Electronic Supplementary Material (ESI) for Toxicology Research.

Supplementary Information

Effect of counter ion and combination of constituents on the toxicity of DODAX:MO nanocarriers *in vitro* and *in vivo*

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Zebrafish embryogenesis statistical analysis

One-way ANOVA results on zebrafish embryos epiboly percentage at 8 hpf showed no significant interaction among different DODAX:MO ratios of liposomes. In fact, with exception of 9 μM of DODAX, which caused a significant decrease of the epibolic arc percentage (comparatively with control group: 0 μM DODAX), no significant interaction was observed among groups, for both the free-compounds and liposomes (see Table S1).

When considering the effects of DODAX surfactants (administered as free lipids) on the HTA of 32 hpf zebrafish, the statistical results showed a significant interaction between the considered factors (Table S1). An increase in DODAX concentration is translated into larger angles, causing atypical acceleration of the embryonic development. On the contrary, MO did not affect the HTA normal pattern. A similar pattern was also visible for the DODAX:MO liposomes.

The cardiac rhythm of zebrafish embryos exposed to the individual surfactants did not differed significantly from the control groups up to 32 hpf (Table S1). Nevertheless, regarding DODAX:MO liposomes, the heartbeat of pre-hatched larvae increased, particularly when exposed to C:M(2:1) and C:M(4:1) liposomes. Interestingly, a normal cardiac rhythm appears to be restored after exposure to increasing MO proportions for the molar fractions DODAX:MO (1:1), (1:2) and (1:4).

ANCOVA results on the effects of DODAX:MO liposomes in zebrafish embryos yolk volume (adjusting for egg size), showed no significant interaction independently of DODAX:MO molar fractions (Table S1). SNK showed that the tested DODAB concentrations did not cause significant alterations in comparison to the control. Although a similar profile was visible with DODAC, the post-hoc test (data not shown) detected differences between the concentration levels. An opposite trend is observed for DODAC 9 μM, with an increasing yolk size. Presence of MO did not affect the zebrafish yolk parameter.

The eye surface of zebrafish larvae exposed to DODAB up to 56 hpf did not differ statistically from the control group, although a decreasing concentration-dependent tendency was recorded (Table S1). At 56 hpf, only zebrafish larvae exposed to the lowest concentration of DODAC survived,

exhibiting a significant reduction of eye surface in comparison to the control group (Table S1). No phenotypic alterations of the eyes were observed for zebrafish larvae exposed to either DODAX:MO liposomes or MO alone. No differences in body size were recorded within the groups of exposed zebrafish embryos at 56 hpf (SNK not shown).

When analyzing the effects of DODAX in spontaneous movements of zebrafish embryos at 32 hpf, a significant interaction was observed (see Table S1), associated with an increased percentage of this behavior, in a concentration-dependent manner. MO individual administration did not affect this parameter (Table S1). For the DODAX:MO liposomes, increasing the molar fraction of DODAX (without varying MO molar fraction) or increasing the molar fraction of MO (without varying DODAX molar fraction) showed potential to lead to a reduction in spontaneous movements. The molar fraction DODAX:MO (1:1) did not differ significantly from the control group (SNK not shown).

One-way ANOVA results on zebrafish larvae free-swimming behavior at 80 hpf, did show any significant interaction among different DODAX:MO ratios of liposomes. In opposition, individual exposure of DODAX led to high embryotoxicity, decreasing the percentage of larvae exhibiting free-life, while MO did not cause any apparent toxic effect.

