SUPPORTING INFORMATION

Screening for DNA Alkylation Mono and Cross-linked Adducts with a Comprehensive LC-MS^3 Adductomic Approach

Alessia Stornetta[1], Peter W. Villalta[2], Stephen S. Hecht[2], Shana J. Sturla[1], and Silvia Balbo[2*]

[1]Department of Health Sciences and Technology, ETH Zurich, Schmelzbergstrasse 9, 8092 Zurich, Switzerland

[2]Masonic Cancer Center, University of Minnesota, 2231 Sixth Street SE, Minneapolis, Minnesota 55455, United States

*E-mail: balbo006@umn.edu. Fax: (612) 624-3869. Tel.: (612) 624-4240.

1. EXPERIMENTAL SECTION

Cell Culture. HT-29 cells were obtained from the Leibniz-Institut DSMZ GmbH (Braunschweig, Germany). Cells were grown in DMEM medium with glutaMAX containing 10% bovine calf serum and 1% penicillin/streptomycin. Cells were incubated in a humidified incubator containing 5% CO_2 at 37 °C.

Reaction of Calf Thymus DNA (ctDNA) with PR104A. PR104A (20 µL, 2.5 mg/mL) in CH_3OH was added to a reaction mixture containing BSA (125 µL, 4 mg/mL) and NADPH (400 µL, 19 mg/mL) dissolved in potassium phosphate buffer (100 mM, pH 7.4). The reaction mixture was incubated at 37 °C for 5 min. CtDNA (400 µL, 2.5 mg/mL) dissolved in potassium phosphate buffer (100 mM, pH 7.4) was incubated at 37 °C for 5 min and added to the reaction mixture containing PR104A. 2 mU of AKR1C3 were added to the reaction mixture, which was subsequently diluted to a final volume of 1 mL with potassium phosphate buffer (100 mM, pH 7.4), and incubated at 37 °C for 24 h. The same reaction mixture without PR104A was used as negative control. DNA isolation was performed by EtOH precipitation. Briefly, 1 mL cold EtOH was added to each sample vial. The precipitated DNA was isolated, washed twice with 1 mL 70% EtOH and 1 mL 100% EtOH, and dried under a nitrogen stream. All steps of the protocol were performed using silanized glass vials. This experiment was performed in three replicates.

Treatment of HT-29 Cells with PR104A. Cells were seeded in 6-well plates at a density of 5.0 x 10^5 cells/well and were allowed to attach and grow for 48 h. The medium was replaced with fresh medium (2 mL) containing 0.1% DMSO (solvent control), or PR104A resulting in final concentrations of 100 µM, 250 µM, or 500 µM. Cells were incubated for 24 h; thereafter, the DNA from intact cells was isolated.

DNA Isolation from HT-29 Cells. DNA was extracted from the incubated cells with the Wizard SV Genomic DNA Purification System (Promega, Switzerland). For each treatment concentration, a total of eight samples, each present in a single well, were extracted. For extractions, cells were washed once with 1X PBS (1 mL) and lysed by adding lysis buffer (450 µL) to each well of the 6-well plate. The lysate was transferred to the Wizard SV mini-column assembly and it was centrifuged for 3 min at 13,000xg.

Thereafter, the column was washed 4 times with wash solution (650 µL) to remove any contaminants (1 min, 13,000xg). The binding matrix then was dried by centrifugation (2 min, 13,000xg), and the column was transferred to a new 1.5 mL DNA LoBind Eppendorf tube. RNase (2 µL, 4 mg/mL), then nuclease-free water (250 µL) was added to the column, and after 2 min at 20 °C, the assembly was centrifuged (1 min, 13,000xg). Extra care should be taken in removing RNA from the sample, since base adducts resulting from DNA have the same mass than those resulting from RNA. The DNA concentration of the resulting solutions was determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific). DNA solutions were concentrated to a final volume of 100 µL under reduced pressure and stored at -20 °C.

