Supporting Information

Discovery of BT8009: a Nectin-4 Targeting Bicycle Toxin Conjugate for the Treatment of Cancer

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# Table of Contents

1. Protein information ................................................................. S3
2. Phage selections ...................................................................... S3
3. *In vitro* methods ................................................................. S3
   3.1. Affinity determination by fluorescence polarization (FP) direct binding
   3.2. Affinity determination by fluorescence polarization (FP) competition
   3.3 Affinity determination by SPR
4. Chemical methods ..................................................................... S8
   4.1 Peptide sequences
   4.2 Fluorescent probes - analytics
   4.3 Bicycles synthesised during peptide optimisation - analytics
   4.4 BT8009 synthesis and analytics
5. Physicochemical profiling / *in vitro* DMPK ................................. S20
   5.1. Lipophilicity measurements (CHIlogP)
   5.2. Plasma protein binding
   5.3. *In vitro* plasma stability
6. *In vivo* studies ......................................................................... S22
   6.1 CDX studies
7. References ................................................................................ S23
1. Protein Information

All proteins (unless otherwise stated) used in this paper were purchased from R&D Systems with the details available in Table S1.

Table S1. Source of Proteins used in the following studies

| Protein (catalogue number) | Species  | Supplier |
|----------------------------|----------|----------|
| Necl-1 (3678-S4 Lot#OAR0109091) | Human    | R&D Systems |
| Necl-2 (3519-S4 Lot#OYA0116021) | Human    | R&D Systems |
| Necl-3 isoform2 (3064-N3 Lot#POF0107041) | Human    | R&D Systems |
| Necl-4 (4164-S4 Lot#NZB0112031) | Human    | R&D Systems |
| Necl-5 (2530-CD/CF Lot#MWZ0315081) | Human    | R&D Systems |
| Nectin-1 (2880-N1 Lot#MYC0616011) | Human    | R&D Systems |
| Nectin-2 (2229-N2/CF Lot#MYP0715082) | Human    | R&D Systems |
| Nectin-3 (3064-N3 Lot#MYA0316041) | Human    | R&D Systems |
| Nectin-4 (2659-N4 Lot#MYJ0417021) | Human    | R&D Systems |
| Nectin-4 (3116-N4 Lot#NPN0114121) | Mouse    | R&D Systems |
| Nectin-4 (3116-N4 Lot#NPN0112021) | Mouse    | R&D Systems |
| Nectin-4 (9996-N4 Lot#DJBE0121021) | Cyno     | R&D Systems |
| Nectin-4 (9997-N4 Lot#DJFP0221041) | Rat      | R&D Systems |

2. Phage selections

Chemically biotinylated Nectin-4, (R&D Systems, 2659-N4) was used as target material in phage panning selections. Naïve libraries cyclized on TATA with differing loop lengths where all residues between the conserved cysteines are randomized (naïve libraries) were exposed to the target material and binders were enriched, isolated and sequenced using methods reported previously.\textsuperscript{1,2} Subsequently affinity maturation Bicycle phage libraries were constructed based on hits identified from the naïve selections by retaining some residues thought to be important for binding and randomizing others. Phage selections using these custom libraries were carried out as before to identify binders with improved affinity over the parent sequences.

3. In vitro methods

3.1. Affinity determination by fluorescence polarization (FP) direct binding.

Peptides with a fluorescent tag were diluted to 2.5nM in 20mM HEPES with 150mM NaCl and 0.01% tween pH 7.4 (referred to as assay buffer). This was combined with a titration of protein in the same assay buffer as the peptide to give 1nM peptide in a total volume of 25µL in a black walled and bottomed low bind low volume 384 well plates, typically 5µL assay buffer, 10µL human Nectin-4 then 10µL fluorescent peptide. One in two serial dilutions were used to give 12 different concentrations with the top concentration of 2000nM. Measurements were conducted on a BMG PHERAstar FS equipped with an “FP 485 520 520” optic module which excites at 485nm and detects parallel and perpendicular emission at 520nm. The PHERAstar FS was set at 25°C with 200 flashes per well and a positioning delay of 0.1 second, with each well measured at 5 to 10 minute intervals for 60 minutes.
The gain used for analysis was determined for each tracer at the end of the 60 minutes where there was no protein in the well. Data was analyzed using Systat Sigmplot version 12.0. mP values were fit to a user defined quadratic equation to generate a Kd value: \[ f = y_{\text{min}} + \frac{(y_{\text{max}} - y_{\text{min}})}{\text{Lig}} \times ((x + \text{Lig} + \text{Kd})/2 - \sqrt{((x + \text{Lig} + \text{Kd})/2)^2 - (\text{Lig} \times x)}). \]

“Lig” was a defined value of the concentration of tracer used.

Table S2. Affinities of naïve peptides of Bicycle families identified through phage display

| Naïve Bicycle number | \(K_d\) (nM) | Geomean ± SD (n=number of replicates) |
|----------------------|--------------|----------------------------------------|
| 1                    | 508.0 ± 130.4 (n=4) |
| 3                    | 207.6 ± 29.3 (n=8) |
| 5                    | 887.8 ± 153.2 (n=4) |

Peptides with a fluorescent tag were diluted to 2.5nM in 20mM HEPES with 150mM NaCl and 0.01% tween pH 7.4 (referred to as assay buffer). This was combined with a titration of protein in the same assay buffer as the peptide to give 1nM peptide in a total volume of 25µL in a black walled and bottomed low bind low volume 384 well plates, typically 5µL assay buffer, 10µL Nectin-4 protein then 10µL fluorescent peptide. One in two serial dilutions were used to give 12 different concentrations with the top concentration of 2000nM. Measurements were conducted on a BMG%PHERAstar%FS equipped with an “FP 485 520 520” optic module which excites at 485nm and detects parallel and perpendicular emission at 520nm. The%PHERAstar%FS was set at 25°C with 200 flashes per well and a positioning delay of 0.1 second, with each well measured at 5 to 10 minute intervals for 60 minutes. The gain used for analysis was determined for each tracer at the end of the 60 minutes where there was no protein in the well. Data was analyzed using Dotmatics. Polarisation data was fit to non-linear regression analysis (using the following calculation: \[ y_{\text{min}} + \frac{(y_{\text{max}} - y_{\text{min}})}{\text{Lig}} \times ((x + \text{Lig} + \text{Kd})/2 - \sqrt{((x + \text{Lig} + \text{Kd})/2)^2 - (\text{Lig} \times x)}). \) in Dotmatics to generate a Kd value.

