Targeted Drug Delivery with Chemically Self-assembled Antibody Nanostructures (CSANs)

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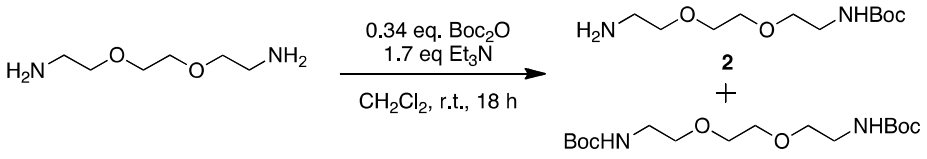
Supporting information

General Methods

All reagents and solvents were of highest quality available and were used directly unless otherwise described. Reactions were performed in an efficient fume hood equipped with continuous argon and vacuum lines. Reactions involving air sensitive material were carried out in an argon atmosphere. All NMR spectra were taken at default temperature (~20º C). 1H NMR spectra were recorded on a Varian 400 MHz NMR spectrometer. Chemical shifts are reported in ppm relative to the solvent signal. ESI-MS analyses were done on a Bruker BioTOF II mass spectrometer. Thin-layer chromatography (TLC) was performed on Analtech (Newark, DE) general purpose silica gel on polyester plates with fluorescent indicator, and spots were visualized with UV light or, by staining with anisaldehyde or Ninhydrin. Flash column chromatography was performed using regular gravity columns or ISCO – Combiflash Companion with Isco Redisep normal phase or reverse phase C18 columns. HPLC purifications were performed on a Beckman Coulter instrument (126 solvent module, 168 PDA detector) equipped with a Higgins Analytical – HAISIL C-8, 5 um Semiprep column (250x10 mm).

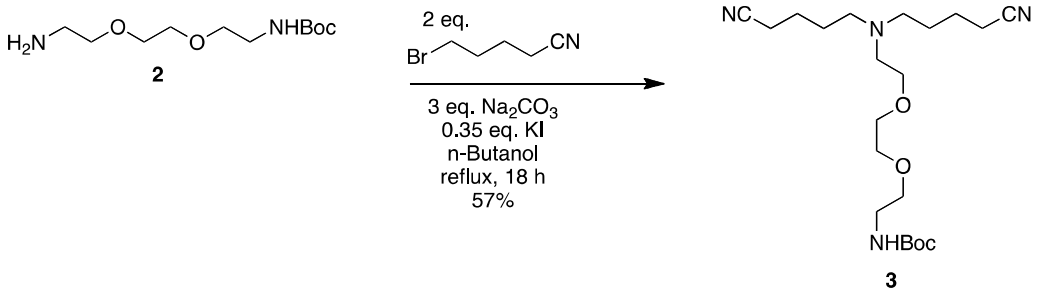
1) Synthesis of *N*-BOC-2,2′-(ethylenedioxy)bis(ethylamine) **(2)**



To a stirring solution of 2,2′-(Ethylenedioxy)bis(ethylamine) (7.26 mL, 50 mmol) and anhydrous triethylamine (11.8 mL, 85 mmol) in dry CH_2Cl_2 (100 mL) in an ice bath was added dropwise a solution of di-*tert*-butyl dicarbonate (3.71 g, 17 mmol) in dry CH_2Cl_2 (20 mL) over 15 minutes. Then the

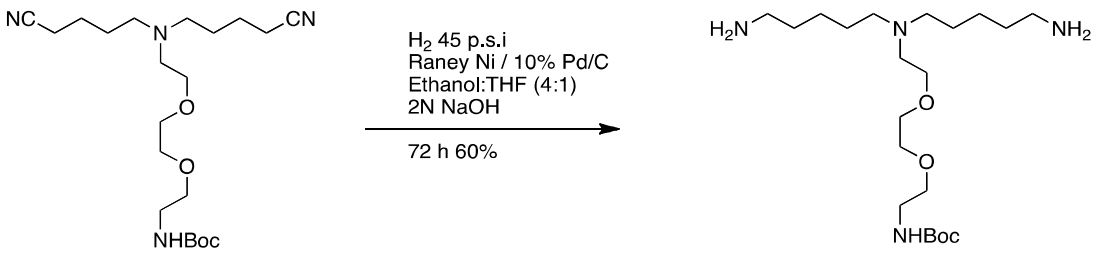
reaction mixture was allowed to reach the room temperature and continued stirring overnight. The resulting white suspension was washed with sat. $NaHCO_3$ (100 mL) and brine (100 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, solvents evaporated and dried under a high vacuum to yield a mixture of mono (major product) and di-protected product (3.86 g). This material was used in the next reaction without further purification. ESI-MS calcd for mono $C_{11}H_{24}N_2O_4$ $[M+H]^+$ 249.1814, found 249.1896; calcd for di-protected $C_{16}H_{32}N_2O_6Na$ $[M+Na]^+$ 371.2158, found 371.2258.

2) Synthesis of compound **3**



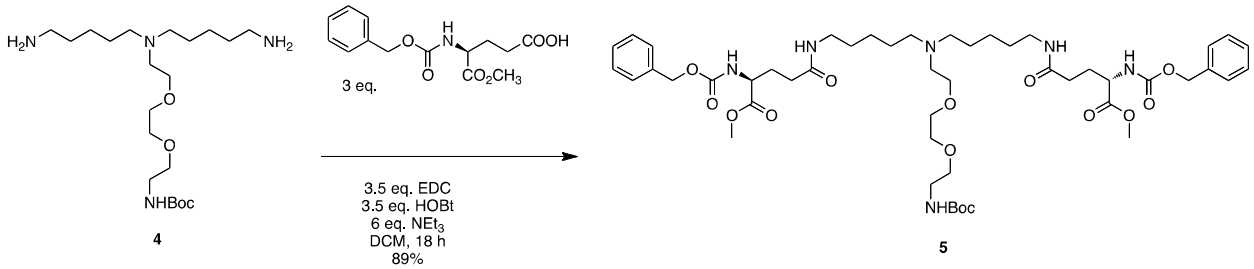
To a stirring suspension of **2** (2.98 g, 12.0 mmol) sodium carbonate (3.82 g, 36.0 mmol), and potassium iodide (0.70 g, 4.2 mmol) in n-butanol (100 mL) at 115° C was added dropwise 5-bromovaleronitrile (2.80 mL, 24.0 mmol) in n-butanol (20 mL). The reaction was refluxed overnight (18 hrs). Then the reaction was filtered and the solid was washed with diethyl ether. The product was then extracted into 1 M HCl, which was then made basic with solid sodium carbonate and the product extracted back into diethyl ether. The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to yield the compound **3** as a colorless oil (2.80 g, 57%, over 2 steps). ^1H NMR (400 MHz, $CDCl_3$) δ 4.972 (br. s, 1H), 3.591 (s, 4H), 3.538 (m, 4H), 3.315 (d, 2H), 2.645 (t, 2H), 2.497 (t, 4H), 2.385 (t, 4H), 1.701 (m, 4H), 1.649 (m, 4H), 1.446 (s, 9H). ESI-MS calcd for $C_{21}H_{39}N_4O_4$ $[M+H]^+$ 411.2966, found 411.2979.

