Supporting Information

Tumor-Cell-Specific Targeting of Ibrutinib: Introducing Electrostatic Antibody-Inhibitor Conjugates (AiCs)

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Supporting Information

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1. Synthesis of Cy3.5-ibrutinib

1.1. General information

All solvents and reagents were used as received from commercial suppliers unless otherwise stated. Reaction progress was monitored by TLC performed on aluminium plates coated with silica gel 60 F254 from Merck (Darmstadt, Germany). Chromatograms were visualized by fluorescence quenching with UV light at λ=254 nm and by staining with iodine vapour or ninhydrin staining solution. Column chromatography was performed using silica gel 60 (230-400 mesh, Merck) or prepacked columns Büchi FlashPure silica 40 µm irregular, using a Grace Revealeris X2 flash purification system. Some reactions were performed in microwave reactor CEM Discover. For solid separation was used Eppendorf Centrifuge 5804R. Melting points were measured on Mettler Toledo MP70 Melting Point System. NMR-spectra were obtained at the Organisch-Chemisches Institut Münster (WWU), using an Agilent DD2 500/Agilent DD2 600 or a Bruker Avance II 400. All measurements were performed at room temperature unless mentioned otherwise. The 1H-NMR and 13C-NMR chemical shifts (δ) of the signals are given in parts per million and are referenced to the residual proton signal in the deuterated solvent. The signal multiplicities are abbreviated as follows: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet.
Exact mass (EM) determination by mass spectrometry (MS) was carried out at the Organisch-Chemisches Institut Münster (WWU) using a LTQ Orbitap LTQ XL (Thermo-Fisher Scientific, Bremen, Germany) with electron spray ionisation (ESI). HPLC chromatograms were made on Knauer (Berlin, Germany) HPLC system equipped with the pump P2.1L and diode array detector DAD6.1L.

1.2 Synthesis

1.2.1 3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidine-4-amine (2) [1]

![Chemical Structure](image)

Iodide 1 (1000 mg, 3.85 mmol, 1.0 eq.), 4-phenoxyphenylboronic acid (823 mg, 3.85 mmol, 1.0 eq.) and tetrakis-(triphenylphosphine)palladium (444 mg, 0.38 mmol, 0.1 eq.) were suspended in dioxane (10 mL) and potassium hydroxide (431 mg, 7.69 mmol, 2.0 eq.) dissolved in water (2 mL) was added. The reaction mixture was irradiated 10 minutes on 180°C in microwave reactor. The resulting reaction mixture was diluted with EtOAc (80 mL) and water (20 mL) and layers were separated. The aqueous layer was additionally extracted with 3 x 20 mL EtOAc. Organic layers were combined, washed with brine and dried over anhydrous MgSO₄. The solvent was removed under reduced pressure and residue was triturated four times with DCM and dried on high vacuum to give 668 mg of product 2 (2.20 mmol, 58%) as a white powder.

$^1$H NMR (400MHz, DMSO-d₆): $\delta$ = 13.58 (s, 1H, NH), 8.22 (s, 1H, CH), 7.67 (d, 2H, $J$=8.7Hz, PhH), 7.45-7.40 (m, 2H, PhH), 7.20-7.10 (m, 5H, PhH).

$^{13}$C NMR (100MHz, DMSO-d₆): $\delta$ = 158.1, 157.0, 156.3, 156.1, 155.8, 143.9, 130.1, 130.0, 128.5, 123.8, 119.0, 118.9, 96.9.

TLC (silica, 10% MeOH / CHCl₃, det.: UV$_{254nm}$ & J2): $R_f$ = 0.37

HRMS (ESI+): exact mass calculated for [M+H]$^+$ (C$_{17}$H$_{14}$N$_5$O) required $m/z$ 304.1193; found $m/z$ 304.1198.

[1] slightly modified from A. Turetsky, E. Kim, R. H. Kohler, M. A. Miller, R. Weissleder, Sci. Rep. 2014, 4, 4782.
1.2.2 (S)-3-MesyI-N-Boc-piperidine (3) \[2\]

(S)-3-hydroxy-N-Boc-piperidine (13.0 g, 65 mmol, 1 eq.) was dissolved in DCM (100 mL) and triethylamine (20 mL, 14.4 g, 143 mmol, 2.2 eq.) was added. With cooling on -5°C methanesulfonyl chloride (6.5 mL, 9.6 g, 84 mmol, 1.3 eq.) was added dropwise and after that the reaction mixture was stirred on room temperature overnight. The reaction mixture was diluted with water (100 mL) and layers were separated. Organic layer was washed with 0.1M HCl until aqueous layer be in the range pH=3-4. Combined aqueous layers were additionally extracted with 2 x 100 mL DCM. Combined organic layers were washed with brine and dried over anhydrous MgSO₄. The solvent was removed under reduced pressure to give 13.9 g of product 3 (49.80 mmol, 77%) as a white solid.

$^1$H NMR (400MHz, CDCl₃): $\delta =$ 4.72-4.67 (m, 1H, CH), 3.66-3.54 (m, 2H, CH), 3.46-3.38 (m, 1H, CH), 3.33-3.25 (m, 1H, CH), 3.02 (s, 3H, CH), 1.96-1.74 (m, 3H, CH), 1.55-1.43 (m, 1H, CH), 1.43 (s, 9H, CH).

