cIRCR201-dPBD, a Novel Pyrrolobenzodiazepine Dimer-Containing Site-Specific Antibody–Drug Conjugate Targeting c-Met Overexpression Tumors

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Cite This: ACS Omega 2020, 5, 25798−25809

ABSTRACT: c-Met, as a receptor expressed on the cell membrane, contributes to the growth and metastasis of tumors, as well as angiogenesis, mainly through the hepatocyte growth factor (HGF)/c-Met axis during tumor progression. Although several c-Met inhibitors, including small molecules and monoclonal antibody inhibitors, are currently being investigated, their clinical outcomes have not been promising. Development of an antibody–drug conjugate (ADC) against c-Met could be an attractive therapeutic strategy that would provide superior antitumor efficacy with broad-spectrum c-Met expression levels. In the present study, site-specific drug–conjugate technology was applied to develop an ADC using the human-mouse cross-reactive c-Met antibody and a prodrug pyrrolobenzodiazepine (PBD). The toxin payload was uniformly conjugated to the light-chain C-terminus of the native cIRCR201 antibody (drug-to-antibody ratio = 2), as confirmed using LC−MS. Using a high-throughput screening system, we found that cIRCR201-dPBD exhibited varying sensitivities depending on the expression levels of c-Met, and it induced receptor-mediated endocytosis and toxin-mediated apoptosis in 47 different cancer cell lines. cIRCR201-dPBD also showed significant antitumor activity on the MET-amplified cancer cells using in vivo xenograft models. Therefore, cIRCR201-dPBD could be a promising therapeutic strategy for tumors with c-Met expression.

1. INTRODUCTION

The hepatocyte growth factor receptor (HGFR, c-Met), a tyrosine kinase encoded by the MET oncogene, plays an essential role in the development and progression of several human cancers at multiple levels. Dysregulation of the hepatocyte growth factor (HGF)/c-Met pathway has been reported to promote metastasis, angiogenesis, and growth, as well as confer resistance to EGFR tyrosine kinase inhibitors (TKIs). MET is found to be amplified, mutated, or overexpressed as part of pathway hyperactivation in various tumors, including non-small-cell lung cancer (NSCLC), where MET exon 14 mutations, MET amplification, and constitutive kinase activation have been reported. The development of treatment strategies for targeting the c-Met/HGF axis would provide novel therapeutic approaches for multiple cancer types. Major classes of c-Met/HGF inhibitors include monoclonal antibodies that bind HGF or compete with HGF for binding to c-Met and selective or nonselective small molecules. Although several c-Met inhibitors are under investigation, either as monotherapy or in combination with other targeted agents or chemotherapy for the treatment of a wide variety of tumors, clinical outcomes of these inhibitors do not seem promising. In the case of antibody therapeutics, the phase III clinical trial of onartuzumab (a “one-armed” anti-c-Met antibody) did not report improved clinical outcomes in patients with MET-positive NSCLC. Such poor clinical outcomes suggest that c-Met inhibition via ligand-blocking antibodies may not be an effective therapeutic strategy. In addition, a strategy for patient selection in order to identify tumors dependent on activated c-Met signaling would be necessary in order to predict the sensitivity to the inhibitors.

The development of an antibody–drug conjugate (ADC) against c-Met could be an attractive therapeutic strategy since efficacy would depend on target expression rather than downstream signaling. The development of c-Met-targeting...
Figure 1. Synthesis of cIRCR201-dPBD and physicochemical analysis. (a) Native IRCR201 was modified with a flexible linker (G7) and a CaaX motif (Cys-Val-Ile-Met) at the light-chain C-terminus (PDB ID: 2IG2), which was prenylated to generate CaaX-modified IRCR201 (cIRCR201) using farnesyltransferase (FTase). Pyrrolobenzodiazepine (PBD) dimers and β-glucuronide-linkage were conjugated to the specific site (Cys214) within the CaaX motif through oxime ligation. (b) Physicochemical purity and DAR (drug-to-antibody ratio) of cIRCR201-dPBD were analyzed by size exclusion chromatography (SEC-HPLC) (upper chromatogram) and hydrophobic interaction chromatography (HIC-HPLC) (lower chromatogram), respectively. (c) Payload conjugation chain was assessed by intact LC-MS for analyzing the heavy chains and light chains of IRCR201 (left) and cIRCR201-dPBD (right). (d) Identification of the payload (PBDs) conjugation site was performed through LC-MS.
ADCs has been recently reported with different strategies for the generation of c-Met ADCs (ABBV-399, AbbVie; SHR-A1403, Hengrui Therapeutics; and TR1081-ADC, Tanabe Research Laboratories). They all exhibited a robust antitumor effect against c-Met overexpression cancers at the preclinical stage. In particular, the clinical phase I data of ABBV-399 has revealed its favorable safety and tolerability profile in patients with c-Met-positive NSCLC. The other therapeutics are still in clinical phase I.