3) Synthesis of compound **4**



A solution of **3** (1.15 g, 2.80 mmol) in EtOH:THF (4:1, 100 mL) in a Parr flask was added Raney Ni (1.5 g, 50% suspension in water), 10% palladium on carbon (0.3 g) and 2 N NaOH (35 mL) and shaken under 45 psi hydrogen pressure for 72 hrs at room temp. The catalyst was filtered off on celite, solvents removed in vacuo and a mixture of water (100 mL) and methylene chloride (100 mL) was added. After phase separation aqueous phase was extracted with methylene chloride (3 x 50 mL) and combined organic phase was dried over anhydrous sodium sulfate, filtered and solvents were removed in vacuo to obtain **4** as a colorless oil (0.7g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.161 (s, 1H), 5.531 (s, 2H), 5.132 (s, 2H), 3.595 (m, 4H), 3.537 (m, 4H), 3.318 (d, J = 5.1 Hz, 2H), 2.75 – 2.57 (m, 6H), 2.50 – 2.38 (m, 4H), 1.66 – 1.39 (m, 17H), 1.35 – 1.15 (m, 4H). ESI-MS calcd for $C_{21}H_{46}N_4O_4Na$ $[M+Na]^+$ 441.3423, found 441.3411.

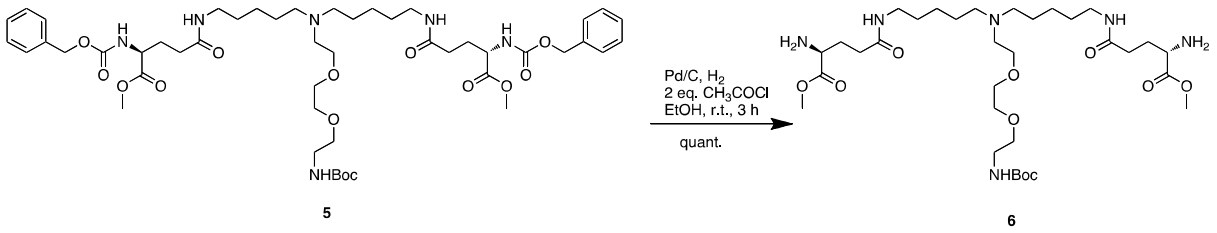
4) Synthesis of compound **5**



To a stirring solution of **4** (1.26 g, 3.01 mmol), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (2.01 g, 10 mmol) and 1-hydroxybenzotriazole hydrate (1.61 g, 10.5 mmol) in DCM was added *N*-Carbobenzyloxy-L-glutamic acid 1-methyl ester (2.65 g, 8.97 mmol) and anhydrous

triethylamine (2.5 mL, 18 mmol) under Ar. The reaction mixture was stirred overnight, diluted with CH_2Cl_2 (40 mL), washed with Sat. $NaHCO_3$ (50 mL), 5% KH_2PO_4 (50 mL), water (50 mL) and brine (50 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered and solvents were removed. The crude product was purified by flash chromatography (5-20 % CH_3OH in CH_2Cl_2) to provide **5** as a white foam (2.6 g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.341 (m, 10H), 6.23 (br. s, 2H), 5.89 (br. s, 2H), 5.095 (s, 4H), 4.33 (m, 2H), 3.728 (s, 6H), 3.572 (m, 6H), 3.515 (t, J = 5.1 Hz, 2H), 3.29 (d, J = 4.8 Hz, 2H), 3.202 (dd, J = 12.7, 6.5 Hz, 4H), 2.76 (br. s, 2H), 2.57 (br. s, 3H), 2.26 (m, 6H), 1.97 (m, 4H), 1.44 (m, 17H), 1.37 – 1.22 (m, 4H). ESI-MS calcd for $C_{49}H_{77}N_6O_{14}$ $[M+H]^+$ 973.5492, found 973.5509.

