Supplementary Material: Reduction of Drug Toxicity Using Dendrimers Based on Melamine

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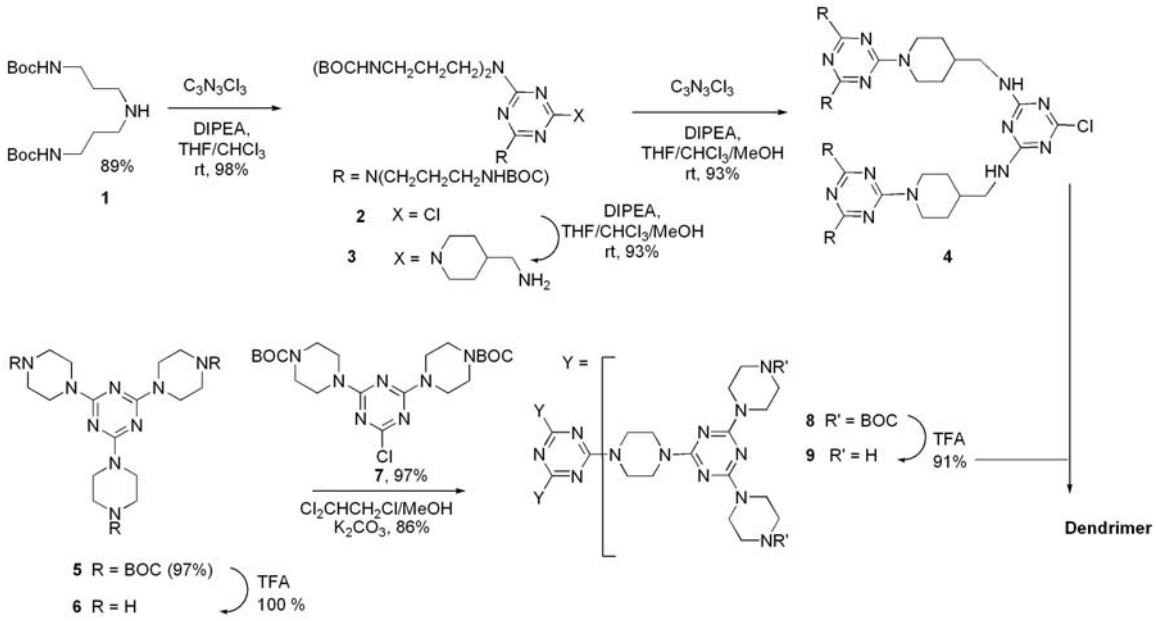
Animals. All animals were approved by the ULACC Committee, Texas A&M University (AUP#2003-68).

Preparation of Drug Solutions. Methotrexate (MTX) and 6-mercaptopurine (6-MP) were dissolved in 100 mM saline and the pH was adjusted to 8 with 1 N NaOH by adding base dropwise to obtain concentrations of 1.25 mg/ml and 2.5 mg/ml, respectively. Dilutions of these stocks were prepared in saline to get final concentrations of 0.12 mg/ml and 0.21 mg/ml of MTX and 6-MP, respectively. Dendrimer was dissolved in saline at a concentration of 22.5 mg/ml.

Preparations of Drug-Dendrimer Solutions. To solubilize the drugs with dendrimer, stock solutions of MTX and 6-MP were prepared in saline. A 50 mg of MTX was dissolved in 40 ml of 100 mM saline to get a concentration of 1.25 mg/ml. 100 mg of 6-MP was dissolved in 100 mM saline to get a concentration of 2.5 mg/ml 1.25 ml of the MTX and 1.09 ml of the 6-MP stocks were each added to 347 ul of a dendrimer solution having a concentration of 22.5 mg/ml. This solutions were sonicated for 5 min. After such time, 11.4 and 11.56 ml of saline were added to the MTX and 6-MP solutions, respectively, to yield a final working concentration of .12 mg/ml MTX and .21 mg/ml 6-MP in .6 mg/ml dendrimer made up in a volume of 13 ml.

