Supporting Information

What do we learn when we study cytotoxicity? Critical shortcomings in the green chemistry context using imidazolium ionic liquids as an example case

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The ionic liquids 1-ethyl-3-methylimidazolium chloride (C_2MIm Cl, purity >98%, abcr GmbH, Germany), 1-butyl-3-methylimidazolium chloride (C_4MIm Cl, purity >98%, abcr GmbH, Germany), and 1-hexadecyl-3-methylimidazolium chloride (C_{16}MIm Cl, purity >98%, abcr GmbH, Germany), and sodium azide (NaN_3, purity >99.5%, Molekula Ltd., Great Britain) were used as supplied. Stock solutions of the individual compounds were prepared in Milli-Q ultrapure deionized water (Millipore Co., USA) or dimethylsulfoxide (biochemical grade, Servicebio Technology, Ltd., China).

Cell lines

The HEK293T (human embryonic kidney) and Jurkat (human T-cell leukemia) cell lines were purchased from the Institute of Cytology RAS, St. Petersburg, Russia. The cells were cultured in 100 mm clear plastic TC-treated Petri dishes (Eppendorf SE, Germany) or 25 cm^3 clear plastic flasks with a vented lid (Corning, Inc., USA) in a Binder CB 53 CO_2 incubator (BINDER GmbH, Germany) at 37°C, 5% CO_2, and 95% humidity. Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, USA) supplemented with 4.50 $g \cdot L^{-1}$ D-glucose and 4.00 mM L-glutamine and Roswell Park Memorial Institute Medium 1640 (RPMI-1640, Gibco, Thermo Fisher Scientific, USA) supplemented with 2.00 $g \cdot L^{-1}$ D-glucose and 2.05 mM L-glutamine supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and 100 $U \cdot mL^{-1}$ penicillin–streptomycin (Gibco, Thermo Fisher Scientific, USA) were used for the cultivation of HEK293T and Jurkat cells, respectively.

Cytotoxicity assay

The cytotoxicity of the ionic liquids was evaluated by propidium iodide (PI) staining via a flow cytometry assay. Prior to the test, the cells were seeded into 24-well flat-bottom microplates (Wuxi NEST Biotechnology Co., Ltd., China), 10^5 cells per well. Following seeding, the HEK293T cells were allowed to reach 70% confluence (48 h), whereas the Jurkat cells were used immediately. The cells were incubated with the test substances at appropriate concentrations for 24 h. A positive control was

prepared by treating the cells with ethanol for 10 s (HEK293T) or heating them at 60°C for 10 min (Jurkat); intact cells were used as a negative control.

After incubation, the cells were harvested with a 0.05% trypsin-EDTA solution (HEK293T; Gibco, Thermo Fisher Scientific, USA) or transferred directly (Jurkat) into 15-mL tubes (Corning, Inc., USA), centrifuged at 1000 rpm for 5 min, and washed with cold phosphate-buffered saline (PBS, Sigma–Aldrich, USA). The cell pellets were resuspended in 300 µL of cold PBS. Then, 100 µL of each suspension was transferred to a dark microcentrifuge tube (Accumax Lab Devices Pvt Ltd., India), and 0.5 µL of a 0.1 $mg \cdot mL^{-1}$ aqueous solution of propidium iodide (PI, Sigma–Aldrich, USA) was added to each tube. The samples were incubated for 15 min in the dark, after which they were diluted with cold PBS to 500 µL, transferred to 15-mL tubes (Corning, Inc., USA) and immediately analyzed by flow cytometry using BD FACSAria III™ Cell Sorter (Becton Dickinson, USA).

The data were processed in BD FACSDiva 9.4 software (Becton Dickinson, USA). All test points were measured in 3–5 replicates. Statistical processing of the obtained values and calculations of the 24-h half-maximal cytotoxicity concentration (24-h CC_{50}) were carried out in Microsoft Excel 2010 (Microsoft, USA) and Prism 5 (GraphPad Software, Inc., USA) software.