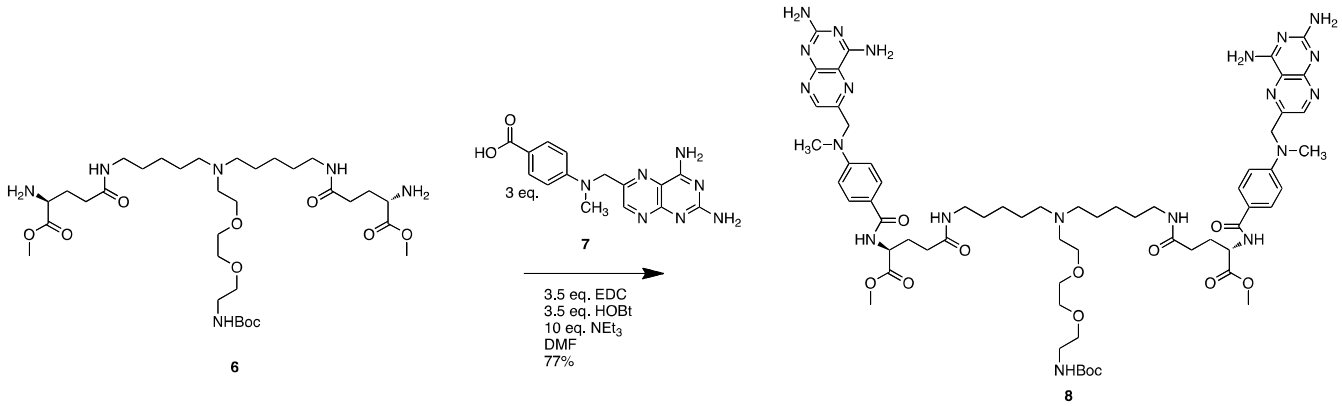
5) Synthesis of compound **6**



5 6

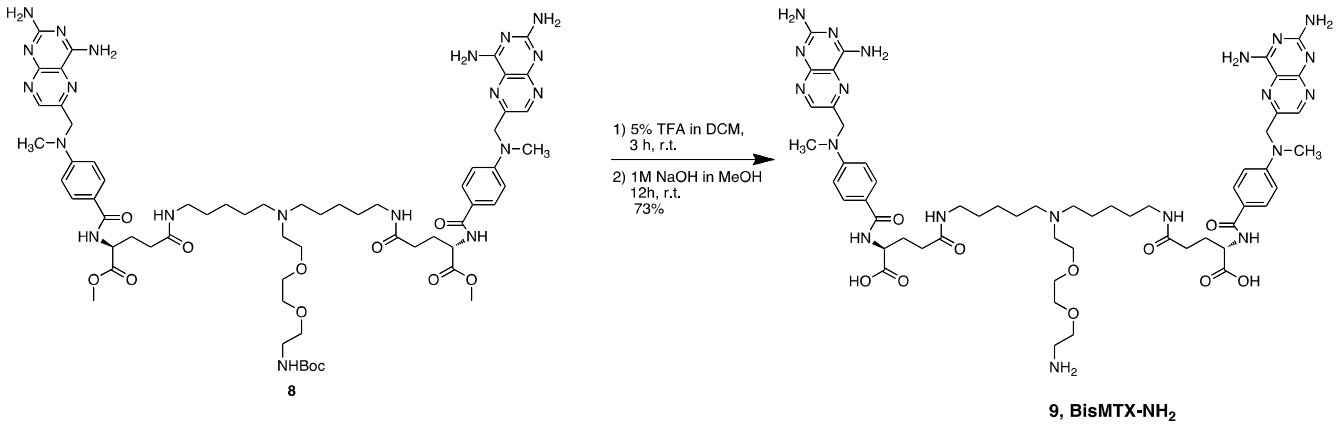
The compound **5** (2.43 g, 2.50 mmol) was dissolved in a solution of acetyl chloride (0.35 mL, 5.0 mmol) in methanol (30 mL) under Ar. Then 10% palladium on carbon was added and flushed with Ar. The mixture was flushed with hydrogen and a H_2 balloon was attached. The reaction mixture was stirred vigorously for 3 hrs (reaction was monitored by TLC). Then the reaction mixture was diluted with 4x methanol and filtered through celite. The filtrate was evaporated and dried under vacuum to yield the compound **6** as a white foam (1.8 g, quant.). %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.976 (br. t, 3H), 3.849 (t, 2H), 3.693 (s, 6H), 3.642 (m, 2H), 3.497 (m, 4H), 3.350 (m, 10H), 3.014 (m, 8H), 2.215 (m, 4H), 1.928 (m, 4H), 1.537 (m, 4H), 1.420-1.320 (overlapping s and m, 13H), 1.225 (m, 4H). ESI-MS calcd for $C_{33}H_{65}N_6O_{10}$ $[M+H]^+$ 705.4757, found 705.4771.

6) Synthesis of compound **8**



To a stirring solution of **6** (0.7g, 1 mmol), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (0.67 g, 3.5 mmol) and 1-hydroxybenzotriazole hydrate (0.54 g, 3.5 mmol) in anhydrous dimethyl formamide (20 mL) was added **7**[1] (0.98 g, 3.0 mmol). Then anhydrous triethylamine (1.4 mL, 10 mmol) was added dropwise to the reaction mixture under Ar and continued stirring for 18 hrs. After evaporating the solvents the resulting slurry was loaded on to silica and purified by flash chromatography (0-20 % CH_3OH/1%Et_3N in CH_2Cl_2/1%Et_3N) to provide **8** as a yellow solid (1.0 g, 77%). Second flash chromatographic purification (0-20 % CH_3OH/1%Et_3N in CH_2Cl_2/1%Et_3N) was necessary to remove minor impurities (0.66 g, 45%). ESI-MS calcd for $C_{63}H_{92}N_{20}O_{12}$ $[M+2H]^{2+}$ 660.3602, found 660.3612.

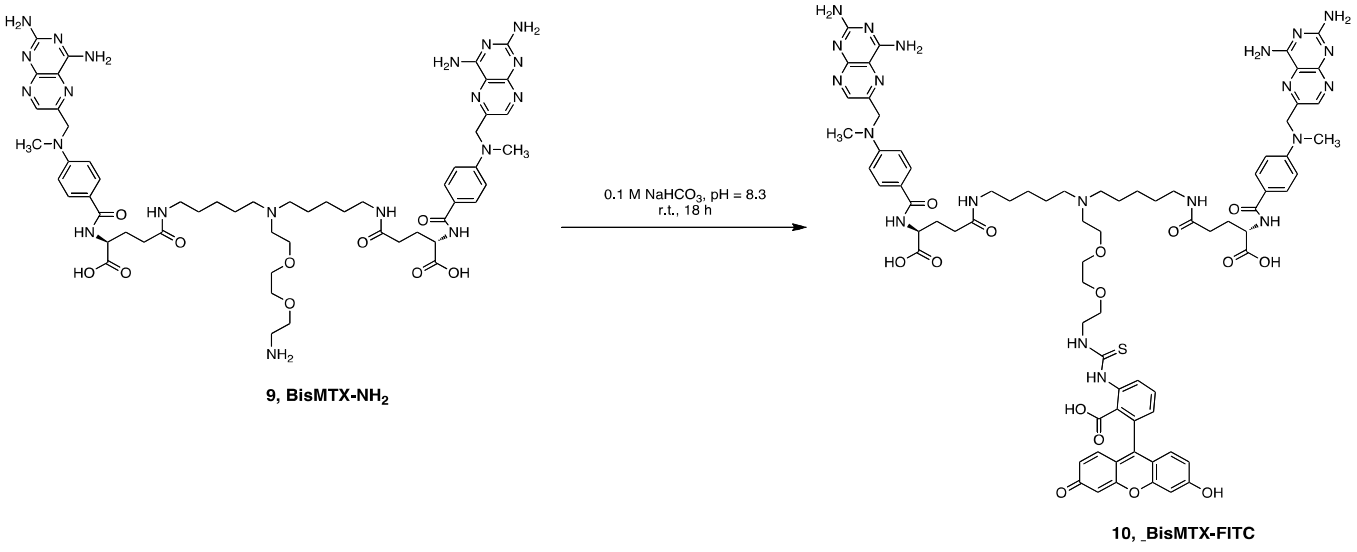
7) Synthesis of **bisMTX-NH$_2$ trilinker** (**9**)



