Supplementary Material

for

The Nitric Oxide Prodrug V-PROLI/NO Inhibits Cellular Uptake of Proline

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General.

DAF-FM DA was obtained from Invitrogen. Acetonitrile (MeCN), dimethyl sulfoxide (DMSO), and sodium nitrite were purchased from Sigma-Aldrich Company. L-[^{14}C] Proline obtained in ethanol:water solution from PerkinElmer. HBSS, Dulbecco's Modified Eagle Medium (DMEM), and Eagle's Minimum Essential Medium (EMEM) were obtained from Gibco. Radio-Immunoprecipitation Assay (RIPA) Buffer was obtained from Thermo Scientific. Scintillation cocktail used was Ecoscint A from National Diagnostics. Hepatocellular carcinoma HepG2 cells and human colorectal adenocarcinoma CaCo-2 cell lines used in this study were obtained from American Type Culture Collection. HepG2 cells were maintained in DMEM supplemented with 10 % fetal calf serum, 100 U/mL penicillin and 2 mM glutamine, at 37 °C and 5 % CO_2. CaCo-2 cells were maintained in EMEM supplemented with 10 % fetal calf serum, 100 U/mL penicillin and 2 mM glutamine, at 37 °C and 5 % CO_2. An Agilent 1100 series HPLC fitted with a C-18 reverse phase column (Phenomenex Luna 250 × 4.60 mm) operating at 262 nm and run isocratically with MeCN:water (75%, v/v) was used to analyze the decomposition profile. Quantification of NO by chemiluminescence was determined by using a Sievers nitric oxide analyzer (NOA) model 280i. Fluorescence spectrometry was performed on a PerkinElmer LS50B luminescence spectrometer. Radiolabeled proline uptake was measured using a Beckman LS 6000TA liquid scintillation counter. V-PYRRO/NO (**1a**), V-PROLI/NO (**1b)**, **1c**, **1d**, V-SARCO/NO (**1e)**, **1f** were synthesized through reported methods.[1, 2]

Table S1. Fluorescence values for CaCo-2 DAF-FM assay

Compound (Concentration)	Fluorescence (AU)		Average Fluorescence (AU)
DMSO	0.534	0.515	0.525
1a (100 μM)	0.531	0.595	0.563
1a (250 μM)	0.696	0.705	0.701
1b (50 μM)	2.168	2.618	2.393
1b (100 μM)	4.24	4.113	4.177
1b (250 μM)	9.433	8.201	8.817
1c (100 μM)	0.761	0.852	0.807
1d (100 μM)	0.666	0.774	0.720
1e (50 μM)	3.819	3.79	3.805
1e (100 μM)	6.637	6.283	6.46
1e (250 μM)	13.62	16.4	15.01
1f (100 μM)	0.644	0.739	0.692

Table S2. Fluorescence values for HepG2 DAF-FM assay

Compound (Concentration)	Fluorescence (AU)		Average Fluorescence (AU)
DMSO	0.394	0.498	0.446
1a (100 μM)	0.480	0.547	0.514
1a (250 μM)	0.563	0.539	0.551
1b (50 μM)	2.704	2.799	2.752
1b (100 μM)	3.638	3.376	3.507
1b (250 μM)	4.738	5.118	4.928
1c (100 μM)	0.622	0.556	0.589
1d (100 μM)	0.554	0.579	0.567
1e (50 μM)	6.915	6.904	6.910
1e (100 μM)	10.770	11.390	11.080
1e (250 μM)	19.620	21.250	20.435
1f (100 μM)	0.602	0.630	0.616

Table S3. Area of HPLC traces (mAU·s)

Time (d)	V-PYRRO/NO (mAU·s)		V-PROLI/NO (mAU·s)		V-SARCO/NO (mAU·s)	
0	1044	1090	978	916	820	855
0.25	1056	1099	825	865	808	855
1	1037	1084	810	848	778	739
4	925	1004	797	835	712	676
5	902	990	727	767	652	602
6	845	947	733	770	639	602
7	803	915	712	747	633	593

Table S4. Standard curve for nitrite determination

Nitrite (μM)	Volume (μL)	pmol	Area (mAU·s)	
200.0	10	2000	4.04E+06	
150.0	15	2250	2.84E+06	
100.0	10	1000	1.93E+06	
75.0	15	1125	1.23E+06	
50.0	10	500	9.27E+05	
25.0	10	250	4.50E+05	
12.5	10	125	2.07E+05	
6.3	10	62.5	1.03E+05	
3.1	10	31	4.63E+04	

