded Micelles. The synthesized mPEG-b-(CB-co-LA) copolymer had an average molecular weight of 9800 Da with carbonate and lactic acid contents of 10 and 29 mol, respectively, and a mPEG content of 51.02% as determined by ^{1}H NMR. On nanoprecipitation, the copolymer self-assembled into nanosized micelles resulting in a clear homogeneous suspension in contrast to a turbid drug suspension when no copolymer was used. Micelles loaded with CYA and GEF alone were prepared at three initial drug loadings, that is, 2.5, 5, and 7.5% w/w. Table 1 shows the particle size, encapsulation efficiency, and drug loading in the micelles. A mean particle size of 50−60 nm with a low polydispersity of <0.2 was obtained, which did not significantly change on altering the initial drug loading. However, on increasing the initial drug loading from 2.5 to 7.5%, the encapsulation efficiency decreased from 94.7 to 85.2% for CYA and from 86.7 to 81.2% for GEF. The multiple drug-loaded micelles were prepared at 1:1 and 1:4 ratios of CYA:GEF with 5% total initial drug loading showing encapsulation efficiencies >90% for CYA and >85% for GEF with a particle size of ~55 nm, indicating the suitability of the micellar system to encapsulate and carry the payload. CYA and GEF release from micelles followed the Higuchi model with 85% cumulative release in 4 days for GEF and 80% cumulative release for CYA in 7 days (Figure 1).

4.2. In Vitro Cytotoxicity Assays. Cytotoxic effects of CYA, GEF, and their combination on the pancreatic cancer cell lines, MIA PaCa-2, and L3.6pl are shown in Figure 2. CYA and GEF were effective in reducing the viability of both cell lines in a concentration-dependent manner. In L3.6pl cells, IC_{50} values of 17.8 and 62.09 μM were obtained for CYA and GEF, respectively, while IC_{50} values of 9.33 and 49.2 μM were obtained in MIA PaCa-2 cells (Table 2). Furthermore, GEF-loaded micelles were more effective than free GEF in both cell lines, while the CYA-loaded formulation was more effective in MIA PaCa-2 cells and showed an equivalent effect in L3.6pl cells to that of free CYA. Empty micelles were nontoxic to the cells.

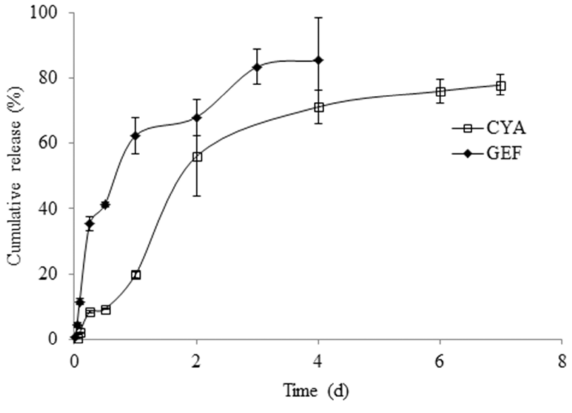

Figure 1. Release profile of CYA and GEF from PEG-b-P(CB-co-LA) micelles coloaded with these two drugs.