A solution of compound **8** (0.30 g, 0.23 mmol) in 5% v/v trifluoroacetic acid/dichlormethane

was stirred at room temperature for 3 hrs. After evaporating the solvents, crude product was re-dissolved in dicholoromethane and evaporated 3 times to remove any excess trifluoroacetic acid. Completion of the reaction was confirmed by ESI-MS (MS calcd for $C_{58}H_{83}N_{20}O_{10}$ $[M+2H]^{2+}$ 610.20, found 610.34). This crude product was then dissolved in 1M NaOH in methanol (10 mL) and stirred overnight at room temperature in the dark. Reaction mixture was neutralized with acetic acid and desalted by loading on to a C18-Sep-Pak reverse phase cartridge and eluting with 50% acetonitrile in water. The resulting solution was lyophilized and further purified by HPLC using a C-8 semi-prep reverse phase column (mobile phase gradient 2% - 50 % acetonitrile/0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid) to obtain the final product **9** (0.20 g, 73%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.518 (s, 2H), 7.75 (d, J = 8 Hz, 4H), 6.85 (d, J = 8 Hz, 4H), 4.905 (s, 4H), 4.526 (m, 2H), 3.801 (m, 2H), 3.686, (m, 6H), 3.366 (m, 3H), 3.264 – 3.060 (overlapping s and m, 17H), 2.460 – 2.26 (overlapping m, 6H), 2.059 (m, 2H), 1.710 (m, 4H), 1.537 (m, 4H), 1.371 (m, 4H). ESI-MS calcd for $C_{56}H_{79}N_{20}O_{10}$ $[M+2H]^{2+}$ 596.3183, found 596.3238.

Synthesis of **bisMTX-FITC trilinker** (**10**)



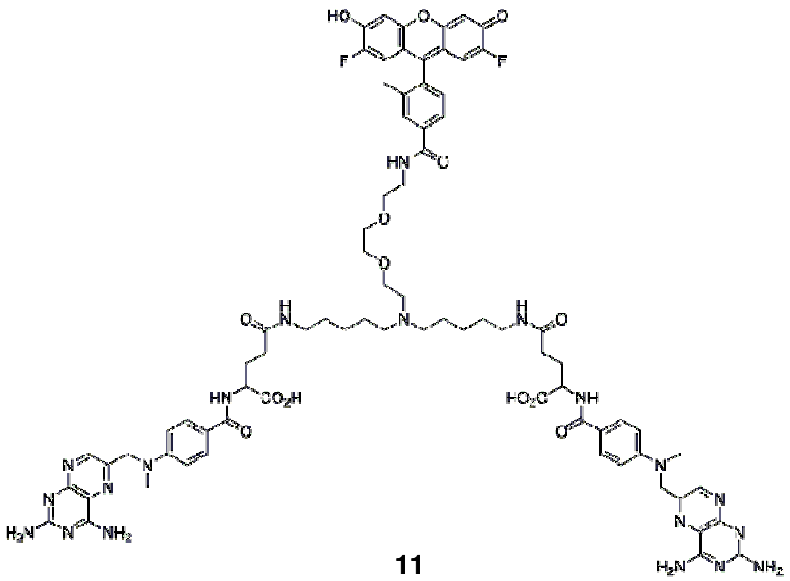
9, BisMTX-NH$_2$ **10, BisMTX-FITC**

A solution of compound **9** (2.5 mg, 2.1 μmol) was dissolved in 2 mL 0.1 M sodium bicarbonate buffer

(pH 8.5). Separately, FITC (1 mg, 2.5 μmol) was dissolved in anhydrous DMF and added to the solution of **9**. After stirring overnight the product was purified by HPLC using a C-8 semi-prep reverse phase column (mobile phase gradient 2% - 50 % acetonitrile/0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid) to obtain the final product **10** (2.1 mg, 63%).

ESI-MS calcd for $C_{77}H_{91}N_{21}O_{15}S_1$ $[M+2H]^{2+}$ 790.8357, found 790.8437.

Synthesis of **bisMTX-PG** trilinker (**11**)



11

9 (2 mg, 1.7 μmol) was dissolved in anhydrous DMF (1 mL) and triethylamine (1.4 uL, 10 μmol) was added followed by Pennsylvania Green N-hydroxysuccinimde ester[2] (0.96 mg, 2 μmol). The solution was stirred overnight at room temperature and the product was purified by HPLC using a C-8 semi-prep reverse phase column (mobile phase gradient 2% - 50 % acetonitrile/0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid) to obtain the final product **11** (1.2 mg, 45%).

