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Patient Bone Marrow Aspiration to Explore the Cyclooxygenases (COXs) Involvement in Multiple Myeloma

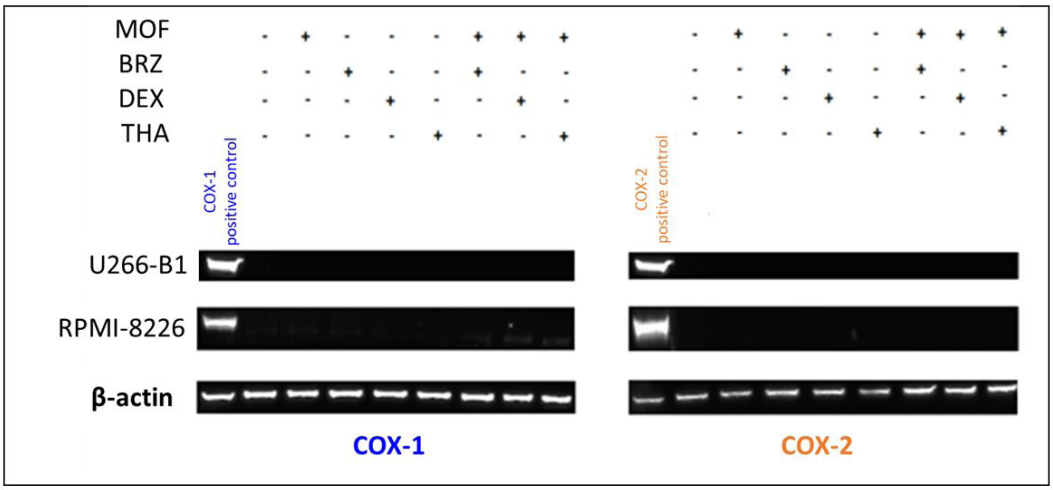

Figure S1: Expressions of COX-1 and COX-2 proteins in U266 and RPMI-8226 cell lines upon treatment with mofezolac alone or in combination with bortezomib dexamethasone or thalidomide. Cells were incubated for 48 h with (+) or without (-) mofezolac (55 µM in U266 and 70 µM in RPMI-8226), dexamethasone (100 µM both in U266 and RPMI-8226) or thalidomide (100 µM both in U266 and RPMI-8226) used at different concentrations on the basis of their EC50 values. The cell homogenates (30 µg protein) were applied to Western blotting. HEK-293 COX-1 and HEK-293 COX-2 were used as positive controls for COX-1 and COX-2 expression, respectively [34]. The protein level of β-actin was used as loading control. The experiments were repeated three times, and essentially the same results were obtained. Marker Biorad Precision Plus Protein™ Dual Color Standards, 500 µl #1610374