Table S3. Affinities of affinity matured peptides of Bicycle families identified through phage display

| Affinity matured Bicycle number | \(K_d\) (nM) | Geometric mean (nM) (Lower 95% CI of geo. mean; Upper 95% CI of geo. mean, n=Number of values) |
|-------------------------------|--------------|------------------------------------------------------------------------------------------------|
| 2                             | 20.0 (16.4;24.4, n=23) |
Table S4. Species cross reactivity and selectivity binding data for Bicycle 2 determined by FP direct binding assay

| Protein               | Geometric mean (nM) (Lower 95% CI of geo. mean; Upper 95% CI of geo. mean, n=Number of values) |
|-----------------------|---------------------------------------------------------------------------------------------|
| Human Nectin 4        | 19.4 (15.6;24.0, n=19)                                                                        |
| Mouse Nectin 4        | 42.9 (24.6;74.9, n=6)                                                                         |
| Rat Nectin 4          | 91.8 (55.9;150.7, n=4)                                                                        |
| Cyno Nectin 4         | 49.2 (32.7;74.2, n=4)                                                                         |
| Nectin-1              | >2000 (n=2)                                                                                  |
| Nectin-2              | >2000 (n=2)                                                                                  |
| Nectin-3              | >2000 (n=2)                                                                                  |
| Nectin-like 1         | >2000 (n=2)                                                                                  |
| Nectin-like 2         | >2000 (n=2)                                                                                  |
| Nectin-like 3         | >2000 (n=2)                                                                                  |
| Nectin-like 4         | >2000 (n=2)                                                                                  |
| Nectin-like 5         | >2000 (n=2)                                                                                  |

3.2. Affinity determination by fluorescence polarization (FP) competition.

*Bicycles* were screened to determine affinity ($K_i$) in a fluorescence polarization assay where in competition with a Bicycle labelled with fluorescein (referred to as tracer) with a known affinity for Nectin-4. Peptides were diluted to an appropriate concentration in 20mM HEPES with 150mM NaCl, 5% DMSO and 0.01% tween pH 7.4 (referred to as assay buffer) then serially diluted 1 in 2. Into black 384 well low volume plates 5µL of diluted peptide was added to the plate followed by 10µL of Nectin-4 at a fixed concentration (40nM final assay concentration), then 10µL tracer added to a final assay concentration of 1nM. Measurements were conducted on a BMG PHERAstar FS equipped with an “FP 485 520 520” optic module which excites at 485nm and detects parallel and perpendicular emission at 520nm. The PHERAstar FS was set at 25°C with 200 flashes per well and a positioning delay of 0.1 second, with each well measured at 5 to 10 minute intervals for 60 minutes. The gain used for measurements was determined at the point of experiment on a tracer alone well. Data analysis was performed in Dotmatics where polarization data were fit to the Cheng-Prusoff correction equation (Yung-Chi and Prusoff, 1973) in order to generate a $K_i$ value. All *Bicycles* in Tables 3, 4, 5, 6, 7, 8, 9, 10 and 11 in the manuscript were assessed for affinity using FP competition using tracer 90.
Table S5. Bicycle tracer (90) affinity as determined by FP direct binding.

| Bicycle number | $K_d$ (nM) | Geometric mean (Lower 95% CI of geo. mean;Upper 95% CI of geo. mean, n=Number of values) |
|----------------|------------|--------------------------------------------------------------------------------------------|
| 90             | 7.07       | (3.74;13.37, n=4)                                                                          |

Table S6. Profiles of hit sequences in physical chemistry and plasma stability assays

| Bicycle number | $K_i$ (nM), Geometric mean (Lower 95% CI of geo. mean;Upper 95% CI of geo. mean, n=Number of values) |
|----------------|-----------------------------------------------------------------------------------------------|
| 7              | 27.3 (25.3;29.5, n=15)                                                                         |
| 8              | 13.5 (1.4;128.7, n=2)                                                                           |
| 9              | 57.6 (53.3;62.2, n=27)                                                                          |
| 10             | 17.6 (15.8;19.6, n=23)                                                                          |

Table S7. Affinities of Bicycles from Ala and D-Ala scan of Bicycle 7 determined by FP competition