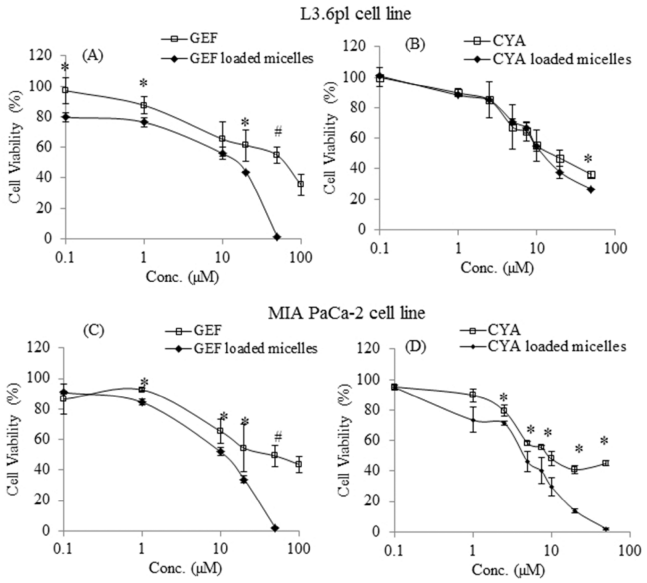

Figure 2. Antiproliferative effect of drugs and their micellar formulation on L3.6pl and MIA PaCa-2 pancreatic cancer cells. (A and B) Cytotoxicity assay of GEF and CYA on L3.6pl cells, respectively. (C and D) Cytotoxicity assay on GEF and CYA, respectively, on MIA PaCa-2 cells. Data represent means ± SDs (N = 4); $^*p < 0.05$, and $^\#p < 0.01$.

Table 2. IC_{50} Values and Isobologram Analysis of CYA and GEF in L3.6pl and MIA PaCa-2 Cell Lines

	IC_{50}[a] (μM)					
	L3.6pl			MIA PaCa-2		
treatment	CYA	GEF	CI[b]	CYA	GEF	CI[b]
CYA	17.83			9.33		
GEF		62.09			49.22	
CYA-loaded micelles	12.70			4.60		
GEF-loaded micelles		14.80			11.00	
CYA−GEF-loaded micelles (1:1 ratio)	3.73	3.73	0.55	3.65	3.65	1.1
CYA−GEF-loaded micelles (1:4 ratio)	1.60	6.50	0.57	1.70	6.70	0.9

[a]Inhibitory concentration 50 (IC_{50}) denotes the drug concentration required to reduce the cell viability by 50% of control. It is determined graphically from the plots of cell viability and drug concentrations. [b]The CI was determined from the IC_{50} value using the formula, CI = $d1/D_{50}1 + d2/D_{50}2$, where $d1$ and $d2$ are the IC_{50} values of CYA and GEF when used in combination and $D_{50}1$ and $D_{50}2$ are IC_{50} values of CYA and GEF when used alone. CI < 1 indicates synergism.

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There was a significant decrease in the IC_{50} values in both cell lines when treated with a CYA and GEF combination (Figure 3 and Table 2). The CI, which is indicative of an interaction in drug activity, was determined from the IC_{50} values, and it was >0.9 for the treatment in the L3.6pl cell line, indicating the synergism, while it was between 0.9 and 1.1 in MIA PaCa-2 cells, indicating an additive effect (Table 2).

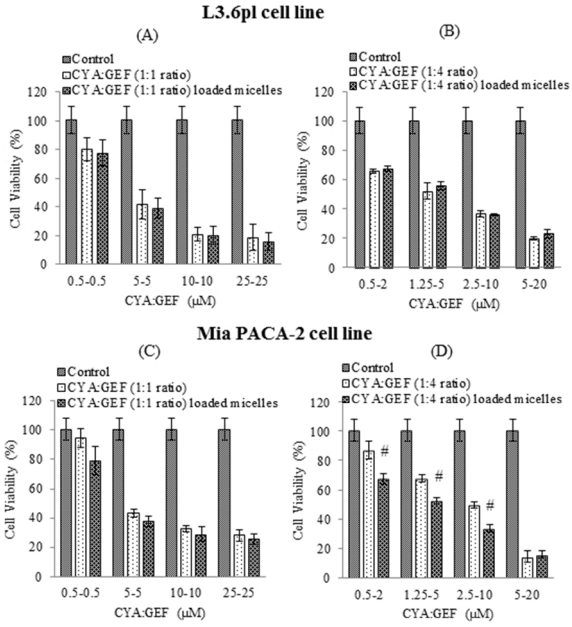

Figure 3. Antiproliferative effect of combination formulations on pancreatic cancer cells. (A and B) Cytotoxicity assay of a combination of CYA:GEF in 1:4 and 1:1 ratios, respectively, on L3.6pl cells. (C and D) Cytotoxicity assay of a combination of CYA:GEF in 1:4 and 1:1 ratios, respectively, on MIA PaCa-2 cells. Data represent means ± SDs (N = 4); $^{\#}p < 0.01$ vs CYA:GEF.