DNA Hydrolysis. DNA (500 µg from ctDNA and 130-240 µg from HT-29-treated cells) was dissolved in 10 mM Tris-HCl/5 mM $MgCl_2$ buffer (pH 7) to a final volume of 1 mL, spiked with 5 pmol labeled standard (D_4-O^2-POB-dT), and digested overnight at 37 °C with 600 U/mg DNA of DNase I. Additional 600 U/mg DNA of DNase I were added together with 20 mU/mg DNA of phosphodiesterase I, and 240 U/mg DNA of alkaline phosphatase, followed by incubation at 37 °C overnight. The enzymes were removed by centrifugation using a centrifree ultrafiltration device (MW cutoff of 30,000; Merck Millipore, Cork, Ireland). An aliquot (20 µL) was taken from each sample for the analysis of deoxyguanosine (dGuo) by high performance liquid chromatoraphy (HPLC). A reaction mixture containing buffer and enzymes served as negative control. All steps of the protocol were performed using silanized glass vials.

Sample Enrichment. Hydrolysates were purified by solid-phase extraction on a Strata-X cartridge (30 µm, Phenomenex, Torrance, CA). The samples were loaded on the cartridge, washed with 1 mL of H_2O, and eluted with 1 mL of 10%, 50%, and 100% CH_3OH in H_2O. The eluted fractions were evaporated to dryness, and dissolved in 1% (ctDNA) or 20% (HT-29 cells) CH_3OH in H_2O to a final volume of 1000 µL or 10 µL, respectively.

dGuo Quantitation by HPLC-UV Analysis. Quantitation of dGuo was carried out with a Dionex UltiMate 3000 RSLCnano System (Thermo Scientific, Waltham, MA) with a UV detector set at 254 nm. A 300 µm x 15 cm C18 column (2 µm, 100 Å) (Thermo Scientific, Waltham, MA) was used with water (A) and CH_3OH (B) as mobile phases, an injection volume of 1 µL, and a flow rate of 5 µL/min. The elution gradient was as follows: an initial condition of 2% B for 3 minutes, a linear gradient from 2% to 30% B over the course of 22 minutes, and then from 30% to 95% B over the course of 8 minutes, followed by a 5 minutes hold, a return to the starting conditions over 5 minutes and re-equilibration for 10 minutes (50 minutes total run time). A calibration curve for dGuo (0.0625-8 ng/µL) was performed in triplicate. The amount of dGuo in each sample was calculated using the peak area, the slope of the calibration curve, and the fraction of the total volume injected. The amount of dGuo was then used to calculate the amount of DNA in each sample using the ratio of 1 mg of DNA per 0.66 µmol of dGuo.

2. SUPPLEMENTARY TABLES

Table S1. Targeted analysis of adducts by triggered MS^3 fragmentation events from the reaction of ctDNA with PR104A in the presence of AKR1C3. Only DNA adduct masses that triggered an MS^3 fragmentation event in all three replicate experiments are reported in Table S1. Measured $[M+H]^+$, mass deviation, retention time, and peak area are reported for one of the three replicates.