| Position | From | Bicycle number | Ala Scan | D-Ala Scan |
|----------|------|----------------|----------|------------|
|          |      |                | $K_i$ (nM) Geometric mean (Lower 95% CI of geo. mean;Upper 95% CI of geo. mean, n=Number of values) | $K_i$ (nM) Geometric mean (Lower 95% CI of geo. mean;Upper 95% CI of geo. mean, n=Number of values) |
| N/A      | Parent | 7              | 27.3 (25.3;29.5, n=15) | - |
| 1        | Pro   | 60             | 132.2 (36.3;481.4, n=2) | 75 (106.1;196.6, n=2) |
| 2        | Phe   | 61             | >10000 (n=2)            | 76 (n=2) |
| 3        | Gly   | 62             | 883.6 (n=1, n2 >10000)  | 77 (9.5;9.8, n=2) |
| 4        | Met   | 63             | 825.2 (n=1, n2 >10000)  | 78 (n=2) |
| 5        | Lys   | 64             | 43.5 (17.4;109.2, n=2)  | 79 (6.5;3939, n=2) |
| 6        | Asn   | 65             | 45.2 (8.5;238.7, n=2)   | 80 (20.5;227.4, n=2) |
| 7        | Trp   | 66             | >10000 (n=2)            | 81 (n=2) |
| 8        | Ser   | 67             | >10000 (n=2)            | 82 (n=2) |
| 9        | Trp   | 68             | 309.8 (174.6;549.8, n=2)| 83 (n=2) |
| 10       | Pro   | 69             | >10000 (n=2)            | 84 (n=2) |
| Bicycle number | Kᵢ (nM) Geometric mean (Lower 95% CI of geo. mean;Upper 95% CI of geo. mean, n=Number of values) |
|----------------|------------------------------------------------------------------------------------------------|
| 10             | 17.6 (15.8;19.6, n=23)                                                                          |
| 11             | 66.9 (34.5;130, n=2)                                                                             |
| 12             | >10000 (n=2)                                                                                    |
| 13             | 737 (198;2738, n=2)                                                                             |
| 14             | 13.4 (11.2;16.1, n=2)                                                                            |
| 15             | 19.4 (7.2;51.9, n=2)                                                                             |
| 16             | 606 (67.4;5446, n=2)                                                                             |
| 17             | 176 (2.4;12949, n=2)                                                                             |
| 18             | >7000 (n=2)                                                                                      |
| 19             | 13.2 (9.3;18.7, n=2)                                                                             |
| 20             | 38.6 (16.1;92.6, n=2)                                                                            |
| 21             | 46.8 (15.1;145, n=2)                                                                             |
| 22             | 64.2 (53.1;77.7, n=2)                                                                             |
| 23             | 68.9 (56.9;83.6, n=2)                                                                             |
| 24             | 44.3 (1.4;1414, n=2)                                                                             |
| 25             | 38.7 (0.1;23191, n=2)                                                                            |
| 26             | 31.4 (0.5;1872, n=2)                                                                             |
| 27             | 37.3 (0.7;2012, n=2)                                                                             |
| 28             | 77.7 (16.5;365, n=2)                                                                             |
| 29             | 29.4 (2.0;444, n=2)                                                                               |
| 30             | 16.3 (0.5;561, n=2)                                                                              |
| 31             | 59.2 (35.1;99.7, n=2)                                                                             |
| 32             | 142 (20.5;990, n=2)                                                                               |
| 33             | >9000 (n=2)                                                                                      |
| Bicycle number | $K_i$ (nM) Geometric mean (Lower 95% CI of geo. mean;Upper 95% CI of geo. mean, n=Number of values) |
|----------------|----------------------------------------------------------------------------------------------|
| 45             | 8.9 (7.9;10.1, n=27)                                                                         |
| 46             | 46.4 (18.1;118.8, n=2)                                                                        |
| 47             | 2.4 (2.1;2.8, n=8)                                                                             |
| 48             | 2.4 (0.5;11.6, n=4)                                                                            |
| 49             | 1065 (n=1), >3000 (n=3)                                                                       |
| 50             | >3000 (n=4)                                                                                    |
| 51             | 4.9 (2.8;8.8, n=2)                                                                             |
| 52             | 201.7 (96.8;420.3, n=2)                                                                        |
| 53             | 158.2 (57.9;432.8, n=2)                                                                        |
| 54             | 6.1 (2.9;12.9, n=4)                                                                            |
| 55             | 5.2 (3.6;7.6, n=4)                                                                             |
| 56             | >3000 (n=4)                                                                                    |
| 57             | 9.7 (7.6;12.3, n=2)                                                                            |
| 58             | 2.8 (2.3;3.5, n=6)                                                                             |

Table S9. Profile of hit Bicycle 10 versus partially optimized lead 45

3.3 Affinity determination by SPR.

3.3.1 Human Nectin-4 protein production and purification
Cloning: Human Nectin-4 (Uniprot: Q96NY8, residues 32-349) with a gp67 signal sequence and C-terminal FLAG tag was cloned into pFastbac-1 and baculovirus using standard Bac-to-Bac™ protocols (Life Technologies).
Expression and purification: Sf21 cells at 1 x 106/ml-1 in Excell-420 medium (Sigma) at 27°C were infected at a Multiplicity of infection (MOI) of 2 with a P1 virus stock. Supernatant was harvested at 72 hours and incubated for 1 hour at 4°C with Anti-FLAG M2 affinity agarose resin (Sigma) followed by a PBS wash. Resin was subsequently transferred to a column and washed extensively with...
physiologically buffered saline (PBS). Protein was eluted with 100µg/ml FLAG peptide concentrated to a volume of 2ml and loaded onto an S-200 Superdex column (GE Healthcare) in PBS at 1ml/min. 2ml fractions were collected and fractions containing Nectin-4 protein were concentrated to 16mg/ml. Biotinylation: The protein was randomly biotinylated using EZ-Link™ Sulfo-NHS-LC-LC-Biotin reagent (Thermo Fisher) as per the manufacturer’s suggested protocol. The protein was extensively desalted to remove uncoupled biotin using spin columns and transferred to PBS.

3.3.1 Affinity determination of BT8009 by SPR
Streptavidin was immobilized on a CM5 (GE Healthcare) or CMD5000 (Xantec) chip using standard amine-coupling chemistry at 25°C with HBS-N (10 mM HEPES, 0.15 M NaCl, pH 7.4) as the running buffer. Briefly, the carboxymethyl dextran surface was activated with a 7 min injection of a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/0.1 M N-hydroxy succinimide (NHS) at a flow rate of 10 µl/min. Streptavidin was diluted to 0.2 mg/ml in 10 mM sodium acetate (pH 4.5) and captured to a level of 1,200-1,800 RU. Bicycles and BTCs were prepared in PBS/0.05% Tween 20 with a final DMSO concentration of 0.5%. SPR analysis was run on a Biacore 3000 instrument at 25°C at a flow rate of 50µl/min with association time of 60 seconds and dissociation time of between 400 and 1,200 seconds depending upon the individual peptide. Data were corrected for DMSO excluded volume effects. All data were double-referenced for blank injections and reference surface using standard processing procedures and 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.

