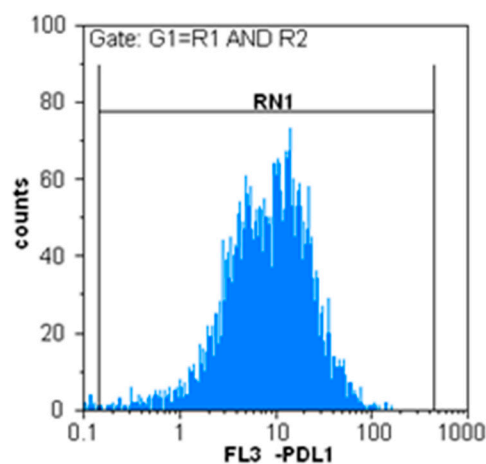


# Supplementary Materials: Effects of a Novel Thiadiazole Derivative with High Anticancer Activity on Cancer Cell Immunogenic Markers: Mismatch Repair System, PD-L1 Expression, and Tumor Mutation Burden

Sofia Sagredou, Panagiotis Dalezis, Eirini Papadopoulou, Maria Voura, Maria V. Deligiorgi, Michail Nikolaou, Mihalis I. Panayiotidis, George Nasioulas, Vasiliki Sarli and Dimitrios T. Trafalis

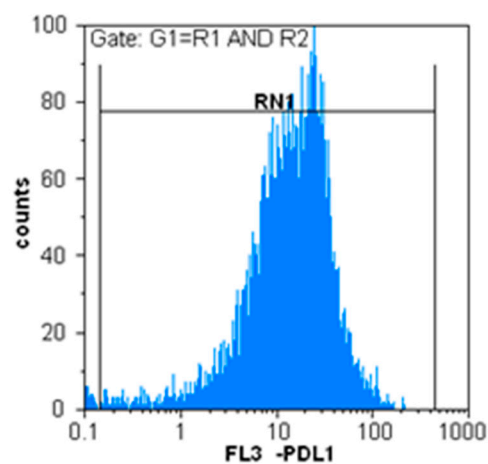
## Flow Cytometric Analysis Data



**Figure S1.** Flow cytometric analysis of PD-L1 expression on untreated DLD-1 cancer cells cultured for 48 h.

**Table S1.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

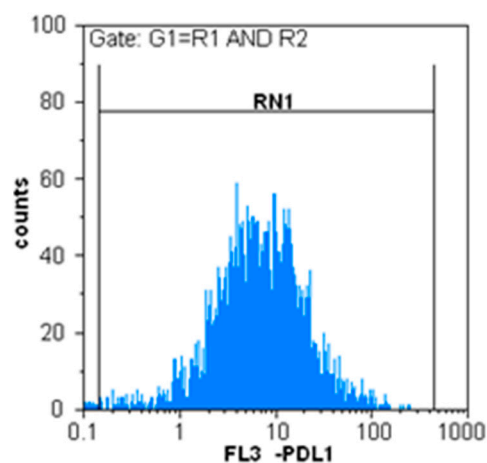
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	6730	6730	-	65.61	2.37	65.80	12.13	47.06
RN1	G1	9784	3989	-	97.34	<b>12.92</b>	104.62	-	-
R2	<None>	4283	4283	-	41.75	0.52	488.28	0.26	10.38



**Figure S2.** Flow cytometric analysis of PD-L1 expression on DLD-1 cancer cells treated with KA39 at IC<sub>50</sub> concentration (µM) for 48 h.

**Table S2.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

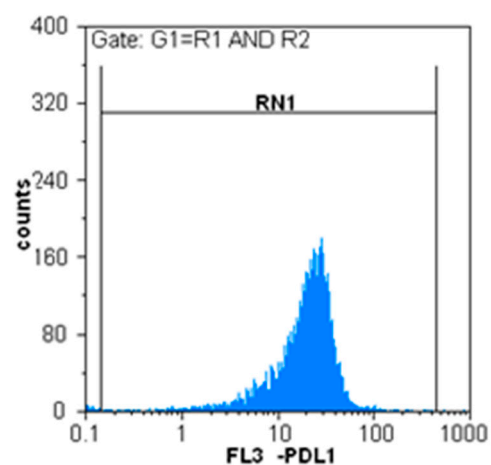
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	7320	7320	-	62.06	2.99	68.31	16.15	40.74
RN1	G1	11050	4991	-	93.69	<b>20.14</b>	97.27	-	-
R2	<None>	5472	5472	-	46.39	0.67	396.09	0.26	9.78



**Figure S3.** Flow cytometric analysis of PD-L1 expression on DLD-1 cancer cells treated with KA39 at IC<sub>50</sub> concentration (µM) for 72 h.

**Table S3.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

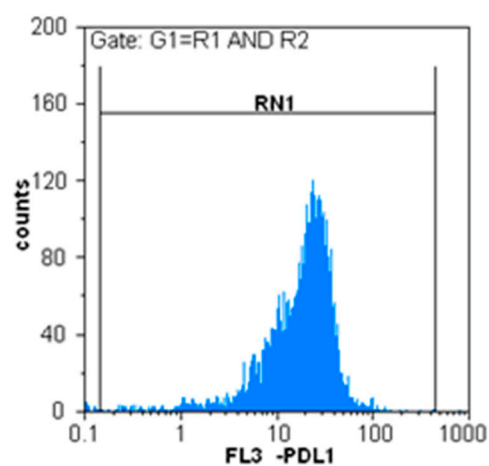
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	6798	6798	-	66.50	2.04	71.35	10.39	44.68
RN1	G1	9803	3383	-	97.05	<b>12.30</b>	144.61	-	-
R2	<None>	3628	3628	-	35.49	0.35	583.91	0.26	9.72



**Figure S4.** Flow cytometric analysis of PD-L1 expression on untreated DLD-1 cancer cells cultured for 72 h.

**Table S4.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

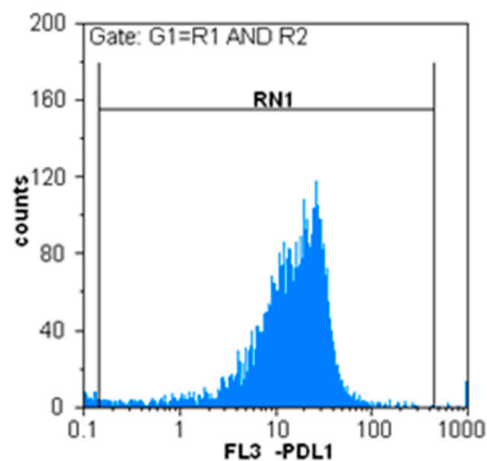
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	11511	11511	-	55.75	3.21	64.50	18.92	42.29
RN1	G1	19656	5599	-	97.14	<b>23.19</b>	75.96	-	-
R2	<None>	6010	6010	-	29.11	2.14	267.75	0.28	10.09



**Figure S5.** Flow cytometric analysis of PD-L1 expression on DLD-1 cancer cells treated with KA39 at TGI concentration ( $\mu$ M) for 72 h.