4.3. Caspase 3/7 Activity. Incubation with CYA, GEF, and their combination resulted in a significant increase in caspase 3/7 activity, indicating apoptosis (Figure 4). Furthermore, the combination of these drugs showed significantly higher apoptosis in the L3.6pl cell line and an equivalent effect in the MIA PaCa-2 cell line at a reduced dose.

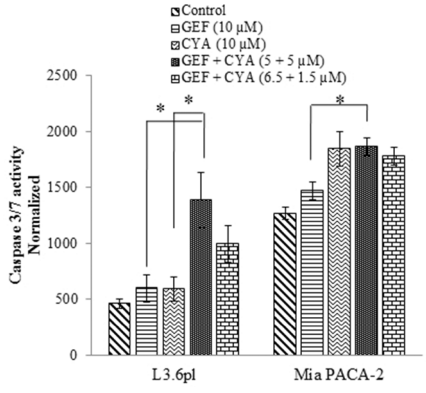

Figure 4. Apoptosis assay of GEF, CYA, and their combination loaded micelles in L3.6pl and MIA PaCa-2 cells. The activity was expressed as relative light units per second (RLU/s) and normalized with total protein concentration. Data represented as the mean ± SD (N = 3); $^{*}p < 0.05$.

4.4. Effect of Drug Treatment on GLI-1 and EGFR Gene Expression. Expression of GLI-1 and EGFR after drug treatments of L3.6pl and MIA PaCa-2 cells was assessed using real-time RT-PCR and calculating the fold changes with respect to the Cp values for S19 (housekeeping gene) (Figure 5).

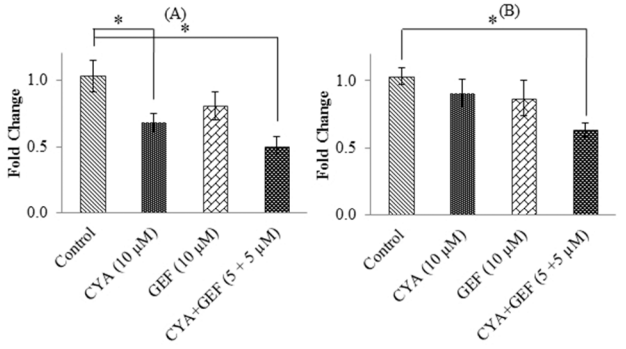

Figure 5. Effect of treatment of CYA, GEF, and their combination loaded micelles on gene expression of GLI-1 in (A) L3.6pl cells and (B) MIA PaCa-2 cells; $^{*}p < 0.05$.

Treatment with CYA or its combination with GEF significantly reduced GLI-1 expression in L3.6pl cells. In contrast, neither CYA nor GEF alone significantly reduced GLI-1 in MIA PaCa-2 cells. However, a combination treatment significantly decreased GLI-1 levels. Furthermore, drug treatments did not significantly affect EGFR expression levels in both cell lines (data not shown).

4.5. Effect of Drug Treatment on Protein Expression of GLI-1, EGFR, and pEGFR. Treatment with GEF at 10 μM dose resulted in a significant decrease in phosphorylation of EGFR protein, while CYA treatment at 10 μM did not affect EGFR phosphorylation. Combination therapy with CYA and GEF at a half dose of 5 μM was effective in knocking down EGFR phosphorylation. Furthermore, both GEF and CYA reduced overall protein expression of GLI-1, and combination therapy resulted in higher activity.