Table S10. Affinity determination of BT8009 by SPR

| Bicycle number | $K_D$ (nM) Geometric mean (Lower 95% CI of geo. mean;Upper 95% CI of geo. mean, n=Number of values) |
|----------------|------------------------------------------------------------------------------------------------|
| BT8009         | 2.50 (2.09;2.98, n=13)                                                                         |

4. Chemical methods

4.1 Peptide Sequences

Table S11. Peptide sequences

| Bicycle number | Sequence                                               |
|----------------|--------------------------------------------------------|
| 1              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 2              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 3              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 4              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 5              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 6              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 7              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 8              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 9              | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 10             | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 11             | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 12             | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
| 13             | ACWPLDSYWCARICA[Sar6][KFl][CONH2]                      |
|   | Sequence                      |
|---|-------------------------------|
| 14| [Ac]CP[3MePhe]GCMKNWSWPIWCTNK[CONH2] |
| 15| [Ac]CP[4MePhe]GCMKNWSWPIWCTNK[CONH2] |
| 16| [Ac]CP[3Pal]GCMKNWSWPIWCTNK[CONH2] |
| 17| [Ac]CP[2Pal]GCMKNWSWPIWCTNK[CONH2] |
| 18| [Ac]CP[4Pal]GCMKNWSWPIWCTNK[CONH2] |
| 19| [Ac]CPF[dD]CMKNWSWPIWCTNK[CONH2] |
| 20| [Ac]CPFGCKKNWSWPIWCTNK[CONH2] |
| 21| [Ac]CPFGCQKNWSWPIWCTNK[CONH2] |
| 22| [Ac]CPFGC[tBuAla]KNWSWPIWCTNK[CONH2] |
| 23| [Ac]CPFGC[HLue]KNWSWPIWCTNK[CONH2] |
| 24| [Ac]CPFGC[MetO2]KNWSWPIWCTNK[CONH2] |
| 25| [Ac]CPFGCKLKNWSWPIWCTNK[CONH2] |
| 26| [Ac]CPFGCM[HAArg]NWSTPIWCTNK[CONH2] |
| 27| [Ac]CPFGCM[KA(c)]NWSTPIWCTNK[CONH2] |
| 28| [Ac]CPFGCMKN[1Nal]NWSTPIWCTNK[CONH2] |
| 29| [Ac]CPFGCMKN[2Nal]NWSTPIWCTNK[CONH2] |
| 30| [Ac]CPFGCMKN[AsaTPr]NWSTPIWCTNK[CONH2] |
| 31| [Ac]CPFGCMKNWS[1Nal]PIWCTNK[CONH2] |
| 32| [Ac]CPFGCMKNWS[2Nal]PIWCTNK[CONH2] |
| 33| [Ac]CPFGCMKNWS[AsaTPr]PIWCTNK[CONH2] |
| 34| [Ac]CPFGCMKNSWSTPIWCTNK[CONH2] |
| 35| [Ac]CPFGCMKNSWST[Oxa]IWC[CONH2] |
| 36| [Ac]CPFGCMKNSWST[HyP]IWC[CONH2] |
| 37| [Ac]CPFGCMKNSWSTPI[33DPA]C[CONH2] |
| 38| [Ac]CPFGCMKNSWSTPI[5FTrp]C[CONH2] |
| 39| [Ac]CPFGCMKNSWSTPI[33DPA]C[CONH2] |
| 40| [Ac]CPFGCMKNSWSTPI[5FTrp]C[CONH2] |
| 41| [Ac]CPFGCMKNSWSTPI[33DPA]C[CONH2] |
| 42| [Ac]CPFGCMKNSWSTPI[5FTrp]C[CONH2] |
| 43| [Ac]CPFGCMKNSWSTPI[33DPA]C[CONH2] |
| 44| [Ac]CPFGCMKNSWSTPI[5FTrp]C[CONH2] |
| 45| [Ac]CP[dD]CM[HAArg]NWSTPIWCTNK[CONH2] |
| 46| [Ac]CP[dD]CM[HAArg]NWSTPIWCTNK[CONH2] |
| 47| [Ac]CP[1Nal][dD]CM[HAArg]NWSTPIWCTNK[CONH2] |
| 48| [Ac]CP[2Nal][dD]CM[HAArg]NWSTPIWCTNK[CONH2] |
| 49| [Ac]CP[44BPA][dD]CM[HAArg]NWSTPIWCTNK[CONH2] |
| 50| [Ac]CP[dD]CM[HAArg][33DPA]STPIWCTNK[CONH2] |
| 51| [Ac]CP[dD]CM[HAArg][5FTrp]STPIWCTNK[CONH2] |
| 52| [Ac]CP[dD]CM[HAArg]NWST[Oxa]IWC[CONH2] |
| 53| [Ac]CP[dD]CM[HAArg]NWST[HyP]IWC[CONH2] |
| 54| [Ac]CP[dD]CM[HAArg]NWSTPPWCTNK[CONH2] |
| 55| [Ac]CP[dD]CM[HAArg]NWSTP[HyP]WC[CONH2] |
| 56| [Ac]CP[dD]CM[HAArg]NWSTP[33DPA]C[CONH2] |
| 57| [Ac]CP[dD]CM[HAArg]NWSTP[5FTrp]C[CONH2] |
| 58| [Ac]CP[dD]CM[HAArg]NWSTP[33DPA]C[CONH2] |
| 59| [Ac]CP[dD]CM[HAArg]NWSTP[5FTrp]C[CONH2] |
| 60| [Ac]CP[dD]CM[HAArg]NWSTP[33DPA]C[CONH2] |
| 61| [Ac]CP[dD]CM[HAArg]NWSTP[5FTrp]C[CONH2] |
| 62| [Ac]CP[dD]CM[HAArg]NWSTP[33DPA]C[CONH2] |
| 63| [Ac]CP[dD]CM[HAArg]NWSTP[5FTrp]C[CONH2] |
| 64| [Ac]CP[dD]CM[HAArg]NWSTP[33DPA]C[CONH2] |
| 65| [Ac]CP[dD]CM[HAArg]NWSTP[5FTrp]C[CONH2] |
| 66| [Ac]CP[dD]CM[HAArg]NWSTP[33DPA]C[CONH2] |
| 67| [Ac]CP[dD]CM[HAArg]NWSTP[5FTrp]C[CONH2] |
68  ACPFGCMKNWSAPIWCTNK[CONH2]
69  ACPFGCMKNWSWAIWCTNK[CONH2]
70  ACPFGCMKNWSWPAWCTNK[CONH2]
71  ACPFGCMKNWSWIACTNK[CONH2]
72  ACPFGCMKNWSWPCANK[CONH2]
73  ACPFGCMKNWSWICTAK[CONH2]
74  ACPFGCMKNWSWIWCTNA[CONH2]
75  AC[dA]FGCMKNWSWIWCTNK[CONH2]
76  ACP[dA]GCMKNWSWIWCTNK[CONH2]
77  ACPF[dA]CMKNWSWIWCTNK[CONH2]
78  ACPFGC[dA]MKnWSWIWCTNK[CONH2]
79  ACPFGCM[dA]NWSWIWCTNK[CONH2]
80  ACPFGCM[dA]WSWIWCTNK[CONH2]
81  ACPFGCMK[dA]SWWIWCTNK[CONH2]
82  ACPFGCMKN[dA]SWWIWCTNK[CONH2]
83  ACPFGCMKNW[dA]WPIWCTNK[CONH2]
84  ACPFGCMKNWS[dA]PIWCTNK[CONH2]
85  ACPFGCMKNWSW[dA]IWIWCTNK[CONH2]
86  ACPFGCMKNWSWP[dA]WCTNK[CONH2]
87  ACPFGCMKNWSWIWC[dA]K[CONH2]
88  ACPFGCMKNWSWIWC[dA]K[CONH2]
89  ACPFGCMKNWSWIWCTN[dA][CONH2]
90  [Fl]ACPFGCMKNWSWIWCTN[CONH2]