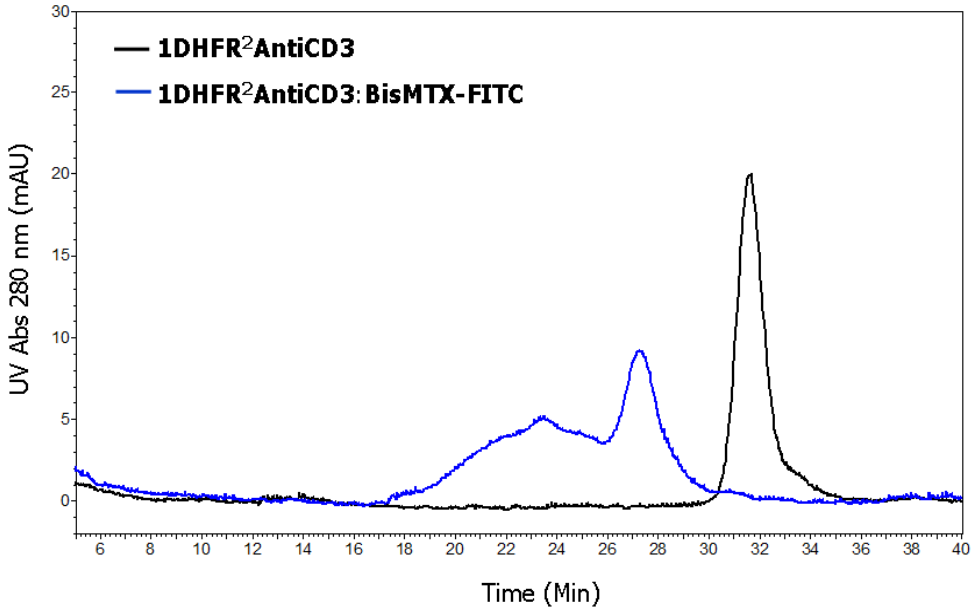
ESI-MS calcd for $C_{77}H_{90}N_{20}O_{14}F_2$ $[M+2H]^{2+}$ 778.3451, found 778.3531.

Reference:

1) Carlson, J. C. T. *et al*, *J. Am. Chem. Soc.* (2003), ***125***, 1501.

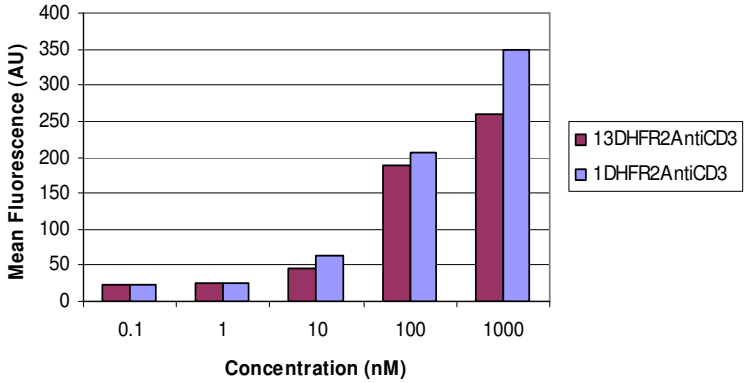
2) Mottram, L. F. *et al*, *Org. Lett* (2007), ***9***, 3741.

Supporting Information Figure S1



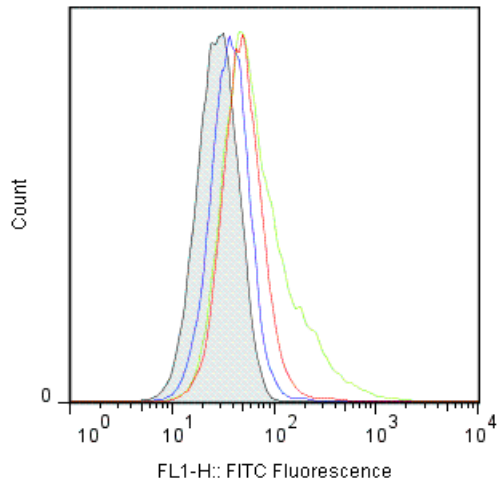
Size Exclusion chromatograms of **1DHFR^{2}AntiCD3** (black) and the mixture of species obtained upon incubation of **1DHFR^{2}AntiCD3** with **bisMTX-FITC** (blue) showing the formation of dimeric to pentameric-hexameric CSANs.

Supporting Information Figure S2



Dependence of HPB-MLT mean fluorescence, as observed by flow cytometry, upon incubation with varying concentrations of **13DHFR^{2}AntiCD3** or **1DHFR^{2}AntiCD3** CSANs with **bisMTX-FITC**.

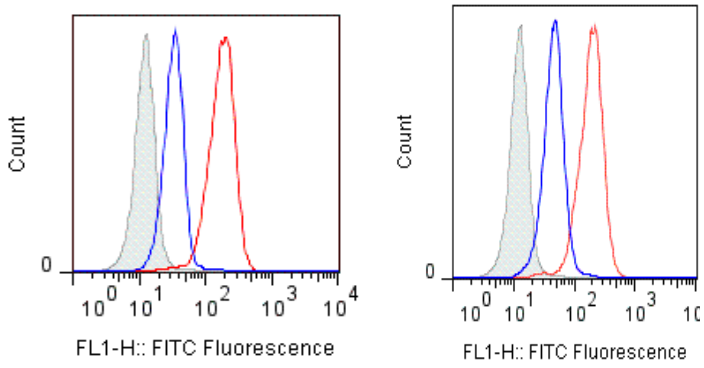
Supporting Information Figure S3



Incubation of **13DHFR^{2}AntiCD3** (red) or **1DHFR^{2}AntiCD3** (green) CSANs containing **bisMTX-FITC** or FITC labeled UCHT-1 (blue) with CD3$^-$ Raji (B) cells results in only minor increases in fluorescence over unstained cells (shaded grey) as would be expected for background non-specific binding.