Table S1 - Statistical analysis of ZET experiments.

	hpf	independent variables	statistical analysis	MO	DODAB	DODAC	M:B liposomes	M:C liposomes
morphometric analysis	8	*epipolic arc*	one-way ANOVA	$F(5,110)= 2.278$; $P= 0.05171$	$F(5,112)= 2.487$; $P< 0.05$	$F(5,112)= 3.900$; $P< 0.05$	$F(5,100)= 1.534$; $P= 0.18614$	$F(5,95)= 1.419$; $P=0.22447$
	8	*yolk volume*	ANCOVA	$F(5,110)= 0.428$; $P= 0.82839$	$F(5,111)= 1.929$; $P= 0.09517$	$F(5,111)= 10.188$; $P<0.05$	$F(5,99)= 0.449$; $P= 0.8131$	$F(5.94)= 1.424$; $P=2.22281$
	32	*head-trunk angle*	one-way ANOVA	$F(5,60)= 0.432$; $P= 0.82466$	$F(5,86)= 2.434$; $P< 0.05$	$F(5,44)= 7.303$; $P< 0.05$	$F(5,57)= 1.752$; $P= 0.13744$	$F(5,70)= 0.533$; $P= 0.75043$
	56	*eye surface*	one-way ANOVA	$\boldsymbol{F}(5,108)= 1.564$; $P= 0.17624$	$F(5,85)= 0.507$; $P= 0.76997$	$F(1,31)= 23.971$; $P< 0.05$	$F(5,92)= 0.358$; $P= 0.87581$	$F(5,92)= 0.358$; $P= 0.87581$
muscular coordination	32	*cardiac rhythm*	one-way ANOVA	$F(5,54)= 0.908$; $P= 0.48266$	$F(5,54)= 0.896$; $P=0.49054$	$F(5.47)= 4.522$; $P< 0.05$	$F(5,54)= 3.948$; $P< 0.05$	$F(5,54)= 7.8433$; $P<0.05$
	32	*spontaneous movements*	chi-square	$\chi2= 9.268$; df = 5 ; $P= 0.09884$	$\chi2= 12.369$; df = 5 ; $P< 0.05$	$\chi2= 19.596$; df = 5 ; $P< 0.05$	$\chi2= 45.165$; df = 5 ; $P< 0.05$	$\chi2= 107.378$; df = 5 ; $P<0.05$
	80	*free-swimming*	one-way ANOVA	$F(5,18)= 2.4619$; $P =0.7172$	$F(5,18)= 9.757$; $P< 0.05$	$F(5,18)= 3.251$; $P< 0.05$	$F(5,18)= 0.148$; $P=0.97809$	$F(5,18)= 0.608$; $P= 0.69520$
	80	*survival*	chi-square	$\chi2= 5.316$; df = 5 ; $P= 0.39951$	$\chi2= 70.891$; df = 5 ; $P< 0.05$	$\chi2= 176.042$; df = 5 ; $P< 0.05$	$\chi2= 9.868$; df = 5 ; $P= 0.07906$	$\chi2= 6.731$; df = 5 ; $P= 0.24142$

	hpf	independent variables	statistical analysis	ethanol	HEPES	DODAB:MO	DODAC:MO
morphometric analysis	8	*epipolic arc*	one-way ANOVA	$F(4,19)= 9.6799$; $P< 0.05$	$F(5,112)= 2.1546$; $P= 0.06413$	$F(5,101)= 0.55411$; $P= 0.73486$	$F(5,93)= 0,07397$; $P= 0.99599$
	8	*yolk volume*	ANCOVA	$F(5,103)= 7.7348$; $P< 0.05$	$F(5,218)= 1.729$; $P= 0.12820$	$F(5,186)= 0.26206$; $P= 0.93326$	$F(5,187)= 1.5853$; $P= 0.16614$
	32	*head-trunk angle*	one-way ANOVA	$F(3,32)= 3.3032$; $P< 0.05$	$F(5,76)= 1.080$; $P=0.37836$	$F(5,61)= 0.78757$; $P=0.56267$	$F(5,69)= 0.38742$; $P=0.86921$
	56	*eye surface*	one-way ANOVA	$F(3,60)= 15.129$; $P< 0.05$	$F(5,99)= 0.525$; $P= 0.75702$	$F(5,97)= 3.753$ $P< 0.05$	$F(5,93)= 2.116$; $P= 0.07031$
muscular coordination	32	*cardiac rhythm*	one-way ANOVA	$F(3,72)= 179.820$; $P< 0.05$	$F(5,108)= 3.5763$; $P< 0.05$	$F(5,108)= 14.227$ $P< 0.05$	$F(5,108)= 1.7113$; $P=0.13809$
	32	*spontaneous movements*	chi-square	$\chi2= 4.380$; df = 3 ; $P= 0.22323$	$\chi2= 13.818$; df = 5 ; $P< 0.05$	$\chi2=10.691$; df = 5 ; $P= 0.05786$	$\chi2= 14,810$; df = 5 ; $P< 0.05$
	80	*free-swimming*	one-way ANOVA	$F(3, 12)= 239.050$; $P< 0.05$	$F(5, 17)= 10.542$; $P< 0.05$	$F(5, 18)= 7.411$; $P< 0.05$	$F(5, 18)= 0.932$; $P= 0.48371$
	80	*survival*	chi-square	$\chi2= 229.535$; df = 5 ; $P < 0.05$	$\chi2=5.286$; df = 5 ; $P= 0.38198$	$\chi2=3.134$; df = 5 ; $P= 0.67934$	$\chi2= 3.833$; df = 5 ; $P= 0.57370$