Synthesis of the Dendrimer. Solvents and reagents were reagent grade and used without further purification. ^{1}H NMR (300 MHz or 500 MHz) and proton decoupled ^{13}C NMR spectra (75 MHz or 125 MHz) were recorded with $CDCl_3$, $CDCl_3$/MeOH-d_4 (10:1), or MeOH-d_4/D_2O (2:1) as internal standards. The synthetic scheme used is shown below:



Intermediate 1. A solution of triamine (13.4 g, 0.1 mol) and triethylamine (42.6 mL, 0.3 mol) in THF (500 mL) was cooled in an ice bath and stirred as a solution of 2-[[*tert*-butoxy-carbonyl]oxy]imino]-2-phenylacetonitrile (BOC-ON: 50 g, 0.2 mol) in THF (500 mL) was added over 40 min. After stirring for 2 hr in the ice bath, the solution was warmed to room temperature

and stirred overnight. The solvent was removed by evaporation to give golden oil which was dissolved in $CHCl_3$ and washed with 5 % HCl solution (x3), 5 % NaOH (W/V) solution (x4) and brine (x3). The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvents were removed. The residue was purified by column chromatography ($MeOH/CH_2Cl_2$=1/10, R_f=0.21) to provide **1**. (29.4 g, 89 %). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 5.18 (brs, NH)), 3.20 (td, *J*=6.0, 6.3 Hz, 4 H), 2.65 (t, *J*=6.6 Hz, 4 H), 1.69 (brs, NH), 1.65 (m, 4 H), 1.43 (s, 18H); ^{13} C NMR (CDCl3, 75 Hz) δ: 156.38, 79.27, 47.57, 39.09, 29.91, 28.64. MS (ESI-TOF): calcd for $C_{16}H_{33}N_3O_4$: 331.25; found 332.25 $(M+H)^+$.

As per Westerberg, D. A.; Carney, P. L.; Rogers, P. E.; Kline, S. J.; Johnson, D. K. *J. Med. Chem.* **1989**, *32*, 236.

Intermediate 2. To an iced solution of **8** (10.0 g, 30.2 mmol) in THF/$CHCl_3$ (1:1, 50 mL), trichlorotriazine (2.7 g, 14.4 mmol) and DIPEA (10.7 mL, 60.0 mmol) were added. The mixture was warmed to room temperature and stirred for 2 hr. The white solid was removed by filtration, and the filtrate was evaporated to give an oil. The oil was dissolved in $CHCl_3$ again and washed by 5 % HCl solution (x3), 5 % NaOH (W/V) solution (x4) and brine (x3). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed to give **2** as foam. (10.9 g, 98 %, $MeOH/CH_2Cl_2$=1/20 R_f=0.25). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 5.65 (brs, NH), 5.30 (brs, NH), 3.60 (t, *J*=6.0 Hz, 4 H), 3.50 (t, *J*=7.2 Hz, 4 H), 3.12 (m, 8 H), 1.76 (m, 8 H), 1.45 (s, 18 H), 1.44 (s, 18 H); ^{13} C NMR ($CDCl_3$, 75 Hz) δ: 169.34, 165.11, 156.66, 79.69, 79.28, 45.15, 44.07, 38.74, 37.22, 28.92, 28.89, 28.48. MS (ESI-TOF): calcd for $C_{35}H_{64}ClN_9O_8$: 773.46; found 774.46 $(M+H)^+$.

