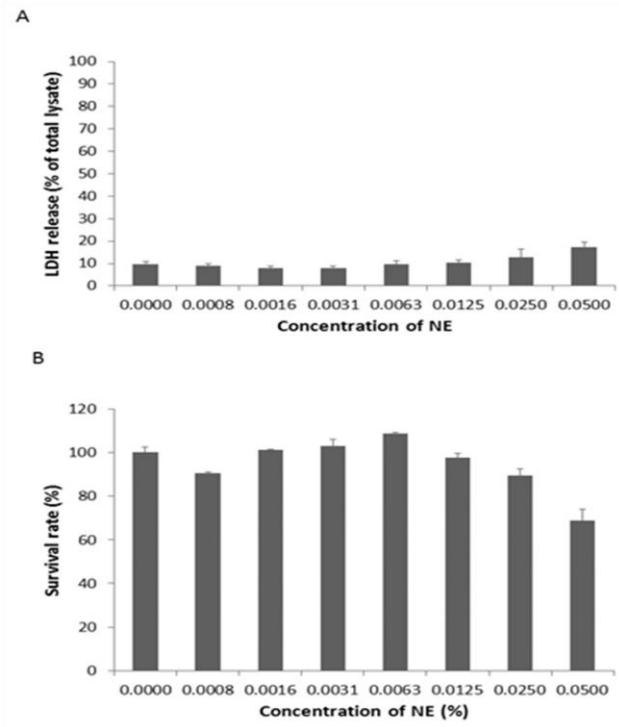
Supplemental Data

Assessment of subcytolytic concentration of NE on TC-1 cells

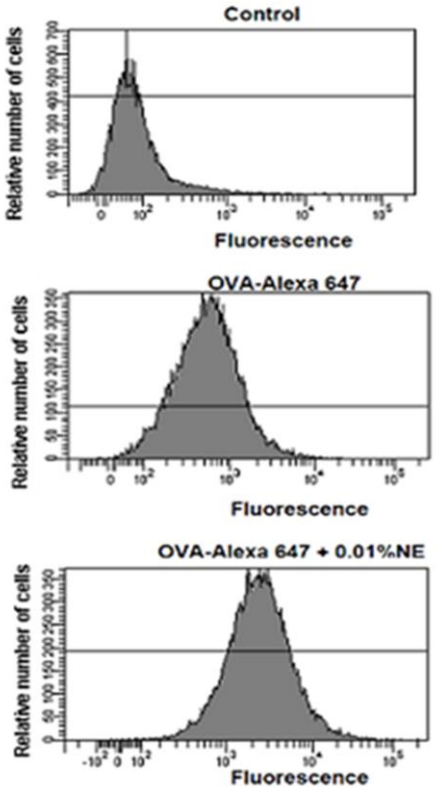
Cell survival rate and cytotoxicity were measured using the XTT and LDH assays, respectively. Briefly, TC-1 cells ($1x10^4$ cells) in 100 µL of RPMI 1640 were added to wells of a 96-well plate and incubated overnight. The next day, the cells were treated with increasing concentrations of $W_{80}5EC$ and incubated for an additional 24 hours before XTT or LDH assays were applied. The optical density of the wells was recorded on an ELISA reader (Spectra Max 340, Molecular Devices, Sunnyvale, CA).

Supplemental Figure 1 shows that the concentrations of $W_{80}5EC$ nanoemulsion used to investigate the effect of NE on epithelial cells (0.025 and 0.05%) were not cytolytic. Based on these two assays, the sub-cytolytic concentration of NE was assessed (see Supplemental Figure 1). Therefore, in subsequent experiments 0.025% and 0.05% concentrations of NE were used to study the effect of NE on cells.



Supplemental Figure 1. Cytotoxic effect of NE on TC-1 cells as measured in LDH (a) and XTT (b) assays.

$W_{80}5EC$ promotes antigen uptake by BMDs in vitro



Supplemental Figure 2. The effect of NE on antigen uptake by mouse BMDCs. The cells were incubated with OVA-Alexa 647 alone or with W_{80}5EC for 4 hours and then analyzed using flow cytometry.

