## Abbreviations:

LLOQ: lower limit of quantification, the lowest standard on the calibration curve.

ULOQ: upper limit of quantification, the highest standard on the calibration curve.

mP: milli-polarization unit (1 Polarization Unit = 1000 mP Units)

## Synthesis of an Epha2 binding antibody drug conjugate

The Epha2 ADC comprises an EphA2-binding antibody (1C1) conjugated to the cytotoxin MMAF. 1C1 antibody was produced using standard methods by Genscript, based on the coding sequence provided in Patent US2011/0280892A1. The ADC was produced by conjugating 1C1 to the cytotoxin MMAF via a maleimidocaproyl (mc) linkage by standard conjugation chemistry (Jackson et al. 2008) at Wuxi AppTec Co. Ltd. (Shanghai). Analysis indicated the presence of single species, with high purity, low endotoxin levels and 1C1 (or MEDI-547) physicochemical characteristics.

## EphA2 binding assays

Bicycle peptide and BTC binding to Eph receptors was quantified using fluorescence polarisation (Fp) and Surface Plasmon Resonance (SPR) assays.

### EphA2 binding Fluorescence polarisation assays

For competition binding, increasing concentrations of test article were incubated with mouse, human or rat EphA2 and fixed concentrations of an unrelated fluorescein labelled Bicycle trace. Fluorescence polarization was quantified on a BMG PHERAstar FS or Perkin Elmer Envision instrument at 5 to 10-minute intervals for 60 minutes. Data analysis was performed using Systat Sigmaplot version 12.0 or 13.0. The mP values at 60 minutes were fit to user defined equations to generate Ki values.

### Eph receptor Surface Plasmon Resonance assays

SPR assays were performed on a Biacore 3000 with streptavidin or Goat Anti-Human IgG antibody immobilized on CM5 chips. Fc or Biotin tagged proteins were captured to generate 200-400 RU on the CM5 chips. Sensorgrams were generated at 25°C, using a 90µl/min flow rate with 60 seconds association and 900-1200 seconds dissociation in PBS with 0.05% Tween 20 and 0.5% DMSO. Bicycle peptides and BTCs were assayed at concentrations between 0.4 and 100nM for EphA2 proteins and 20 to 5000nM or 0.6 to 25µM for other Eph proteins in multi kinetic mode. All data were double-referenced for blank injections and reference surface using standard processing procedures. Data processing and kinetic fitting were performed using Scrubber software, version 2.0c (BioLogic Software). Data were fitted using simple 1:1 binding model allowing for mass transport effects where appropriate.

## PET imaging studies with BT5528

Imaging studies were conducted at Bioprobe Ltd, London, UK using mice bearing subcutaneous HT-1080 xenografts . The labelled binder was synthesised by conjugating the Bicycle binder BCY6099 to a DOTA chelator which was subsequently incubated with Ga-68 chloride to generate radiolabelled BCY6164. Mice were anaesthetized and administered BCY6164 intravenously (~150pmol, ~1.5-2.5MBq) as a 200µL bolus over 15-25s. Imaging was performed using PET/CT scanning and images constructed using imaging software.

### Quantification of EphA2 Expression on CDX Cell Lines

Cells were stained in staining buffer at 4°C with a live/dead stain and anti-human EphA2 or the isotype control conjugated with Phycoerythrin (PE) for 30 minutes. Cells were washed twice and analysed on an Intellicyt iQue screener or BD FACS Canto II. The mean fluorescence intensity of PE for single live cells was determined and compared to known standard QuantiBRITE beads (BD) to determine the number of antibody binding sites by linear regression. The antibody binding sites for the isotype control was subtracted from that obtained for EphA2. QuantiBRITE beads were used as per manufactures instructions and made up in staining buffer. Beads contain between 474 and 62336 molecules PE per bead, if the total number of antibody binding sites is outside of this range then LLOQ or ULOQ quoted as appropriate.