We developed a novel c-Met antibody (IRCR201) that successfully bound to both human and mouse c-Met proteins with high affinity and specificity in a previous study. IRCR201 depleted c-Met protein from the cell surface via receptor-mediated endocytosis. The next-generation c-Met antibody–drug conjugate (named “cIRCR201-dPBD”) was designed by introducing a site-specific drug conjugation modification into IRCR201. In the first step of site-specific drug conjugation, a flexible glycine linker (G7) and a CaaX motif (Cys-Val-Ile-Met) sequence were inserted into the light-chain C-terminus of the IRCR201 antibody through genetic engineering. We synthesized geranyl ketone pyrophosphate (GKPP), which introduced a bioorthogonal reaction group to cIRCR201 for the site-specific chemoselective drug conjugation, followed by orthogonal functionalization of the antibody through prenylation using farnesyltransferase (FTase). Chemoselective oxime ligation was then performed to bind the β-glucuronide-linked pyrrolobenzodiazepine dimer to the prenylated antibody, where the imine group of dPBD was modified as prodrug to provide a hydrophilic masking of the chemical moiety. The structures of the dPBD used in the conjugation process and the resulting ADC are shown in Figure 1a. The oxime bond contributes to the improved physicochemical stability of the ADC because it has a high tolerance for hydrolytic cleavage in aqueous media at physiological pH. In addition, the glucuronide linkage is known to be stable in blood and specifically cleaved by lysosomal β-glucuronidase, it allows for the selective cleavage of the ADC in cells. The serum stability of the purified cIRCR201-dPBD in mouse serum was monitored by LC−MS.

## 2. RESULTS

### 2.1. Generation of cIRCR201-dPBD and Physicochemical Characterization Analysis.

The IRCR201 antibody against human and mouse c-Met was developed in a previous study. In addition, it inhibits the c-Met-dependent signaling pathway via c-Met internalization through receptor-mediated endocytosis. The next-generation c-Met antibody–drug conjugate (named “cIRCR201-dPBD”) was designed by introducing a site-specific drug conjugation modification into IRCR201. In the first step of site-specific drug conjugation, a flexible glycine linker (G7) and a CaaX motif (Cys-Val-Ile-Met) sequence were inserted into the light-chain C-terminus of the IRCR201 antibody through genetic engineering (cIRCR201). We synthesized geranyl ketone pyrophosphate (GKPP), which introduced a bioorthogonal reaction group to cIRCR201 for the site-specific chemoselective drug conjugation, followed by orthogonal functionalization of the antibody through prenylation using farnesyltransferase (FTase). Chemoselective oxime ligation was then performed to bind the β-glucuronide-linked pyrrolobenzodiazepine dimer to the prenylated antibody, where the imine group of dPBD was modified as prodrug to provide a hydrophilic masking of the chemical moiety. The structures of the dPBD used in the conjugation process and the resulting ADC are shown in Figure 1a. The oxime bond contributes to the improved physicochemical stability of the ADC because it has a high tolerance for hydrolytic cleavage in aqueous media at physiological pH. In addition, the glucuronide linkage is known to be stable in blood and specifically cleaved by lysosomal β-glucuronidase, it allows for the selective cleavage of the ADC in cells. The serum stability of the purified cIRCR201-dPBD in mouse serum was monitored by LC−MS.

![Figure 2](https://example.com/figure2.png)

Figure 2. In vitro characterization of cIRCR201 and cIRCR201-dPBD. (a) The binding abilities of cIRCR201 and cIRCR201-dPBD to human c-Met protein were assessed by ELISA. Data are presented as mean ± SD; **P < 0.01 using the Pearson correlation test. (b) c-Met-specific binding of cIRCR201 and cIRCR201-dPBD to c-Met-negative (MCF7) and c-Met amplification cancer cell lines (MKN45) was determined by flow cytometry. (c) MCF7, MKN45, and EBC-1 cells were incubated with the indicated concentrations of human IgG control, IRCR201, cIRCR201-dPBD, or free dPBD (100 nM) for 72 h. Cell viability was determined using the CTG luminescence analysis.
Figure 3. Apoptotic cell death and cell cycle arrest induced by cIRCR201-dPBD. (a) Expression of apoptosis-related proteins such as cleaved PARP and cleaved caspase-3 was analyzed in the cell lines (MCF7, MKN45, and EBC-1) treated with 0.16, 0.8, 4, 20, or 100 nM cIRCR201-PBD. (b) Cell apoptosis was assessed by caspase-3/7 activity 24 h after incubation of MCF7 (c-Met-negative cell), MKN45, and EBC-1 (c-Met amplification cells) with various concentrations of cIRCR201-dPBD and free dPBD at 1 nM. (c) Cell cycle analysis was determined in the untreated (0 nM) cells, cells treated with cIRCR201-dPBD (0.8 nM), and cells treated with free dPBD at 1 nM by propidium iodide staining. Data represent mean ± SD; **, P < 0.01; ***, P < 0.001 using one-way ANOVA.
measuring the deconjugated dPBD as a free from up to 7 days. The deconjugated dPBD was detected at a level of 0.03% (1333.34 nM from 666.67 nM equivalent of conjugated dPBD (DAR = 2)): 100 μg/mL cIRCR201-dPBD) and up to 0.27% at day 7 showing the serum stability without significant degradation or cleavage of the conjugation site (Table S1). Therefore, due to the high linker stability, cIRCR201-dPBD is considered to have an improved therapeutic window since the payload is not released into the circulation, thus reducing the risk of developing severe systemic toxicity.