Apoptosis assay

Apoptosis was detected by joint propidium iodide (PI) and Annexin V staining via a flow cytometry assay. Prior to the test, the cells were seeded into 24-well flat-bottom microplates (Wuxi NEST Biotechnology Co., Ltd., China), 10^5 cells per well. Following seeding, the HEK293T cells were allowed to reach 70% confluence (48 h), whereas the Jurkat cells were used immediately. The cells were incubated with the test substances at their 24 h CC_{50} concentrations for 4, 8, 16, 24, or 48 h. A positive control was prepared by treating the cells with ethanol for 10 s (HEK293T) or heating at 60°C for 10 min (Jurkat); intact cells were used as a negative control.

After incubation, the cells were harvested with a 0.05% trypsin-EDTA solution (HEK293T; Gibco, Thermo Fisher Scientific, USA) or transferred directly (Jurkat) into 15-mL tubes (Corning, Inc., USA), centrifuged at 1000 rpm for 5 min, and washed with cold phosphate-buffered saline (PBS, Sigma–Aldrich, USA). The samples were subsequently resuspended in Annexin binding buffer, stained with propidium iodide and Annexin V according to the manufacturer's protocol (FITC Annexin V/Dead Cell Apoptosis Kit, Invitrogen, Thermo Fischer Scientific, USA, or AF488 Cell Apoptosis Kit with Annexin V/PI,

SciStoreLab LLC, Russia), and subsequently analyzed by flow cytometry using BD FACSAria III™ Cell Sorter (Becton Dickinson, USA).

The data were processed in BD FACSDiva 9.4 software (Becton Dickinson, USA). All test points were measured in 4–11 replicates. Statistical processing of the obtained values was carried out using built-in functions (*t*-Test: Two-Sample Assuming Unequal Variances, $p<0.05$) of Microsoft Excel 2010 (Microsoft, USA).

Western blot analysis

The following antibodies were used: murine monoclonal anti-CASP3 antibody (2G7, FNab09938, 1:1000, FineTest, China); rabbit polyclonal anti-CASP8 antibodies (1:500, FNab01293, FineTest, China); murine monoclonal anti-CASP9 antibody (1F9, FNab01297, 1:1000, FineTest, China); murine monoclonal anti-PARP1 antibody (4A9, FNab09939, 1:5000, FineTest, China); rabbit polyclonal anti-γ-H2A.X antibodies (GB111841, 1:200, Servicebio, China); rabbit polyclonal anti-SOD1 antibodies (FNab08103, 1:500, FineTest, China); mouse monoclonal anti-CAT antibody (FNab1065, 1:1000, FineTest, China); murine monoclonal anti-GAPDH antibody (4A2, 1:10000, FineTest, China); goat anti-mouse IgG (H + L) and goat anti-rabbit IgG (H + L) HRP conjugates (1:3000, Bio-Rad, USA) were used as secondary antibodies.

After incubation with the test substances for 4, 8, or 24 h, the cells were harvested, counted, dissolved in the corresponding volumes of RIPA buffer (50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 1% protease inhibitor cocktail (Servicebio Technology, Ltd., China), and 1% phosphatase inhibitor cocktail (Servicebio Technology, Ltd., China)), and incubated on ice for 15 min upon gentle mixing. The samples were subsequently centrifuged for 15 min at 14000 × g at 4 °C, after which the supernatants were transferred to clean 1.5 mL plastic tubes (SSI Bio, USA). The protein content in the samples was measured via the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, USA) in accordance with the manufacturer's protocol. Samples for SDS–PAGE were prepared by diluting the lysates with an equal volume of Laemmli buffer (Bio-Rad, USA) supplemented with 100 mM DTT (Sigma–Aldrich, USA); these samples were subsequently incubated at 95 °C for 10 min. Then, the samples were resolved by SDS–PAGE (10 μg per lane), and blotted onto a PVDF Immobilon-P membrane (Millipore, USA). The blots were blocked with I-Block™ Protein-Based Blocking Reagent (Invitrogen, USA), incubated with corresponding antibodies, and developed using the Clarity Western ECL substrate (Bio-Rad, USA) in a ChemiDoc XRS+ gel documenting system (Bio-Rad, USA). All test points were studied 2-3 times.

Table S1. 24-h CC_{50} values of C_2MIm Cl, C_4MIm Cl, and C_{16}MIm Cl measured in Jurkat and HEK293T cells.