$^{13}$C NMR (100MHz, CDCl₃): $\delta =$ 154.8, 80.2, 75.5, 47.5, 43.1, 38.8, 30.5, 28.4, 21.7.

TLC (silica, cyclohexane / EtOAc 1:2 v/v, det.: UV$_{254}$nm & J₂): R$_f$ = 0.60

HRMS (ESI+): exact mass calculated for [M+Na]$^+$ (C$_{11}$H$_{21}$NO$_5$SNa) required m/z 302.1038; found m/z 302.1048.

\[2\] according to WO2017/137446 (PCT/EP2017/052773).
1.2.3 (R)-**tert-butyl 3-(4-amino-3-(4-phenoxyphe nyl)-1H-pyrazolo[3,4-d]pyrimi
dine-1-yl)-piperidine-1-carboxylate (4)** [3]

Amine 2 (1 g, 3.30 mmol, 1.0 eq.) and mesylate 3 (1.84 g, 6.60 mmol, 2.0 eq.) were dissolved in DMF (40 mL) and reaction mixture was stirred on 80°C overnight and then cooled to the room temperature and poured into water (1 L). Obtained emulsion was saturated with crude NaCl and extracted with 3 x 200 mL EtOAc. Combined organic layers were washed with brine and dried over anhydrous MgSO₄. The solvent was removed under reduced pressure and residue was triturated with 3 x 20 mL pentane. Obtained crude product was purified by flash chromatography on 80g silica column with 0->60% EtOAc / cyclohexane as eluent. Fraction, eluted with EtOAc / cyclohexane 1:1, was evaporated to give 730 mg of product 4 (1.50 mmol, 46%) as a white foam.

**1H NMR** (500MHz, CDCl₃): δ = 8.28 (s, 1H, CH), 7.58 (d, 2H, J=8.6Hz, PhH), 7.32-7.29 (m, 2H, PhH), 7.09-7.07 (m, 3H, PhH), 7.01-6.99 (m, 2H, PhH), 5.80 (bs, 2H, NH), 4.79-4.73 (m, 1H, CH), 4.06-4.02 (m, 1H, CH), 3.41-3.38 (m, 1H, CH), 2.24-2.08 (m, 2H, CH), 1.84-1.81 (m, 1H, CH), 1.67-1.56 (m, 1H, CH), 1.37 (s, 9H, CH).

**13C NMR** (125MHz, CDCl₃): δ = 158.5, 157.9, 156.4, 155.6, 154.7, 154.2, 143.8, 130.0, 130.0, 127.9, 124.0, 119.5, 119.1, 98.6, 79.9, 53.0, 47.5, 43.5, 30.2, 28.4.

**TLC** (silica, cyclohexane / EtOAc 1:1 v/v, det.: UV₂₅₄nm & ninhydrin sol.): Rᵣ = 0.32 (blue fluorescence at UV₂₅₄nm)

**HRMS** (ESI+): exact mass calculated for [M+H]+ (C₂₇H₃₁N₆O₃) required m/z 487.2458; found m/z 487.2461.

[3] slightly modified from A. Turetsky, E. Kim, R. H. Kohler, M. A. Miller, R. Weissleder, *Sci. Rep.* **2014**, *4*, 4782.
1.2.4. N-Boc-2-aminoacetaldehyde (5) [4]

\[ \text{O} \ \text{NHBoc} \]

Into suspension of tert-butyl N-(2,3-dihydroxypropyl)carbamate (2.00 g, 10.47 mmol, 1 eq.) in water (17.5 mL), NaIO4 (2.69 g, 12.564 mmol, 1.2 eq.) was added and reaction mixture was stirred on room temperature, protected from the light. After 1 hour reaction mixture was filtered and filtrate was extracted with 4 x 50 mL DCM. Combined organic layers were washed with brine and dried over anhydrous MgSO4. The solvent was removed under reduced pressure to give 1.40 g of product 5 (8.80 mmol, 84%) as a colorless oil.

\(^1\)H NMR (400MHz, CDCl3): \( \delta = 9.58 \) (s, 1H, CHO), 5.20 (bs, 1H, NH), 4.00 (d, 2H, \( J_{H,H}=5.1 \)Hz, CH), 1.39 (s, 9H, CH).

TLC (silica, cyclohexane / EtOAc 1:1 v/v, det.: UV254nm & ninhydrin): \( R_f = 0.43 \)

1.2.5. Ethyl (E)-4-(((tert-butoxycarbonyl)amino)but-2-enoate (6) [4]

\[ \text{BocHN} \rightarrow \text{COOEt} \]

Sodium hydride (60% dispersion in mineral oil) (387 mg, 9.69 mmol, 1.1 eq.) was suspended in dry THF. With cooling on 0°C, solution of triethyl phosphonoacetate (1.92 mL, 9.69 mmol, 1.1 eq.) in 5 mL dry THF was added dropwise. After 20 minutes, solution of aldehyde 5 (1.40 g, 8.81 mmol, 1 eq.) dissolved in 5 mL dry THF was added slowly, and reaction was allowed to warm to the room temperature with tracking by TLC. After about 1 hour, when all starting aldehyde was consumed, reaction mixture was evaporated. Residue was dissolved in mixture 50 mL EtOAc + 50 mL water and layers were separated. Aqueous layer was extracted with 3 x 50 mL EtOAc. Combined all organic layers were washed with brine and dried over anhydrous MgSO4. The solvent was removed under reduced pressure to give 2.30 g of crude product in form of yellow oil. Purification was performed by flash chromatography on 40 g column with

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[4] According to US2017/0152228A1
0->50% EtOAc / cyclohexane as eluent. Collected fractions were evaporated to give 707 mg of pure product 6 (3.09 mmol, 35%) as a colourless oil.