The purity of cIRCR201-dPBD was analyzed using size exclusion chromatography (SEC-HPLC). Hydrophobic interaction chromatography (HIC-HPLC) was used to confirm that the toxin payload was uniformly attached to the antibody. SEC-HPLC analysis confirmed the homogeneous purity of cIRCR201-dPBD (>95% monomeric), and an aggregate of 3% or less was observed. The HIC-HPLC analysis confirmed a drug-to-antibody ratio (DAR) of 2 at >99% (Figure 1b).

2.2. Identification of the cIRCR201-dPBD PBD Toxin Conjugation Site Using LC−MS. After reducing the antibody with Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), the payload conjugation site of cIRCR201-dPBD was identified by intact LC−MS. The deconvaluted monoisotopic masses of the light chains of IRCR201 and cIRCR201-dPBD demonstrated accurate assignment of the attached toxin drugs and linkers to the antibody (IRCR201: 23649.261 Da and cIRCR201-dPBD: 25202.757 Da), while the masses of the heavy chains in both IRCR201 and cIRCR201-dPBD remained the same (Figure 1c). The mass difference (1871.50 Da) of light chains between IRCR201 and cIRCR201-dPBD was in agreement with the combined masses of drug and linker (prenylation + linker-toxin: 192.32 + 1678.7 = 1871.02).

The payload conjugation site was also confirmed by peptide mapping. To confirm the linker-toxin conjugation site of the antibody, reduced and alkylated trypsin-digested samples were analyzed using LC−MS/MS on a Q-Exactive mass spectrometer by HCD. Theoretical masses of peptides, including two modification sites (carbamidomethylation: 57.02 Da and trypsin: 192.32 Da), were calculated manually and matched to experimental masses (peptide: 604.63300 Da, +3 charge). We observed a cleavage site for the peptide-linker (b15 ion: 1453.73 Da, +1 charge), which corresponds to the peptide-retaining fragment pair (b15 ion: 1453.81 Da, +1 charge; peptide with the prenylation reaction site), with an accuracy of 55 ppm (Figure 1d). This shows that the linker-toxin was conjugated to Cys 222 of the light chain (the asterisk represents the theoretical value).

2.3. In Vitro Binding Property Analysis of cIRCR201 and cIRCR201-dPBD. It is important that the existing antibody binding pattern is maintained after antibody–drug conjugate production. An ELISA-based binding assay was performed to confirm that the binding properties of cIRCR201 are retained in cIRCR201-dPBD. It is expected that cIRCR201 and cIRCR201-dPBD should exhibit similar increases in binding to the human c-Met protein, depending on the concentration (Figure 2a). Analysis via the Pearson correlation test revealed that the two antibodies had a significant binding pattern. Moreover, in order to confirm that the binding properties were maintained in cell binding, direct binding of cIRCR201 and cIRCR201-dPBD to cell surface c-Met was assessed by flow cytometry. cIRCR201 and cIRCR201-dPBD exhibited similar cell binding at the same concentration in c-Met amplification cancer cells (MKN45). Further, the two antibodies did not bind to c-Met-negative cancer cells (MCF7) (Figure 2b).

2.4. In Vitro Cytotoxicity of cIRCR201-dPBD. In vitro cytotoxicity of cIRCR201-dPBD was determined in a panel of c-Met amplification cancer cell lines (MKN45; gastric cancer, EBC-1; NSCLC) and a c-Met-negative cell line (MCF7; breast cancer). Sensitivity to cIRCR201-dPBD was assessed by calculating IC50 and maximum killing in each cell line. cIRCR201-dPBD elicited potent cell cytotoxicity in c-Met amplification cell lines with IC50 values of 4.74 ± 0.05 (MKN45, maximal killing: >99.00%) and 0.58 ± 0.09 nM (EBC-1, maximal killing: 94.10%). In contrast, cIRCR201-dPBD did not exhibit cytotoxic effects in the c-Met-negative cell line (MCF7, maximal killing: 27.40%) (Figure 2c). These data demonstrated that conjugation of PBD toxin to cIRCR201 induced strong cytotoxicity and further confirmed that cell line sensitivity to cIRCR201-dPBD depended on c-Met expression.

2.5. Apoptosis and Cell Cycle Arrest Induced by cIRCR201-dPBD. The mechanism of cIRCR201-dPBD-induced cytotoxicity is initiated with the release of the PBDs upon internalization. During toxin release, PBDs can be incorporated into the DNA minor groove to induce the DNA damage response and subsequently trigger cell apoptosis. Markers of cell apoptosis include caspase-3 and PARP. If the DNA repair mechanisms fail, cell death is initiated and cleavage of cellular proteins, such as PARP and caspase, is induced.

c-Met amplification cells and c-Met-negative cells were treated with cIRCR201-dPBD at several concentrations, and levels of cleaved caspase-3 and PARP were detected after 24 h. It was confirmed that as the concentration of cIRCR201-dPBD increased, the protein levels of cleaved caspase-3 and cleaved PARP also increased (Figure 3a). Similar to the Western blot results, cIRCR201-dPBD significantly increased caspase-3/7 activity by about 2-fold at 100 nM, which was indicative of apoptosis in c-Met amplification cancer cells, as revealed by the Caspase-Glo assay (Figure 3b). The cells were treated with unconjugated free dPBD at 1 nM as a positive control showing nonspecific cytotoxicity at a level of 90% regardless of c-Met expression (Figure 3b).