IL	Mw, g·mol^{-1}	24-h CC_{50}, mM*	
		HEK293T	Jurkat
C_2MIm Cl	146.618	160.560 (114.937-206.183)	67.357 (44.335-90.379)
C_4MIm Cl	174.672	64.016 (33.760-94.272)	22.143 (16.253-28.034)
C_{16}MIm Cl	342.996	0.015 (0.012-0.0180	0.002 (0.001-0.003)

* 95% confidence intervals are shown in parentheses.

Table S2. Induction of apoptosis/necrosis in Jurkat cells upon exposure to C_2MIm Cl, C_4MIm Cl, C_{16}MIm Cl, and NaN_3.*

Substance	Jurkat																			
	Q1 (PI+, Annexin-), %					Q2 (PI+, Annexin+), %					Q3 (PI-, Annexin-), %					Q4 (PI-, Annexin+), %				
	4 h	8 h	16 h	24 h	48 h	4 h	8 h	16 h	24 h	48 h	4 h	8 h	16 h	24 h	48 h	4 h	8 h	16 h	24 h	48 h
C_2MIm Cl	**1.82** (0.51-3.12)	**1.52** (0.88-2.16)	**0.86** (0.26-1.46)	**2.08** (0.84-3.32)	**2.07** (0.68-3.46)	**6.48** (4.59-8.38)	**7.48** (3.88-11.08)	**11.66** (8.86-14.46)	**22.30** (16.73-27.87)	**49.22** (42.68-55.75)	**69.83** (54.51-85.15)	**72.06** (58.28-85.84)	**69.04** (65.70-72.38)	**58.88** (53.60-64.16)	**32.67** (27.26-38.07)	**21.85** (8.84-34.86)	**18.92** (5.80-32.04)	**18.42** (16.97-19.87)	**16.78** (13.91-19.65)	**16.02** (11.96-20.07)
C_4MIm Cl	**0.80** (0.28-1.32)	**1.32** (0.65-1.99)	**0.60** (0.13-1.07)	**0.52** (0.38-0.66)	**0.80** (0.42-1.18)	**3.58** (1.64-5.53)	**4.72** (2.43-7.01)	**10.86** (9.81-11.91)	**14.60** (9.43-19.77)	**54.40** (51.04-57.76)	**77.90** (63.61-92.19)	**76.72** (68.33-85.11)	**64.08** (60.25-67.91)	**58.86** (50.30-67.42)	**16.12** (14.52-17.71)	**17.72** (5.35-30.08)	**17.28** (7.77-26.79	**24.44** (21.30-27.58)	**26.04** (21.03-31.05)	**28.68** (25.40-31.97)
C_{16}MIm Cl	**0.80** (0.21-1.39)	**0.88** (0.46-1.30)	**0.58** (0.42-0.74)	**1.06** (0.49-1.63)	**0.92** (0.00-1.96)	**3.98** (2.27-5.70)	**6.28** (3.37-9.19)	**12.58** (10.51-14.65)	**22.12** (15.48-28.76)	**20.47** (15.56-25.37)	**80.17** (72.56-87.77)	**74.36** (70.16-78.56)	**74.48** (71.57-77.39)	**59.70** (47.34-72.06)	**66.97** (60.14-73.79)	**15.03** (8.81-21.26)	**18.44** (16.48-20.40)	**12.34** (8.77-15.91)	**17.14** (9.77-24.51)	**11.63** (9.37-13.89)
NaN_3	**0.40** (0.22-0.58)	**0.60** (0.45-0.75)	**0.50** (0.14-0.86)	**0.33** (0.17-0.48)	**0.57** (0.42-0.71)	**6.75** (4.68-8.82)	**31.54** (27.08-36.00)	**52.32** (49.72-54.92)	**49.35** (40.52-58.18)	**54.35** (49.31-59.39)	**33.05** (28.80-37.30)	**29.50** (25.99-33.01)	**26.12** (24.08-28.16)	**30.98** (26.71-35.24)	**31.98** (28.18-35.79)	**59.78** (54.12-65.43)	**38.38** (32.66-44.10)	**21.04** (18.74-23.34)	**19.38** (13.73-25.02)	**13.13** (8.08-18.19)
Living cells	**0.77** (0.10-1.43)	**0.88** (0.00-1.80)	**0.62** (0.19-1.05)	**0.56** (0.17-0.95)	**0.73** (0.24-1.22)	**3.40** (1.09-5.71)	**4.12** (0.80-7.44)	**5.12** (4.32-5.92)	**4.98** (2.23-7.73)	**6.35** (2.85-9.85)	**83.80** (71.27-96.33)	**85.10** (75.64-94.56)	**84.30** (79.14-89.46)	**90.12** (86.40-93.84)	**87.60** (80.35-94.85)	**12.07** (1.62-22.51)	**9.68** (1.52-17.84)	**9.92** (5.49-14.35)	**4.36** (3.19-5.53)	**5.32** (1.41-9.22)