$^1$H NMR (400MHz, CDCl$_3$): $\delta = 6.84$ (dt, 1H, $J_{H,H}=15.7$Hz, 4.9Hz, CH), 5.86 (dt, 1H $J_{H,H}=15.7$Hz, 1.9Hz, CH), 4.74 (bs, 1H, NH), 4.12 (q, 1H, $J_{H,H}=7.1$Hz, CH), 3.84 (bs, 2H, CH), 1.33 (s, 9H, CH), 1.21 (t, 3H, $J_{H,H}=7.2$Hz, CH).

$^{13}$C NMR (100MHz, CDCl$_3$): $\delta =$ 166.1, 155.6, 144.7, 121.3, 79.8, 60.4, 41.3, 28.3, 14.3.

TLC (silica, cyclohexane / EtOAc 1:1 v/v, det.: UV$_{254}$nm & ninhydrin): $R_f = 0.75$

HRMS (ESI+): exact mass calculated for [M+Na]$^+$ (C$_{11}$H$_{19}$NO$_4$Na) required $m/z$ 252.1212; found $m/z$ 252.1212.

1.2.6. (E)-4-((tert-butoxycarbonyl)amino)but-2-enoic acid (7) \[^5\]

![BocHN=C=COOH](image)

Ester 6 (670 mg, 2.93 mmol, 1 eq.) and lithium hydroxide (280 mg, 11.70 mmol, 4 eq.) were dissolved in mixture THF / water 2:1 (v/v) (30 mL) and the reaction mixture was stirred on room temperature overnight. Reaction mixture was concentrated on rotavapor to about 1/3 of volume and then extracted with 1 x 20 mL EtOAc. Aqueous layer was then acidified to pH=3-4 with saturated solution NH$_4$Cl and 5% aq.sol. citric acid, then saturated by addition of crude NaCl and extracted with 3 x 100 mL EtOAc. That last three EtOAc extracts were combined, washed with brine and dried over anhydrous MgSO$_4$. The solvent was removed under reduced pressure to give 488 mg of product 7 (2.43 mmol, 83%) as a white solid.

$^1$H NMR (400MHz, CDCl$_3$): $\delta = 9.92$ (bs, 1H, COOH), 6.93 (dt, 1H, $J_{H,H}=15.8$Hz, 4.8Hz, CH), 5.87 (dt, 1H, $J_{H,H}=15.6$Hz, 1.9Hz, CH), 4.78 (bs, 1H, NH), 3.88 (bs, 2H, CH), 1.39 (s, 9H, CH).

$^{13}$C NMR (100MHz, CDCl$_3$): $\delta =$ 170.8, 155.7, 147.2, 120.7, 80.1, 41.4, 28.3.

TLC (silica, MeOH/CHCl$_3$ 1:9 v/v, det.: UV$_{254}$mm & ninhydrin): $R_f = 0.32$

HRMS (ESI+): exact mass calculated for [M+Na]$^+$ (C$_9$H$_{15}$NO$_4$Na) required $m/z$ 224.0893; found $m/z$ 224.0900.

\[^5\] slightly modified from A. Turetsky, E. Kim, R. H. Kohler, M. A. Miller, R. Weissleder, Sci. Rep. 2014, 4, 4782.
1.2.7. (\textit{R})-3-(4-phenoxyphenyl)-1-(piperidine-3-yl)-1H-pyrazolo[3,4-d]pyrimidine-4-amine hydrochloride (8) \[^{[6]}\]

Carbamate 4 (690 mg, 1.42 mmol, 1 eq.) was dissolved in mixture EtOAc / MeOH 1:1 (v/v) (40 mL) and 4M solution of HCl\((\text{g})\) in dioxane (2.1 mL, 8.52 mmol, 6 eq.) was added. Reaction mixture was stirred on room temperature overnight. Obtained white suspension was evaporated, residual solid was triturated with pentane and ether and dried under high vacuum to give 637 mg of product 8 (1.51 mmol, 98\%) as a white crystalline solid was directly used for the next step

TLC (silica, MeOH/EtOAc 1:4 v/v, det.: UV\textsubscript{254nm} & ninhydrin): \(R_I = 0.09\)

HRMS (ESI\(^{+}\)): exact mass calculated for \([\text{M+H}]^{+}\) (C\textsubscript{22}H\textsubscript{23}N\textsubscript{6}O) required \(m/z\) 387.1928; found \(m/z\) 387.1927.

\[^{[6]}\] slightly modified method from WO2017/137446 (PCT/EP2017/052773)
**1.2.8. Ibrutinib derivative (9)** [7]