Cell cycle analysis revealed that an increase in S-phase accumulation and G2-M arrest could be observed in the c-Met amplification cancer cells after treatment with 0.8 nM cIRCR201-dPBD. However, cIRCR201-dPBD did not induce significant changes in the cell cycle in c-Met-negative cell line (Figure 3c). Similar to the apoptosis assay, the unconjugated free dPBD at 1 nM showed S-phase accumulation and G2-M arrest regardless of c-Met expression (Figure 3c).

These results indicate that the cell killing effect of cIRCR201-dPBD is mediated by the induction of apoptosis and cell cycle arrest in a c-Met expression level-dependent manner.

2.6. Bystander Cell Killing Effect. ADCs generate intracellular catabolites that can diffuse out of the targeted cells into proximal antigen-negative cells and induce the killing of these bystander cells, provided that the released catabolite is sufficiently hydrophobic to penetrate cell membranes.24−26

To evaluate the bystander effect of cIRCR201-dPBD using a co-culture system, c-Met amplification cells and c-Met-negative cells were cultured at a ratio of 1:1 and treated with 100 nM control IgG, free toxin (PBDs), and cIRCR201-dPBD. The agents were administered for 3 days, and live cells were...
measured through the CellTiter-Glo (CTG) luminescent cell viability assay. When 100 nM cIRCR201-dPBD was adminis-
tered to co-cultures of EBC-1/MCF7 and MKN45/MCF7, c-
Met amplification cells and c-Met-negative cells were
simultaneously killed at magnitudes of 60.7 and 62.6%,
respectively (Figure 4a,b). Based on the observation that
cIRCR201-dPBD suppressed proliferation of total cells by 50%
or more under the 1:1 co-culture condition, it can be inferred
that a bystander effect had occurred. The difference in the
extent of the bystander effect could be due to differences in
the growth rates between cell lines and their sensitivity to
cIRCR201-dPBD.

2.7. Analysis of Correlation between cIRCR201-dPBD
Cytotoxicity and c-Met Expression through High-
throughput screening (HTS) analysis of in vitro cytotoxicity of cIRCR201-dPBD in 47 cancer cell lines. (a) Human cancer cell lines
used in HTS analysis are listed above. (b) The cell viability of multiple 47 cancer cell lines against cIRCR201-dPBD was analyzed by estimating the
AUC and z-score. (c) Violin plot represents the expression of c-Met by the c-Met mRNA expression level (RPKM) values in the top 10 cIRCR201-
dPBD-responsive cancer cell lines compared to the top 10 nonresponsive cancer cell lines. Data are presented as mean ± SEM. (d) The correlation
between c-Met expression in all 47 cancer cell lines and the AUC value for cIRCR201-dPBD. Data represent the Pearson coefficient (r) and P value
(P).
A high-throughput screening (HTS) system was used to analyze the cytotoxicity of cIRCR201-dPBD in 47 cancer cell lines. The antitumor effect of cIRCR201-dPBD was also assessed in relation to the c-Met expression level (Figure 5a). The AUC (area under the curve) of cIRCR201-dPBD was calculated for each cancer cell line, and the \( z \)-score was estimated based on the AUC. A lower AUC would imply higher sensitivity of the cell lines to cIRCR201-dPBD and a lower \( z \)-score. Conversely, the higher the AUC is, the lower the sensitivity of cells to cIRCR201-dPBD is, resulting in a higher \( z \)-score.

The cell line that exhibited the strongest response to cIRCR201-dPBD, as observed through HTS, was EBC-1 (lung) followed by T24 (bladder), 769-P (kidney), and U251MG (brain). The cIRCR201-dPBD response of EBC-1 cells presented an IC\(_{50}\) of 0.19 nM, AUC of 94.87, and a \( z \)-score of \(-3.56\) (Figure 5b). The cancer cell lines with the lowest cIRCR201-dPBD response were identified as HepG2 (liver), KMS-11 (myeloma), PANC-1 (pancreas), and U-2OS (sarcoma). In the case of the HepG2 cell line, even at the highest concentration of ADC, no tumor inhibitory effect was observed, and IC\(_{50}\) was not calculated. The AUC was 456.9, and the \( z \)-score was 1.93 (Figure 5b). In conclusion, it was possible to select cIRCR201-dPBD responder and non-responder cell lines based on the AUC and \( z \)-score.