**Table S5.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

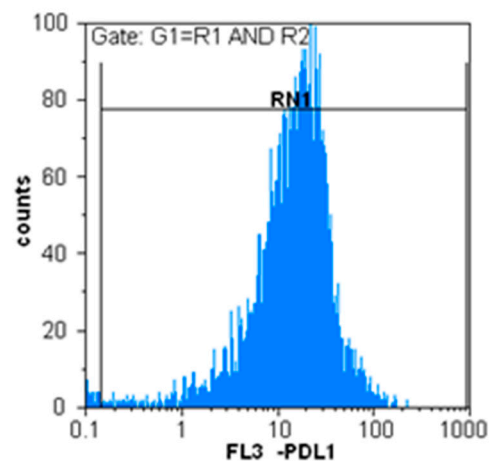
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	8954	8954	-	44.08	2.85	56.68	17.68	39.68
RN1	G1	19308	4172	-	97.20	<b>22.27</b>	77.37	-	-
R2	<None>	4711	4711	-	23.19	1.39	328.99	0.28	9.77



**Figure S6.** Flow cytometric analysis of PD-L1 expression on untreated HT-29 cancer cells cultured for 48 h.

**Table S6.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

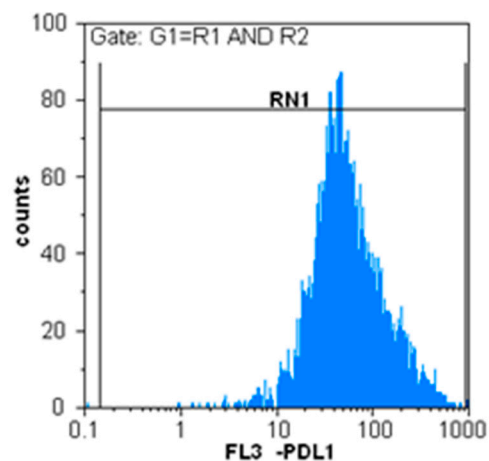
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	7454	7454	-	72.54	3.21	58.94	16.89	40.95
RN1	G1	9513	4681	-	91.03	<b>19.56</b>	104.78	-	-
R2	<None>	5277	5277	-	51.35	1.05	364.10	0.26	10.61



**Figure S7.** Flow cytometric analysis of PD-L1 expression on HT-29 cancer cells treated with KA39 at IC<sub>50</sub> concentration (μM) for 48 h.

**Table S7.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

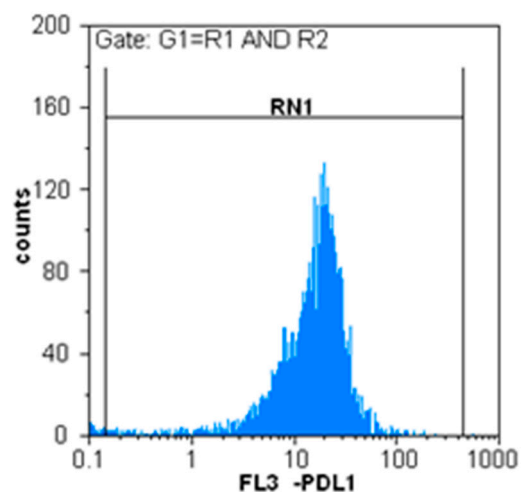
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	7237	7237	-	69.64	4.31	59.31	15.58	40.30
RN1	G1	9855	4524	-	93.88	<b>20.13</b>	92.52	-	-
R2	<None>	4908	4908	-	47.23	0.61	424.39	0.26	9.36



**Figure S8.** Flow cytometric analysis of PD-L1 expression on HT-29 cancer cells treated with KA39 at IC<sub>50</sub> concentration ( $\mu$ M) for 72 h.

**Table S8.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

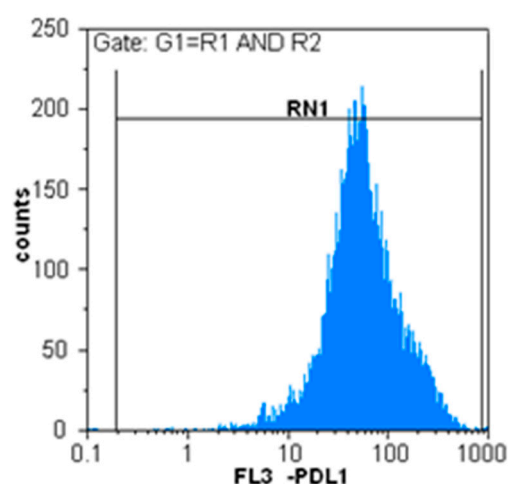
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	6746	6746	-	64.78	7.51	62.31	13.81	39.76
RN1	G1	10280	3698	-	99.76	<b>80.82</b>	108.17	-	-
R2	<None>	3752	3752	-	36.03	0.71	513.16	0.25	8.38