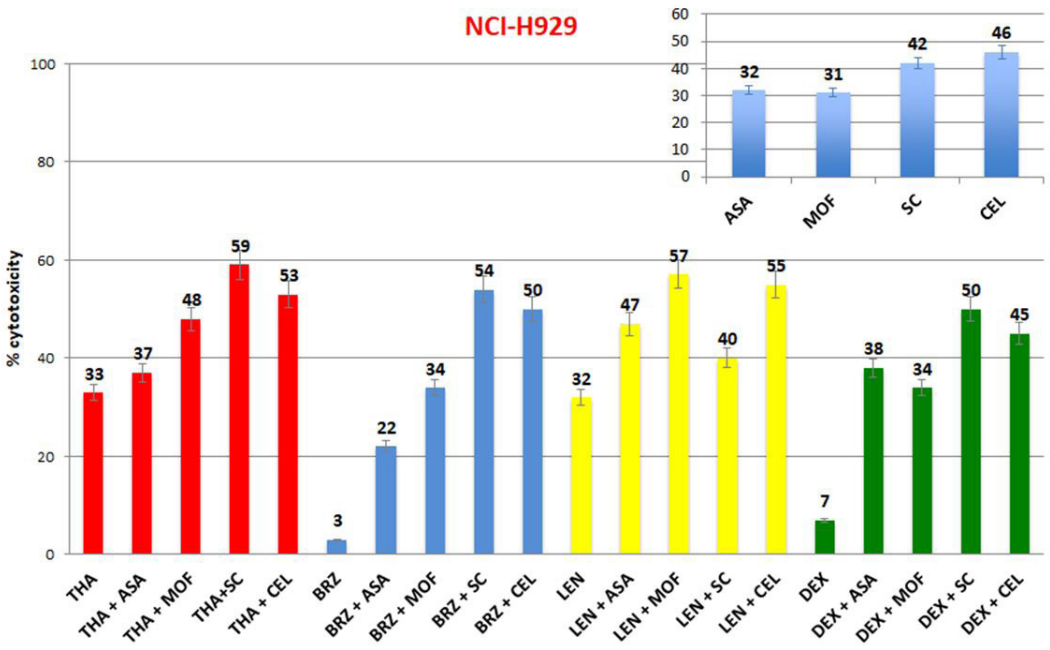

Figure S2: Antiproliferative activity of anti-MM drugs alone and in combination with COX inhibitors after 48h incubation time with NCI-H929 cells. Error bars represent mean ± SD of three experiments in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: $p < 0.05$ excepted for THA vs THA + ASA

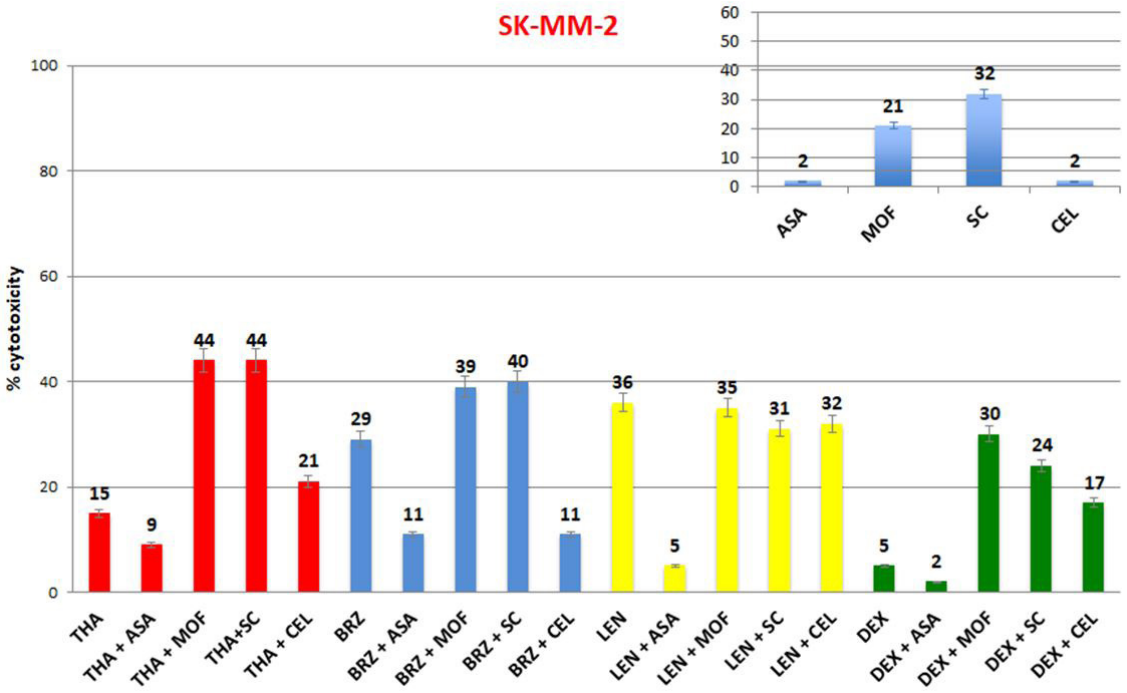

Figure S3: Antiproliferative activity of anti-MM drugs alone and in combination with COX inhibitors after 48h incubation time with SK-MM-2 cell lines. Error bars represent mean ± SD of three experiments in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: $p < 0.05$ excepted for LEN vs LEN + MOF