To confirm the correlation between cIRCR201-dPBD efficacy and cell c-Met expression level, analysis was performed on AUC data obtained from the HTS analysis and the c-Met mRNA expression levels (RPKM) of cell lines reported in the CCLE (Broad Institute Cancer Cell Line Encyclopedia). The averages of the c-Met RPKM values for the cIRCR201-dPBD response group (top 10 cell lines) and the nonresponse group (top 10 cell lines) were 255.3 and 24.1, respectively, meaning that ADC sensitivity was proportionally related to the c-Met mRNA expression level (Figure 5c).

The Pearson coefficient (\( r \)) for the correlation between AUC and c-Met RPKM in 47 cell lines was \(-0.5\), indicating a significant (\( p = 0.0007 \)) correlation. Therefore, cell lines with high c-Met mRNA expression levels were significantly more sensitive to cIRCR201 (Figure 5d).

2.8. In Vivo Antitumor Effect of cIRCR201-dPBD. The in vivo efficacy of cIRCR201-dPBD was evaluated on mouse xenograft models implanted with c-Met amplification cancer cell lines EBC-1 and MKN45. BALB/c nude mice were implanted with tumors through subcutaneous injection. ICR201-dPBD was administered through intraperitoneal injection. In the EBC-1 model, doses of 0.1, 0.2, 0.4, and 0.8

![Figure 6. In vivo efficacy of cIRCR201-dPBD in human tumor xenograft models. BALB/c nude mice were subcutaneously implanted with c-Met amplification tumor cells (EBC-1, NSCLC; MKN45, gastric cancer). (a) Mice received either vehicle or cIRCR201-dPBD through peritoneal injection at a given dose (0.1, 0.2, 0.4, or 0.8 mg/kg), twice, or (b) at doses of 1, 3 (once a week, q1w), or 1 mg/kg, (three times a week, q3w). Tumor volumes are shown as mean ± SD; *** \( P < 0.001 \) using two-way ANOVA. (c,d) EBC-1 and MKN45; body weight change from post treatment in mice treated with cIRCR201-dPBD. Body weight is shown as mean ± SD.](https://dx.doi.org/10.1021/acsomega.0c03102)
mg/kg were administered twice (with an interval of a week) in order to evaluate the tumor growth inhibitory effect. cIRCR201-dPBD-mediated tumor inhibition was observed in a dose-dependent manner. Further, tumor growth was completely suppressed at a dose of 0.8 mg/kg (Figure 6a). In the MKN45 model, 1 or 3 mg/kg injections were given once a week (q1w) or a 1 mg/kg injection was given three times a week (q3w). It was observed that cIRCR201-dPBD completely suppressed tumor growth in all formulations (Figure 6b). In order to monitor the in vivo toxicity, the body weight of the individual animal was measured during the various treatment schedules. At day 0, and day 7 in the EBC-1 xenograft model, there was no change in body weight after twice administration of cIRCR201-dPBD up to 0.8 mg/kg (Figure 6c). The repeated administration of cIRCR201-dPBD at the 1 mg/kg (q1w) in MKN45 xenograft model showed decreased body weight about 17.5% level at day 14 and maintained afterward (Figure 6d). Meanwhile, the repeated administration of 3 mg/kg (q1w) showed the continued decreased body weight, which was similar to the administration of 1 mg/kg (q3w) demonstrating the cumulative toxicity in vivo depending on the amount of administered cIRCR201-dPBD (Figure 6d). At the 1 mg/kg (q3w) group, one object died 27 days after administration.

3. DISCUSSION

Aberrant c-Met/HGF signaling activity and dysregulation have been reported in multiple cancers and play important roles in tumor progression, angiogenesis, and metastasis. It was reported that the expression of c-Met in tumors is closely related to the resistance to VEGF and EGFR targeted therapies. The development of c-Met/HGF axis inhibitors such as small molecules (e.g., crizotinib, cabozantinib, capmatinib, and tepotinib) and antibodies (e.g., rivoltumumab, ficlatuzumab, onartuzumab, emibetuzumab, telisotuzumab, and SAIT301) has been an active field within cancer research. Several therapeutics targeting c-Met have been investigated for various cancer indications, yet only limited benefits have been achieved in clinical settings. In the case of telisotuzumab (ABT-700, AbbVie) clinical trials, patients with MET gene amplification were the only group exhibiting clinical benefits. Clinical studies of the anti-c-Met antibody onartuzumab and the anti-HGF antibody rivoltumumab also revealed limited benefits and suggested that MET amplification, mutation, or HGF-dependent activation of c-Met could be better predictors of outcome than MET overexpression alone. The potential application of c-Met-targeting ADC could provide a broader spectrum of efficacy regardless of c-Met pathway dependency. There have been a few reports related to the development of c-Met ADCs using microtubule inhibitors (e.g., monomethyl-aurestatin E) and DNA-alkylating agents (e.g., pyrrolobenzodiapine). Recently, the clinical data of ABBV-399, a c-Met ADC, have revealed strong antitumor effects, and phase I clinical trial data have shown an acceptable tolerance and preliminary clinical benefits, especially in patients overexpressing c-Met.