### Quantification of EphA2 Expression on PDX Cell Lines

Briefly, single cell suspensions were obtained from tumor tissue via mechanical cutting and enzymatic digestion with Accumax; and purified density gradient centrifugation with Histopaque. Cells were stained in PBS + 2% BSA at 4°C, non-specific binding of antibodies to the cells was blocked with a rat anti-mouse CD16/CD32 for 15 minutes, after which a staining solution containing a live/dead stain, a rat anti-mouse CD45 conjugated to Alexa Fluor® 700 and with PE conjugated anti-human EphA2 or the isotype control for 30 minutes. Cells were washed twice and analyzed on an Intellicyt iQue screener or BD FACS Canto II. The mean fluorescence intensity of PE for single live cells which stained negative for CD45 was determined and compared to known standard QuantiBRITE beads (BD) to determine the number of antibody binding sites by linear regression. The antibody binding sites for the isotype control was subtracted from that obtained for EphA2. QuantiBRITE beads were used as per manufactures instructions and made up in PBS + 2% BSA. Beads contain between 474 and 62336 molecules PE per bead, if the total number of antibody binding sites is outside of this range then LLOQ or ULOQ quoted as appropriate.

### Immunohistochemical detection of EphA2 Expression on Tumor Xenografts

Anti-EPhA2 antibody (1:200 AF3035, R&D Systems) was used on 4-6 µm FFPE sections in conjunction with Ventana’s OmniMap anti-Rabbit HRP and DISCOVERY ChromoMap DAB detection system after standard CC1 (Ventana) antigen retrieval. Incubation time for the primary antibody was 60 minutes (at room temperature) and 16 minutes for the OmniMap anti-Rb HRP secondary antibody. Sections were counterstained with hematoxylin (Ventana).

## BT5528 (or MMAE) binding to HT1080 and PC-3 cells

BT5528 (or free MMAE) binding to HT1080 cells was determined by quantifying cell surface bound MMAE using an anti-MMAE antibody and confocal High Content Screening on a Yokogawa CQ1 instrument.

Cells were seeded in Greiner 96 well black walled, clear bottomed plates and incubated at 37°C overnight. All cell staining was performed in ice cold MEM at 4°C and cells were rinsed in ice cold MEM between test article additions. Cells were incubated for 45 minutes with or without increasing concentrations of compound (1.5nM -10µM) and stained with 5µg/mL anti-MMAE antibody for 30 minutes. Detection was performed following a second 30-minute incubation with anti-mouse IgG conjugated with Alex Fluor® 488 (4µg/mL), blue stain nuclearmask (1µg/mL) and wheat germ agglutinin conjugated with Alexa Fluor® 633 (2µg/mL). Wheat germ agglutinin was used to define the plasma membrane and to distinguish BT5528 bound to the plasma membrane from BT5528 bound to the surface of the plate wells and other non-specific binding. After rinsing with D-PBS cells were fixed with 4% paraformaldehyde for 30 minutes and stored on ice in D-PBS prior to imaging using a Yokogawa CQ1. Software was used to determine the fluorescence intensity of Alex Fluor® 488 that was co-localised with Alex Fluor® 633. The data were then fit to a one-site binding model in GraphPad Prism version 5. to generate affinity measurements (KD).

## Plasma protein binding and stability studies in plasma and hepatocytes

Plasma protein binding and stability was assessed in CD1 mouse, Sprague Dawley rat, Cynomolgus monkey and human plasma. Stability was also assessed in human hepatocytes. All studies were conducted at Wuxi AppTec Co. Ltd. (Shanghai).

Plasma Protein Binding studies were conducted on BT5528 incubated with plasma (2µM compound) for 30min at 37°C for 30minutes and then either incubated for a further 2h or ultracentrifuged (470,000g) for 2h to separate unbound drug. The concentration of BT5528 was measured by LC-MS/MS analysis using a modified generic method.