4.6. In Vivo Efficacy Study in Xenograft Model. We tested the combination formulation in comparison to monotherapy given intratumorally in xenograft tumor-bearing nude mouse model. Tumors were generated by subcutaneous injection of L3.6pl pancreatic cancer cells. Treatment with the formulations (CYA, GEF, and combination) was started when a tumor size of 150 mm^3 was reached. Figure 7 shows the changes in tumor volume when treated with the formulation. The tumor growth rate was significantly lower for the group treated with the combination therapy at half dose levels (10 mg/kg each) in contrast to the groups treated with GEF (20 mg/kg) or CYA (20 mg/kg) alone. The group treated with the combination formulation showed a mean tumor weight of 0.22 g as compared to 0.54 g in the GEF-treated group and 0.70 g in the CYA-treated group, while in vehicle-treated animals, a mean tumor weight of 1.27 g was observed at 21 day post-tumor implantation.

5. DISCUSSION

Cross-talk between Hh and EGFR pathways during cancer cell proliferation is known to occur, and this establishes the rationale of combining inhibitors of these pathways for treating pancreatic cancer.[14,16,17] CYA and GEF play an important role in treating tumors with aberrant activation of Hh and EGFR

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pathways. These drugs have been tested individually as well as in combination for prostate cancer,[14−16] and recent studies have shown their possible synergism in pancreatic cancer as well.[17] However, these drugs are primarily dissolved in either DMSO or ethanol or given in the form of suspensions. Such formulations are not viable for clinical translation, and thus, there is a need to formulate these drugs in a biocompatible delivery system. Hydroxypropylβ-cyclodextrin(HP-β-CD) also has been used for CYA delivery; however, effective solubilization in HP-β-CD requires an acidic pH of 3.5.[22,23] At this pH, CYA has been shown to degrade and isomerizes to less potent forms.[18]

In this study, we have used an amphiphilic mPEG-b-(CB-co-LA) copolymer synthesized and characterized as reported earlier by our group.[19] This polymer consists of mPEG (5000 Da) block that forms the hydrophilic outer shell, while the inner core is formed by carbonate and lactic acid-based hydrophobic block. In our previous report, we have successfully loaded a hydrophobic anticancer molecule, bicalutamide.[19] Furthermore, the polymer showed a low critical micelle concentration (CMC), which varied on varying the carbonate to lactide ratio. In the present report, we have simultaneously loaded GEF and CYA in mPEG-b-(CB-co-LA) copolymer micelles and tested their efficacy in pancreatic cancer cell lines and in a xenograft tumor model.

The nanoprecipitation method was employed for the preparation of drug-loaded micelles since the process occurs rapidly without needing sonication or homogenization. The copolymer was able to encapsulate both CYA and GEF with high efficiency (Table 1), which could be attributed to the ability of the hydrophobic core to solubilize these drugs. In addition to the appreciable drug loading, the outer hydrophilic mPEG shell could prevent the aggregation by steric hindrance and could possibly provide the stealth properties necessary to reduce uptake by the RES. We observed that an increase in initial drug loading decreases the encapsulation efficiency for both CYA and GEF that could be attributed to the holding capacity of the hydrophobic core reaching saturation at higher initial drug loadings with a subsequent decrease in encapsulation efficiency.[24,25] Multiple drug-loaded micelles were then prepared at 5% w/w initial drug loading at 1:1 and 1:4 ratios of CYA:GEF, and a high encapsulation efficiency was obtained for both of the molecules (Table 1). Drug-loaded micelles had a mean particle size of 50−60 nm. A small particle size with a mPEG outer shell will improve the mean residence time in the central compartment and hence the EPR effect in the tumor tissues after systemic administration.