Statistics

Prior to data analysis, all assumptions were met testing for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test).

To investigate the influence of DODAX:MO liposomes on ROS production and on mitochondrial membrane potential of the BJ-5ta cell line, a one-way ANOVA (seven levels: NT - non-treated cells; B:M(1:0), B:M(2:1), B:M(1:2), C:M(1:0), C:M(2:1) and C:M(1:2) liposomes treated-cells) was conducted. Regarding ROS production, 40 μg mL^{-1} and 80 μg mL^{-1} liposomes were analyzed separately, as were the timepoints considered for each test variable. Results were expressed as mean ± standard error of the mean (S.E.M.).

In order to detect differences among concentrations in the overall survival of zebrafish embryos, a chi-square test was conducted with the observed values for each test condition (hpf were analyzed separately). The null hypothesis of "no differences among concentrations" was considered for the establishment of the expected values (average survival of all treatments, for a given hpf).

To investigate the effect of DODAX and MO exposure (as free-compounds and in combination, as liposomes) on the epibolic arc (8 hpf), on the HTA (32 hpf) and on the cardiac rhythm (32 hpf) of zebrafish embryos, six-level one-way ANOVA analysis were conducted.

To avoid biases associated with covariates, ANCOVA model was considered to determine the influence of the DODAX:MO liposomes on zebrafish yolk volume at 8 hpf (egg volume was used as co-variable). To investigate differences among groups on eye surface of zebrafish larvae at 56 hpf, a six-level one-way ANOVA was applied. In order to guaranty that the morphometric results emerging from the proposed experiments were not a result of larvae size differences between groups, a six-level one-way ANOVA was also conducted on zebrafish body size at 56 hpf.

In order to detect differences among groups in the percentage of spontaneous movements of zebrafish embryos at 32 hpf, a chi-square test was conducted with the observed values for

each test condition. The null hypothesis of “no differences among groups” was considered for the establishment of the expected values (average percentage of spontaneous movements of all treatments). The percentage of zebrafish larvae exhibiting free-swimming behavior at 80 hpf, was used as dependent variable in a six-level one-way ANOVA design, to test for differences between treatments.

In vitro data statistical analysis was performed with Prism 5 (GraphPad software Inc. *v5*), while the remaining analyses were conducted in STATISTICA (StatSoft *v7*, Tulsa, OK). For *in vitro* data, *post hoc* comparisons were conducted using Dunnett’s multiple comparison test, while for *in vivo* data Student-Newman-Keuls analysis were considered. Statistical significance is highlighted as follow: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

DODAX:MO liposomes have small size and positive surface charge

Table S2 shows the mean size and surface charge of DODAX:MO liposomes prepared by ethanolic injection in HEPES buffer.