Intemediate 3. To the solution of **2** (9.8 g, 12.7 mmol) in THF/$CHCl_3$/MeOH (5:5:1, 110 mL) was added 4-(aminomethyl) piperidine (4.5 g, 29.0 mmol). The suspension was stirred at room temperature for 20 hr. A white solid was removed by filtration and the filtrate was evaporated to give oil. The oil was dissolved in $CHCl_3$ again and washed by 5 % HCl solution (x3), 5 % NaOH (W/V) solution (x4) and brine (x3). The organic layer was dried over $MgSO_4$, filtered and condensed to afford foam as desired compound **3**, ($MeOH/CH_2Cl_2$=1/10, R_f=0.13) yield 10.0 g. (93 %) ^{1}H NMR (MeOH-d_4+$CDCl_3$, 500 MHz) δ: 5.39 (brs, NH), 4.67 (d, *J*=13.0 Hz, 2H), 3.47 (br, 8 H), 3.05 (br, 8 H), 2.77 (t, *J*=12.3 Hz, 2 H), 2.56 (d, *J*=5.5 Hz, 2H), 1.69 (br, 10 H), 1.53 (br, 1 H), 1.38 (s, 36 H), 1.09 (m, 2 H); ^{13} C NMR ($CDCl_3$, 75 Hz) δ: 165.31, 164.71, 155.95, 78.84, 43.26, 37.98, 37.37, 29.36, 28.39. MS (ESI-TOF): calcd for $C_{41}H_{77}N_{11}O_8$: 851.60; found 852.60 $(M+H)^+$.

Intermediate 4. To the solution of **3** (6.1 g, 7.2 mmol) in THF/$CHCl_3$/MeOH (5:5:1, 110 mL) was added trichlorotriazine (664.0 mg, 3.6 mmol) and DIPEA (2.6 mL, 14.3 mmol). The mixture was stirred at room temperature for 24 hr. After removing volatile components, the foam was purified through column chromatography to afford desired compound **11** as white foam. (6.0 g, 93 %, EA/DCM=1/1, R_f=0.33). ^{1}H NMR ($CDCl_3$+MeOH-d_4, 500 MHz) δ: 4.58 (m, 4 H), 3.39 (brs, 16 H), 3.17 (m, 4 H), 2.95 (brs, 16 H), 2.67 (m, 4 H), 1.62 (m, 22 H), 1.29 (s, 72 H), 1.06 (m, 4 H); ^{13}C NMR ($CDCl_3$+MeOH-d_4, 75 Hz) δ: 165.19, 164.65, 156.25, 79.02, 45.98, 43.00, 42.58, 37.76, 36.50, 29.59, 28.15. MS (MALDI-TOF): calcd for $C_{85}H_{152}ClN_{25}O_{16}$: 1815.73; found 1814.19 $(M)^+$, 1836.73 $(M+Na)^+$.

Intermediate 5. Boc-protected piperazine (1.57 g, 8.3 mmol) and trichlorotriazine (0.5 g, 2.5 mmol) were mixed in 60 mL of THF/DMF (10/1). DIPEA (2.2 mL, 12.5 mmol) was added and the reaction was stirred at room temperature for 2 hr, then at 80^{o}C for 5 hr. After being cooled to room temperature, a white solid was collected by filtration and washed with THF before drying under vacuum to yield compound 1.53 g of **5** (97 %, $MeOH/CH_2Cl_2$=1/50, R_f=0.26). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 3.74 (m, 12 H), 3.44 (m, 12 H), 1.47 (s, 27 H); ^{13} C NMR (CDCl3, 75 Hz) δ: 165.32, 154.83, 79.89, 42.90, 28.41. MS (ESI-TOF): calcd for $C_{30}H_{51}N_9O_6$: 633.40; found 634.40. $(M+H)^+$.

Intermediate 6. Intermediate **5** (2.1 g, 3.3 mmol) was dissolved in 20mL TFA/DCM (1:1). After 4 hr stirring at room temperature, the volatile components were removed by evaporation. The residue was dissolved in MeOH and neutralized by adding trimethylamine, and the solvent was subsequently evaporated. After the procedure was repeated by three times, a yellow oil containing **6** was obtained. (2.22 g, quantitive) ^{1}H NMR (MeOH-d_4, 500 MHz) δ: 4.042 (t, 5.3 Hz, 12 H), 3.235 (t, 5.3 Hz, 12 H); ^{13} C NMR (MeOH-d_4, 125 Hz) δ: 166.61, 44.41, 41.16. MS (ESI-TOF): cacld for $C_{15}H_{27}N_9$: 333.24; found 334.25 $(M+H)^+$.