We also assessed whether BMDCs respond to W_{80}5EC NE with increased antigen uptake. BMDCs were incubated four hours with OVA-Alexa Fluor 647 (Life Technologies) (5 μg/mL) alone or with 0.01% W_{80}5EC, washed and analyzed using flow cytometer. BMDCs treated with W_{80}5EC had MFI values 5 times higher than BMDCs treated with OVA-AlexaFluor 647 alone (**Supplemental Figure 2**).

NE causes cell cycle arrest at G_2/M phase and apoptosis of TC-1 cells

Cell-cycle analysis was performed as described previously [1]. Briefly, TC-1 cells ($5x10^4$ cells/mL) were treated with indicated concentrations of W_{80}5EC or staurosporine (Roche Diagnostics GmbH) for the indicated periods of time. After the treatment cells were fixed in 75% cold ethanol and stored at -20°C. For analysis cells were resuspended in 500 μL of staining solution containing 0.1% (v/v) Triton X-100, 10 μg PI (Roche Diagnostics GmbH), and 100 μg DNase free RNase (Sigma-Aldrich) in PBS, incubated for 40 minutes at 37°C, and analyzed using flow cytometry.

The TC-1 cells were cultured and treated as described for cell cycle analysis. After treatment cells were harvested and stained with annexin V-FITC (BioVison) and PI and analyzed using flow cytometry.