All compounds cyclised on TATA (1,3,5-Triacyloxyhexahydro-1,3,5-triazine) scaffold; Sar6 – 6 Sarcosine residues; KFl - Lysine(6-Carboxyfluorescein); Fl – 6-Carboxyfluorescein; dA – D-alanine; Ac – Acetyl; tBuAla – t-butyl alanine; HLeu – homo-leucine; 1Nal - 3-(1-naphthyl)-L-alanine; 3MePhe – 3-methyl-phenylalanine; 4MePhe – 4-methyl-phenylalanine; dD – D-aspartic acid; AzaTrp – azatryptophan; 2Nal – 3-(2-naphthyl)-L-alanine; Aze – azetidine-2-carboxylic acid; Pip – piperolic acid; 3Pal – 3-(3-pyridyl)-L-alanine; MetO2 – methionine sulfone; K(Ac) – lysine(acetyl); 2Pal – 3-(2-pyridyl)-L-alanine; 4Pal – 3-(4-pyridyl)-L-alanine; HArg – homo-arginine; HyP – hydroxyproline; Oxa – oxazolidine-4-carboxylic acid; 5FTrp – 5-fluoro-L-tryptophan; 33DPA – 3,3-diphenylalanine; 44BPA – 4,4-biphenylalanine; dA – D-alanine; dC – D-cysteine; dP – D-proline; dF – D-phenylalanine; dM – D-methionine ; dK – D-lysine; dN – D-asparagine; dW – D-tryptophan; dS – D-serine; dI – D-isoleucine; dT – D-threonine;

4.2 Fluorescent probes - analytics
Compounds were synthesized via the general methods outlined in the manuscript. SPPS was performed using the Biotage SyroII and purification was carried out using a Phenomenex Kinetex C18 column (2.6μm, 100 Å, 50mm × 21.2mm) with H2O/0.1% (v/v) trifluoroacetic acid and acetonitrile/0.1% (v/v) trifluoroacetic acid as solvents. Purity/identity was determined using LCMS.

Table S12. Fluorescent peptide analytical data

| Bicycle number | Calculated molecular weight | m/z found [M+2H]2+ | m/z found [M+3H]3+ |
|----------------|-----------------------------|--------------------|--------------------|
| 1              | 3141.5                      | 1571.61            | 1048.13            |
| 2              | 3433.92                     | 1717.47            | 1145.60            |
| 3              | 2919.27                     | 1460.44            | 974.28             |

S11
LCMS was performed on an Acquity UPLC CSH C18 Column (130Å, 1.7 µm, 2.1 mm X 30 mm) at a flow rate of 0.6 ml/min. The gradient was started at 95% buffer A (0.1% formic acid in water) to 95% buffer B (0.1% formic acid in acetonitrile) in A in 5 min. Percent purity was determined at 220 nm.