* Q1, necrotic cells; Q2, dead cells; Q3, living cells; Q4, apoptotic cells. C_2MIm Cl: 67.36 mM; C_4MIm Cl: 22.14 mM; C_{16}MIm Cl: 0.002 mM; NaN_3: 100 mM.

Table S3. Induction of apoptosis/necrosis in HEK293T cells upon exposure to C_2MIm Cl, C_4MIm Cl, C_{16}MIm Cl, and NaN_3.*

Substance	HEK293T																			
	Q1 (PI+, Annexin-), %					Q2 (PI+, Annexin+), %					Q3 (PI-, Annexin-), %					Q4 (PI-, Annexin+), %				
	4 h	8 h	16 h	24 h	48 h	4 h	8 h	16 h	24 h	48 h	4 h	8 h	16 h	24 h	48 h	4 h	8 h	16 h	24 h	48 h
C_2MIm Cl	**1.30** (0.67-1.93)	**3.90** (1.81-5.99)	**13.70** (8.23-19.17)	**9.89** (5.44-14.33)	**9.35** (6.38-12.32)	**16.24** (11.47-21.00)	**29.48** (23.44-35.53)	**28.94** (24.06-33.81)	**45.04** (40.95-49.13)	**58.00** (50.87-65.13)	**61.01** (50.38-71.65)	**28.13** (20.79-35.48)	**40.05** (32.92-47.18)	**22.94** (18.32-27.56)	**15.24** (9.13-21.34)	**21.45** (14.95-27.95)	**38.48** (33.25-43.71)	**17.31** (11.64-22.99)	**22.11** (18.33-25.90)	**17.39** (14.24-20.53)
C_4MIm Cl	**1.01** (0.62-1.40)	**1.13** (0.46-1.81)	**19.54** (13.35-25.72)	**17.20** (9.35-25.05)	**12.78** (4.39-21.16)	**13.14** (7.08-19.20)	**14.32** (11.94-16.69)	**34.76** (25.85-43.67)	**60.61** (48.71-72.51)	**54.65** (36.35-72.95)	**63.31** (50.43-76.19)	**49.18** (40.31-58.06)	**30.21** (23.41-37.01)	**10.31** (5.73-14.89)	**11.36** (2.60-20.12)	**22.54** (15.05-30.03)	**35.38** (26.83-43.94)	**15.46** (12.60-18.33)	**11.92** (9.79-14.05)	**21.18** (9.34-33.01)
C_{16}MIm Cl	**12.27** (6.69-17.86)	**11.53** (3.51-19.55)	**23.08** (11.27-34.88)	**26.59** (19.15-34.02)	**11.30** (4.63-17.97)	**44.84** (32.56-57.13)	**52.32** (43.41-61.23)	**38.71** (20.59-56.84)	**55.99** (46.85-65.12)	**60.63** (43.98-77.27)	**34.69** (23.98-45.39)	**28.77** (19.57-37.96)	**32.49** (15.19-49.79)	**11.56** (8.40-14.72)	**17.19** (5.40-28.97)	**8.19** (4.81-11.57)	**7.38** (4.07-10.69)	**5.70** (3.25-8.15)	**5.89** (2.53-9.24)	**10.90** (5.46-16.34)
NaN_3	**0.83** (0.50-1.16)	**1.08** (0.56-1.61)	**11.29** (6.83-15.74)	**6.82** (2.46-11.17)	**17.35** (7.70-27.00)	**11.60** (10.31-12.89)	**14.15** (6.41-21.89)	**23.39** (16.31-30.46)	**45.61** (33.47-57.75)	**41.54** (31.76-51.32)	**69.83** (64.60-75.05)	**61.58** (47.46-75.71)	**52.45** (44.07-60.83)	**29.33** (16.16-42.49)	**26.14** (13.85-38.42)	**17.73** (13.19-22.26)	**23.18** (16.63-29.74)	**12.86** (10.26-15.47)	**18.23** (15.37-21.09)	**14.98** (7.98-21.97)
Living cells	**1.43** (0.59-2.27)	**0.98** (0.62-1.33)	**2.47** (1.18-3.76)	**5.35** (1.63-9.07)	**3.28** (2.12-4.43)	**7.37** (5.02-9.72)	**6.84** (4.84-8.84)	**7.67** (3.76-11.57)	**9.38** (5.46-13.29)	**10.23** (8.24-12.21)	**81.44** (76.18-86.71)	**78.28** (73.60-82.95)	**80.57** (72.45-88.68)	**76.24** (67.53-84.95)	**76.40** (73.43-79.37)	**9.76** (6.03-13.48)	**13.85** (10.60-17.10)	**9.28** (4.23-14.33)	**8.99** (6.39-11.59)	**10.09** (8.40-11.78)