**Figure S9.** Flow cytometric analysis of PD-L1 expression on untreated HT-29 cancer cells cultured for 72 h.

**Table S9.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

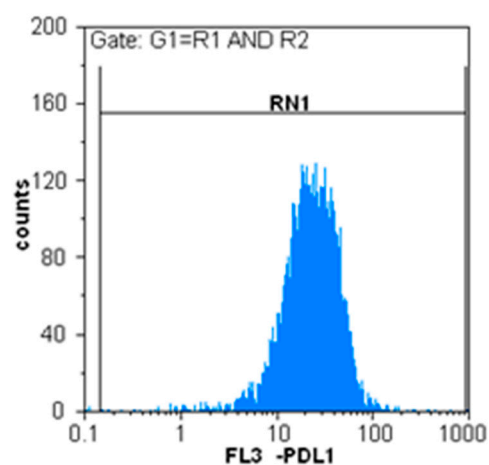
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	10905	10905	-	53.67	3.14	51.40	16.50	35.98
RN1	G1	19242	4057	-	95.28	<b>19.40</b>	75.77	-	-
R2	<None>	4516	4516	-	22.22	0.93	361.94	0.27	8.62



**Figure S10.** Flow cytometric analysis of PD-L1 expression on HT-29 cancer cells treated with KA39 at TGI concentration ( $\mu\text{M}$ ) for 72 h.

**Table S10.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

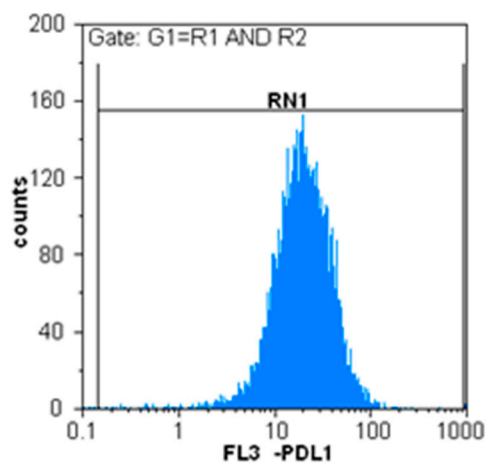
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	15069	15069	-	59.69	6.62	51.50	13.82	35.37
RN1	G1	24557	9083	-	99.67	<b>78.56</b>	95.78	-	-
R2	<None>	9409	9409	-	37.27	0.38	507.46	0.25	8.12



**Figure S11.** Flow cytometric analysis of PD-L1 expression on untreated DU-145 cancer cells cultured for 48 h.

**Table S11.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

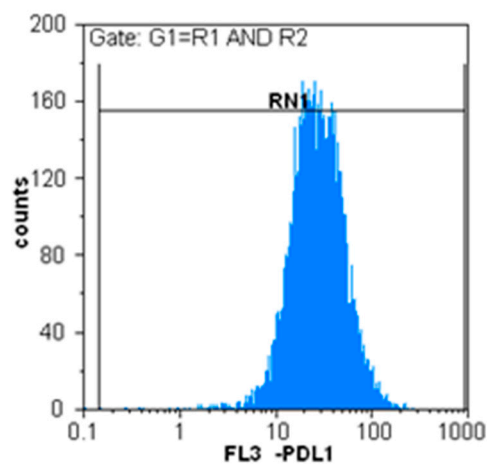
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	6999	6999	-	68.60	3.68	55.48	18.82	36.42
RN1	G1	9957	5387	-	99.57	<b>27.99</b>	79.41	-	-
R2	<None>	5591	5591	-	54.80	1.08	403.67	0.26	10.67



**Figure S12.** Flow cytometric analysis of PD-L1 expression on DU-145 cancer cells treated with KA39 at IC<sub>50</sub> concentration (μM) for 48 h.

**Table S12.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

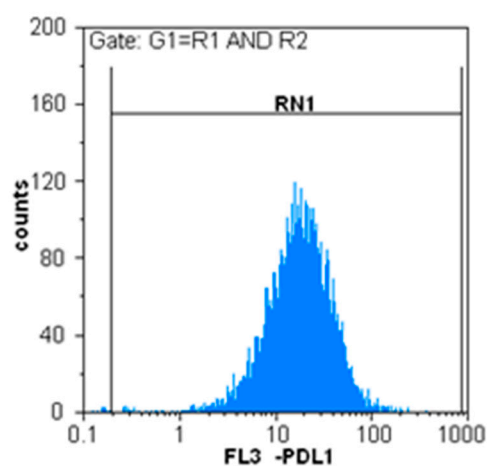
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	7513	7513	-	71.72	3.43	49.02	18.89	32.36
RN1	G1	10201	6007	-	99.29	<b>24.48</b>	77.58	-	-
R2	<None>	6175	6175	-	58.95	0.75	419.37	0.26	9.44



**Figure S13.** Flow cytometric analysis of PD-L1 expression on DU-145 cancer cells treated with KA39 at IC<sub>50</sub> concentration (μM) for 72 h.

**Table S13.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

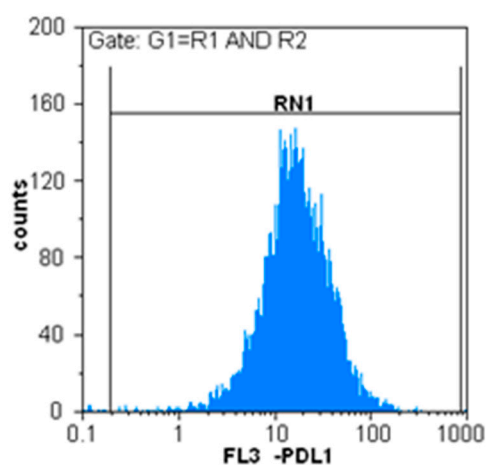
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	8257	8257	-	75.15	5.15	55.46	21.17	35.94
RN1	G1	10802	7069	-	99.80	<b>33.55</b>	69.66	-	-
R2	<None>	7199	7199	-	65.52	1.50	387.07	0.27	11.54



**Figure S14.** Flow cytometric analysis of PD-L1 expression on untreated DU-145 cancer cells cultured for 72 h.

**Table S14.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	6907	6907	-	34.03	3.59	61.76	16.72	39.44
RN1	G1	19285	5088	-	99.10	<b>23.46</b>	86.03	-	-
R2	<None>	5726	5726	-	28.21	0.74	444.21	0.26	9.41

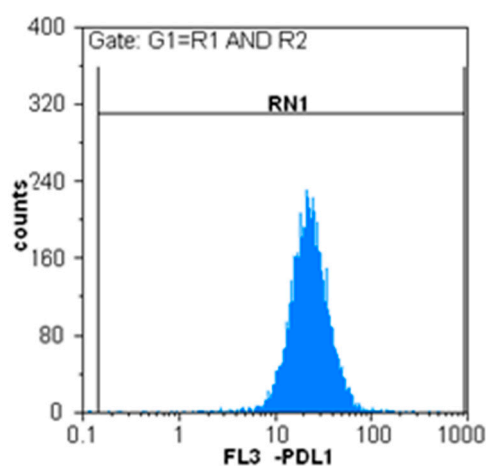


**Figure S15.** Flow cytometric analysis of PD-L1 expression on DU-145 cancer cells treated with KA39 at TGI concentration ( $\mu$ M) for 72 h.