Table S2 – Physicochemical characterization of DODAX:MO liposomes. Averaged mean size (nm) and ζ -potential (mV) of liposomes (1 mM) prepared in HEPES buffer (25 mM pH 7.4). Values are the average ± S.D. of measurements of three independent experiments.

	B:M (1:0)	**B:M (2:1)**	**B:M (1:2)**	**C:M (1:0)**	**C:M (2:1)**	**C:M (1:2)**
Z-avg (nm)	170 ± 8	160 ± 10	180 ± 8	162 ± 7	133 ± 7	102 ± 11
ζ-potential (mV)	55 ± 2	57 ± 4	59 ± 5	58 ± 4	55 ± 3	57 ± 3

Effect of DODAX:MO liposomes on the cellular viability of several cell lines

The effects of DODAX:MO liposomes on cell viability were evaluated in five other cell lines, in order to understand if the cytotoxic response was consistent along a variety of cells, and to allow stronger conclusions about nanocarriers' toxicity.

Human embryonic kidney cell line 293T (ATCC® CRL-3216™), human melanoma cell line MDA-MB-435 and human breast carcinoma cell line MDA-MB-468 (ATCC® HTB-132™) were routinely gown in DMEM® cell culture medium supplemented with 1 % L-Glu, 10 % FBS, 1 % pyruvate sodium solution and 1 % P/S solution. Human myelogenous leukemia cell line K-562 (ATCC® CCL-243™) and human monocytic cell line THP1 (ATCC® TIB-202™) were grown in RPMI 1640® cell culture medium supplemented with 1 % L-Glu, 10 % FBS and 1 % P/S solution. All cell lines were kept in a humidified incubator (37 °C and 5 % CO_2) and sub-cultured every 3-4 days in order to maintain sub-confluency.

For the MTS assay 293T, MDA-MB-435, MDA-MB-468, K562 and THP1 cells were seeded into 96-multiwell plates (TPP, Switzerland) at a density of 20×10^3, 5×10^3, 7.5×10^3, 20×10^3 and 50×10^3 cells per well, respectively, in complete cell culture medium. For the SRB assay 293T, MDA-MB-435, MDA-MB-468 were seeded in 96-multiwell plates (TPP, Switzerland) at a density of 20×10^3, 5×10^3, 7.5×10^3 cells per well, respectively, in complete cell culture medium. For the LDH assay cells were seeded at a density of 100×10^3 cells per well for 293T, MDA-MB-435 and MDA-MB-468, and at a density of 200×10^3 cells per well for K562 and THP1 cells, in 24-multiwell plates (TPP, Switzerland).

MTS, LDH and SRB assays were performed as described in Materials and Methods.

Figure S1 shows the results of the MTS assay after a 48 h-period incubation of 293T, MDA-MB-435, MDA-MB-468, K562 and THP1 cells with 5, 20, 40 and 80 μg mL^{-1} B:M(1:0), B:M(2:1), B:M(1:2), C:M(1:0), C:M(2:1) and C:M(1:2) liposomes.