		Measured $[M+H]^+$ [a]	Mass deviation (ppm)[b]	Retention time (min)	Peak area	Neutral loss[c]	Adduct type	Proposed alkylating compound	Alkylation position(s)	R_1 [d]	R_2 [d]
50% CH_3OH fraction	**A**	446.1892	0.8	13.2	4.25E+06	A	Base monoadduct	PR104M	*N*7-A, *N*3-A	OH	NH_2
	B	595.2242	-1.6	18.94	4.69E+05	G	Base crosslink	PR104H or PR104M	*N*7-G, *N*7-A, *N*3-A	A or G	NHOH or NH_2
	C	462.1845	-0.2	19.07	1.48E+07	G	Base monoadduct	PR104M	*N*7-G	OH	NH_2
	D	492.1589	-0.7	22.39	6.37E+05	G	Base monoadduct	PR104A	*N*7-G	OH	NO_2
100% CH3OH fraction	**E**	592.2110	0.0	20.46	2.45E+05	dR	Nucleoside monoadduct	PR104A	*N*1-dA, *N*3-dA, *N*6-dA, *N*7-dA	OH	NO_2
	F	476.1636	0.2	21.18	2.06E+06	A	Base monoadduct	PR104A	N7-A, N3-A	OH	NO_2
	D	492.1586	-0.1	22.55	2.69E+06	G	Base monoadduct	PR104A	*N*7-G	OH	NO_2
	G	741.2448	0.0	22.95	2.60E+05	dR	Nucleoside crosslink	PR104A	*N*1-dG, *N*2-dG, O^6-dG, *N*7-dG	G	NO_2
	E	592.2110	0.0	23.76	2.17E+05	dR	Nucleoside monoadduct	PR104A	*N*1-dA, *N*3-dA, *N*6-dA, *N*7-dA	OH	NO_2
	H	570.1360	0.3	24.18	1.91E+05	G	Base monoadduct	PR104A	*N*7-G	OSO_2CH_3	NO_2
	I	608.2062	-0.5	24.77	2.16E+05	dR	Nucleoside monoadduct	PR104A	*N*1-dG, *N*2-dG, O^6-dG, *N*7-dG	OH	NO_2
	J	510.1244	0.6	26.42	4.43E+05	G	Base monoadduct	PR104A	*N*7-G	Cl	NO_2
	K	538.0790	0.4	27.01	3.83E+05	A	Base monoadduct	PR104A	*N*7-A, *N*3-A	Br	NO_2
	L	554.0736	1.0	27.16	2.01E+06	G	Base monoadduct	PR104A	*N*7-G	Br	NO_2

[a]All $[M+H]^+$ indicated were not present in control samples (unreacted ctDNA, buffer and enzymes used for DNA hydrolysis); [b]Difference between the theoretical and the measured mass; [c]Neutral loss of base or dR; [d]Refer to Scheme 1 in the manuscript.

Table S2. Untargeted analysis of adducts by triggered MS^3 fragmentation events for DNA extracted from PR104A-treated HT-29 cells. The "X" represents an MS^3 fragmentation triggered for the corresponding $[M+H]^+$.

			HT-29 cells exposed to PR104A					
			100 μM	250 μM	500 μM	100 μM	250 μM	500 μM
	Measured $[M+H]^+$ [a]	Neutral loss[b]	50% CH_3OH fraction			100% CH_3OH fraction		
1	221.1173	dR				X	X	X
2	239.1643	dR		X				
3	251.1642	dR				X		
4	258.1688	dR					X	X
5	270.9580	A	X					
6	277.1412	dR	X					
7	291.1959	dR				X		
8	293.1750	dR				X	X	
9	295.1906	dR	X	X				
10	295.2270	dR				X		
11	304.1018	A	X	X	X			
12	325.0360	C		X				
13	327.1804	dR		X				
14	343.2592	dR					X	
15	359.0287	A			X			
16	371.2278	T		X				
17	408.2956	G				X		
18	445.1200	dR			X			
19	452.3322	A				X	X	
20	474.1474	G				X		
21	476.1636[c]	A					X	
22	492.1588[c]	G					X	X
23	522.7865	C					X	
24	574.3041	dR				X		X
25	608.2058[c]	dR				X	X	
26	611.1717	A		X				
27	629.3121	dR			X			
28	641.1821	A	X	X	X			
29	655.3409	A			X			
30	664.8304	dR		X	X	X		
31	708.4332	dR		X				
32	762.8685	C						X
33	892.4407	C				X		
34	893.9455	C		X				
35	897.3582	A			X			
36	897.3669	dR		X				
37	897.3671	A	X					
38	960.8556	A		X	X			
39	986.4601	C					X	
40	1002.0806	A				X		
41	1026.7473	C		X				
42	1027.4731	G	X					
43	1028.4764	G	X					
44	1046.3981	A			X			
45	1150.4822	A	X	X				
46	1150.9841	A		X				
47	1151.4847	A			X			
48	1718.8429	C			X			
		MS^3 events (total)	9	16	12	13	10	5

[a] All $[M+H]^+$ indicated had a retention time greater than 10 minutes and were not present in control samples (DMSO control, buffer and enzymes used for DNA hydrolysis); [b] Neutral loss of base or dR; [c] These masses were also triggered by the targeted analysis (Table 1).