**Table S15.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	8572	8572	-	42.07	3.65	65.18	18.34	41.79
RN1	G1	19546	6736	-	99.44	<b>23.02</b>	94.70	-	-
R2	<None>	7311	7311	-	35.88	1.18	405.80	0.26	9.86

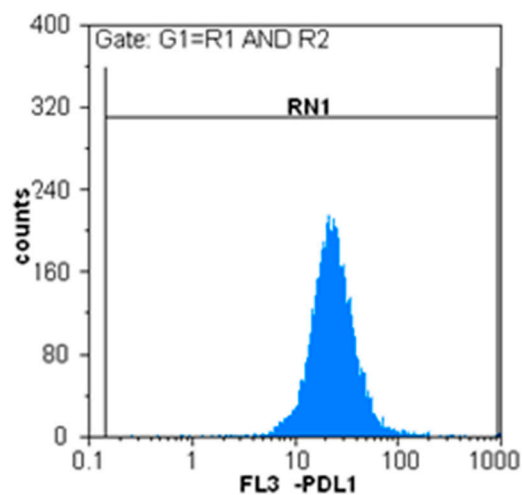




**Figure S16.** Flow cytometric analysis of PD-L1 expression on untreated PC-3 cancer cells cultured for 48 h.

**Table S16.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

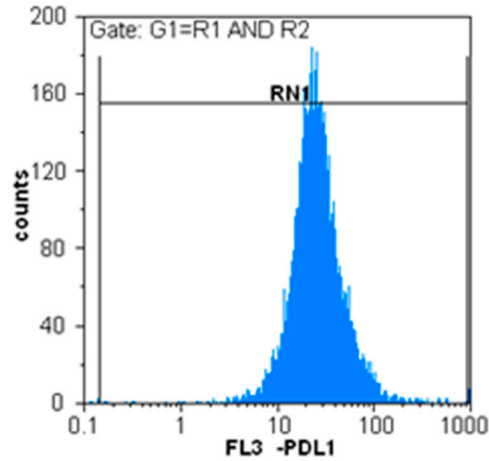
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	8410	8410	-	81.44	3.17	55.75	16.59	32.58
RN1	G1	10263	6134	-	99.89	<b>25.91</b>	64.58	-	-
R2	<None>	6212	6212	-	60.16	0.35	521.31	0.25	6.99



**Figure S17.** Flow cytometric analysis of PD-L1 expression on PC-3 cancer cells treated with KA39 at IC<sub>50</sub> concentration (µM) for 48 h.

**Table S17.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

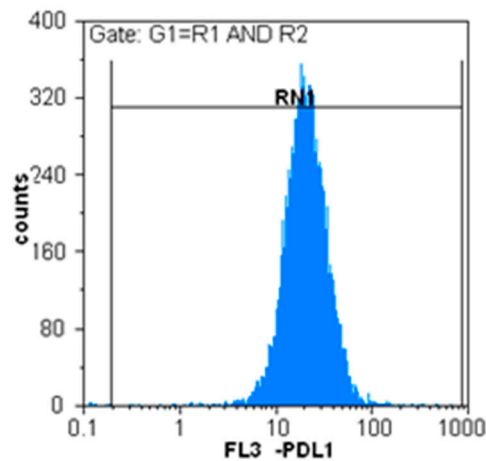
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	8404	8404	-	80.95	4.19	61.38	17.63	34.04
RN1	G1	10257	6087	-	99.90	<b>27.66</b>	83.06	-	-
R2	<None>	6169	6169	-	59.42	0.42	444.45	0.25	7.22



**Figure S18.** Flow cytometric analysis of PD-L1 expression on PC-3 cancer cells treated with KA39 at IC<sub>50</sub> concentration (μM) for 72 h.

**Table S18.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

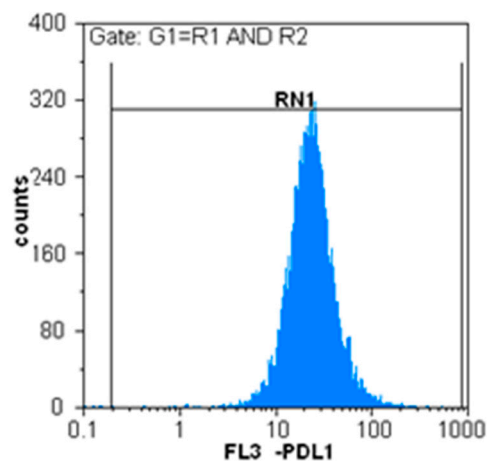
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	8381	8381	-	80.02	6.37	53.97	16.00	36.76
RN1	G1	10305	5663	-	99.68	<b>32.98</b>	108.90	-	-
R2	<None>	5751	5751	-	54.91	0.34	475.01	0.25	7.03