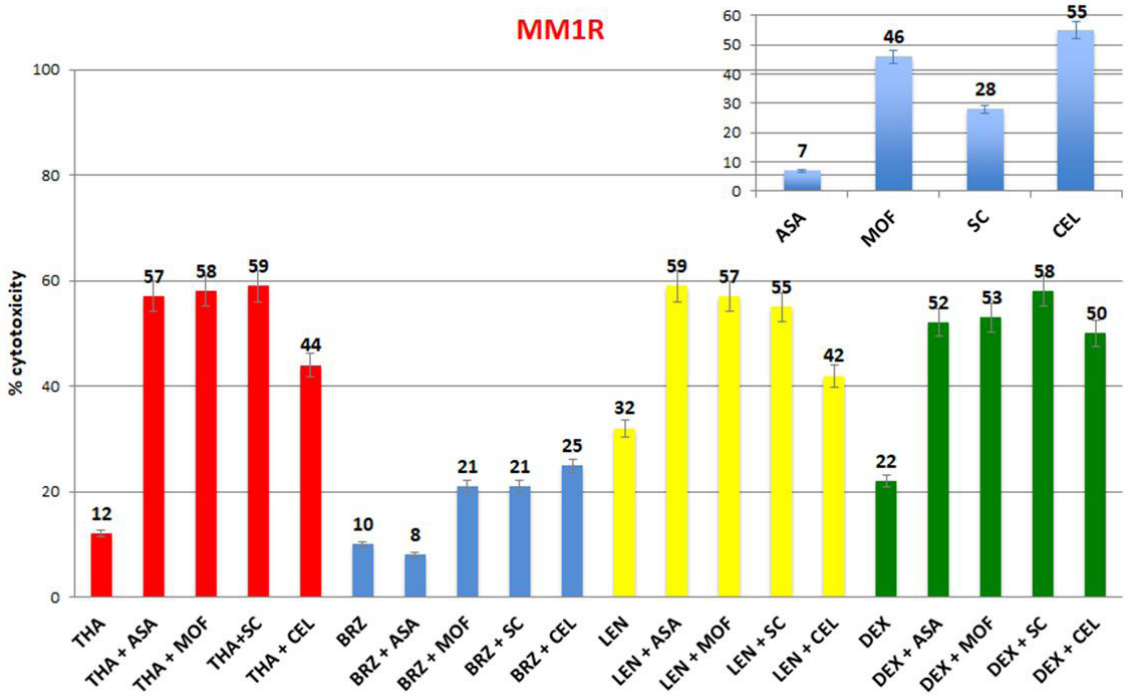

Figure S4: Antiproliferative activity of thalidomide (100 μM), lenalidomide (100 μM), dexamethasone (100 μM) and bortezomib (2 nM) alone or in combination with aspirin (75 μM), celecoxib (75 μM), mofezolac (75 μM) or SC 560 (70 μM) after 48h treatment of MM1R. Error bars represent mean ± SD of three experiments in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: $P<0.05$ except for BRZ vs BRZ + ASA.

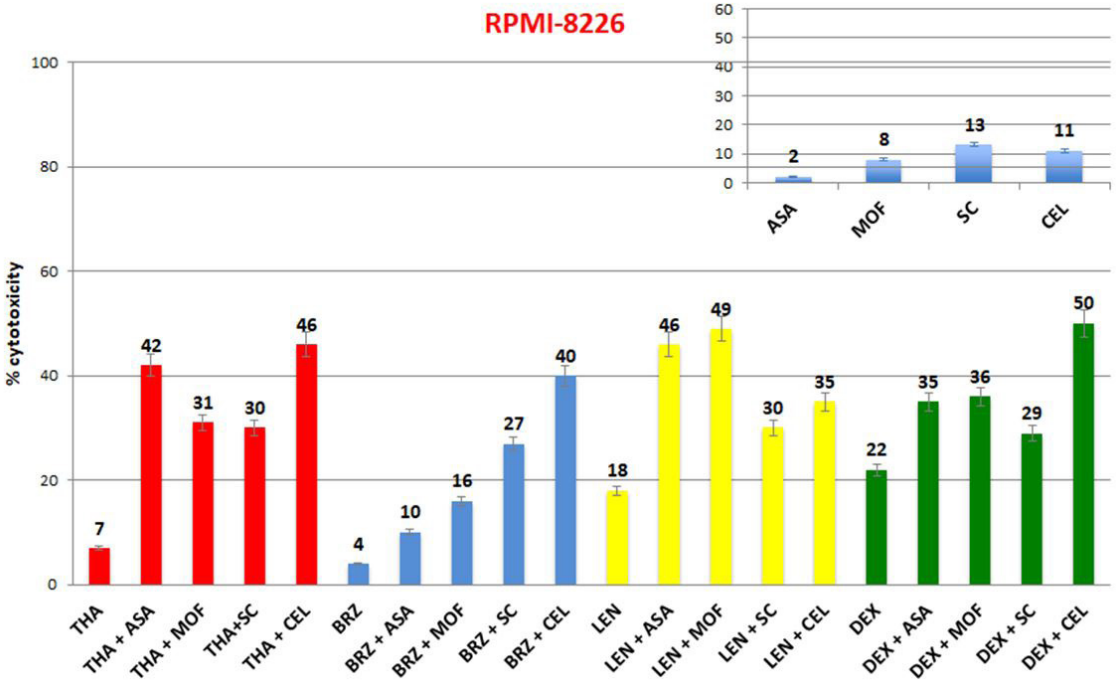

Figure S5: Antiproliferative activity of thalidomide, lenalidomide, dexamethasone (100 μM) and bortezomib (3 nM) alone or in combination with aspirin (75 μM), celecoxib (65 μM), mofezolac (70 μM) or SC 560 (40 μM) after 48h treatment on RPMI-8226. Error bars represent mean ± SD of three experiments in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: P<0.05