Intermediate 7. Boc-protected piperazine (3.99 g, 21 mmol) and trichlorotriazine (1.86 g, 10.0 mmol) were mixed in THF (100 mL). DIPEA (3.7 mL, 30.0 mmol) was added and the reaction was stirred at room temperature for 24 hr. A white solid was removed by filtration and the filtrate was evaporated to provide a residue. Following chromatography ($MeOH/CH_2Cl_2$=1/50, R_f=0.36) **7** was obtained as a white solid in 97% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 3.755 (br s, 8 H), 3.444 (br s, 8 H), 1.456 (s, 18 H); ^{13} C NMR ($CDCl_3$, 75 Hz) δ: 169.92, 164.69, 154.86, 80.48, 43.50, 28.61. MS (ESI-TOF): calcd for $C_{21}H_{34}ClN_7O_4$: 483.24; found 484.25 $(M+H)^+$, 486.26 $(M+H)^+$.

Intermediate 8. Intermediate **6** (0.58 g, 1.74 mmol) was mixed with DIPEA (1 mL) in 20 mL of 1,1,2-trichloroethane/MeOH (1:1) for 5 min. Intermediate **7** (3.0 g, 6.2 mmol) and K_2CO_3 (5.0 g, 36 mmol) were added to the mixture. The mixture was stirred under reflux for 24 hr. After cooling to room temperature, the solid was removed by filtration and washed with CH_2Cl_2 for several times. The filtrate was collected and evaporated to obtain solid. Purification by column chromatography ($MeOH/CH_2Cl_2$=1/20, R_f=0.5) afforded **8** as a white solid (2.51 g, 86 %). ^{1}H NMR ($CDCl_3$+MeOH-d_4, 500 MHz) δ: 3.67 (br, 24 H), 3.37 (br, 24 H), 1.40 (s, 54 H); ^{13} C NMR ($CDCl_3$+MeOH-d_4, 125 Hz) δ: 165.11, 154.93, 80.15, 42.93, 42.8, 28.1. MS (MALDI-TOF): calcd for $C_{78}H_{126}N_{30}O_{12}$: 1676.03; found 1677.70 $(M+H)^+$.

Intermediate 9. Intermediate **8** (1.86g, 1.1 mmol) was stirred in CH_2Cl_2/MeOH (15 mL/2 mL). Trifluoroacetic acid (12 mL) was added to the iced solution. After stirring for 24 hr, the volatiles were evaporated to provide an oil. The oil was partitioned between $CHCl_3$ and 5% NaOH *(aq)*. The organic layer was washed by 5% NaOH (x3) and brine (x3), and then dried over $MgSO_4$. After filtering, the solvent was evaporated to afford **9** as a light yellow solid (1.08 g, 91%). ^{1}H NMR ($CDCl_3$+MeOH-d_4, 300 MHz) δ: 3.68 (br, 12 H), 3.63 (m, 12 H), 2.73 (m, 12 H); ^{13} C NMR ($CDCl_3$+MeOH-d_4, 75 Hz) δ: 165.11, 165.01, 45.17, 43.62, 42.81. MS (ESI-TOF): calcd for $C_{48}H_{78}N_{30}$: 1074.70; found 538.37 $(M+2H)^{2+}$, 1075.75 $(M+H)^+$.

Dendrimer. Intermediate **9** (0.54 g, 0.5 mmol) and polymer-supported BEMP (2.7 g, 6.0 mml) were mixed in 1,1,2 trichloroethane/*i*-PrOH (5/1, 30 mL) for 10 min at room temperature. To the mixture was added compound **4** (5.54 g, 3.05 mmol) and the reaction temperature was raised to 80 ^{o}C. After stirring for 3 days, the supported base was removed, the filtrate was evaporated and purified by column chromatography ($MeOH/CH_2Cl_2$=1/10, R_f=0.35) to obtain **Dendrimer** (5.3 g, 90 %). ^{1}H NMR (MeOH-d_4+$CDCl_3$, 500 MHz) δ: 5.63 (brs, NH), 5.34 (brs, NH), 4.57 (d, *J*=8.5 Hz, 24 Hz), 3.69 (br, 72 H), 3.37 (br, 96 H), 3.16 (br, 24 H), 2.94 (br, 96 H), 2.68 (br, 24 H), 1.59 (br, 132 H), 1.28 (s, 432 H), 1.10 (br, 24 H); ^{13} C NMR (MeOH-d_4+$CDCl_3$, 125 MHz) δ: 165.07, 164.55, 156.19, 78.85, 45.77, 43.15, 42.96, 42.72, 42.47, 37.81, 37.02, 29.55, 28.05, 27.62. MS (MALDI-TOF): calcd for $C_{558}H_{984}N_{180}O_{96}$: 11750.9320; found 5869.7 $(M+2H)^{2+}$, 11764.54 $(M+H)^+$.