**Figure S19.** Flow cytometric analysis of PD-L1 expression on untreated PC-3 cancer cells cultured for 72 h.

**Table S19.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

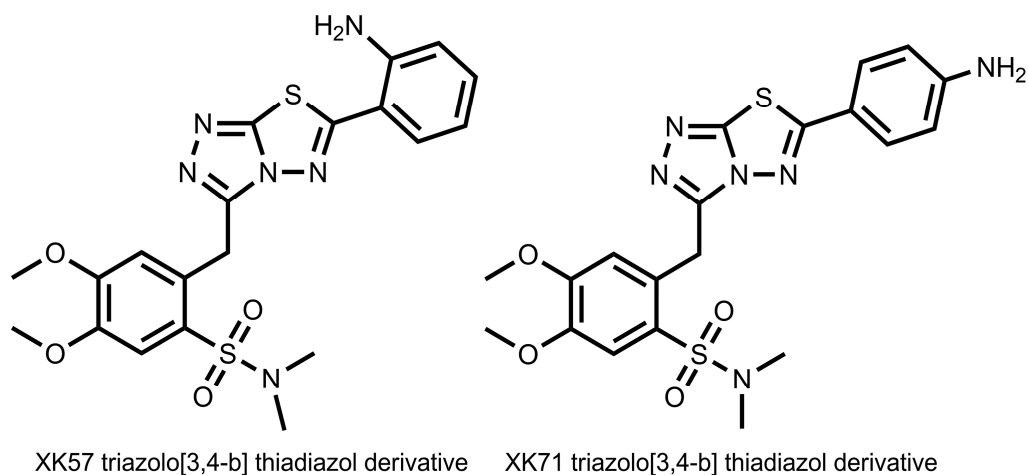
Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	14475	14475	-	61.79	3.23	47.03	17.16	27.35
RN1	G1	22965	10842	-	99.83	<b>23.93</b>	69.66	-	-
R2	<None>	11321	11321	-	48.33	0.33	460.49	0.25	6.47



**Figure S20.** Flow cytometric analysis of PD-L1 expression on PC-3 cancer cells treated with KA39 at TGI concentration ( $\mu\text{M}$ ) for 72 h.

**Table S20.** Region analysis – calculations as resulted from the CyFlow® Partec Report software.

Region	Gate	Ungated	Count	Count/mL	%Gated	Mean-x	CV-x%	Mean-y	CV-y%
R1	<None>	12827	12827	-	61.02	5.46	51.65	17.35	33.49
RN1	G1	20598	9903	-	99.84	<b>28.21</b>	74.87	-	-
R2	<None>	10297	10297	-	48.98	0.36	469.83	0.26	7.40



**Figure S21.** Chemical structures of XK 57: 2-((6-(2-aminophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-3-yl)methyl)-4,5-dimethoxy-*N,N*-dimethylbenzenesulfonamide and XK 71: 2-((6-(4-aminophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-3-yl)methyl)-4,5-dimethoxy-*N,N*-dimethylbenzenesulfonamide.

**Table S21.** Cytostatic (GI<sub>50</sub>, TGI) and cytotoxic effects (IC<sub>50</sub>) induced by XK57 and XK71 on the tested human cancer cell lines, DLD-1, HT-29, LS174T, DU-145 and PC-3.

Cancer Cell Lines	XK57 GI <sub>50</sub>	XK57 TGI	XK57 IC <sub>50</sub>	XK71 GI <sub>50</sub>	XK71 TGI	XK71 IC <sub>50</sub>
DLD-1	90 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M
HT-29	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M
LS174T	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M
DU-145	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M
PC-3	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M	>100 $\mu$ M

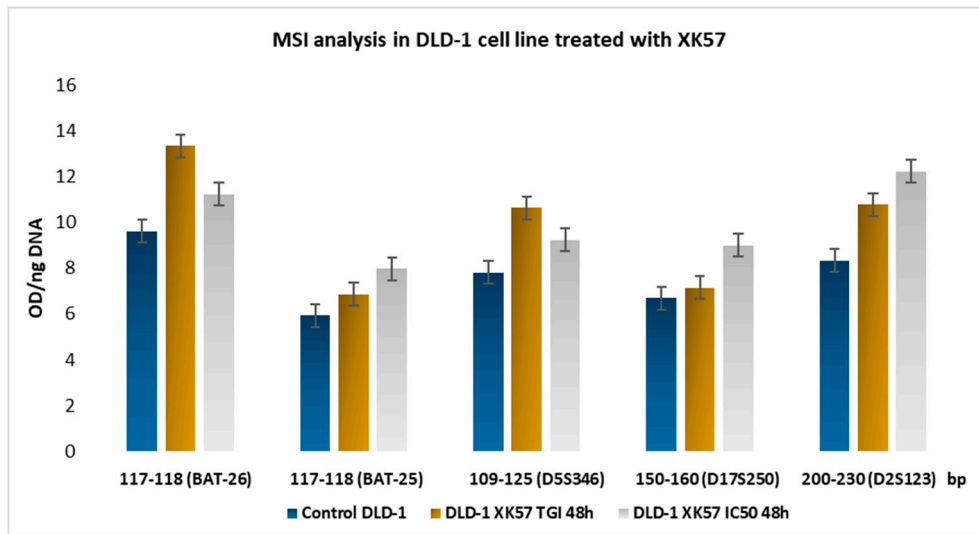
**Table S22.** The absolute values of PD-L1 expression as defined by flow cytometric analysis in untreated (control) and treated cells with XK57 derivative at IC<sub>50</sub> concentration ( $\mu$ M) for 48 and 72 h, as well as at TGI concentration ( $\mu$ M) for 72 h. The units of measurements are expressed as mean of immuno-fluorescence intensity counted per cell.

Cancer cell lines	PD-L1 Expression				
	Control 48 h	XK57 IC <sub>50</sub> 48 h	XK57 IC <sub>50</sub> 72 h	Control 72 h	XK57 TGI 72 h
DLD-1	16.89 $\pm$ 0.84	19.88 $\pm$ 0.99	15.77 $\pm$ 0.94	25.09 $\pm$ 1.25	26.54 $\pm$ 1.59
HT-29	23.34 $\pm$ 1.4	24.79 $\pm$ 1.98	26.34 $\pm$ 1.31	21.23 $\pm$ 2.12	23.19 $\pm$ 1.62
DU-145	28.89 $\pm$ 2.02	27.19 $\pm$ 2.17	32.09 $\pm$ 2.24	24.56 $\pm$ 1.96	25.18 $\pm$ 1.25
PC-3	27.14 $\pm$ 2.44	28.44 $\pm$ 1.42	29.08 $\pm$ 2.03	27.88 $\pm$ 1.39	29.67 $\pm$ 2.07