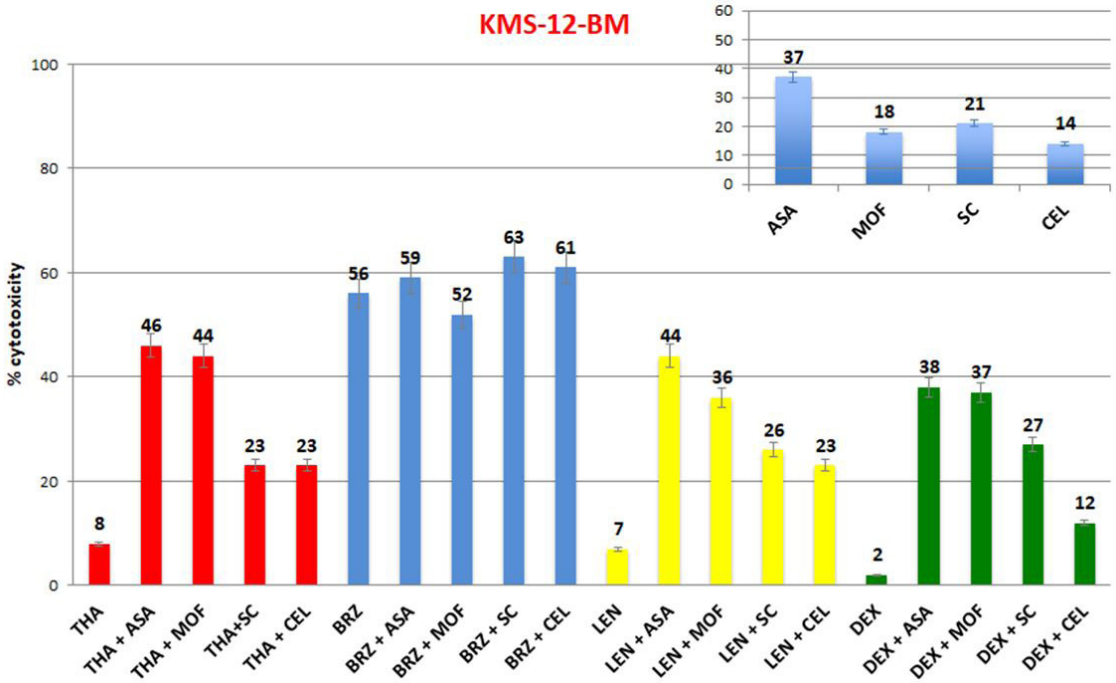

Figure S6: Antiproliferative activity of thalidomide (50 μM), lenalidomide (50 μM), dexamethasone (50 μM) and bortezomib (7 nM) alone or in combination with aspirin (75 μM), celecoxib (70 μM), mofezolac (70 μM) or SC 560 (50 μM) after 48h treatment of KMS-12-BM. Error bars represent mean ± SD of three experiments in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: P<0.05