In this study, we developed a third-generation ADC in which a strong payload was conjugated to a human/mouse anti-c-Met cross-reactive antibody (IRCR201). We demonstrated IRCR201’s robust anticancer effects in vitro and in vivo. Early ADC technology has heterogeneous DAR properties and unstable physical properties because the payload is conjugated to lysine residues (~70 to 90) or interchain disulfide cysteines (e.g., 8 in IgG1) exposed on the surface of the antibody. To overcome these drawbacks, we performed genetic engineering on the antibody to insert a flexible linker (G7) and a CaaX motif (Cys-Val-Ile-Met) into the C-terminus of the light chain. We then applied novel site-specific drug conjugation to attach the payload only to this specific motif. Among the various payloads, PBD dimers are known to demonstrate strong antitumor cytotoxic potency in vitro (IC₅₀ values with the mid to low pM ranges) against a broad range of tumor cell lines. In addition, PBD dimers demonstrated acceptable activity in MDR1 and refractory tumors because of the fact that they are normally not substrates of MDR1, potentially avoiding the commonly observed drug resistance. We have conjugated PBDs to develop ADCs using the mAb (cIRCR201) with the cross-reactive human and mouse c-Met. Since cIRCR201-dPBD uses the β-glucuronide-linkage as a cleavable linker, it is stable in the blood and could be cleaved specifically by lysosomal β-glucuronidase in cells with high c-Met expression inside the tumor, resulting in excellent tumor selectivity. We have evaluated the homogeneous DAR and the high-purity physical properties of cIRCR201-dPBD using SEC-HPLC and HIC-HPLC, respectively. We demonstrated that the PBD payload was bound to the light-chain C-terminus-specific residue (CaaX motif, cysteine) based on LC–MS analysis (Figure 1c,d). We confirmed that cIRCR201-dPBD induced selective antiproliferative effects and apoptosis in c-Met amplification cancer cell lines (Figure 2). In contrast, these effects were not observed in c-Met-negative cells, such as the MFC7 cell line. Further, the direct killing effect of cIRCR201-dPBD on cancer cells was demonstrated through cell cycle arrest and apoptosis biomarker analysis (Figure 3). cIRCR201-dPBD was internalized through receptor-mediated endocytosis in c-Met-positive tumor cells, which induced tumor cell killing through release of the payload. Further, payload release also had the capacity to kill adjacent c-Met-negative tumor cells (Figure 4). To reinforce the notion that cIRCR201-dPBD has c-Met expression level-dependent activity, we evaluated the in vitro cytotoxicity tests of 47 cancer cell lines through high-throughput screening. As a result, a considerable correlation was established between RPKM and cIRCR201-dPBD-induced cytotoxicity (Figure 5). General c-Met antibody treatment has a limited effect only on cancers dependent on the c-Met dependent signaling pathway, but cIRCR201-dPBD exerts a cytotoxic effect in proportion to the c-Met expression level irrespective of the c-Met pathway dependence. c-Met-targeting ADCs present a risk of toxicity to normal tissue where there is expression of c-Met. However, c-Met expression is significantly higher in various cancers than in normal tissues suggesting that such ADCs could have a wide enough therapeutic index. cIRCR201-dPBD was significantly effective in our in vivo xenograft model. The main observation of the in vivo study was that cIRCR201-dPBD completely suppressed the tumor growth of the NSCLC xenograft model (EBC-1) after injection of 0.8 mg/kg twice (with a week in between). Growth was also suppressed in the gastric cancer xenograft model (MKN45) after 1 mg/kg injection once a week (Figure 6).

While there are several potential advantages of ADC, the narrow therapeutic window has been a major limitation for clinical development. In order to design a proper dosing schedule, the pharmacodynamics and potential toxicity have to be investigated in preclinical settings. The c-Met-specific monoclonal antibody used in this study has the unique
property of almost equal reactivity to the human and murine versions of the c-Met protein. This allows us to assess the potential therapeutic window through off-target and on-target toxicity profiles. In the xenograft gastric cancer model (MKN45), preliminary toxicity data suggest that significant weight loss and sudden death were observed at a concentration of 1 mg/kg (three times a week) of cIRCR201-dPBD. The application of fractionated dosing could improve the preclinical therapeutic index of PBD toxin-conjugated ADCs, including c-Met-targeting ADCs.57 The accurate toxicity profile and dose regimen analysis of cIRCR201-dPBD have not been understood and will be the subject of further studies. Some c-Met-targeting antibodies have been reported to promote tumor proliferation by exerting an agonistic effect through c-Met dimerization and downstream signaling stimulation.35,48 In previous studies, IRCR201 was reported to have nonagonistic effects. Further, side effects associated with agonism were not observed during treatment with cIRCR201-dPBD in the current study.

In conclusion, we have developed and characterized an anti-c-Met site-specific antibody–drug conjugate (cIRCR201-dPBD) that applies a powerful cytotoxic payload to achieve potent antitumor activity in preclinical models of c-Met amplification tumors. It is expected that cIRCR201-dPBD will satisfy unmet clinical needs in the future after a more thorough drug evaluation, dose analysis, and in vivo pharmacokinetic investigation.