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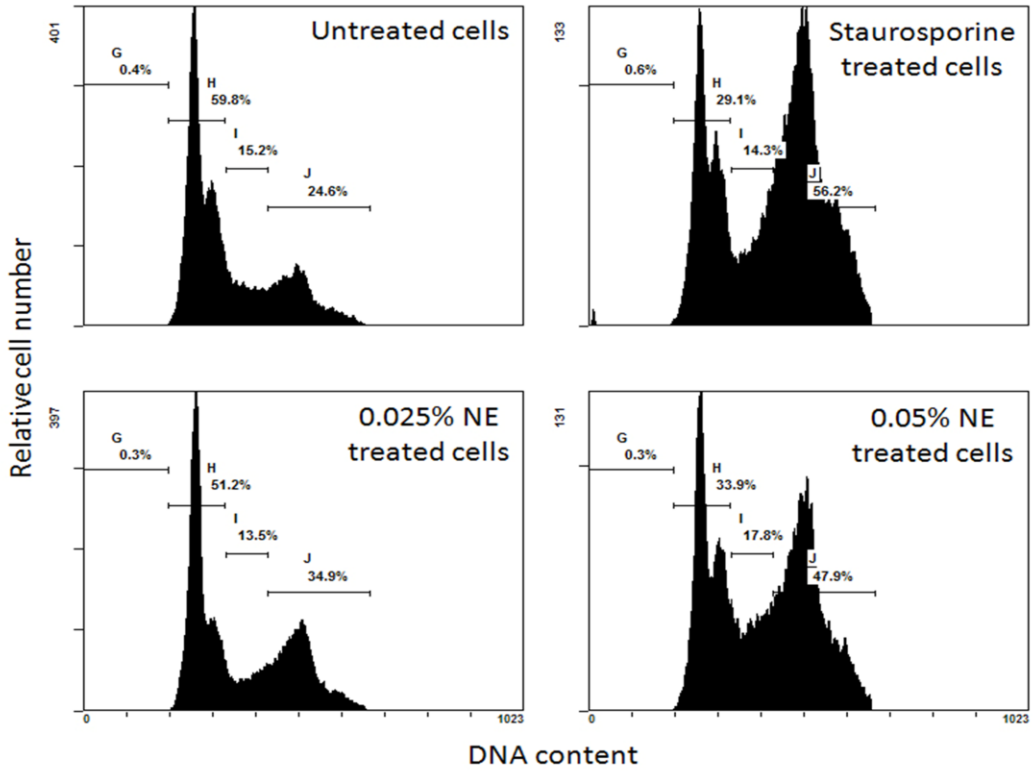
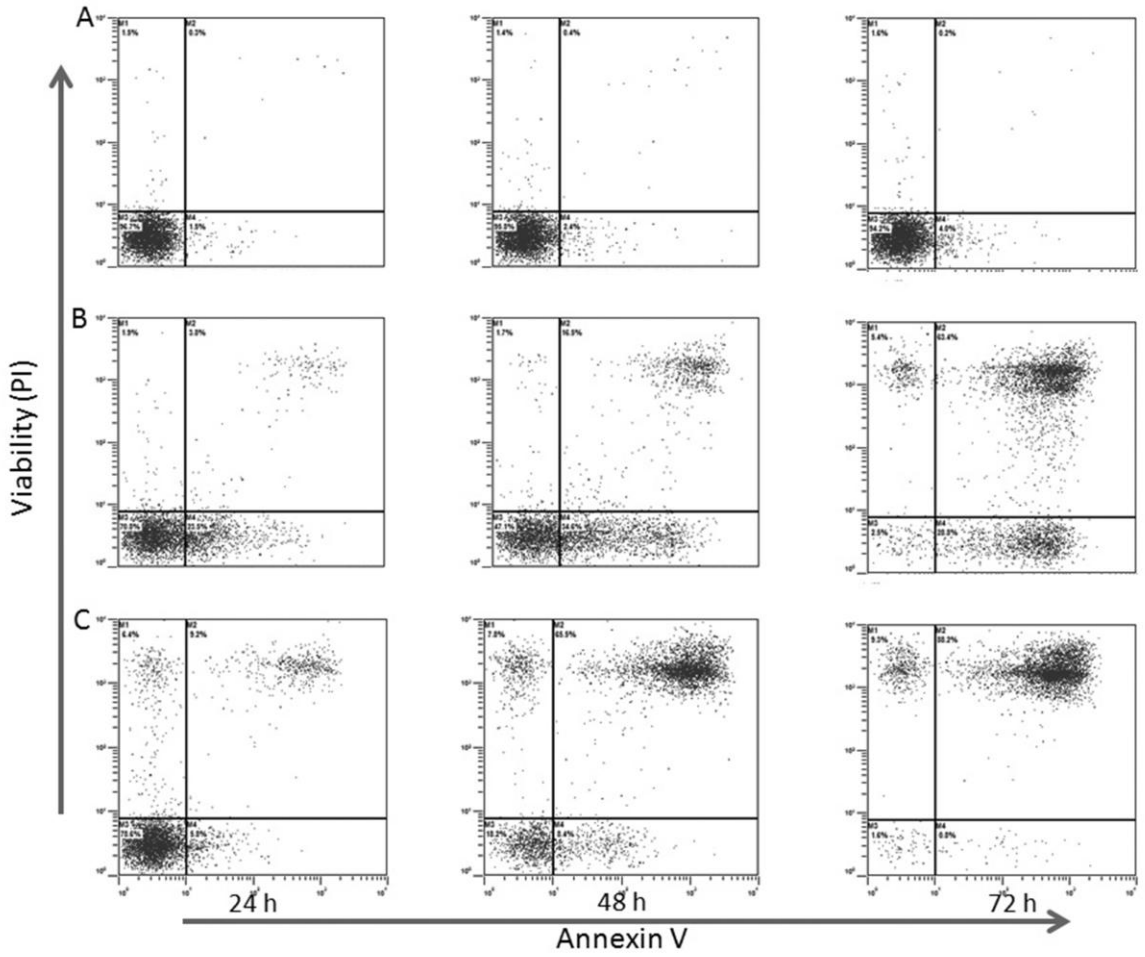


Supplemental Figure 3. Cell cycle analysis TC-1 cells. The cells were treated 24 hours with staurosporine (1μM) or NE. Data shown are from one of two independent experiments.

determine whether $W_{80}5EC$ could induce cell-cycle aberration, TC-1 cells were treated with $W_{80}5EC$ overnight and cell-cycle analysis was performed using flow cytometry. We observed an increase of cells in the G_2/M phase from 24.6% in untreated cells to 34.9% in cells treated with 0.025% $W_{80}5EC$, and 47.9% in cells treated with 0.05% $W_{80}5EC$ (**Supplemental Figure 3A, C, and D**). Treatment of cells with 1 μM staurosporine used as a positive control also caused cell-cycle arrest at G_2/M (**Supplemental Figure 3B**).