![Chemical Structure](image)

Hydrochloride 8 (300 mg, 0.71 mmol, 1 eq.) and DIPEA (740 µL, 4.27 mmol, 6 eq.) were dissolved in ACN (20 mL) and reaction mixture was stirred for 10 minutes on room temperature. PyAOP (741 mg, 1.42 mmol, 2 eq.) and acid 7 (143 mg, 0.71 mmol, 1 eq.) were dissolved separately in ACN (10 mL) and after 10 minutes that solution was added to reaction mixture with amine. Stirring was continued on room temperature with tracking by TLC. When reaction was completed, reaction mixture was evaporated, residue was dissolved in 100 mL EtOAc and washed with: 1 x 20 mL water, 1 x 20 mL 5% aq.sol. citric acid, 1 x 20 mL sat.sol. NaHCO₃, brine and dried over anhydrous MgSO₄. The solvent was removed under reduced pressure to give 896 mg of crude product 9 as brown oil. Crude product was purified by flash chromatography on 40 g silica column with 0->10% MeOH in EtOAc as eluent. Collected fractions were evaporated to give 291 mg of pure product 9 (0.511 mmol, 72%) as a white solid [8].

\[ \text{1H NMR (300MHz, CDCl₃): } \delta = 8.28 \text{ (bd, 1H, NH), 7.59-7.54 (m, 2H, PhH), 7.35-7.28 (m, 2H, PhH), 7.13-6.99 (m, 5H, PhH), 6.67 (bs, 1H, CH), 6.32 (t, 1H, } J_{H,H}=14.2\text{Hz, CH), 5.73 (bs, 2H, NH), 4.78-4.74 (m, 2.5H, CH), 4.52-4.47 (m, 0.5H, CH), 4.12-3.63 (m, 3.5H, CH), 3.32-3.23 (m, 0.5H, CH), 3.14-3.04 (m, 0.5H, CH), 2.84-2.76 (m, 0.5H, CH), 2.23-2.10 (m, 2H, CH), 1.94-1.89 (m, 1H, CH), 1.66-1.62 (m, 1H, CH), 1.39, 1.30 (2s, 9H, CH).} \]

[7] only method from *J. Am. Chem. Soc.*, (2012), 134, 18388-18400; solvent choice from *J. Pept. Sci.*, (2009), 15, 629-633.
[8] colorless oil obtained after evaporation was converted into white solid by precipitation from DCM/pentane
13C NMR (75MHz, CDCl3): δ = 165.5, 157.9, 155.7, 155.6, 144.0, 130.0, 127.2, 124.1, 121.0, 119.6, 119.1, 110.0, 53.4, 50.1, 46.2, 41.8, 34.2, 28.3.

TLC (silica, MeOH/EtOAc 1:9 v/v, det.: UV254nm & ninhydrin): Rf = 0.39 (dark blue fluorescence at UV254nm)

HRMS (ESI+): exact mass calculated for [M+Na]+ (C31H36N7O4) required m/z 570.2823; found m/z 570.2822.

1.2.9. Ibrutinib-Cy3.5

Ibrutinib derivative 9 (8.2 mg, 0.014 mmol, 1.05 eq.) was dissolved in dry DCM (0.5 mL) (dried over mol. sieves 4A), 4M solution of HCl(g) in dioxane (41.4 µL, 0.166 mmol, 12 eq.) was added and reaction mixture was stirred on room temperature until complete conversion of starting material (tracking by TLC9). Reaction mixture was evaporated with heating on 35°C. Residual white solid was dissolved in anhydrous DMF (0.5 mL) and NHS-ester of sulfo-cyanine3.5 dye (15 mg, 0.014 mmol, 1.0 eq.) dissolved in anhydrous DMF (0.5 mL) and DIPEA (72.0 µL, 0.414 mmol, 30 eq.) were added. Reaction mixture was stirred protected from light on room temperature until the

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9 TLC (silica, 10% MeOH / EtOAc, det.: UV254nm & ninhydrin staining)
completion of the reaction (tracking by TLC\textsuperscript{10}) and then was evaporated with heating on 35°C. Residue was triturated with pentane, Et\textsubscript{2}O and EtOAc, and after that was dried under high vacuum at room temperature to give 21 mg of crude product \textit{ibrutinib-Cy3.5} in form of violet solid.

Analytically pure sample was prepared by SPE purification of crude product \textit{ibrutinib-Cy3.5} on 12g C\textsubscript{18}-RP flash cartridge. The cartridge was preconditioned by washing with water (10 mL). Crude product \textit{ibrutinib-Cy3.5} (10 mg, 0.007 mmol) was dissolved in water (0.5 mL) and loaded on the cartridge. After that, the cartridge was washed with water (10 mL) and further with acetonitrile (10 mL) to remove impurities and side products of the reaction. Compound \textit{ibrutinib-Cy3.5} was eluted with mixture ACN / H\textsubscript{2}O 1:1 (v/v) in a few fractions containing exclusively pure product. Collected eluates with the product were combined and lyophilised to give 8 mg of pure final product \textit{ibrutinib-Cy3.5} (0.006 mmol, 80%) as violet solid.