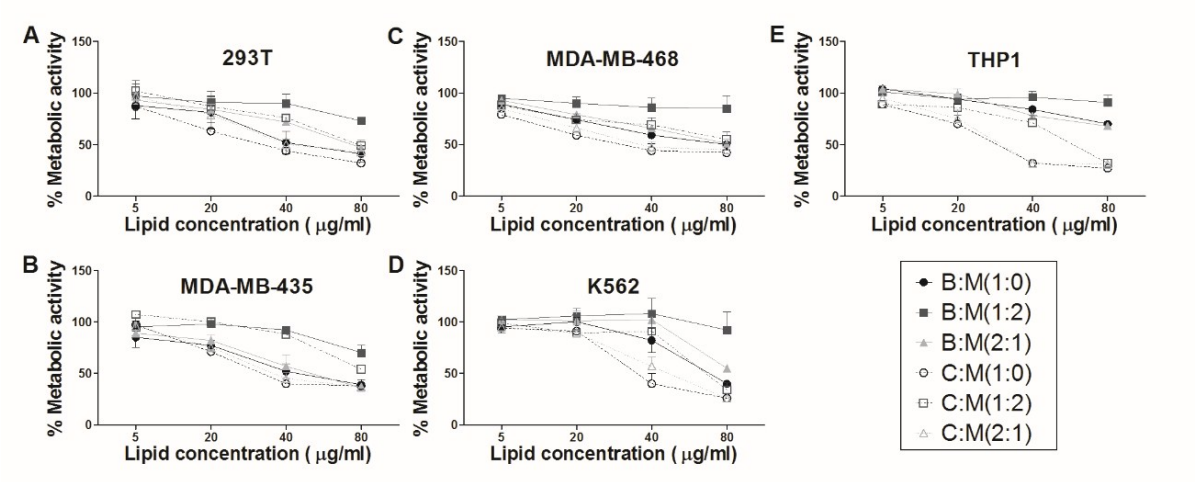

Figure S1 - Metabolic activity of different cell lines after incubation with DODAX:MO liposomes. 293T (**A**), MDA-MB-435 (**B**), MDA-MB-468 (**C**), K562 (**D**) and THP1 (**E**) cells were exposed to 5, 20, 40 and 80 μg mL^{-1} of B:M(1:0), B:M(2:1), B:M(1:2), C:M(1:0), C:M(2:1) and C:M(1:2) liposomes, and MTS was performed after 48 h. The % of metabolic activity was expressed in relation to non-treated cells (NT), set to 100 %. Results are expressed as mean ± S.E.M. of three independent experiments.

The metabolic activity after exposure to DODAX:MO liposomes was cell-line dependent, but similar in the five tested cell lines, with DODAC-based liposomes inducing higher cytotoxicity than DODAB-based liposomes, for the same molar fraction. B:M(1:2) formulation had always a less negative effect on cellular metabolism when compared to the other liposomes.

Figure S2 shows the results of the SRB assay after a 48 h-period incubation of 293T, MDA-MB-435, and MDA-MB-468 cells with 5 and 40 μg mL^{-1} B:M(1:0), B:M(2:1), B:M(1:2), C:M(1:0), C:M(2:1) and C:M(1:2) liposomes. For higher concentrations, liposomes interfered with the assay.