4.3 Bicycles synthesised during peptide optimisation - analytics
Compounds were synthesised via the general methods outlined in the manuscript. SPPS was performed using the Biotage SyroII and purification was carried out using a Phenomenex Kinetex C18 column (2.6µm, 100 Å, 50mm × 21.2mm) H₂O/0.1% (v/v) trifluoroacetic acid and acetonitrile/0.1% (v/v) trifluoroacetic acid as solvents. Purity/identity was determined using LCMS.

| Bicycle number | Calculated molecular weight | m/z found [M+2H]²⁺ | m/z found [M+3H]³⁺ |
|----------------|-----------------------------|---------------------|---------------------|
| 7              | 2520.98                     | 1261.8              | 841.29              |
| 8              | 2491.93                     | 1246.75             | 831.58              |
| 9              | 2248.67                     | 1125.56             | 750.6               |
| 10             | 2148.55                     | 1075.2              | 717.8               |
| 11             | 2134.52                     | 1068.2              | 712.52              |
| 12             | 2162.57                     | 1082.26             | 721.9               |
| 13             | 2164.54                     | 1082.98             | 722.49              |
| 14             | 2162.57                     | 1082.06             | 721.9               |
| 15             | 2162.57                     | 1082.06             | 721.63              |
| 16             | 2149.53                     | 1076.19             | 717.34              |
| 17             | 2149.53                     | 1075.53             | 717.94              |
| 18             | 2149.53                     | 1075.92             | 717.41              |
| 19             | 2206.58                     | 1104.3              | 736.74              |
| 20             | 2130.51                     | 1066.15             | 711.34              |
| 21             | 2145.48                     | 1073.55             | 716.42              |
| 22             | 2144.53                     | 1073.22             | 715.82              |
| 23             | 2144.53                     | 1073.08             | 715.89              |
| 24             | 2180.54                     | 1091.37             | 728.1               |
| 25             | 2130.51                     | 1066.62             | 711.34              |
| 26             | 2190.58                     | 1096.71             | 731.4               |
| 27             | 2190.58                     | 1096.71             | 731.53              |
| 28             | 2135.46                     | 1068.46             | 712.79              |
| 29             | 2149.53                     | 1075.72             | 717.41              |
| 30             | 2171.58                     | 1086.75             | 724.86              |
| 31             | 2159.56                     | 1080.61             | 721.3               |
| 32             | 2159.56                     | 1080.74             | 721.24              |
| 33             | 2149.53                     | 1075.72             | 717.41              |
|  34 | 2159.56 | 1081  | 721.04 |
|  35 | 2159.56 | 1080.54 | 721.3 |
|  36 | 2149.53 | 1075.72 | 717.61 |
|  37 | 2063.44 | 1032.62 | 689.23 |
|  38 | 2134.52 | 1068.4 | 712.19 |
|  39 | 2162.57 | 1082.46 | 722.03 |
|  40 | 2132.5 | 1067.08 | 711.53 |
|  41 | 2150.48 | 1076.25 | 718.2 |
|  42 | 2159.56 | 1080.67 | 720.97 |
|  43 | 2159.56 | 1080.81 | 720.84 |
|  44 | 2149.53 | 1075.86 | 717.47 |
|  45 | 2163.51 | 1082.9 | 722.42 |
|  46 | 2165.49 | 1084.17 | 722.49 |
|  47 | 2213.57 | 1108.07 | 739.25 |
|  48 | 2213.57 | 1107.54 | 739.19 |
|  49 | 2239.61 | 1120.74 | 747.37 |
|  50 | 2200.57 | 1101.53 | 734.3 |
|  51 | 2181.5 | 1091.63 | 728.1 |
|  52 | 2165.49 | 1083.51 | 722.56 |
|  53 | 2179.51 | 1090.64 | 727.83 |
|  54 | 2147.47 | 1074.54 | 716.48 |
|  55 | 2163.46 | 1082.52 | 722.03 |
|  56 | 2200.57 | 1101.6 | 734.9 |
|  57 | 2181.5 | 1091.63 | 728.86 |
|  58 | 2214.5 | 1108.63 | 739.05 |
|  59 | 2494.94 | 1248.4 | 832.57 |
|  60 | 2444.88 | 1223.58 | 815.94 |
|  61 | 2535 | 1268.34 | 845.97 |
|  62 | 2460.86 | 1231.83 | 821.29 |
|  63 | 2463.88 | 1232.89 | 822.61 |
|  64 | 2477.95 | 1239.82 | 827.03 |
|  65 | 2405.84 | 1203.84 | 802.87 |
|  66 | 2504.98 | 1253.55 | 836.14 |
|  67 | 2405.84 | 1204.37 | 803.01 |
|  68 | 2494.94 | 1248.27 | 832.7 |
|  69 | 2478.9 | 1240.28 | 827.16 |
|  70 | 2405.84 | 1203.78 | 803.07 |
|  71 | 2490.95 | 1246.42 | 831.45 |
|  72 | 2477.95 | 1239.75 | 826.96 |
|  73 | 2463.88 | 1232.89 | 822.34 |
|  74 | 2494.94 | 1248.07 | 832.64 |
|  75 | 2444.88 | 1223.71 | 816.01 |
|  76 | 2535 | 1268.54 | 846.1 |
|  77 | 2460.86 | 1231.17 | 821.22 |
|  78 | 2463.88 | 1233.09 | 822.28 |
|  79 | 2477.95 | 1239.82 | 826.9 |
|   |    |    |    |
|---|----|----|----|
| 81| 2405.84 | 1204.18 | 803.01 |
| 82| 2504.98 | 1253.09 | 836.2  |
| 83| 2405.84 | 1204.11 | 802.74 |
| 84| 2494.94 | 1248.67 | 832.71 |
| 85| 2478.9  | 1240.22 | 827.56 |
| 86| 2405.84 | 1203.65 | 802.87 |
| 87| 2490.95 | 1246.29 | 831.39 |
| 88| 2477.95 | 1240.22 | 826.96 |
| 89| 2463.88 | 1232.96 | 822.41 |