Drug release studies showed a controlled release for 7 and 4 days for CYA and GEF, respectively, from drug-loaded micelles (Figure 1). From such systems, drug release was mainly controlled by diffusion and/or degradation of the polymer. Several factors can influence the drug release pattern, including the nature of hydrophobic core, physicochemical properties of incorporated drugs, and the strength of the interaction between the drug and the hydrophobic core.[26] CYA and GEF showed different release patterns, which were expected since both molecules have different hydrophobicities. CYA is more hydrophobic (predicted Log *P*, 5.439) and released at a slower rate than GEF (predicted Log *P*, 2.702). Because both CYA and GEF have low water solubility (<5 μg/mL), 50% ethanol was added as a cosolvent in the release media to maintain sink conditions. Ethanol was added at a concentration where these drugs have appreciable solubility (>200 μg/mL), thereby preventing any chances of drug precipitation inside the dialysis membrane. Because ethanol does not dissolve the polymer, disruption of micelles is unlikely. This is in line with the previous reports where PBS containing 50% ethanol was used as a release media for drug-loaded PLA-PEG micelles for the Hh pathway inhibitor.[27]

After fully characterizing drug-loaded micelles, we tested these for activity against pancreatic cancer cell lines. A CI value of <0.9 was obtained in the L3.6pl cell line, indicating synergism, while an additive effect was seen in MIA PaCa-2 cells (CI between 0.9 and 1.1) (Table 2). The extent of cross-talk between Hh and EGFR pathways could determine the effect of selective inhibition. Because these pathways work constitutively in metastatic cancer, inhibition by selective inhibitors may be expected to have a synergistic effect.[14] Similarly, combination therapy had shown a significant increase in apoptosis in L3.6pl cells at a half-dose level (Figure 4).

We have tested the CYA and GEF combination at two different ratios (1:1 and 1:4) for in vitro cytotoxicity assays to determine the IC_{50} values and synergism in both L3.6pl and MIA PaCa-2 cell lines (Table 2). Caspase 3/7 assay was then performed at concentrations near the calculated IC_{50} values (i.e., 5 + 5 and 6.5 + 1.5 μM) of the combination to determine their effect on apoptotic cell death.

To elucidate the potential interaction between the Hh and the EGFR pathway and their subsequent inhibition by treatment, we assessed the mRNA expression levels of GLI-1 and EGFR (Figure 5). Our observation that CYA or its combination with GEF is able to significantly decrease GLI-1 in L3.6pl cells is in accordance with previous literature.[22,23] In contrast, CYA did not decrease GLI-1 expression in MIA PaCa-2 cells, and only its combination with GEF had a significant impact. Furthermore, both drugs were effective in decreasing protein levels of transcription factor GLI-1 in L3.6pl cell line (Figure 6). This downregulation of GLI-1 expression by drug

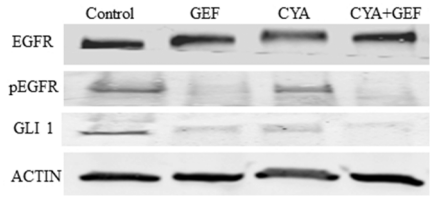

Figure 6. Effect of CYA (10 μM), GEF (10 μM), and their combination (5 μM each) loaded micelles on protein expression of Gli1, EGFR, and phospho-EGFR in L3.6pl cells.

combination points not only to the extent of cross-talk between the two signaling cascades in pancreatic cancer progression but also toward the efficacy of our combination approach over monotherapy with either CYA or GEF. In neither of the cell lines was treatment with either drug able to reduce EGFR gene and protein expression, confirming that the activity of this signaling cascade is dependent on protein phosphorylation. EGFR cascades are chiefly regulated at this level, and treatment with both GEF and CYA was able to decrease phospho-EGFR levels (Figure 6).

In summary, our in vitro studies have indicated the potential synergism of CYA and GEF in the L3.6pl cell line. For in vivo assessment of the formulations, we used the L3.6pl cell line-derived xenograft nude mice model. We used the ratio of 1:1 (i.e., 10 + 10 mg/kg of each drug) since at this ratio, the combination had lower CI (and thus demonstrates synergism).