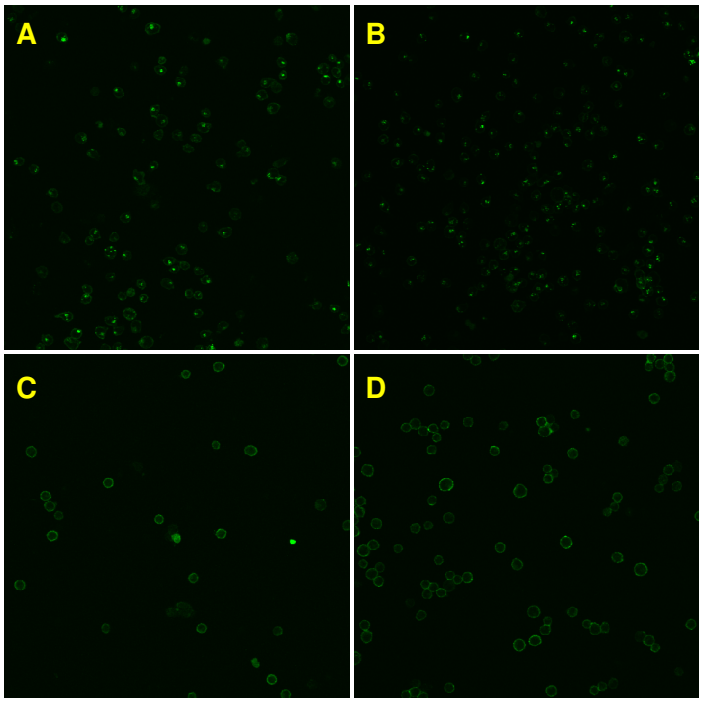
Supporting Information Figure S4

A **B**



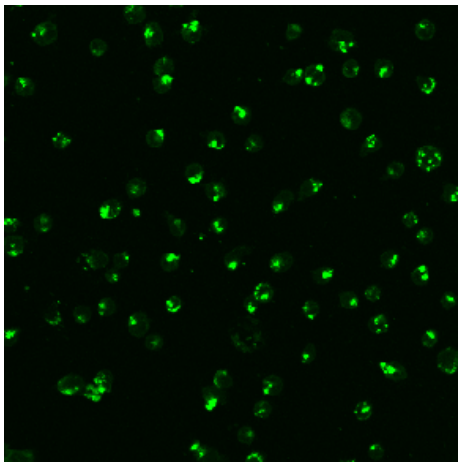
Preincubation of HPB-MLT cells with unlabeled UCHT-1 (40 nM) prior to addition of A) **13DHFR^{2}AntiCD3** or B) **1DHFR^{2}AntiCD3** with **bisMTX-FITC** CSANs (100 nM) results in an 86% and 80% decrease, respectively, in observed fluorescence (blue) as compared to cells treated CSANs in the absence of UCHT-1 (red). Unstained HPB-MLT cells are shaded grey.

Supporting Information Figure S5



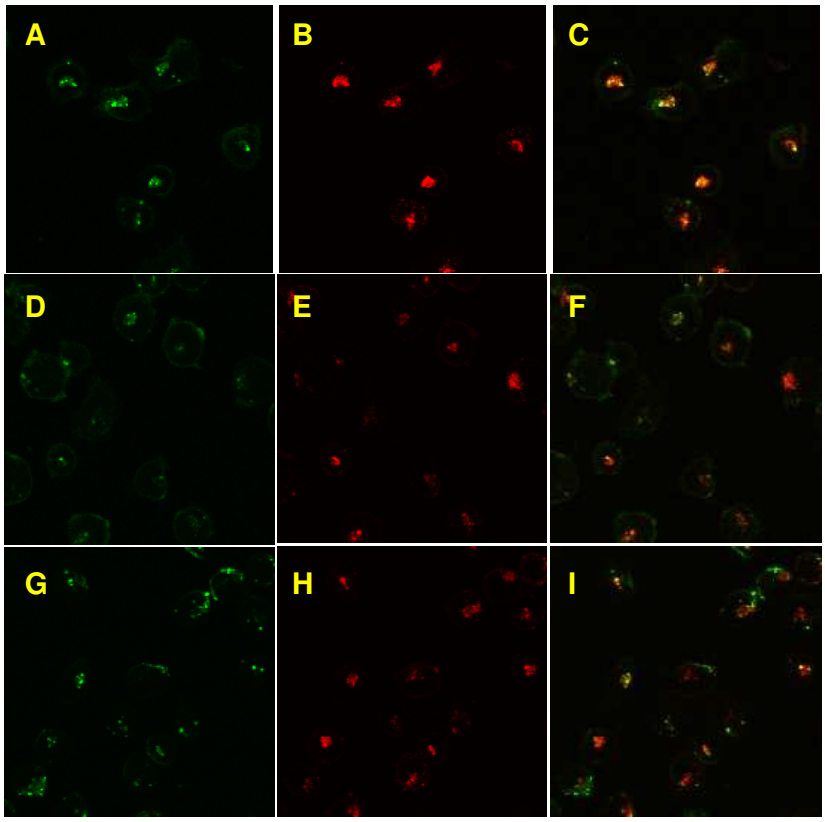
Fluorescence confocal microscopy images HPB-MLT cells incubated with purified monomeric **13DHFR^{2}AntiCD3**:**bisMTX-FITC** at 37 °C (A) and 4 °C (C) and dimeric **13DHFR^{2-}AntiCD3**:**bisMTX-FITC** at 37 °C (B) and 4 °C (D) with both showing a similar level of internalization at 37 °C, as apparent by the green punctates, and cell surface binding at 4 °C.

Supporting Information Figure S6



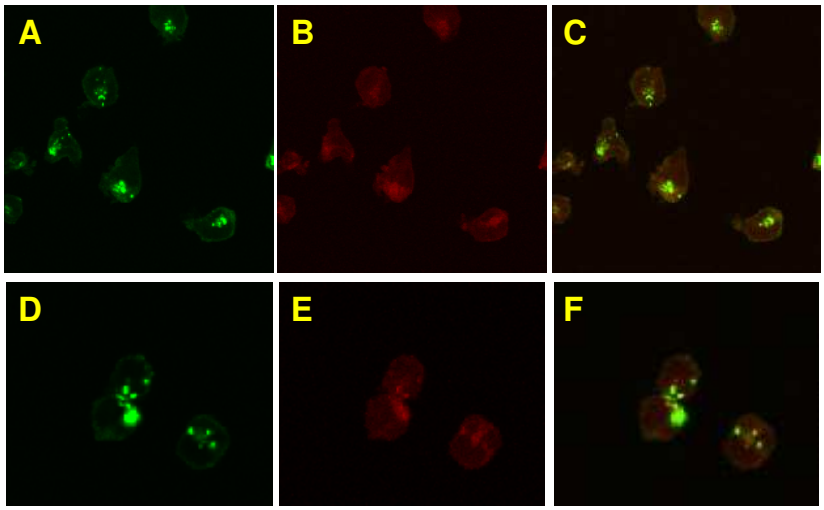
Fluorescence confocal microscopy images for HPB-MLT cell incubated with **1DHFR^{2-}AntiCD3**:**bisMTX-FITC** at 37 °C showing CSAN internalization, as visualized by the intracellular green punctates.