* Q1, necrotic cells; Q2, dead cells; Q3, living cells; Q4, apoptotic cells. C_2MIm Cl: 160.56 mM; C_4MIm Cl: 64.02 mM; C_{16}MIm Cl: 0.015 mM; NaN_3: 100 mM.

Table S4. Overview of the induction of apoptosis/necrosis, DNA damage, and oxidative stress in Jurkat and HEK293T cells upon exposure to C_2MIm Cl, C_4MIm Cl, C_{16}MIm Cl, or NaN_3.

Process	Marker	Exposure time	Jurkat				HEK293T			
			C_2MIm Cl	C_4MIm Cl	C_{16}MIm Cl	NaN_3	C_2MIm Cl	C_4MIm Cl	C_{16}MIm Cl	NaN_3
Apoptosis	PS externalization/ Annexin V	*4 h*	-	-	-	++++	+	+	-	+
		8 h	-	-	+	+++	++	++	-*	+
		16 h	+	+	-	++	+	+	-	-
		24 h	+	+	+	++	+	-	-	+
		48 h	+	++	+	+	+	-	-	-
	CASP3	*4 h*	-	-	-	-	-	-	-	-
		8 h	-	-	-	-	-	-	-	-
		24 h	-	-	-	-*	-	-	-	
	CASP8	*4 h*	-	-	+	++	-	-	++	-
		8 h	-	+	+	+	-	+	+	-
		24 h	-	+	+	-*	-	-	-	-
	CASP9	*4 h*	+	+	+	+	-	-	-	-
		8 h	-	-	-	-	-	-	-	-
		24 h	-	-	-	-*	-	-	-	-
Necrosis	Membrane perforation/PI	*4 h*	-	-	-	-	-	-	+	-
		8 h	-	-	-	-	+	-	+	-
		16 h	-	-	-	-	++	+	+	+
		24 h	+	-	-	-	-	+	++	-
		48 h	-	-	-	-	++	+	+	+
DNA damage	PARP1	*4 h*	-	-	+	+	-	+	-	+
		8 h	-	-	+	+	-	+	+	-
		24 h	-	+	-	-*	-	-	-	-
	γ-H2A.X (DSBs)	*4 h*	-	-	+	+	+	+	-	+
		8 h	-	-	+	+	+	+	+	+
		24 h	-	+	+	-	+	+	-	+
Oxidative stress	SOD1	*4 h*	-	-	-	-	-	-	-*	-
		8 h	-	-	-	-	-	+	-	-
		24 h	-	-	-	-*	-	-	-	-
	CAT	*4 h*	-	-	-		-	-	++	-
		8 h	-	-	-	-	+	+	+	-
		24 h	-	-	-	+	-	-	-	-

* Below control level. DSBs, double-strand breaks.