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Because other workers have tested the efficacy of GEF and CYA at doses ranging from 10 to 100 mg/kg,[28−30] we decided to use the dose of 20 mg/kg for monotherapy and half-dose levels (10 + 10 mg/kg) for testing drug combination. In line with the in vitro results, our in vivo results too confirmed the enhanced efficacy of the combination formulation. The growth rate of tumor was significantly slower in the group treated with combination therapy when compared to monotherapy (Figure 7). Previous reports have demonstrated efficacy of CYA[5,31] and

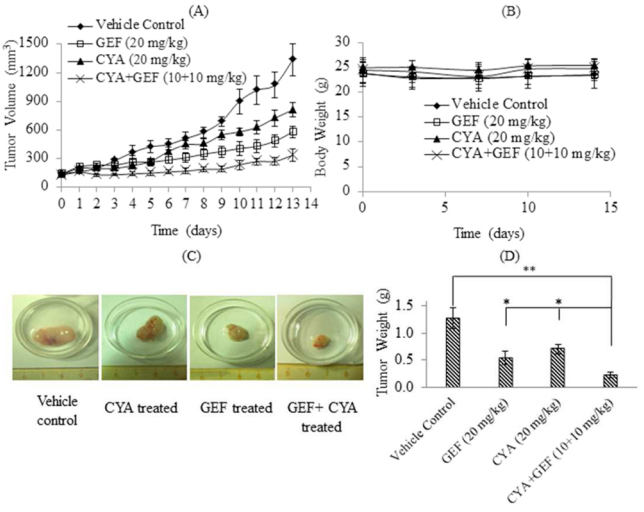

Figure 7. Anticancer activity of CYA, GEF, and their combination loaded micelles on the growth of tumors derived from L3.6pl cells in nude mice. (A and B) Tumor volumes and body weights, (C) pictures of tumors excised from the mice, and (D) tumor weights at the end of the study. Data represented as the mean ± SE; $*p < 0.05$, and $**p < 0.01$.

GEF[28,32] as monotherapy in various cancer models. For GEF, the oral route was more frequently explored, whereas for CYA, the subcutaneous route was used, and drugs were given as suspensions.[5,31] Our results clearly indicate the use of micelles for efficient delivery of a Hh pathway and an EGFR pathway inhibitor for combination chemotherapy for the treatment of pancreatic cancer.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Siegel, R.; Ward, E.; Brawley, O.; Jemal, A. Cancer Statistics, 2011. *CA Cancer J. Clin.* **2011**, *61*, 212−236.

(2) Li, D.; Xie, K.; Wolff, R.; Abbruzzese, J. L. Pancreatic cancer. *Lancet* **2004**, *363*, 1049−1057.

(3) Warshaw, A. L.; Fernandez-del Castillo, C. Pancreatic carcinoma. *N. Engl. J. Med.* **1992**, *326*, 455−465.

(4) Berman, D. M.; Karhadkar, S. S.; Maitra, A.; Oca, R. M. d.; Gerstenblith, M. R.; Briggs, K.; Parker, A. R.; Shimada, Y.; Eshleman, J. R.; Watkins, D. N.; Beachy, P. A. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* **2003**, *425*, 846−851.

(5) Thayer, S. P.; Magliano, M. P. d.; Heiser, P. W.; Nielsen, C. M.; Roberts, D. J.; Lauwers, G. Y.; Qi, Y. P.; Gysin, S.; Castillo, C. F. n.-d.; Yajnik, V.; Antoniu, B.; McMahon, M.; Warshaw, A. L.; Hebrok, M. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* **2003**, *425*, 851−856.

(6) Wang, Z.; Sengupta, R.; Banerjee, S.; Y., L.; Zhang, Y.; Rahman, K. M. W. Epidermal growth factor receptor-related protein inhibits cell growth and invasion in pancreatic cancer. *Cancer Res.* **2006**, *66*, 7653−7660.

(7) Magliano, M. P. d.; Hebrok, M. Hedgehog signalling in cancer formation and maintenance. *Nature Rev.* **2003**, *3*, 903−911.