Supporting Information Figure S7



Fluorescence confocal microscopy images obtained from HPB-MLT cells after co-incubation with Alexa Fluor 594 labeled transferin and either FITC labeled UCHT-1 (A-C), **13DHFR^{2-}AntiCD3**:**bisMTX-FITC** (D-F) or **1DHFR^{2}AntiCD3**:**bisMTX-FITC** (G-I) at 37 °C for 30 mins showing co-localization of fluorescent species. FITC fluorescence from labeled UCHT-1, **13DHFR^{2-}AntiCD3** CSANs and **1DHFR^{2}AntiCD3** CSANs are shown in figures A, D and G respectively. The red fluorescence from Alexa Fluor 594 labeled transferin is shown in figures B, E and H while the overlays are shown in the final column (figures C, F and I).

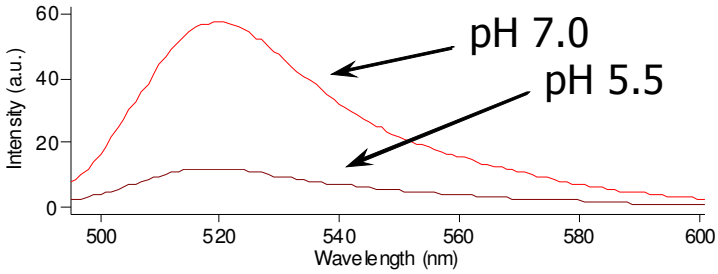
Supporting Information Figure S8



Fluorescence confocal microscopy images obtained from HPB-MLT cells after co-incubation with Lysotracker red DND-99 and either **13DHFR^{2}AntiCD3**:**bisMTX-FITC** (A-C) or **1DHFR^{2-}AntiCD3**:**bisMTX-FITC** (D-F) at 37 °C for 1 hour showing localization of CSANs to lysosomes. FITC fluorescence **13DHFR^{2}AntiCD3** CSANs and **1DHFR^{2}AntiCD3** CSANs are shown in figures A,

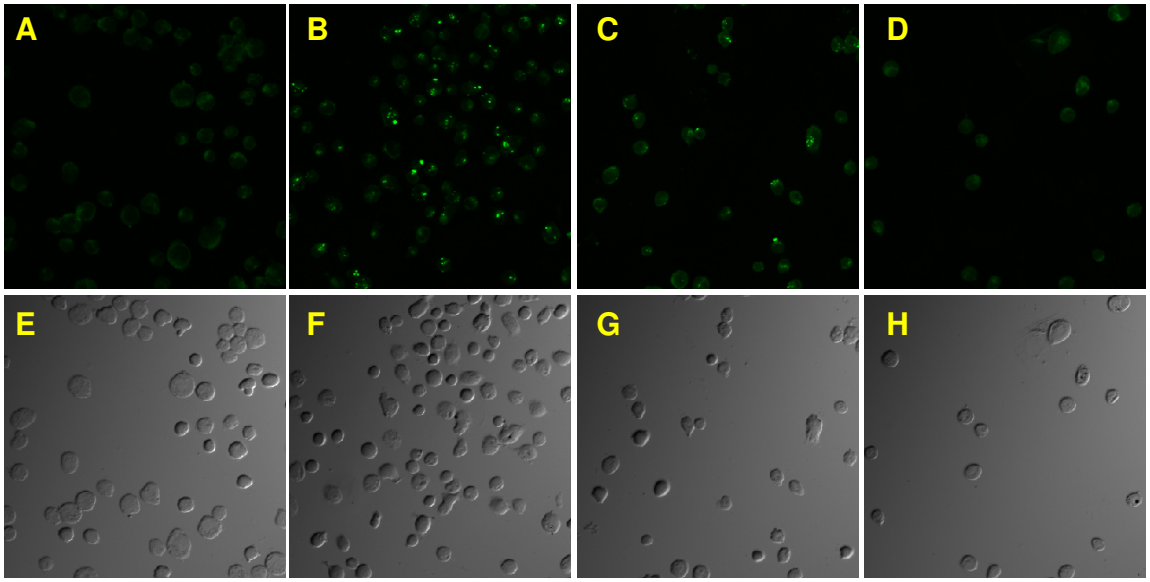
and D respectively. The red fluorescence from Lysotracker red DND-99, showing lysosomes is shown in figures B and E while the overlays are shown in the final column (figures C and F).

Supporting Information Figure S9



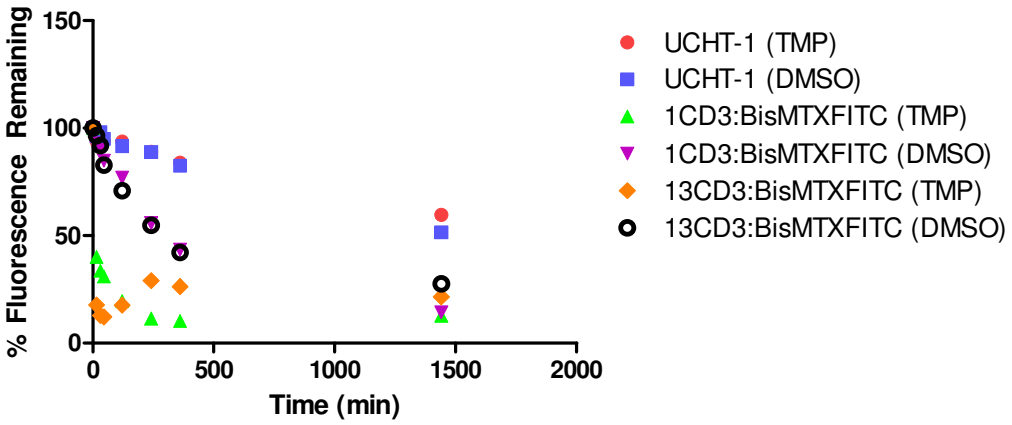
Effect of pH on the measured fluorescence of **bisMTX-FITC** containing AntiCD3 CSANs formed with **1DHFR^{2}AntiCD3**.