4. EXPERIMENTAL SECTION

4.1. Production of Modified IRCR201 with the CaaX Motif. The IRCR201 antibody against human and mouse c-Met was isolated via 6 rounds of bio-panning by the conventional method of immobilized antigen coating on immune-tubes using the synthetic human single chain variable fragment (scFv) phage libraries from previous reports.18,19 The isolated scFv (native IRCR201) was reformatted to full-length IgG1. A CaaX motif was inserted into the C-terminus of the light chain of the native IRCR201 antibody sequence with a flexible glycine linker (G7) and a CaaX motif (Cys-Val-His-Met) DNA sequence at the position of cysteine 214 (Kabat numbering). The antibodies (native IRCR201, IRCR201 and CaaX-modified IRCR201, cIRCR201) were produced in an Expi293 transient mammalian expression system (Gibco) and purified by a HiTrap MabSelect SuRe (GE Healthcare).

4.2. Production of the Site-Specific Anti-c-Met Antibody–Drug Conjugate (cIRCR201-dPBD) and Physicochemical Property Analysis Using SEC-HPLC and HIC-HPLC. To conjugate the toxin to the specific site of the anti-c-Met antibody, prenylation was performed using the geranyl ketone pyrophosphate (GKPP) on cIRCR201 followed by a chemoselective oxime reaction to synthesize the cIRCR201-dPBD (ADC), with the β-glucuronide linker connected to the pyrrolobenzodiazepine dimer.20 The homologous ADC with a defined DAR (DAR = 2) underwent secondary purification using HIC-HPLC, and endotoxin was then removed.

The ADC was analyzed by SEC-HPLC and hydrophobic interaction chromatography (HIC-HPLC) using a Waters Alliance 2695 HPLC, equipped with a 7.5 × 300 mm BioSuite High Resolution SEC Column with 10 μm particle size (Waters Alliance) and a 4.6 × 100 mm MAbPac HIC-Butyl HPLC Column with 5 μm particle size.

4.3. Intact Mass Analysis. For analysis of the molecular weights of the light and heavy chains of IRCR201 and cIRCR201, intact mass analysis was performed. To reduce the sample, TCEP-HCl (50 mM) was added to antibodies followed by incubation for 45 min at 56 °C. The reduced sample was immediately loaded onto a liquid chromatography–mass spectrometry (LC–MS) column at a flow rate of 0.2 mL/min. Separation was accomplished using a Waters ACQUITY I class UPLC system (Milford) with a Thermo MabPac RP column (2.1 mm × 50 mm, 4 μm particle size) at 65 °C. Buffers used included eluent A, consisting of 0.1% formic acid in water, and eluent B, consisting of 0.1% formic acid in 100% acetonitrile. The gradient was fixed with 25% eluent B for 2 min. A linear gradient was applied with 25 to 45% eluent B for 8 min. The effluent was analyzed with a Thermo LTQ Orbitrap system.

4.4. In-Solution Digestion and Mass Spectrometry Analysis of the Toxin Conjugation Site. cIRCR201 or cIRCR201-dPBD was denatured with 8 M urea and reduced with 10 mM DTT (for 30 min at 37 °C), which had been treated with 25 mM iodoacetamide (for 30 min at room temperature) for alkylation. Two enzymatic digestions followed: Glu-C and Trypsin (1:50). After digestion, the solution was desalted by a C18 SPE cartridge and analyzed using a Q-Exact mass spectrometer coupled to an Easy-nLC system (Thermo Fisher Scientific). Peptides were reconstituted in 0.1% formic acid, and samples were separated on an analytical column (C18, 2 μm particle size, 50 μm id × 15 cm length, Thermo Fisher Scientific). Samples were eluted with a linear gradient of solvent B (100% ACN, 0.1% formic acid) from 5–40 (45 min) to 40–80% (2 min), at a flow rate of 300 nL/min. All MS/MS spectra were obtained in a data-dependent mode for fragmentation of the 20 most abundant peaks from the full MS scan with a normalized collision energy of 25%. The full MS spectra of IRCR201 and cIRCR201-dPBD were analyzed using the Thermo Scientific Protein Deconvolution software (v2.0), which utilizes the ReSpect algorithm for molecular mass determination. In order to identify the peptide-linker binding site, acquired MS/MS spectra were searched with the Sequest algorithm in Proteome Discoverer 1.4 (Thermo Fisher Scientific).

4.5. ELISA Binding and Cell Binding Assays. To analyze the c-Met-specific binding of cIRCR201 and cIRCR201-dPBD, 96-well EIA/RIA plates were coated with 1 μg/mL human c-Met (Sino Biological, 10,692-H03H) at 4 °C overnight and then blocked using 3% skim milk in PBS, pH 7.4, for 1 h at room temperature. The plate was incubated with prediluted cIRCR201 or cIRCR201-dPBD followed by incubation with the secondary antibody conjugated to HRP. After the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution, the reaction was quenched with a stop solution and optical density (OD) was determined at 450 nm. To assess the cell binding abilities of cIRCR201 and cIRCR201-dPBD, MCF7 and MKN cells were treated with the antibodies. Cells were then incubated with the anti-human IgG secondary antibody conjugated to Alexa Fluor 594 (Thermo Fisher Scientific, A-11014). Mean fluorescence intensity was analyzed by flow cytometry (BD FACSAria III).