Statistical Analysis. To calculate the standard deviation for each set of pooled data, the following equation was used:

$$s = \sqrt{\frac{\sum(x_i - \bar{x})^2}{(n - 1)}}$$

Where x_i = control value, **X-bar** = average, **n** = sample size

An example is performed from the G3/MTX treatment group:

$$\frac{(35\text{-}58.66)^2 + (65.2\text{-}58.66)^2 + (73\text{-}58.66)^2 + (64.8\text{-}58.66)^2 + (55.3\text{-}58.66)^2}{5\text{-}1}$$

$$\frac{214.297}{4} = \text{sqrt of } 53.57425 = \mathbf{14.638}$$

To calculate whether there existed significant differences between two means, the Student's t-test was used:

$$t = |\bar{x}_1 - \bar{x}_2| \div \sqrt{A * B}$$

Where,

$$A = (n_1 + n_2) \div n_1 n_2 ,$$

and

$$B = [(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2] \div [n_1 + n_2 - 2]$$

Where **X-bar** is the sample mean, **s** is the sample standard deviation and **n** is the sample size

An example is performed using data from the controls and 6-MP treated groups to see if a significant difference is present between these two means:

A = (5 + 5) ÷ (5)(5) = **.4**

B = $[(5\text{-}1)9.982^2 + (5\text{-}1)22.82^2] \div [5 + 5\text{-}2]$ = **311.567**

So **t** = |51.6 – 88.68| ÷ sqrt of (.4)(311.567) = **3.3215**

To intrepret this t value we use a probability table:

Degrees of Freedom	p=0.05	p=0.025	p=0.01	p=0.005
1	12.71	25.45	63.66	127.32
2	4.30	6.20	9.92	14.09
3	3.18	4.17	5.84	7.45
4	2.78	3.50	4.60	5.60
5	2.57	3.16	4.03	4.77
6	2.45	2.97	3.71	4.32
7	2.36	2.84	3.50	4.03
8	2.31	2.75	3.36	3.83
9	2.26	2.68	3.25	3.69
10	2.23	2.63	3.17	3.58
11	2.20	2.59	3.11	3.50
12	2.18	2.56	3.05	3.43
13	2.16	2.53	3.01	3.37
14	2.14	2.51	2.98	3.33
15	2.13	2.49	2.95	3.29
16	2.12	2.47	2.92	3.25
17	2.11	2.46	2.90	3.22
18	2.10	2.44	2.88	3.20
19	2.09	2.43	2.86	3.17
20	2.09	2.42	2.84	3.15
21	2.08	2.41	2.83	3.14
22	2.07	2.41	2.82	3.12
23	2.07	2.40	2.81	3.10
24	2.06	2.39	2.80	3.09
25	2.06	2.38	2.79	3.08
26	2.06	2.38	2.78	3.07
27	2.05	2.37	2.77	3.06
28	2.05	2.37	2.76	3.05
29	2.04	2.36	2.76	3.04
30	2.04	2.36	2.75	3.03
40	2.02	2.33	2.70	2.97
60	2.00	2.30	2.66	2.92
120	1.98	2.27	2.62	2.86
infinity	1.96	2.24	2.58	2.81

We determine the degrees of freedom by the number of data points in the two groups combined minus 2. So,

$10 - 2 = 8$.

At 8 degrees of freedom we have a value of 2.31 at $p = 0.05$. If **t** exceeds the tabled value of p value (0.05), the means are significantly different at the probability level that is listed. Since our t value, 3.3215 is higher than 2.31, our means are significant different.