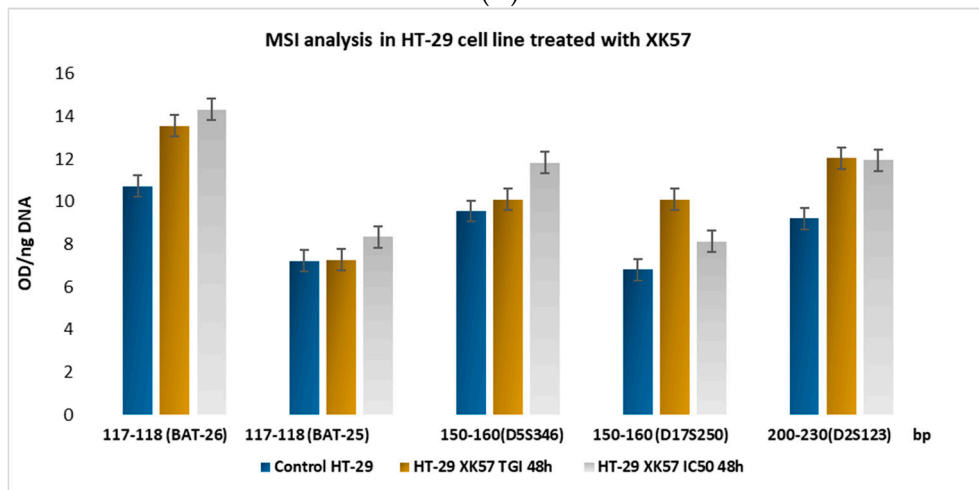
**Table S23.** The absolute values of PD-L1 expression as defined by flow cytometric analysis in untreated (control) and treated cells with XK71 derivative at IC<sub>50</sub> concentration ( $\mu$ M) for 48 and 72 h, as well as at TGI concentration ( $\mu$ M) for 72 h. The units of measurements are expressed as mean of immuno-fluorescence intensity counted per cell.

Cancer cell lines	PD-L1 expression				
	Control 48 h	XK71 IC <sub>50</sub> 48 h	XK71 IC <sub>50</sub> 72 h	Control 72 h	XK71 TGI 72 h
DLD-1	14.09 $\pm$ 0.7	17.45 $\pm$ 1.04	13.43 $\pm$ 0.67	22.81 $\pm$ 1.14	21.19 $\pm$ 1.05
HT-29	22.78 $\pm$ 1.36	21.31 $\pm$ 1.06	27.32 $\pm$ 1.63	20.59 $\pm$ 1.23	24.64 $\pm$ 1.47
DU-145	25.35 $\pm$ 1.26	27.53 $\pm$ 1.65	28.77 $\pm$ 2.01	26.89 $\pm$ 2.15	25.04 $\pm$ 1.75
PC-3	28.19 $\pm$ 1.97	30.98 $\pm$ 2.78	26.19 $\pm$ 1.83	25.12 $\pm$ 1.75	27.23 $\pm$ 2.17

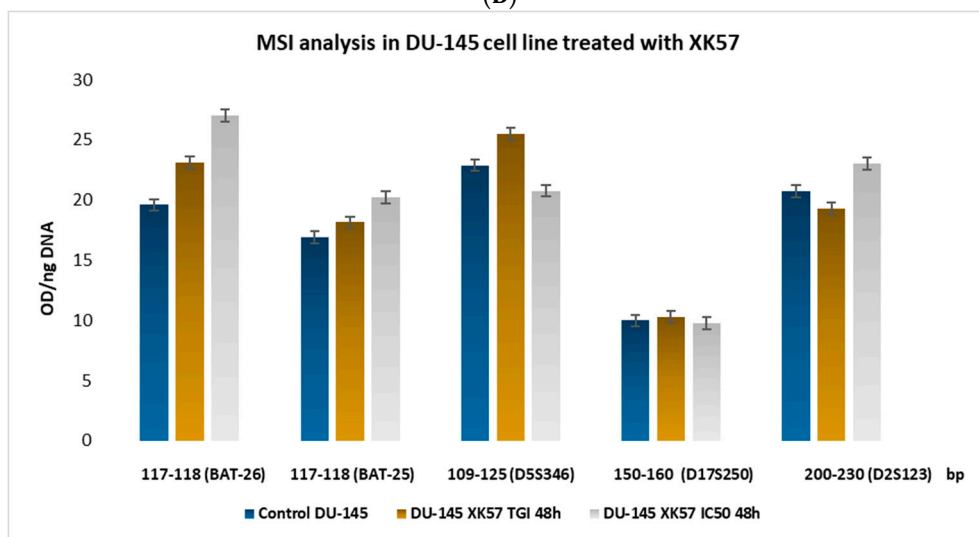
**MSI analysis in DLD-1, HT-29, DU-145, PC-3 and LS174T cancer cells treated with XK71 and XK57 derivatives**



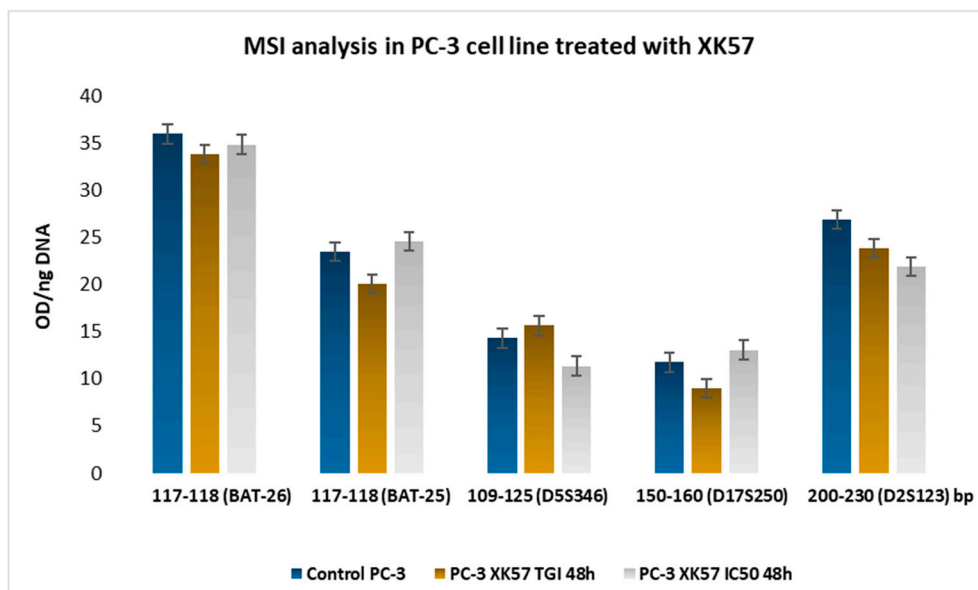
(A)