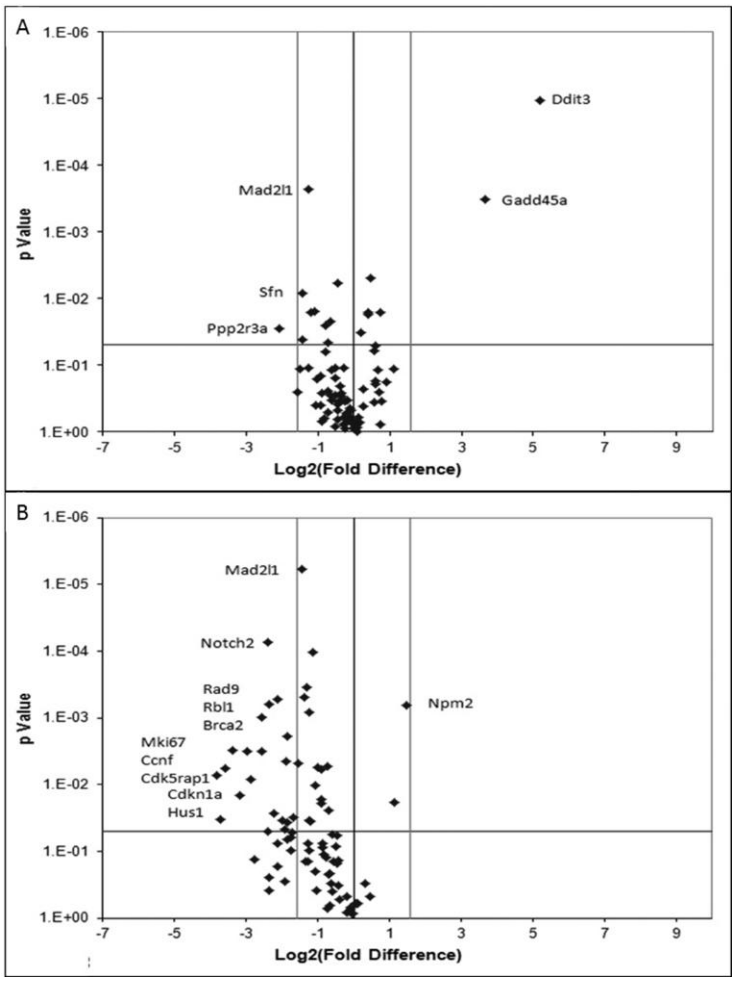
To detect apoptosis TC-1 cells were treated with $W_{80}5EC$ for 24, 48 and 72 hours, stained with



Supplemental Figure 4. Flow cytometry scatter plot of TC-1 cells untreated (A), treated with 1μM staurosporine (B), and treated with 0.05% $W_{80}5EC$. The cells were incubated for indicated periods of time, and then harvested, washed, stained with annexin V and PI, and analyzed using flow cytometry. Data shown are from one of five independent experiments.

annexin V (apoptosis) and PI (necrosis), and analyzed by flow cytometry. Treatment of TC-1

cells with W_{80}5EC resulted in approximately 8% early apoptotic cells at 48 hr (annexin V fluorescence alone) and 65% late apoptotic cells (annexin V and PI fluorescence). At 72 hr time point the fraction of late apoptotic cells increased to 88% and at that time point no early apoptotic cells were recorded (**Supplemental Figure 4C**). In contrast, staurosporine-treated cells (**Supplemental Figure 4B**) showed only 16% late apoptotic/necrotic cells, while one third of the cells were early apoptotic after 48-hour treatment. At 72 hours, the fraction of late apoptotic/necrotic cells increased up to 63%. The progression of NE-treated TC-1 cells from early apoptosis to late apoptosis occurs faster than in the cells treated with staurosporine.



Supplemental Figure 5. The RT-PCR analysis of 84 genes involved in the cell-cycle pathway. The TC-1 cells were treated with 0.05% NE (A) or 1 μM staurosporine (B) for 6 hours before the RT-PCR analysis. The vertical line indicates a fold-change in gene expression of 1. The two vertical side lines indicate 3 fold-change in gene expression threshold. The horizontal line indicates the 0.05 threshold for the p value of the t-test. Data shown are representative of one experiment. In order to get statistical significance each sample (i.e., untreated control, NE and staurosporine) in this comparison was run 3 times.