Supporting Information Figure S10



Fluorescence confocal (A-D) and the corresponding differential inference contrast microscopy images (E-H) for HPB-MLT cell incubated with **bisMTX-PenGreen** for 3hours at 37 °C (A and E) or **1DHFR^{2}AntiCD3**: **bisMTX-PenGreen** for 1 hour at 37 °C followed by a further 3hours with either media (B & F), 2 mM TMP (C and G) or 0.5 mM Pyr (D and H) at 37 °C.

Supporting Information Figure S11

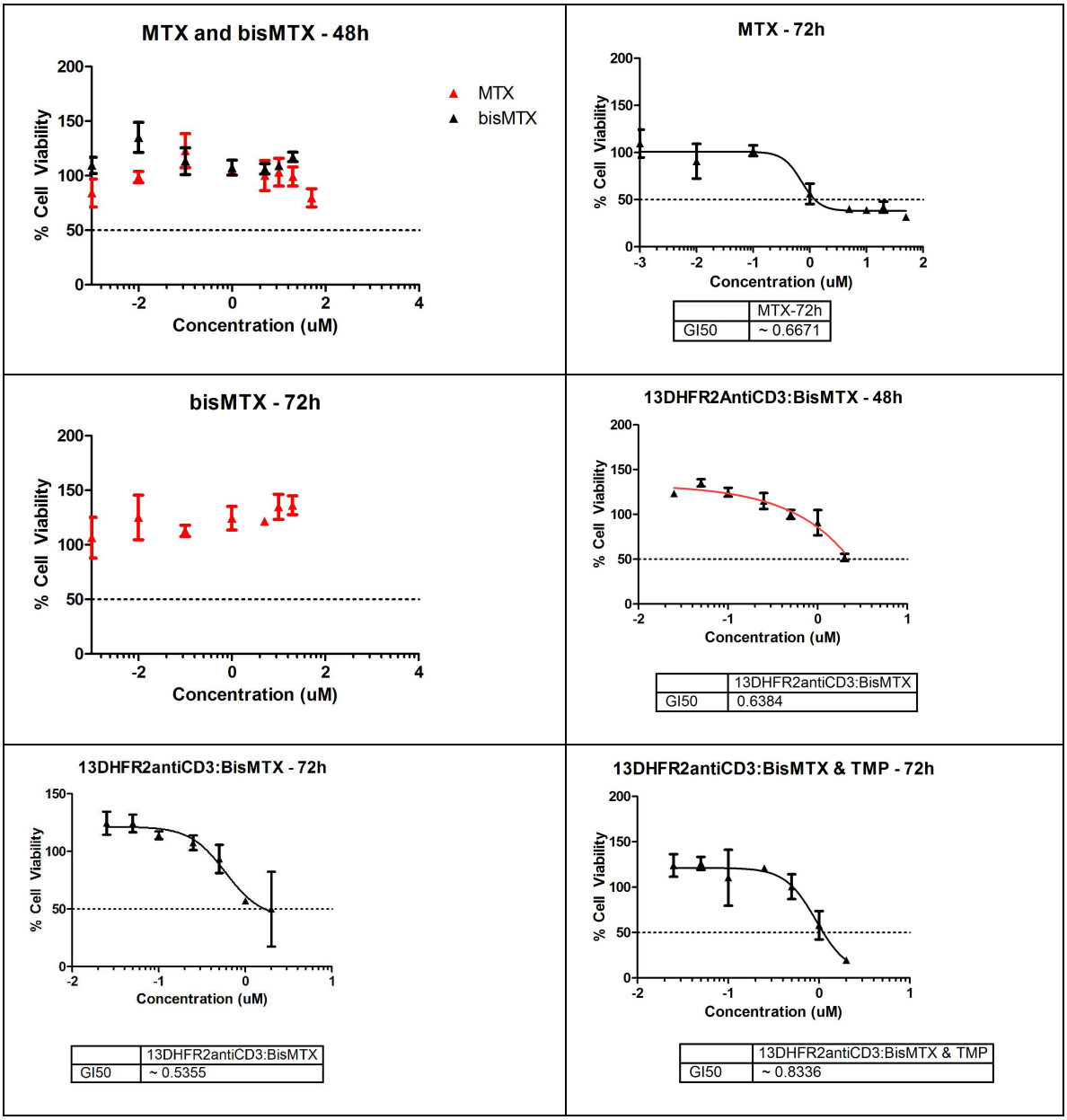


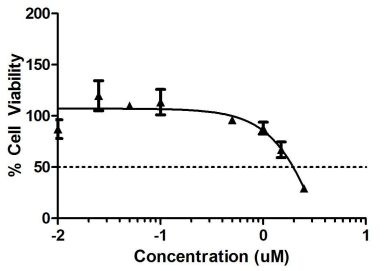
Flow cytometry analysis of the time dependent decrease in fluorescence of HPB-MLT cells previously treated with either FITC labeled UCHT-1 or **bisMTX-FITC** containing CSANs in media containing TMP or DMSO (control) over 24 hours**.**

Supporting Information Figure S12

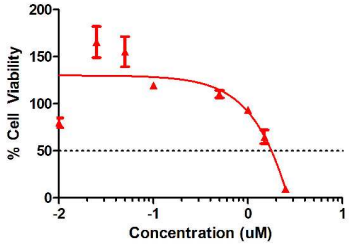
Cytotoxicity of MTX, bisMTX, 13DHFR2AntiCD3 and 1DHFR2AntiCD3 in HPB-MLT cells at 48 and 72 hrs, with and without TMP as determined by MTS assay.

GI50 is defined as the concentration at which 50% of the cell viability was observed. Each graph with a curve indicates the GI50 value in micro molar (uM). GI50 values were calculated by plotting the curves by non-linear regression analysis using Prism 5 software.

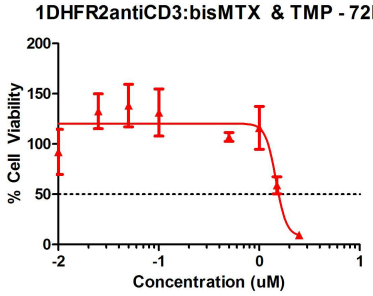




	1DHFR2antiCD3:bisMTX 48h
GI50	~ 1.426



	1DHFR2antiCD3:bisMTX
GI50	~ 1.299



	1DHFR2antiCD3:bisMTX & TMP
GI50	~ 1.471