(8) Rubin, L. L.; Sauvage, F. J. d. Targeting the Hedgehog pathway in cancer. *Nature Rev. Drug Discovery* **2006**, *5*, 1026−1033.

(9) Krasinskas, A. M. EGFR Signaling in Colorectal Carcinoma. *Pathol. Res. Int.* **2011**, *2011*, 1−6.

(10) Mangelberger, D.; Kern, D.; Loipetzberger, A.; Eberl, M.; Aberger, F. Cooperative Hedgehog-EGFR signaling. *Front. Biosci.* **2012**, *17*, 90−99.

(11) Palma, V.; Altaba, A. R. i. Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. *Development* **2004**, *131*, 337−345.

(12) Palma, V.; Lim, D. A.; Dahmane, N.; Sanchez, P.; Brionne, T. C.; Herzberg, C. D.; Gitton, Y.; Carleton, A.; Alvarez-Buylla, A.; Altaba, A. R. i. Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. *Development* **2005**, *132*, 335−344.

(13) Bigelow, R. L.; Jen, E. Y.; Delehedde, M.; Chari, N. S.; McDonnell, T. J. Sonic hedgehog induces epidermal growth factor dependent matrix infiltration in HaCaT keratinocytes. *J. Invest. Dermatol.* **2005**, *124*, 457−465.

(14) Mimeault, M.; Moore, E.; Moniaux, N. Cytotoxic effects induced by a combination of cyclopamine and gefitinib, the selective hedgehog and epidermal growth factor receptor signaling inhibitors, in prostate cancer cells. *Int. J. Cancer* **2006**, *118*, 1022−1031.

(15) Mimeault, M.; Johansson, S. L.; Henichart, J.-P.; Depreux, P.; Batra, S. K. Cytotoxic Effects Induced by Docetaxel, Gefitinib, and Cyclopamine on Side Population and Nonside Population Cell Fractions from Human Invasive Prostate Cancer Cells. *Mol. Cancer Ther.* **2010**, *9*, 617−630.

(16) Mimeault, M.; Johansson, S. L.; Vankatraman, G.; Moore, E.; Henichart, J.-P.; Depreux, P.; Lin, M.-F.; Batra, S. K. Combined targeting of epidermal growth factor receptor and hedgehog signaling by gefitinib and cyclopamine cooperatively improves the cytotoxic effects of docetaxel on metastatic prostate cancer cells. *Mol. Cancer Ther.* **2007**, *6*, 967−978.

(17) Hu, W.; Liu, T.; Xiong, J.; Wang, C. Blockade of sonic hedgehog signal pathway enhances antiproliferative effect of EGFR inhibitor in pancreatic cancer cells. *Acta Pharmacol. Sin.* **2007**, *28*, 1224−1230.

(18) Wilson, S. R.; Strand, M. F.; Krapp, A.; Rise, F.; Petersen, D.; Krauss, S. Hedgehog antagonist cyclopamine isomerizes to less potent forms when acidified. *J. Pharm. Biomed. Anal.* **2010**, *52*, 707−713.

(19) Danquah, M.; Fujiwara, T.; Mahato, R. I. Self-assembling methoxypoly(ethylene glycol)-b-poly(carbonate-co-L-lactide) block copolymers for drug delivery. *Biomaterials* **2010**, *31*, 2358−2370.

(20) Danquah, M.; Li, F.; Duke, C. B., III; Miller, D. D.; Mahato, R. I. Micellar Delivery of Bicalutamide and Embelin for Treating Prostate Cancer. *Pharm. Res.* **2009**, *26*, 2081−2092.

(21) Steel, G. G.; Peckham, M. J. Exploitable mechanisms in combined radiotherapy-chemotherapy: The concept of additivity. *Int. J. Radiat. Oncol. Biol. Phys.* **1979**, *5*, 85−91.