LCMS was performed on an Acquity UPLC CSH C18 Column (130Å, 1.7 µm, 2.1 mm X 30 mm) at a flow rate of 0.6 ml/min. The gradient was started at 95% buffer A (0.1% formic acid in water) to 95% buffer B (0.1% formic acid in acetonitrile) in A in 5 min. Percent purity was determined at 220 nm.
Example LC/MS traces for compounds in Table 6

**Bicycle 10**

| Mobile Phase:           | A: 0.1% Formic Acid in H2O B: 0.1% Formic Acid in MeCN |
|------------------------|--------------------------------------------------------|
| Flow:                  | 0.6ml/min                                              |
| Column:                | CSH C18 Column (130Å, 1.7 µm, 2.1 mm X 30 mm)          |
| Retention Time:        | 1.95 min                                               |
| m/z found              | [M+2H]^2+ = 1075.4; [M+3H]^3+ = 717.2                  |
**Bicycle 45**

| Mobile Phase:         | A: 0.1% Formic Acid in H2O B: 0.1% Formic Acid in MeCN |
|-----------------------|---------------------------------------------------------|
| Flow:                 | 0.6ml/min                                                |
| Column:               | CSH C18 Column (130Å, 1.7 µm, 2.1 mm X 30 mm)            |
| Retention Time:       | 1.86 min                                                 |
| m/z found             | [M+2H]^2+ = 1082.9; [M+3H]^3+ = 722.4                   |
Bicycle 58

| Parameter          | Value |
|--------------------|-------|
| Mobile Phase       | A: 0.1% Formic Acid in H2O B: 0.1% Formic Acid in MeCN |
| Flow               | 0.6ml/min |
| Column             | CSH C18 Column (130Å, 1.7 µm, 2.1 mm X 30 mm) |
| Retention Time     | 1.88 min |
| m/z found          | [M+2H]^{2+} = 1108.6; [M+3H]^{3+} = 739.1 |
4.4 BT8009 synthetic scheme and analytics

Figure S1: BT8009 synthetic scheme
Glutarate-Val-Cit-PAB-MMAE

Mobile Phase: A: 0.1% TFA in H2O B: 0.1% TFA in MeCN
Flow: 1.0ml/min
Column: Gemini-NX C18 5um 110A 150*4.6mm
Instrument: Agilent 1200 HPLC-BE(1-614)
Method: 40% B for 4 minutes, then 40-70% B over 20 minutes, then 70-90% B over 3 minutes
Retention Time: 8.0 min

Area Percent Report

| Peak | RT  | Height | Height % | Width | Area   | Area % |
|------|-----|--------|----------|-------|--------|--------|
| #    | [min]|        |          | [min] |        |        |
| 1    | 4.927 | 1.548  | 0.191    | 0.097 | 9.687  | 0.133  |
| 2    | 7.069 | 0.849  | 0.105    | 0.248 | 16.585 | 0.227  |
| 3    | 8.038 | 798.282 | 98.545   | 0.137 | 7160.584 | 98.218 |
| 4    | 8.747 | 8.364  | 1.032    | 0.162 | 89.135 | 1.223  |
| 5    | 9.298 | 1.029  | 0.127    | 0.189 | 14.690 | 0.201  |
**NHS-Glutamate-Val-Cit-PAB-MMAE**

![Chemical Structures](image)

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**Mobile Phase:**
- A: 0.1% TFA in H$_2$O
- B: 0.1% TFA in MeCN

**Flow:**
1.0 ml/min

**Column:**
Gemini-NX 5u C18 110A 150*4.6mm

**Instrument:**
Agilent 1200 HPLC-BE(1-614)

**Method:**
- 10% B for 4 minutes, then 10-80% B over 20 minutes, then 80-90% B over 2 minutes

**Retention Time:**
15.8 min

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### Area Percent Report

| Peak | RT [min] | Height | Height % | Width [min] | Area | Area % |
|------|----------|--------|----------|-------------|------|--------|
| 1    | 14.025   | 3.776  | 0.259    | 0.084       | 21.362 | 0.244  |
| 2    | 14.141   | 3.105  | 0.213    | 0.086       | 17.648 | 0.201  |
| 3    | 14.430   | 3.158  | 0.216    | 0.084       | 17.305 | 0.197  |
| 4    | 14.969   | 60.377 | 4.682    | 0.099       | 452.648 | 5.164 |
| 5    | 15.075   | 19.737 | 1.351    | 0.069       | 89.716 | 1.023  |
| 6    | 15.553   | 9.756  | 0.668    | 0.094       | 58.929 | 0.672  |
| 7    | 15.788   | 1317.692 | 90.219  | 0.092       | 7885.647 | 89.917 |
| 8    | 16.040   | 10.221 | 0.700    | 0.096       | 63.373 | 0.723  |
**Bicycle 59**