Stability studies were conducted on BT5528 or MMAE incubated with plasma (2µM compound, for up to 6h) or hepatocytes (1µM compound, 0.5x106/mL cells, for up to 90minutes). The incubation was stopped at prespecified timepoints to produce a time course, and concentration of BT5528 measured by LC-MS/MS analysis using a modified generic method.

Measurement of BT5528 and MMAE in plasma

Mouse (CD-1), Rat (Sprague Dawley) and Cynomolgus monkey plasma samples were analyzed for BT5528 and MMAE (free toxin) using a qualified bioanalytical methods based on protein precipitation followed by liquid chromatographic triple quadrupole mass spectrometric (LC-MS/MS) analysis. Prepared plasma samples were spiked with internal standard and analyzed after methanol extraction. Standards and controls were prepared in tumor or plasma matrices in an identical manner.

## Measurement of pHH3 in tumour samples

Samples of tumour from in vivo xenograft studies were fixed with 10% neutral buffered formalin and processed and embedded in paraffin. 4-5M sections were cut and stained for pHH3 (Cell Signaling Technology #9701, 1:200 dilution) using a Ventana XT autostainer with OMap anti-Rb HRP and ChromMap DAB detection system and counterstained with hematoxylin. The percentage of pHH3 positive nuclei were quantified using HALO image analysis software.

## Xenograft models

*Cell-line Derived Xenograft (CDX) models:* mice (6-8 week old female Balb/c nude mice for OE-21, SK-OV-3, NCI-N87, MDA-MB-231, NCI-H1975, HT1080, male Balb/c nude mice for PC-3 or female CB17-SCID mice for MOLP-8) were inoculated with ~107 cells/mouse in 0.2ml PBS subcutaneously to the right flank. Animals were randomized when the average tumour volume reached the pre-designated start size. Group size ranged from n=3 to n=6 depending on the model.

*Patient-Derived Xenograft (PDX) models:* Lu-01-0251 and Lu-01-0486, 6-8 week old female Balb/c nude were inoculated with ~30mm3 fragments of a source tumor.

Test articles were administered by intravenous bolus dosing at 10mL/kg. 50mM acetate, 10% sucrose pH5 was used as vehicle throughout the studies. Tumor volumes were measured in two dimensions using a caliper, and the volume was expressed in mm3 using the formula: V = 0.5 a x b2 where a and b are the long and short diameters of the tumor, respectively. All xenograft studies were conducted at Wuxi AppTec Co. Ltd. (Shanghai).

## Bone metastasis model

5-6 week old Balb/c nude male mice were inoculated via an intracardiac injection with 3x106 PC-3M-Luc-C6 cells/mouse in 0.1ml PBS (day 0). 14 days after tumor implantation the animals were randomized to 3 treatment cohorts of 5 animals based on their total bone bioluminescence signal (quantified from right and left hind limbs as well as mandible) and minimum bioluminescence signal in the thoracic cavity. Total bone signal was quantified weekly thereafter. The vehicle group was dosed weekly with vehicle (50mM acetate, 10% sucrose pH5, i.v. 10 mL/kg). The two treatment groups received 1.5 mg/kg BT5528 (i.v., 10 mL/kg) starting on day 14 or day 21 following tumour implantation. The final dose was administered on day 35. Bioluminescence imaging was performed 10 minutes after administration of 150 mg/kg (i.p. at 10 mL/kg) D-luciferin with IVIS Spectrum (PerkinElmer, MA) and Images were analyzed using Living Image 4.7.1 (PerkinElmer, MA) software.

## Toxicology studies

Toxicology studies were conducted at Envigo Ltd, Huntingdon, UK, in accordance with the recommendations of ICH S9 Nonclinical Evaluation for Anticancer Pharmaceuticals. Rats and Non-Human Primates (cynomolgus monkeys) were dosed with intravenous BT5528 once weekly for 5 doses on days 1, 8, 15, 22 and 29 in 32 day studies. Evaluations included clinical signs, body weight and food consumption, macroscopic and microscopic pathology, haematology, clinical chemistry, urinalysis, coagulation and toxicokinetics.