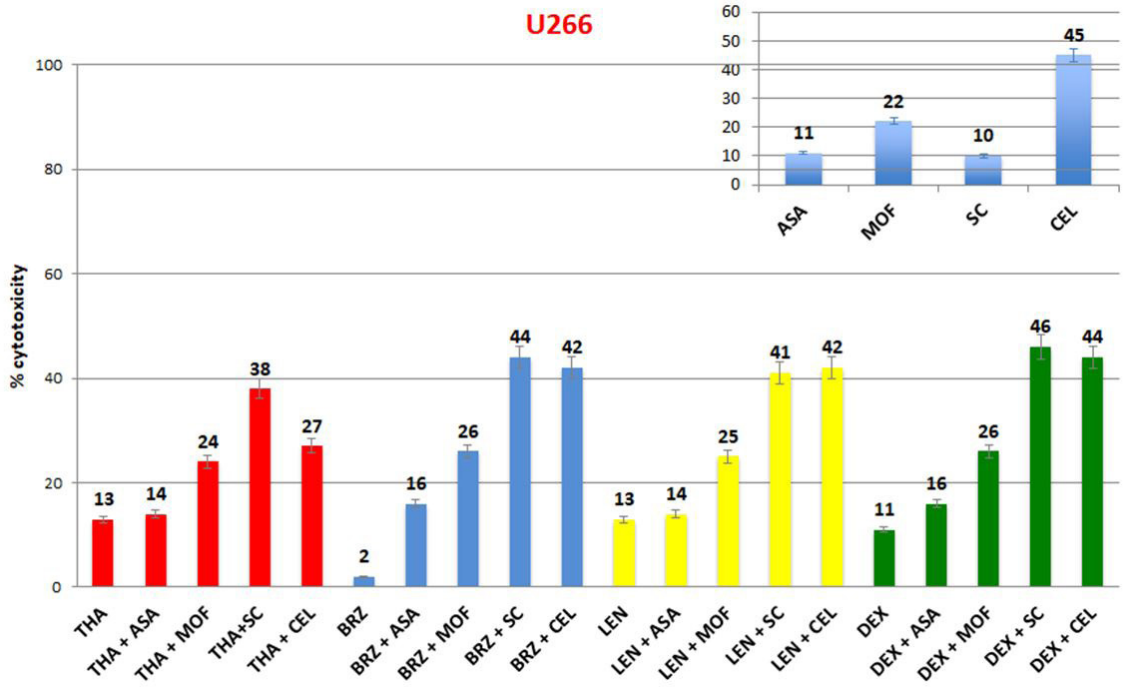

Figure S7: Antiproliferative activity of thalidomide (100 μM), lenalidomide (100 μM), dexamethasone (100 μM) and bortezomib (3 nM) alone or in combination with aspirin (65 μM), celecoxib (75 μM), mofezolac (55 μM) or SC 560 (80 μM) after 48h treatment on U266. Error bars represent mean ± SD of three experiments in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: $P<0.05$ except for THA vs THA + ASA and LEN vs LEN + ASA