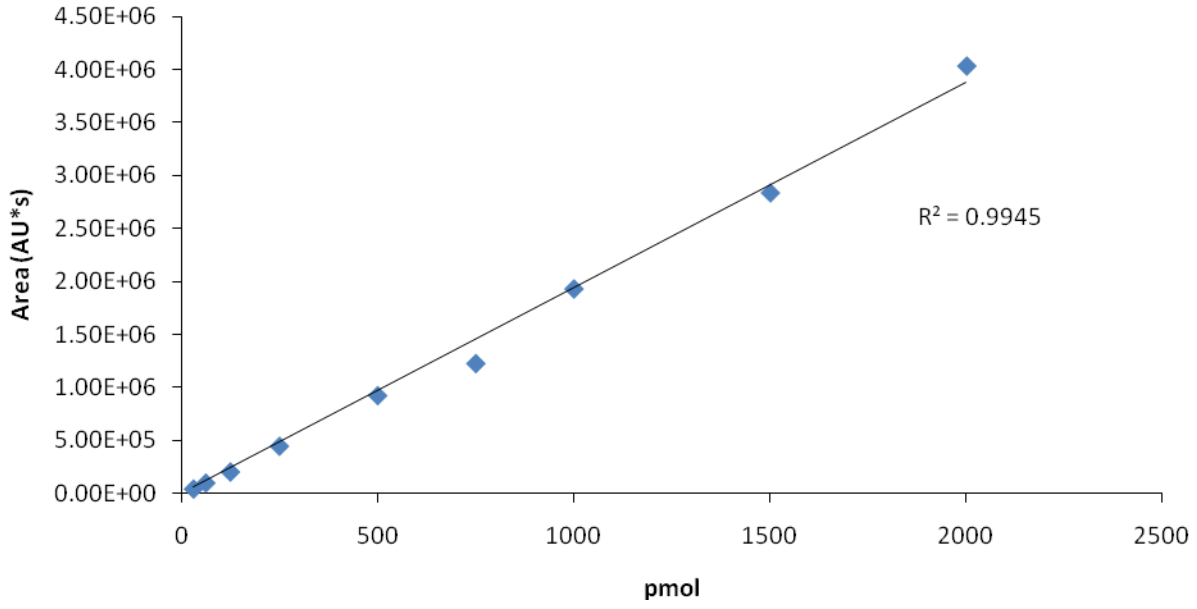

Figure S1. Standard curve for nitrite determination

Table S5. Nitrite Levels found using 50 uL injections of cell culture medium

Compound	Area (mAU·s)	Nitrite (pmol)	Injection Volume (µL)	Concentration (µM)
V-SARCO/NO	2.04E+06	1053.771	50	21.08
V-SARCO/NO	2.16E+06	1113.574	50	22.27
V-PROLI/NO	3.35E+05	172.7071	50	3.45
V-PROLI/NO	3.69E+05	190.2356	50	3.80
V-PYRRRO/NO	0.00E+00	0	50	0.00
V-PYRRO/NO	0.00E+00	0	50	0.00

Table S6. Radiolabeled proline uptake data.

Compound	CPM			Average CPM	Average DPM	σ
^{14}C-Proline Only	919	963	924	935	843	24
^{14}C-Proline + Proline	242	303	336	294	202	48
^{14}C-Proline +V-PYRRO/NO	604	575	652	610	518	39
^{14}C-Proline + V-PROLI/NO	231	202	226	220	128	16
^{14}C-Proline + V-SARCO/NO	434	422	455	437	345	17

References

1. Hong, S. Y.; Saavedra, J. E.; Keefer, L. K.; Chakrapani, H. Improved synthesis of V-PYRRO/NO, a liver-selective nitric oxide prodrug, and analogues. *Tetrahedron Lett.* **2009**, *50*, 2069-2071.
2. Hong, S. Y.; Nandurdikar, R. S.; Keefer, L. K.; Saavedra, J. E.; Chakrapani, H. An improved synthesis of V-PROLI/NO, a cytochrome P450-activated nitric oxide prodrug. *Tetrahedron Lett.* **2009**, *50*, 4545-4548.