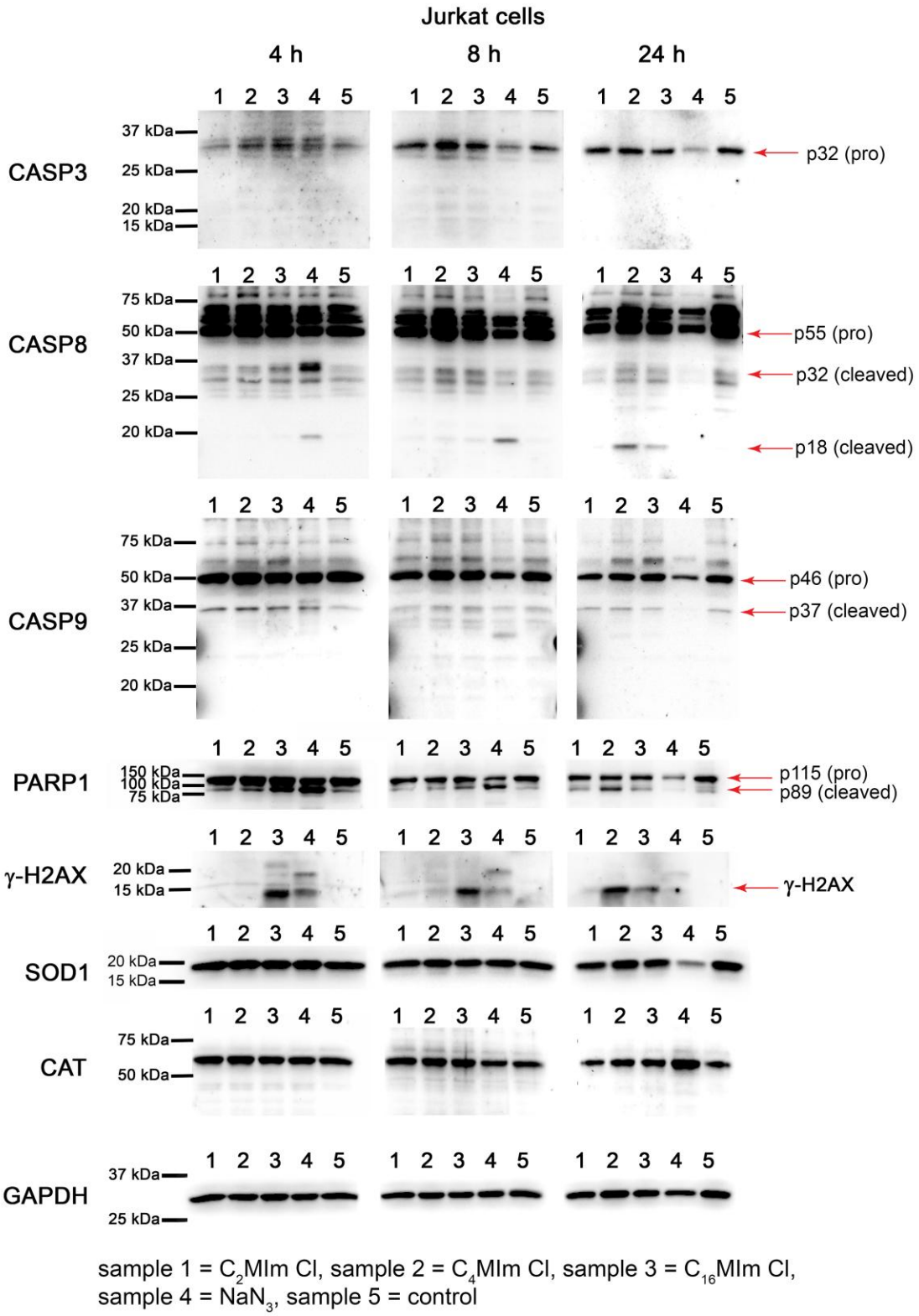

Figure S1. Western blot analysis of the induction of apoptosis, DNA damage, and oxidative stress by C_2MIm Cl, C_4MIm Cl, and C_{16}MIm Cl in Jurkat cells after 4 h, 8 h and 24 h of exposure. 100 mM NaN_3 was used as a positive control of apoptosis. GAPDH was used as a loading control.