RT-PCR analysis of genes involved in cell cycle arrest and apoptosis regulation

Gene expression analysis was performed with real-time transcription RT-PCR. The mRNA was isolated from TC-1 cells treated with 0.05% W_{80}5EC or 1 μM staurosporine for 6 hours and from control cells using RNAzol®RT, following the protocol from Molecular Research Center, Inc. The analysis was performed using the RT^2 First Strand Kit, RT^2 Profiler ^{TM}PCR Array, and RT^2 SYBR^R Green qPCR Master Mix from SABiosciences (Qiagen Company). The PCR array was run on an Eppendorf realplex^2 Mastercycler epgradient S. Data analysis was performed using the DDC_t Method [2]. The fold-change for each gene from the control to the experimental group was calculated as 2^(-DDC_t).

RT-PCR analysis was performed to examine whether W_{80}5EC and staurosporine treatments have a similar effect on genes involved in cell-cycle arrest and apoptosis. As shown in **Supplemental Figure 5**, 0.05% W_{80}5EC had a significant effect of gene transcription of two genes involved in cell-cycle checkpoints and cell-cycle arrest: Ddit3 (36 fold) and Gadd45a (13 fold) as compared to untreated control cells (**Supplemental Figure 5A**). These genes are members of a group of genes that increase transcript levels following growth arrest conditions or treatment with DNA-damaging agents. In contrast, the pattern of gene expression in cells treated with staurosporine was different. Staurosporine-treated cells showed significant down-regulation of 10 genes: G_1 phase and G_1/S transition gene - Nfatc1; S phase and DNA replication gene - Mki67; cell-cycle checkpoint and cell-cycle arrest genes - Brca2, Cdk5rap1 and Notch2; regulation of cell-cycle genes - Ccna2, Ccnb1, Ccnf; and negative regulation of the cell-cycle gene – Rbl1. RT-PCR

analysis was also performed to examine whether genes are differentially regulated in apoptotic cells with staurosporine and NE.

Supplemental Table 1. Down-regulation of genes involved in apoptosis as measured using RT-PCR. TC-1 cells were treated with either 1μM staurosporine or 0.05% W_{80}5EC. The mRNA was isolated and was subjected to real-time reverse transcription PCR .

Staurosporine			**Nanoemulsion**		
Symbol	Log2(FC)	p Value	Symbol	Log2(FC)	p Value
Bok	**-5.81**	**0.017**	**Bok**	**-2.38**	**0.031**
Casp6	**-4.21**	**0.003**	**Casp6**	**-2.09**	**0.026**
Tnfrsf11b	**-2.02**	**0.030**	**Tnfrsf11b**	**-2.80**	**0.018**
Bcl2l1	-2.76	0.003	Casp14	-2.71	0.012
Bnip3	-5.33	0.020	Casp8	-2.09	0.045
Bnip3l	-2.59	0.018	Trp73	-2.23	0.002
Casp1	-2.49	0.022			
Casp4	-3.89	0.026			
Casp7	-3.37	0.003			
Casp9	-3.66	0.013			
Cradd	-2.90	0.012			
Dapk1	-2.34	0.033			
Dffb	-3.74	0.011			
Hells	-2.69	0.038			
Ltbr	-3.75	0.021			
Polb	-2.57	0.033			
Ripk1	-5.11	0.005			
Sphk2	-2.68	0.033			
Tnfrsf1a	-3.87	0.013			
Trp53bp2	-2.69	0.005			

RT-PCR analysis was performed to examine gene expression in cells treated with staurosporine and NE. After treatment with staurosporine 20 of 84 measured genes involved in apoptosis were down-regulated, while cells treated with NE showed only 6 genes down-regulated. Three of the six genes down-regulated by NE were also down-regulated by staurosporine (BCL2-related ovarian killer protein, Caspase 6 and Tumor Necrosis Factor; see **Supplemental Table 1**).

References

1. Darzynkiewicz Z: Nucleic Acid Analysis. In: *Current Protocols in Cytometry.* Edited by Robinson J. New York J Wiley & Sons, Inc; 1997.
2. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001, 25(4):402-408.