4.6. Cell Lines and High-Throughput Cell Cytotoxicity Screening. The human cancer cell lines (MKN45 gastric cancer cells and EBC-1 lung cancer cells) were obtained from the JCRB Cell Bank (Japan), and the breast cancer MCF7 cell line was purchased from ATCC (USA). The 47 cell lines used in the HTS were seeded in 384-well plates at a density of 500 cells per well in duplicate or triplicate. Cells were treated with a
4-fold concentration of cIRCR201-dPBD at a seven-point serial dilution series from 167 nM. After 72 h of incubation, cell viability was determined using an adenosine triphosphate (ATP) monitoring system based on firefly luciferase (PerkinElmer, ATPLite 1step) and EnVision Multilabel Reader (PerkinElmer). Dose–response curve (DRC) fitting was performed by GraphPad Prism 5 to measure the area under the curve (AUC). To evaluate drug sensitivity, the AUC was transformed into a z-score. Cell lines with a z-score lower than −0.5 were considered as sensitive to the target drug. All cell lines were authenticated by short tandem repeat analysis and were tested for mycoplasma prior to use. Cell lines were maintained in a culture medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C and 5% CO2.

4.7. Cell Proliferation, Apoptosis, and Bystander Effect Assays. For the cell proliferation assay, MCF7, MKN45, and EBC-1 cells were treated with the control antibody or cIRCR201-dPBD ranging from 0 to 500 nM, in triplicate, for 72 h at 37 °C. For the apoptosis assay, cells were treated with cIRCR201-dPBD (0.8, 4, 20, 100 nM). Cell viability and apoptosis were determined using the CTG treated with cIRCR201-dPBD (0.8, 4, 20, 100 nM). Cell suspension was mixed with Matrigel (10× v/v FBS). Co-cultured cells were then treated with positive cells (MKN45 and EBC-1) were co-cultured with M200 Pro (Tecan). To estimate the bystander effect, MET-positive cells (MKN45 and EBC-1) were co-cultured with MET-negative cells (MCF7) in an RPMI medium containing 10% v/v FBS. Co-cultured cells were then treated with cIRCR201-dPBD at a concentration of 100 nM at 37 °C for 72 h. Cell viability was determined through the CTG assay, and the relative luminescence intensity was analyzed with an Infinite M200 Pro (Tecan). To estimate the bystander effect, MET-positive cells (MKN45 and EBC-1) were co-cultured with MET-negative cells (MCF7) in an RPMI medium containing 10% v/v FBS. Co-cultured cells were then treated with cIRCR201-dPBD at a concentration of 100 nM at 37 °C for 72 h. Cell viability was determined through the CTG assay, and the relative luminescence intensity was analyzed with GraphPad Prism 5.

4.8. Western Blot Analysis. Whole cell lysates were prepared from MCF7, MKN45, and EBC-1 cells and treated with 0.16, 0.8, 4, 20, or 100 nM cIRCR201-dPBD for 48 h. The following series of antibodies purchased from Cell Signaling Technology were used as primary antibodies: rabbit anti-Met (#8198), rabbit anti-PARP (#9532), rabbit anticaspase 3 (#9661), and rabbit anti-β-actin (#4970). For detection, the PVDF membrane was treated with goat anti-rabbit IgG HRP as a secondary antibody, which is able to react with ECL detection reagents (GE healthcare). ECL-based chemiluminescence signals were analyzed by ImageQuant LAS 4000.

4.9. In Vivo Antitumor Activity. BALB/c nude mice were subcutaneously injected in the flank with 1.0 × 106 cells (EBC-1 and MKN-45). The cell suspension was mixed with Matrigel at a ratio of 1:1. The xenograft tumor models were used to evaluate the antitumor efficacy of cIRCR201-dPBD. When tumors reached an average volume of 150 mm3 (day 0), mice were divided into several groups (n = 5 per group), and cIRCR201-dPBD was intraperitoneally administered once a week for 2 weeks. Tumor volume was measured periodically using a digital caliper, and the volume was calculated using the formula TV = 0.5 × length × width2. Mice were sacrificed when the tumor volume reached a maximum size of 1500 mm3. All in vivo experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and conducted at the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) internationally accredited facility at Samsung Medical Center (SMC).

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03102.

Analysis of serum stability for cIRCR201-dPBD using LC–MS (Table S1) (PDF)

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MSIT) (No. 2017M3A9C8064720). This research was partly supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2020R1I1A1A0107559).

■ ABBREVIATIONS

ADC, antibody-drug conjugate; HGFR, hepatocyte growth factor receptor; HGF, hepatocyte growth factor receptor; TKIs, tyrosine kinase inhibitors; NSCLC, non-small-cell lung cancer; PBDs, pyrrolobenzodiazepine dimers; GKPP, geranyl ketone pyrophosphate; FTase, farnesyltransferase; SEC, size exclusion chromatography; HIC, hydrophobic interaction chromatography; DAR, drug-to-antibody ratio; TCEP, Tris (2-carboxyethyl) phosphate hydrochloride; HTS, high-throughput screening; LC−MS, liquid chromatography−mass spectrometry

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