**Mobile Phase:**  
A: 0.1% TFA in H2O  
B: 0.1% TFA in MeCN

**Flow:**  
1.0 ml/min

**Column:**  
Gemini-NX C18 5um 110A 150*4.6mm

**Instrument:**  
Agilent 1200 HPLC-BE(1-614)

**Method:**  
20% B for 4 minutes, then 20-50% B over 20 minutes, then 70-90% B over 2 minutes

**Retention Time:**  
11.0 min
5. Physicochemical profiling / in vitro DMPK

5.1. Lipophilicity measurements (CHIlogP). As a lipophilicity measure the peptide chromatography hydrophobicity indexes (CHI) were determined by comparing the peptide retention behaviour on RP-HPLC stationary phase at three different pHs and with this for a set of standards with known CHI [1,2,3]. The peptides and the standards were analysed on Gemini NX C18 column (5 μm, 110 Å, 50 x 3 mm, Phenomenex) and Agilent 1260 HPLC system using 0 - 100% acetonitrile gradient elution at three different pH (2.6, 7.4 and 10.5) over 3 min. As aqueous phase 0.01M formic acid (pH 2.6) or 50mM ammonium acetate (pH 7.4 or pH 10.5) was used. The CHI values determined at pH 2.6, 7.4 or 10.5 (based on CHI value vs retention time standard curve) were converted to the widely used logarithmic partition coefficient (logP) by linear conversion: CHIlogD = 0.0525 CHI – 1.467. The reported CHIlogP value was the highest of the CHIlogD values at pH 2.6, 7.4 or 10.5.3-5
5.2. Plasma protein binding. Frozen plasma with EDTA-K2 used as anticoagulant was pooled from male, female or mixed gender (minimum of 3 donors) and stored at -80 °C before use. This was thawed and warmed in a water bath, then centrifuged at 4,000 rpm for 5 min to remove the clots. The pH of the plasma was adjusted to 7.4 ± 0.1 using NaOH or phosphoric acid, if needed. Working solutions of peptides were generated using DMSO as solvent (400 μM, 200-fold of final test concentration). These were spiked into blank plasma to achieve final concentration of 2 μM in plasma and a final organic solvent concentration of less than 1%. Samples were thoroughly mixed and an aliquot of 30 μL taken in duplicate for time zero stability check (T0 samples). The ultracentrifuge rotor was warmed to 37°C before centrifugation. Plasma samples were incubated at 37 °C with 5% CO2 in a humidified incubator for 30 min (pre-incubation). Aliquots of 400 μL plasma samples were transferred to polycarbonate tubes in duplicate and subjected to ultracentrifugation at 470000g for 2 hours at 37°C (OptimaTM TLX). Meanwhile, aliquots of 30 μL spiked plasma in duplicate were transferred into a 96 well plate, then incubated for 2 hours at 37°C for the % remaining measurement (T2.5 samples). At the end of ultracentrifugation, 30 μL aliquots of protein-free sample (F sample) from the second layer beneath top layer of the supernatant was collected and transferred into a 96-well polypropylene plate (sample collection plate). Stop solution containing internal standards (IS - warfarin) was added to precipitate protein (300μL) then the samples were vortexed vigorously and centrifuged at 4,000 rpm for at least 15 minutes. Supernatant was removed and diluted with an appropriate volume of dilution solution (such as 200 μL deionized water). Samples were again shaken at 1000 rpm at least 6 min. Samples were subjected to bioanalysis by LC/MS/MS. Semi-quantitative analysis was carried out using peak area ratios of analyte to internal standards without standard curves. The values are interrelated and the calculations for them are given below:

\%

Unbound = 100 * F / T2.5

\%

Bound = 100 - % Unbound

\%

Remaining=100 * T2.5/ T0

T0 = Total compound concentration as determined by the plasma concentration at time zero

T2.5 = Total compound concentration as determined by the calculated concentration after 2.5-hr incubation

T4.5 = Total compound concentration as determined by the calculated concentration after 4.5-hr incubation

F = Free compound concentration as determined by the calculated concentration of the plasma-free layer after ultracentrifugation

Plasma protein binding studies were carried out at Wuxi AppTec Co. Ltd. (Shanghai).

5.3. In vitro plasma stability. Pooled frozen plasma (heparin anticoagulant) was thawed in a water bath at 37°C. Plasma was centrifuged at 4000 rpm for 5 min and the clots were removed if any. The pH will be adjusted to 7.4 ± 0.1 if required. 1 mM stock solution was prepared with DMSO. Propantheline (positive control) was prepared by making a 100 μM working solution by diluting 5 μL of the stock solution (10 mM) with 495 μL ultra pure water. 100 μM working solutions of test compounds were made up by diluting 10 μL of the stock solution (1 mM) with 90 μL DMSO. 98 μL of blank plasma was spiked with 2 μL of dosing solution (100 μM) to achieve 2 μM of the final concentration in duplicate and samples were incubated at 37°C in a water bath. At each time point (0,1,2,4,6 and 24hr), 400 μL of 200ng/mL tolbutamide and Labetalol in 100% MeOH was added and mixed thoroughly to precipitate proteins. Sample plates were centrifuged at 4,000 rpm for 15 min. An aliquot of supernatant (150 μL) was transferred from each well before submitting for LC-MS/MS analysis. The % remaining test compound after incubation in plasma was calculated using following equation:
% Remaining = 100 x (PAR at appointed incubation time / PAR at T0 time)

where PAR is the peak area ratio of analyte versus internal standard (IS). The appointed incubation time points are T0 (0 hr), Tn (n=0,1,2,4,6,24hr). The half-life (T1/2) was calculated from a log linear plot of concentration versus time.

When the % remaining value at the maximal incubation time, which was 24 hr in this study, was higher than 75%, it is considered to be within the acceptable experimental variation. Therefore, a corresponding t1/2 of >57.8 hr was reported. Plasma stability studies were carried out at Wuxi AppTec Co. Ltd. (Shanghai).

6. In vivo studies

6.2. CDX studies. Mice (balb/c nude, female, 18-22g at study start) were inoculated subcutaneously at the right flank with human cancer cells (5% x 10^6 to 1% x 10^7 cells dependent on cell line). When tumors reached approximately 150 to 200 mm³ (or larger tumor volume as specified in the respective study), mice were randomized by tumor volume and body weight to their respective treatment groups (typically 3 animals per group). BTCs were injected as an IV bolus once weekly unless otherwise specified. Tumor volume was expressed in mm³ using the formula V=0.5 a x b² where a and b were the long and short diameters of the tumor respectively. All studies included a vehicle-treated control. All Xenograft studies were conducted at Wuxi AppTec Co. Ltd. (Shanghai).

![Body weights of mice following dosing of BT8009](image)

**Figure S2:** Body weights of mice following dosing of BT8009
7. References

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