TLC (RP-C\textsubscript{18}, MeOH / H\textsubscript{2}O / AcOH 10:0.5:0.2 v/v/v, det.: UV/Vis & ninhydrin staining): \( R_f = 0.90 \) (violet spot)

HPLC (RP-C\textsubscript{18}, CH\textsubscript{3}CN/H\textsubscript{2}O): >95%

HRMS (ESI, neg): HRMS (ESI, CH\textsubscript{3}CN/H\textsubscript{2}O):

\[ m/z \text{ calc. for } C_{64} H_{62} N_9 O_{15} S_4^3\text{ } [M-H] (z=3): 441.44216; \text{ found: } 441.44160; \]
\[ m/z \text{ calc. for } C_{64} H_{62} N_9 O_{15} S_4 H^2\text{ } [M-H] (z=2): 662.66687; \text{ found: } 662.66630; \]
\[ m/z \text{ calc. for } (C_{64} H_{62} N_9 O_{15} S_4 H)_{24}\text{ } [M-H] (z=4): 662.66687; \text{ found: } 662.66630. \]

\[^{10}\] TLC (RP-C\textsubscript{18}, MeOH / H\textsubscript{2}O / AcOH 10:0.5:0.2 v/v/v, det.: UV/Vis & ninhydrin staining)
Supporting Figure 1: A: High resolution mass spectrometry of Cy3.5-ibrutinib, B: HPLC-analysis of the final compound to ensure purity. Method: Mobile phase A = H₂O + 0.1% TFA, Mobile phase B = CH₃CN + 0.1% TFA, Column: Eurospher II 100-10 C₁₈, 250 x 4 mm, Flow: 3 mL / min, Detector: DAD (210 – 780 nm), λ₁ = 254 nm, λ₂ = 590 nm. Gradient: 0 min - 90% A; 2 min - 90% A; 12 min - 5% A; 15 min - 5% A; 18 min - 90% A.
**DAD6.1L [Channel 1] Results**

| Retention Time | Area   | Area % |
|----------------|--------|--------|
| 5,950          | 194740 | 2.94   |
| 6,083          | 6318360| 95.40  |
| 7,100          | 74847  | 1.13   |
| 8,650          | 35130  | 0.53   |
| **Totals**     | **6623077**| **100.00** |
2. Nanocarrier formation

2.1. Coupling of anti-CD20-mAB rituximab to protamine-sulfate

Coupling of the anti-CD20-mAB rituximab (Truxima®, Mundipharma) (αCD20-mAB) to protamine-sulfate was performed in a two-step synthesis (see Fig. 2 A). First, the positive charged protamine-sulfate (cat. No. 110123, Merck) was amino-terminally coupled to the bifunctional crosslinker sulfo-SMCC (Sulfosuccinimidy 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, cat.no. 13415-1, CovacyChem,Loves Park IL, USA) at a molar ratio of 1:5 in ddH₂O. pH was adjusted to pH 6.0 with 0.1 M carbonate buffer (pH 8.3). After incubation for 1 hour at 37°C uncoupled sulfo-SMCC was removed by gel filtration chromatography in Zeba spin desalting columns (Pierce No. 89891). Then, the sulfo-SMCC-protamine-complex was then coupled to cysteine residues of rituximab in a 32:1 molar ratio according to protamine-sulfate. The mixture was left to react over night at 4°C. Rituximab-protamine conjugates, here referred to as αCD20-mAB/P) were stored at 4°C and stable for several weeks.

2.2. Ibrutinib-Cy3.5 and rituximab-protamine/protamine (αCD20-mAB/P/P) complex formation

Negative charged ibrutinib-Cy3.5 was bound in 20 times molar excess to αCD20-mAB/P with free protamine present, if not stated otherwise, for ibrutinib at room temperature in the dark for 30-60 min. Complexes were prepared freshly before use.

2.3. Determination of ibrutinib-Cy3.5 load capacity

For the electromobility shift assay, a constant concentration of ibrutinib-Cy3.5 (15 µM) was incubated as described above with a decreasing amount of αCD20-mAB/P/P complexes from 10-fold up to 100-fold molar excess according to ibrutinib-Cy3.5. After incubation, the mixture was subjected to a 2% agarose gel electrophoresis stained with Red Safe.

2.4. Nanoparticle formation

For the analysis of in vitro-formation of nanoparticles, 1,200 nM ibrutinib-Cy3.5 was complexed in 60 nM αCD20-mAB/P (= 1:20 molar ratio) and free protamine as described above in a final volume of 200 µl PBS, RPMI medium with 10% FCS, or 50% FCS in PBS, respectively, and incubated in chamber slide (cat.no. 94.6140.202,
Sarstedt) overnight at 37°C. The slides were then washed with PBS, fixed with cold 4% PFA, mounted with mounting medium (cat.no. 10121691, Dako North America) and analysed via fluorescence using a Nikon Eclipse 50i upright microscope.

3. In vitro evaluation

3.1. Cell culture

Human DLBCL cell line HBL1 was maintained in RPMI-1640 medium (cat.no. 21875-034, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin. Human DLBCL cell line TMD8 was cultivated in IMDM medium (cat.no. 21980-032, Thermo Fisher) supplemented with 10% FBS, 1% sodium pyruvate and 1% penicillin and streptomycin. Both cell lines were cultivated at a cell density of 0.2 – 1 x 10⁶ cells/ml and incubated at 37°C with 5% CO₂ in high humidity.