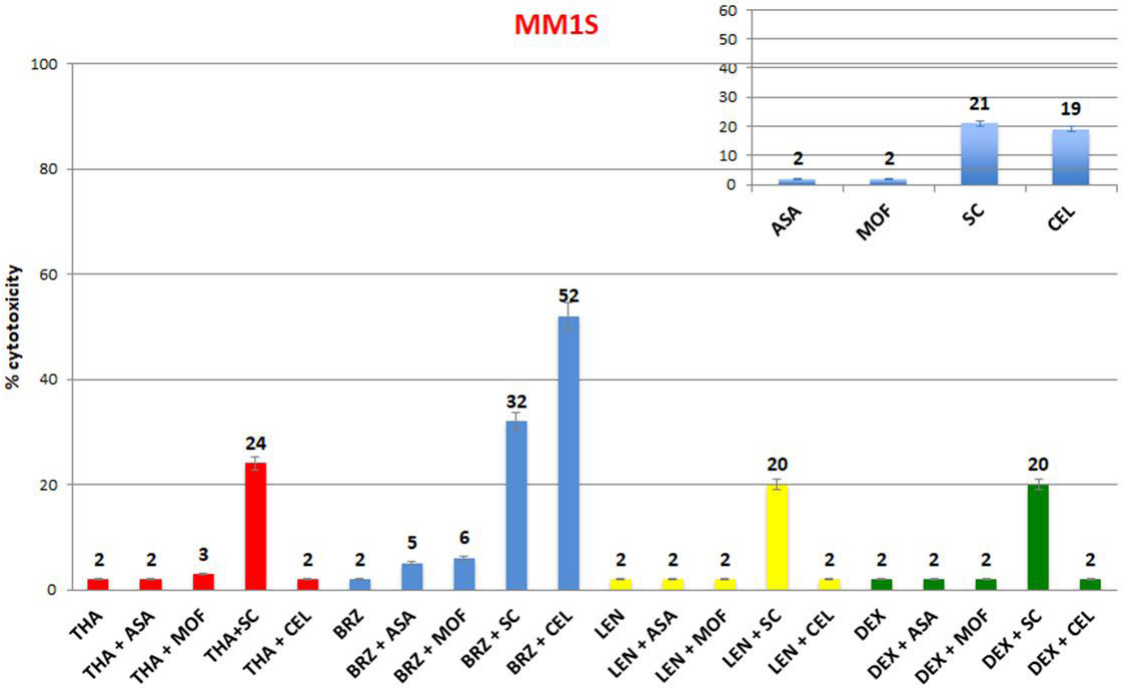

Figure S8: Antiproliferative activity of thalidomide (100 μM), lenalidomide (100 μM), dexamethasone (100 μM) and bortezomib (8 nM) alone or in combination with aspirin (75 μM), celecoxib (75 μM), mofezolac (100 μM) or SC 560 (50 μM) after 48h treatment of MM1S. Error bars represent mean ± SD of three experiments in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: $P>0.05$ except for THA vs THA + SC; BRZ vs BRZ + SC; BRZ vs BRZ + CEL; LEN vs LEN + SC and DEX vs DEX + SC

COX activity by PGE_2 and TXB_2 production measurement and effect on NF-kB activation

PGE_2 and TXB_2 biosynthesis was evaluated in supernatants of NCI-H929 cells at 48 hours incubation time in the presence of the different drugs. To evaluate how anti-MM drugs and COX inhibitors could affect PGE_2 and TXB_2 production and NF-κB activation, cells were treated with bortezomib, dexamethasone and thalidomide alone or in combination with the highly selective COX-1 inhibitor mofezolac, or celecoxib, a selective COX-2 inhibitor. In resting cells, NF-κB is sequestered in the cytoplasm in complexes with its endogenous inhibitor IκB. In response to various stimuli, IκB undergoes phosphorylation by IκB kinases (IKK), ubiquitination, and subsequent proteasome-dependent degradation. Then, free NF-κB heterodimer (p65/p50) rapidly translocate to the nucleus to initiate transcription activity by binding to regulatory κB motifs on target genes [26,37]. To determine NF-κB activation, p65 protein was extracted from NCI-H929 cell nucleus after treatments with the drugs. PGE2 and TXB2 biosynthesis, and NF-κB activation in treated cells did not change respect to the untreated cells content (control) (Table S1).

Moreover, NF-κB activation was evaluated in the seven MM cell lines with (+) or without (-) LPS (Table S2). In LPS-stimulated RPMI-8226, MM1R and MM1S an higher NF-κB activation was registered, whereas in the other MM cell lines, NF-κB activation did not changed in the LPS-treated counterparts.

Table S1: Percentage (%) effect of bortezomib (BRZ, 2nM), dexamethasone (DEX, 35 μM), thalidomide (THA, 45 μM), celecoxib (CEL, 70 μM) and mofezolac (MOF, 75 μM) on PGE2 and TXB2 biosynthesis, and NF-κB (ng/ml) activation at 48 hours in NCI-H929 cells. Values are the means ± SEM of three independent experiments carried out in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: $p > 0.05$

	PGE_2 (%)	TXB_2 (%)	NF-κB (%)
Control	**65±1.2**	**51±2**	**43±1.4**
BRZ	65±1.3	40±0.7	44±0.1
BRZ + MOF	59±1.4	47±1.2	33±1.4
BRZ + CEL	56±0.2	46±1.5	30±1.8
DEX	63±0.4	43±1.8	58±1.5
DEX + MOF	60±0.5	42±1.9	30±1.8
DEX + CEL	51±1.2	47±0.4	37±0.4
THA	67±0.7	45±0.5	45±0.1
THA + MOF	61±0.4	40±0.9	38±0.7
THA + CEL	61±0.3	45±0.4	37±1.5
MOF	60±1.1	46±0.1	46±0.7
CEL	56±0.2	46±0.2	57±3.1

Table S2: Percentage (%) NF-κB activation (ng/ml) at 48 hours with (+) or without (-) lipopolysaccharide (LPS) in seven different cell lines derived from human myeloma (NCI-H929, RPMI-8226, U266-B1, MM1R, MM1S, KMS-12-BM, and SK-MM-2). Values are the means ± SEM of three independent experiments carried out in triplicate; one-way ANOVA followed by Bonferroni's post-hoc comparison test: $p < 0.05$ excepted for KMS-12-BM and SK-MM-2

	NCI-H929		RPMI-8226		U266-B1		MM1R		MM1S		KMS-12-BM		SK-MM-2	
LPS	-	+	-	+	-	+	-	+	-	+	-	+	-	+
NF-κB	43 ±1.2	68 ±0.4	58 ±0.3	100 ±1.3	44 ±0.4	69 ±0.3	50 ±0.2	100 ±1.3	40 ±0.7	100 ±0.1	100 0.2±	100 ±0.1	42 ±0.4	40 ±1.2