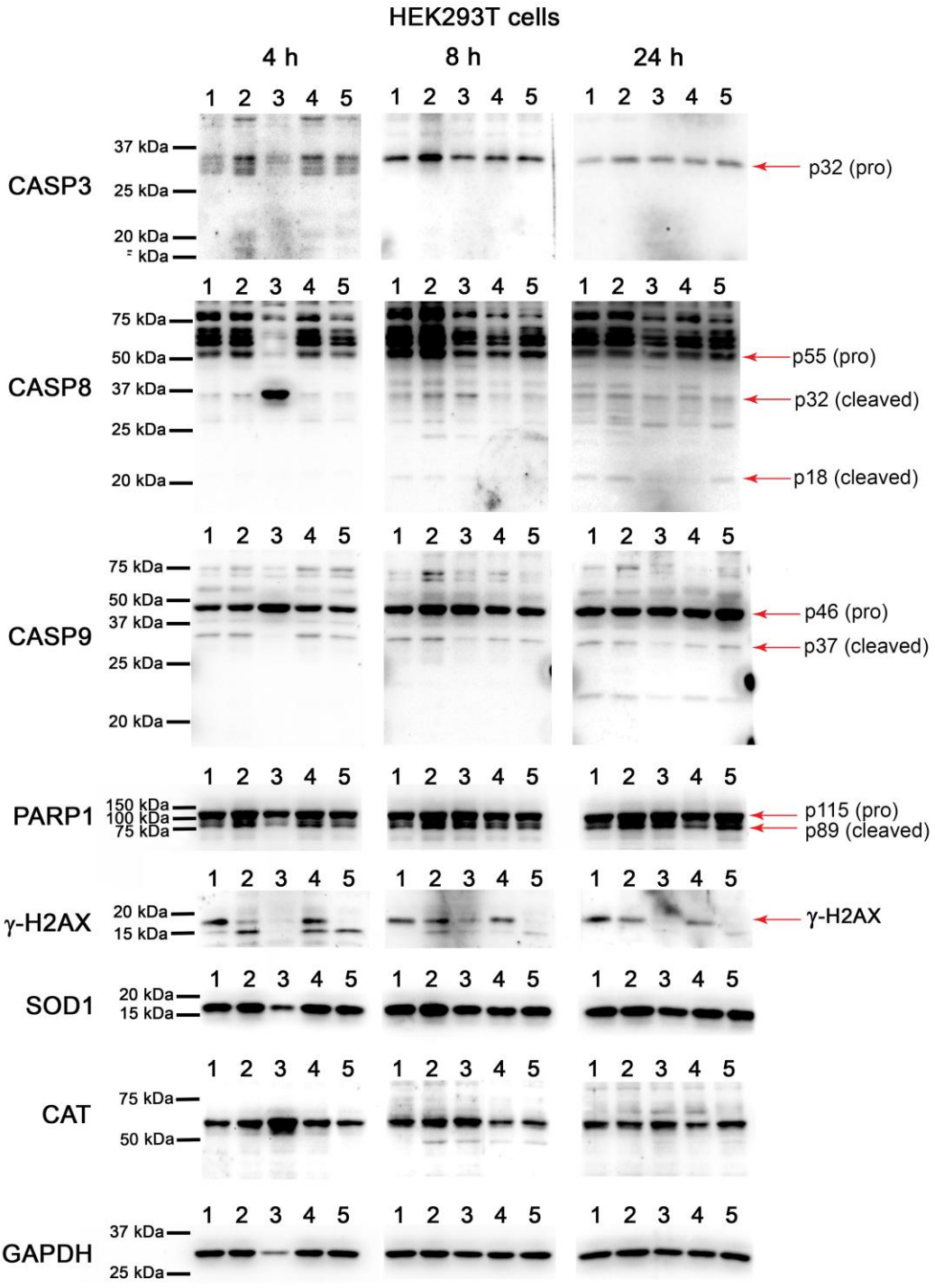

Figure S2. Western blot analysis of the induction of apoptosis, DNA damage, and oxidative stress by C_2MIm Cl, C_4MIm Cl, and C_{16}MIm Cl in HEK293T cells after 4 h, 8 h and 24 h of exposure. 100 mM NaN_3 was used as a positive control of apoptosis. GAPDH was used as a loading control.