(22) Feldmann, G.; Dhara, S.; Fendrich, V.; Bedja, D.; Beaty, R.; Mullendore, M.; Karikari, C.; Alvarez, H.; Iacobuzio-Donahue, C.; Jimeno, A.; Gabrielson, K. L.; Matsui, W.; Maitra, A. Blockade of

Hedgehog Signaling Inhibits Pancreatic Cancer Invasion and Metastases: A New Paradigm for Combination Therapy in Solid Cancers. *Cancer Res.* **2007**, *67*, 2187−2196.
(23) Feldmann, G.; Habbe, N.; Dhara, S.; Bisht, S.; Alvarez, H.; Fendrich, V.; Beaty, R.; Mullendore, M.; Karikari, C.; Bardeesy, N.; Ouellette, M. M.; Yu, W.; Maitra, A. Hedgehog inhibition prolongs survival in a genetically engineered mouse model of pancreatic cancer. *Gut* **2008**, *57*, 1420−1430.
(24) Li, F.; Danquah, M.; Mahato, R. I. Synthesis and Characterization of Amphiphilic Lipopolymers for Micellar Drug Delivery. *Biomacromolecules* **2010**, *11*, 2610−2620.
(25) Li, F.; Lu, Y.; Li, W.; Miller, D. D.; Mahato, R. I. Synthesis, formulation and in vitro evaluation of a novel microtubule destabilizer, SMART-100. *J. Controlled Release* **2010**, *143*, 151−158.
(26) Allen, C.; Maysinger, D.; Eisenberg, A. Nano-engineering block copolymer aggregates for drug delivery. *Colloids Surf., B* **1999**, *16*, 3−27.
(27) Chenna, V.; Hu, C.; Pramanik, D.; Aftab, B. T.; Karikari, C.; Campbell, N. R.; Hong, S. M.; Zhao, M.; Rudek, M. A.; Khan, S. R.; Rudin, C. M.; Maitra, A. A polymeric nanoparticle encapsulated small-molecule inhibitor of Hedgehog signaling (NanoHHI) bypasses secondary mutational resistance to Smoothened antagonists. *Mol. Cancer Ther.* **2012**, *11*, 165−173.
(28) Shintani, S.; Li, C.; Mihara, M.; Nakashiro, K.-i.; Hamakawa, H. Gefitinib ('Iressa'), an epidermal growth factor receptor tyrosine kinase inhibitor, mediates the inhibition of lymph node metastasis in oral cancer cells. *Cancer Lett.* **2003**, *201*, 149−155.
(29) Varnat, F.; Duquet, A.; Malerba, M.; Zbinden, M.; Mas, C.; Gervaz, P.; Ruiz i Altaba, A. Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. *EMBO Mol. Med.* **2009**, *1* (6−7), 338−351.
(30) Wang, S.; Guo, P.; Wang, X.; Zhou, Q.; Gallo, J. M. Preclinical pharmacokinetic/pharmacodynamic models of gefitinib and the design of equivalent dosing regimens in EGFR wild-type and mutant tumor models. *Mol. Cancer Ther.* **2008**, *7* (2), 407−417.
(31) Lee, J.; Wu, X.; Magliano, M. P. d.; Peters, E. C.; Wang, Y.; Hong, J.; Hebrok, M.; Ding, S.; Cho, C. Y.; Schultz, P. G. A Small-Molecule Antagonist of the Hedgehog Signaling Pathway. *ChemBioChem* **2007**, *8*, 1916−1919.
(32) Schiff, B. A.; McMurphy, A. B.; Jasser, S. A.; Younes, M. N.; Doan, D.; Yigitbasi, O. G.; Kim, S.; Zhou, G.; Mandal, M.; Bekele, B. N.; Holsinger, F. C.; Sherman, S. I.; Yeung, S.-C.; El-Naggar, A. K.; Myers, J. N. Epidermal Growth Factor Receptor (EGFR) Is Overexpressed in Anaplastic Thyroid Cancer, and the EGFR Inhibitor Gefitinib Inhibits the Growth of Anaplastic Thyroid Cancer. *Clin. Cancer Res.* **2004**, *10*, 8594−8602.