3.2. Flow cytometry

To approve the internalisation of the αCD20-mAb/P/P complexes with ibrutinib-Cy3.5, cells were treated overnight with 60 nM αCD20-mAb/P/P, 60 nM uncoupled αCD20-mAb, or PBS, respectively. Cells were washed with PBS and analysed by flow cytometry using a BD FACSCalibur.

3.3. Fluorescence microscopy

Cell lines were seeded at 2 x 10⁵ cells/ml and treated with PBS, 60 nM αCD20-mAb/P/P complexed with 1,200 nM ibrutinib-Cy3.5, or 1,200 nM uncomplexed ibrutinib-Cy3.5 overnight at 37°C and 5% CO₂. For BTK occupancy analysis, HBL-1 cells were treated with PBS, 2,400 nM ibrutinib-Bodipy-FL (PCI-33380, cat. no. HY-100335, Hölzel-Diagnostika, Cologne, Germany) or 2,400 nM ibrutinib for 2 h, washed with medium and treated for 4 h or 24 h with 120 nM αCD20-mAb/P/P complexed with 2,400 nM ibrutinib-Cy3.5.

Cells were washed twice with cold PBS, transferred to adhesion slides (ImmunoSelect Adhesion Slides, cat.no. SQ-IS-10100, MoBiTec) bordered with a liquid blocker pen. Cells were incubated on ice for 20 minutes to stick to surface, fixed with ice-cold 4% paraformaldehyde (PFA), stained with Hoechst33342 (cat.no. H1399, Thermo Fisher),
mounted with mounting medium (cat.no. 10121691, Dako, Santa Clara, CA, USA). Slides were photographed on a Nikon Eclipse 50i upright microscope or on a Keyence BZ 9000 (Figure 2).

3.4. Electron microscopy

Freshly prepared nanoparticles were sedimented on a formvar-coated, carbon-sputtered copper grid. After negative staining with 1% phosphotungstic acid, pH 7, the samples were analyzed at 80kV on a Tecnai 12 electron microscope (Fei, Eindhoven, The Netherlands). Images of selected areas were documented with Veleta 4k CCD camera (Emsis, Münster, Germany).

3.5. Apoptosis assay

To determine apoptosis, cells were treated with uncoupled rituximab, ibrutinib-Cy3.5, free ibrutinib (Imbruvica®) and or αCD20-mAB/P/P-ibrutinib-Cy3.5 at a final concentration of 60nM relating to rituximab and 1200nM relating to ibrutinib. Treatments were repeated twice at consecutive days. After 72 h, cells were stained for AnnexinV expression (APC AnnexinV, cat.no. 550474, BD Pharmingen) and analysed by flow cytometry.

3.6. Colony formation assays

For HBL1 colony formation, 2,500 cells were seeded in 150 μl culture medium per sample. Cells were incubated with different conditions of αCD20-mAB/P/P (60 nM) coupled in a 1:20 molar ratio to ibrutinib-Cy3.5 in a final volume of 750 μl for at least two hours at 37°C. Afterwards, cell suspension was mixed with 600 μl methylcellulose M3231 (cat. No. 03231, Stemcell Technologies) and seeded in triplicates for colony formation in 96-well format (150 μl/well). For TMD8 colony formation, 2,000 cells were seeded in 150 μl culture medium per sample. Cells were treated with αCD20-mAB/P/P-complexes coupled and incubated as described above. Then, 432 μl culture medium and 168 μl 2% agar (agar noble, cat.no. 214220, BD) was added and mixture was also divided in triplicates in 96-well format (180 μl/well). After 5-7 days, the assays were stained with 20 μL 4 mg/ml iodonitrotetrazolium chloride (INT) solution and incubated overnight at 37°C. The assays were counted for colony numbers using a binocular.
3.7. SDS-Page and Western blot

2x10^5 cells each cell line were seeded and treated over night with ibritinib-Cy3.5 or αCD20-mAB/P/P-ibrutinib Cy3.5. Cells were harvested after 24, 48 or 72 hours, washed with PBS and resuspended directly in NuPage™ SDS sample buffer (cat.no. NP0007, Invitrogen) supplemented with 1x NuPage™ Sample reducing agent (cat.no. NP0004, Invitrogen, Thermo Fisher), boiled for 5 minutes at 95°C, applied to a 10% SDS-PAGE. Before blotting, gel was photographed with Intas ECL Chemostar Imager. Western blot analysis was performed using standard procedures. Analyses were performed using following antibodies: anti-BTK (Cell Signaling #3532), anti Phospho-BTK-Tyr223 (Cell Signaling, #5082), anti-phospho-ERK (Cell Signaling, #4370), anti-total-ERK (Cell Signaling, #4696) and anti-β-Actin (Clone AC-15, Sigma Aldrich).

4. In vivo evaluation

All animal experiments in this study were carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee “Landesamt fuer Natur, Umwelt und Verbraucherschutz NRW” (LANUV). This study was performed with permission of the Institutional Animal Care and Use Committee and of the local veterinary administration of Münster (Permit no. 81-02.04.2017.A439).