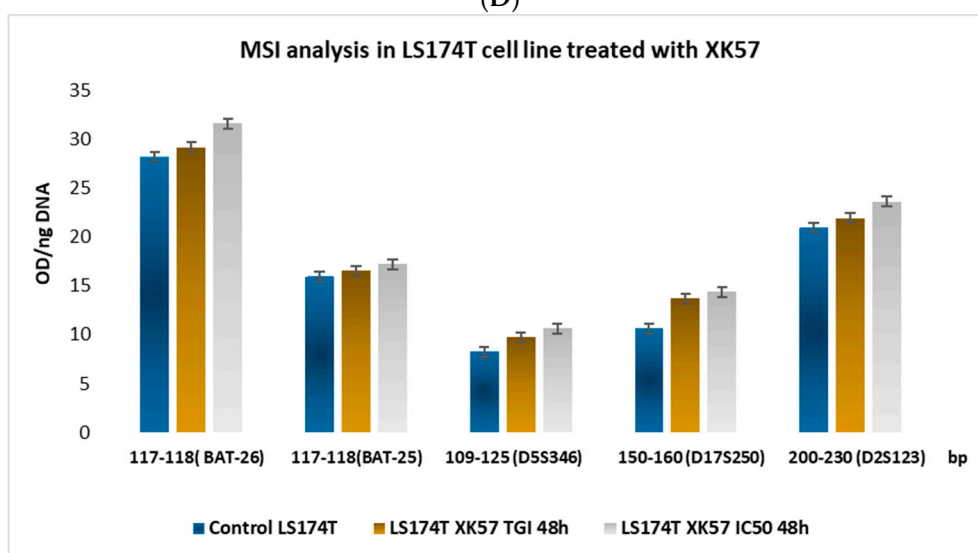
(B)



(C)

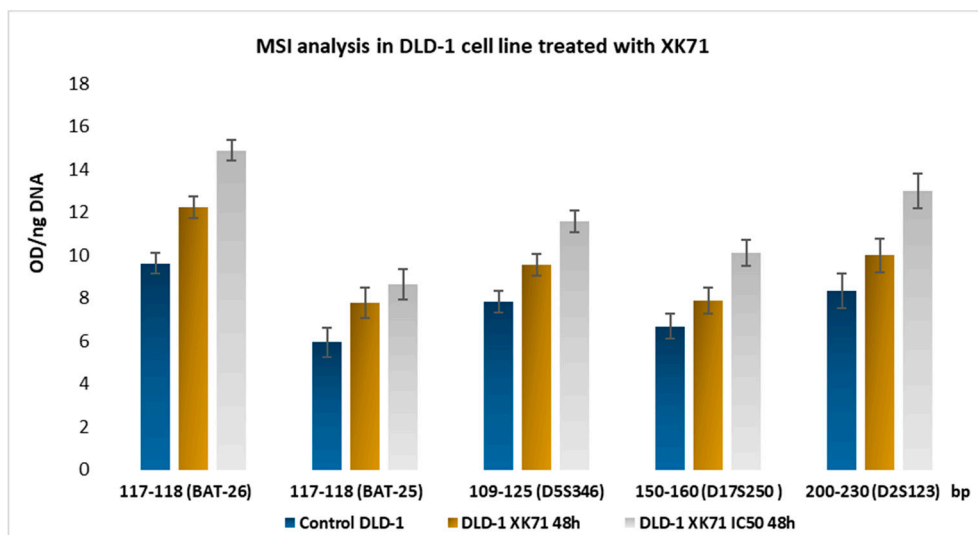


(D)

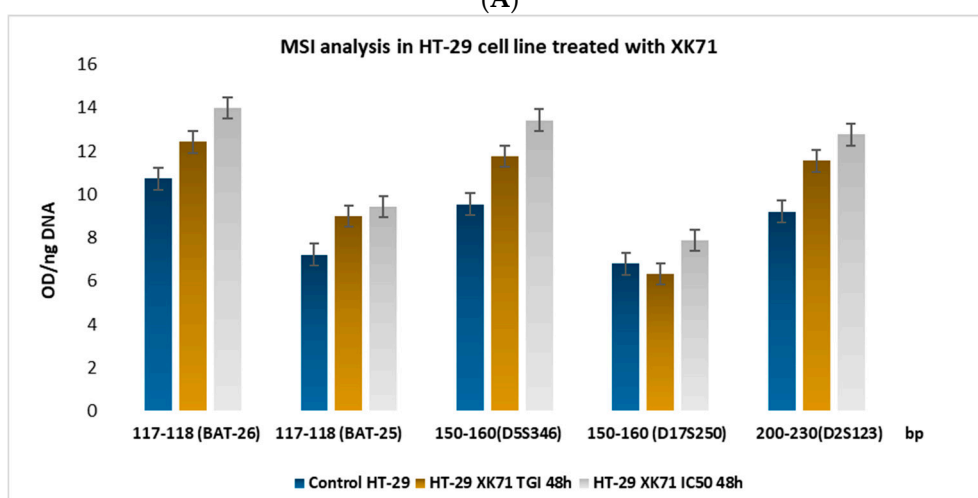


(E)