4.1. Mouse xenograft tumor model

1x10^7 HBL1 cells resuspended in 1:1 PBS and matrigel were injected subcutaneously in the flank of 50 NSG-mice. When tumors reached a measurably size of at least 150 mm^3 mice were randomized divided into treatment groups.

αCD20-mAB-protamine (calculated for the mAB) and unmodified αCD20-mAB antibody were applied intra-peritoneal, twice a week at 4 mg antibody/kg mouse weight in the first experimental line, which equals a 120 µg or 0.75 nmol single dose for a 30 g mouse. This was complexed before in a 1:20 molar ratio to ibritinib-Cy3.5, which leads to 15 nmol single dose of ibritinib-Cy3.5. As a control, un-complexed ibritinib-Cy3.5 was given in the same dose. In the second line of in vivo experiments, all doses were doubled. Uncharged ibritinib (cat.no. HY-10997, MedChem-Express) was
applied at 15 nmol single dose in the same routine as above. Mouse weight was determined before each treatment.

Tumor growth was measured with a caliper and tumor volume was calculated by the formula length x width² x 0.52. When the tumor volume reached an intolerable size >1,500 mm³, the respective mouse was euthanized, tumor, spleen, liver, lung, sternum, femur, heart and kidney were isolated and prepared for further analysis and serum was prepared from blood samples for determination of liver enzyme activity of GOT (glutamate-oxalacetate-transaminase) and GPT (glutamate-pyruvate-transaminase) using standard procedures. The survival was statistically evaluated in a Kaplan-Meier plot. For the second trial, double amount of αCD20-mAB/P was tested (8 mg/kg αCD20-mAB/P, 1.44 mg/kg ibrutinib).

4.2. Ex vivo fluorescence imaging (biodistribution)

Excised organs and tumors were placed at a petri dish and imaged in a Fluorescence Reflectance Imager (in vivo MS FX Pro, Bruker Biospin MRI GmbH, Ettlingen, Germany). First, a white light image was taken, followed by a fluorescence image with an exposure time of 30 sec. The fluorescent protein was excited with light at a wavelength of 530 nm (±10 nm) and fluorescence was registered within the wavelength of 600 nm (± 17.5 nm). The data was analysed with the imaging software MISE (Bruker, v 7.5.2).
Supporting Figure 2: Properties of the αCD20-mAB-protamine. A: Flow cytometric analysis HBL1 cells stained for CD20 surface expression. When cells were incubated with αCD20-mAB-P/P, the CD20-receptor disappears from the cell surface. Dark grey curve: CD20-expression on untreated cells (positive control), light grey: incubation with unconjugated αCD20-mAB, green: incubation with αCD20-mAB-protamine (-P); black: unstained cells (negative control). FACS staining was performed with a labelled anti-CD20 antibody binding a separate epitope from the one bound by the targeting anti-CD20 antibody used. B: electromobility shift assays showing the electrostatic loading capacity of ibrutinib-Cy3.5 to conjugates.
**Supporting Figure 3:** A: Affinity chromatographic purification (HPLC) of the αCD20-mAB-protamine conjugate. αCD20-mAB-protamine conjugation mix was applied to protein G sepharose column equilibrated with PBS and eluted with buffer pH 2.5. Elution fractions were collected and subjected to SDS-PAGE and Coomassie stain. The last fractions 25/26 contained αCD20-mAB-protamine conjugate purified from excess free SMCC-protamine, which was used in further analysis. B: The SMCC-protamine purified fraction 25 conjugate (left; “after HPLC”) was compared to the input control with free SMCC-protamine (right) in terms of ibrutinib-Cy3.5 complexation ability. Whereas the input control with free SMCC-protamine was able to complex ibrutinib-Cy3.5 in all tested conditions, the variant purified from free SMCC-protamine showed no distinct binding affinity to ibrutinib-Cy3.5.
Supporting Figure 4: Cellular targeting of Bruton’s kinase BTK by αCD20-mAB-P/P loaded ibrutinib-Cy3.5. A-F: Fluorescence microscopy of TMD8 DLBCL cells treated with targeted nanocarriers and controls showing a marked intracellular enrichment of Cy3.5-signals upon the incubation with αCD20-mAB-P/P loaded ibrutinib-Cy3.5. G-H: Lysates from cells treated for 24 h (G) and 48 h (H) with targeted nanocarriers and controls were subjected to SDS PAGE and illuminated for Cy3.5 signals. A clear band of 70 kDa, identified as BTK by parallel immunoblot was covalently marked by ibrutinib-Cy3.5, indicating binding and functionality of the ibrutinib-Cy3.5 derivate. tBTK, total BTK.
Supporting Figure 5: A-I. Fluorescence microscopy of HBL1 DLBCL cells pre-treated with ibrutinib-bodipy (green, G and I) do not show intracellular enrichment of Cy3.5-signals after αCD20-mAB-P/P-ibrutinib-Cy3.5 treatment (F, compared to E).

**fixation after 26 h**

- **2 h ibrutinib-bodipy only**
  - A
  - D
  - G

- **24 h αCD20-mAB-P/P-ibrutinib-Cy3.5**
  - B
  - E
  - H

- **2 h ibrutinib-bodipy / 24 h αCD20-mAB-P/P-ibrutinib-Cy3.5**
  - C
  - F
  - I

50 μm
Supporting Figure 6: A-F. Fluorescence microscopy of HBL1 DLBCL cells pre-treated with ibrutinib (G and I) do not show intracellular enrichment of Cy3.5-signals after αCD20-mAB-P/P-ibrutinib-Cy3.5 treatment (F, compared to E).

**fixation after 6 h**

|                | 2 h ibrutinib | 4 h αCD20-mAB-P/P-ibrutinib-Cy3.5 | 2 h ibrutinib / 4 h αCD20-mAB-P/P-ibrutinib-Cy3.5 |
|----------------|---------------|-----------------------------------|--------------------------------------------------|
| Hoechst        | A             | B                                 | C                                                 |
| Cy3.5          | D             | E                                 | F                                                 |

**fixation after 26 h**

|                | 2 h ibrutinib | 24 h αCD20-mAB-P/P-ibrutinib-Cy3.5 | 2 h ibrutinib / 24 h αCD20-mAB-P/P-ibrutinib-Cy3.5 |
|----------------|---------------|-----------------------------------|--------------------------------------------------|
| Hoechst        | G             | H                                 | I                                                 |
| Cy3.5          | J             | K                                 | L                                                 |

50 µm
Supporting Figure 7: Physiological and functional consequences of BTK-inactivation by αCD20-mAB-P/P-ibrutinib-Cy3.5 nanocarrier treatment in DLBCL cell lines. A: TMD8 cells were treated by the respective conjugates shown for 72 hrs, lysed and subjected to SDS-PAGE and Western blotting for phospho-BTK (pBTK), total BTK (tBTK), phospho-ERK (p-ERK), total-ERK (t-ERK) and actin as a loading control. Free ibrutinib-Cy3.5 inhibited the phosphorylation of BTK a bit less than αCD20-mAB-P/P-ibrutinib-Cy3.5 nanocarrier. The difference of expected downstream phosphorylation targets such as ERK was more pronounced: Only αCD20-mAB-P/P-ibrutinib-Cy3.5 treatment was able to reduce ERK phosphorylation significantly. B: In colony formation assays, targeted αCD20-mAB-P/P/ibrutinib-Cy3.5 strongly reduced colony growth of TMD8 cells.
Supporting Figure 8: Induction of apoptosis of BTK targeting by αCD20-mAB-P/P-ibrutinib-Cy3.5 nanocarrier treatment in DLBCL cell lines. TMD8 cells were treated by the respective conjugates shown for 72 hrs and subjected to AnnexinV-staining. Apoptotic cells were detected by AnnexinV-expression (X-axis) by flow cytometry (upper panels), while Cy3.5-dependent fluorescence was detected on the Y-axis. Values from upper right and lower right gates were counted. Lower panel: AnnexinV-positive cells in three independent experiments were summarized. P<0.05, 2-sided T-test.
Supporting Figure 9: Determination of toxicity parameters in transplanted and treated mice. **A:** In the \textit{in vivo} experimental trial presented in Figure 6 A (4 mg/kg), mouse weight values were determined on day one and after the treatment on day 11. The values varied insignificantly in all groups exposing no obvious signs of toxicity. **B:** Blood plasma was withdrawn from mice on the day of treatment termination and subjected to test for clinical liver parameters \textit{GOT} (glutamate-oxalacetate-transaminase) and \textit{GPT} (glutamate-pyruvate-transaminase). Here, the values clearly differed and showed that αCD20-mAB-P/P-ibrutinib-Cy3.5 treated mice exposed \textit{GOT} values comparable to PBS and antibody control groups, whereas the untargeted compounds showed significantly elevated \textit{GOT} values. A similar picture is seen, when \textit{GPT} plasma values of αCD20-mAB-P/P-ibrutinib-Cy3.5 treated mice are compared to untargeted ibrutinib and ibrutinib-Cy3.5: Also here, \textit{GOT} levels was significantly lower in the complex treatments vs ibrutinib-treatment. **C:** Here, mouse weights for the \textit{in vivo} trial presented in Figure 6 B (8 mg/kg) are determined with no significant differences in the treatment groups. \textit{GOT/GPT} liver parameters have not been recorded in this trial. P<0.05, 2-sided T-test.

![Figure 9: Determination of toxicity parameters in transplanted and treated mice.](image)

A. Mouse weight development, 4 mg/kg trial

B. Blood plasma withdrawn from mice on the day of treatment termination and subjected to test for clinical liver parameters. Here, the values clearly differed and showed that αCD20-mAB-P/P-ibrutinib-Cy3.5 treated mice exposed \textit{GOT} values comparable to PBS and antibody control groups, whereas the untargeted compounds showed significantly elevated \textit{GOT} values. A similar picture is seen, when \textit{GPT} plasma values of αCD20-mAB-P/P-ibrutinib-Cy3.5 treated mice are compared to untargeted ibrutinib and ibrutinib-Cy3.5: Also here, \textit{GPT} levels was significantly lower in the complex treatments vs ibrutinib-treatment.

C. Mouse weight development, 8 mg/kg trial

![Figure 9: Determination of toxicity parameters in transplanted and treated mice.](image)