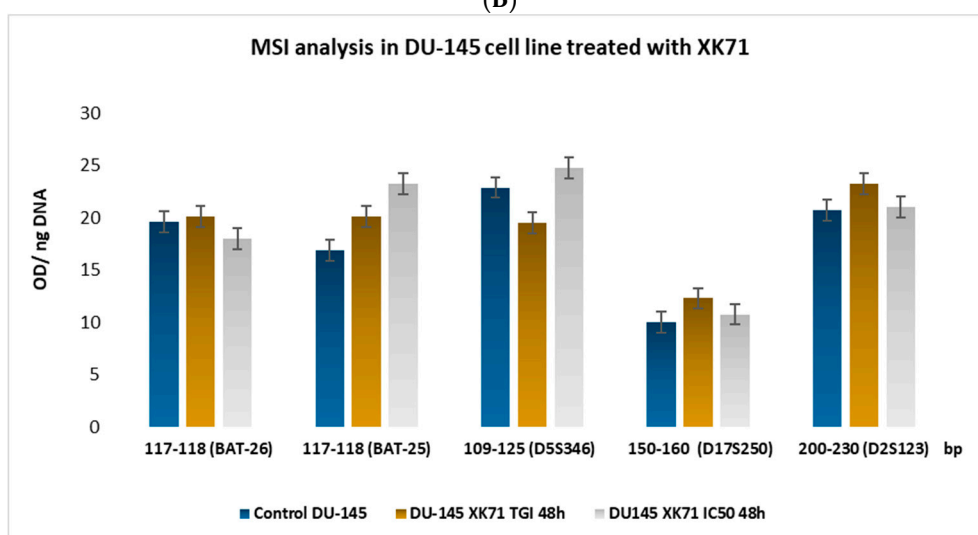
**Figure S22.** MSI alterations (mean  $\pm$  SEM) induced by XK57 triazolo[3,4-b]thiadiazole derivative (per ng DNA) in five human cancer cell lines. (A), (B), (C), (D) and (E) demonstrate the MSI alterations in DLD-1, HT-29, DU-145, PC-3, and LS174T cancer cells, treated with XK57 at the TGI concentration and IC<sub>50</sub> ( $\mu$ M) for 48h, respectively. Quantitative MSI fragments analysis conducted in Bethesda panel (BAT-26, BAT-25, D5S346, D17S250 and D2S123). Student t-test;  $p > 0.01$ ; no statistical significance level.



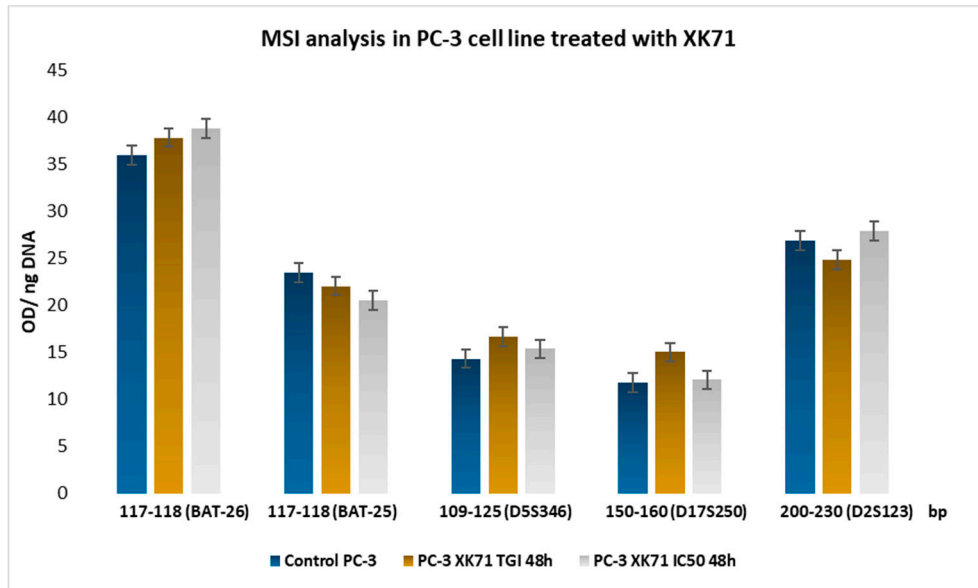
(A)



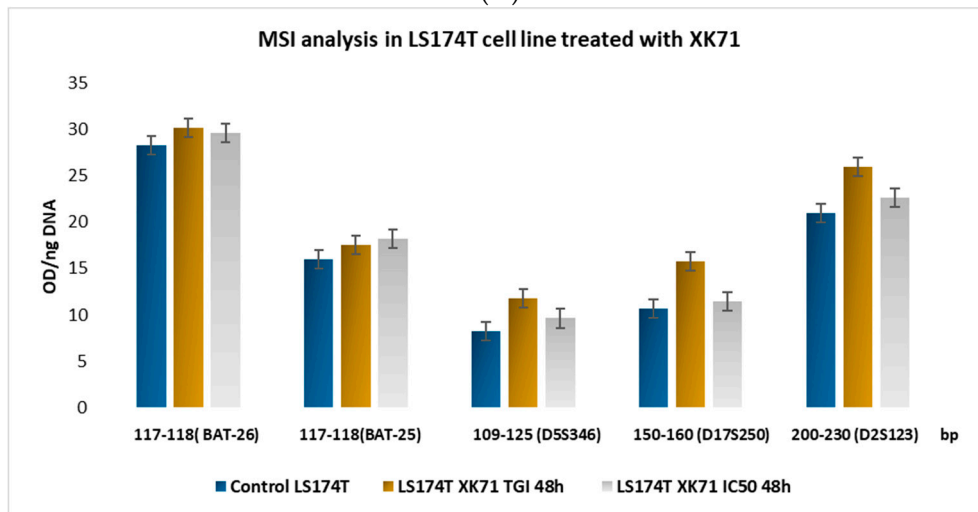
(B)



(C)



(D)



(E)

**Figure S23.** MSI alterations (mean  $\pm$  SEM) induced by XK71 triazolo[3,4-*b*]thiadiazole derivative (per ng DNA) in five human cancer cell lines. (A), (B), (C), (D) and (E) show the MSI alterations in DLD-1, HT-29, DU-145, PC-3, and LS174T cancer cells, treated with XK71 at the TGI concentration and IC<sub>50</sub> ( $\mu$ M) for 48h, respectively. Quantitative MSI fragments analysis conducted in Bethesda panel (BAT-26, BAT-25, D5S346, D17S250 and D2S123). Student t-test;  $p > 0.01$ ; no statistical significance level.