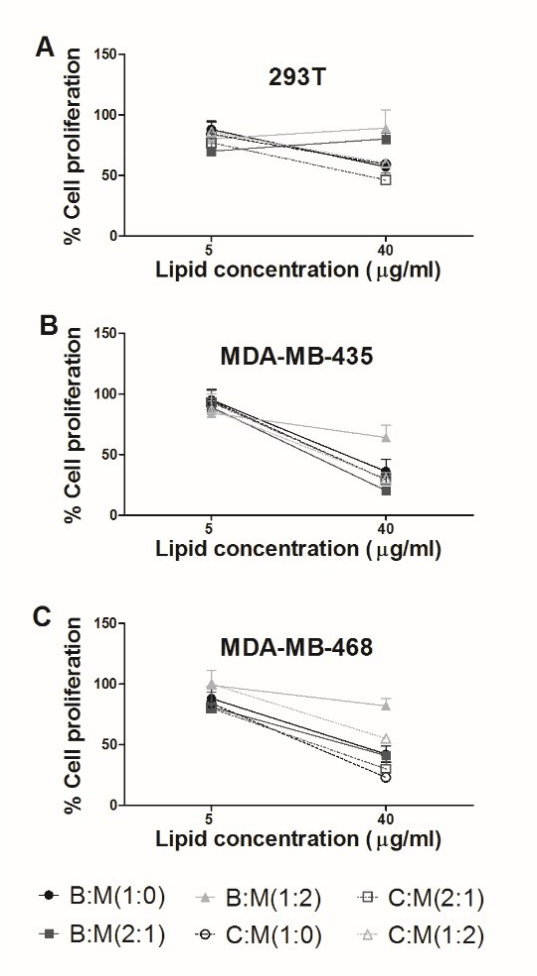

Figure S2 - Cellular proliferation of different cell lines after 48 h exposure to DODAX:MO liposomes. 293T (**A**), MDA-MB-435 (**B**), MDA-MB-468 (**C**) cells were incubated with 5 and 40 μg mL^{-1} of B:M(1:0), B:M(2:1), B:M(1:2), C:M(1:0), C:M(2:1) and C:M(1:2) liposomes and SRB assay was performed after 48 h. Time point 0 h (before addition of liposomes) was considered as 100 % cell proliferation, and the % of cell proliferation was expressed relative to non-treated cells (NT). Results are expressed as mean ± S.E.M. of three independent experiments.

The effects of DODAX:MO liposomes on cellular proliferation were cell line-dependent, with MDA-MB-435 being the most severely affected cell line. A clear correlation between DODAC and DODAB, or the presence of MO and cellular proliferation, was not found although B:M(1:2) seemed to generally interfere less with cells' proliferation.

Figure S3 shows the results of the LDH assay after a 4 h-period incubation of 293T, MDA-MB-435, MDA-MB-468, K562, THP1 and BJ5-ta cells with 80 and 160 μg mL^{-1} B:M(1:0), B:M(2:1), B:M(1:2), C:M(1:0), C:M(2:1) and C:M(1:2) liposomes. This timepoint was chosen in order to observe an immediate effect of the interaction of the nanocarriers with plasma cell membranes.

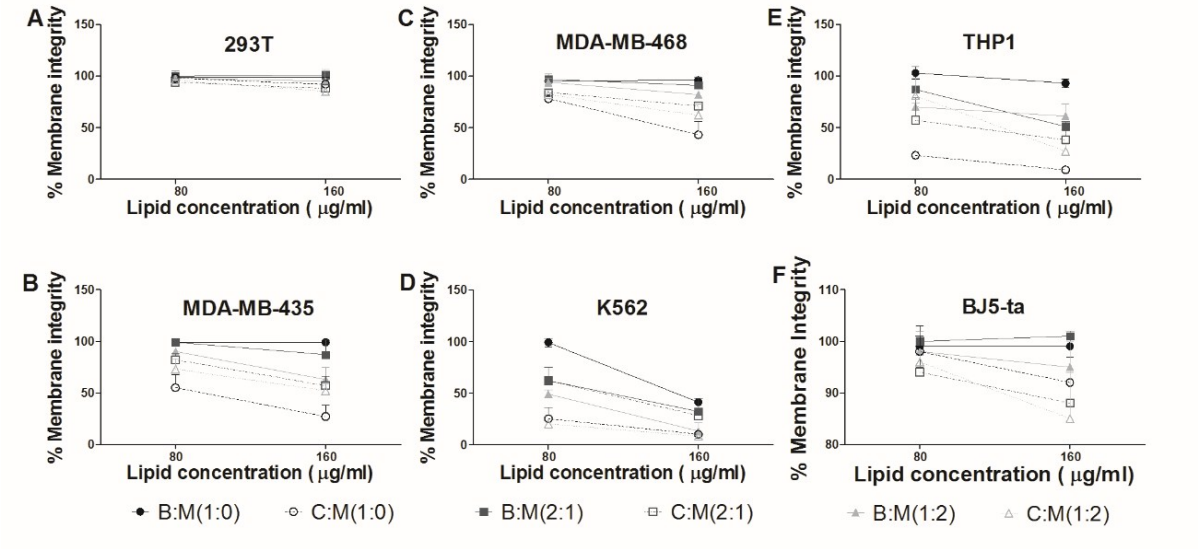

Figure S3 – Plasma membrane integrity of different cell lines after contact with DODAX:MO liposomes. 293T (**A**), MDA-MB-435 (**B**), MDA-MB-468 (**C**), K562 (**D**) THP1 (**E**) and BJ5-ta (**F**) cells were incubated with 80 and 160 μg mL^{-1} of B:M(1:0), B:M(2:1), B:M(1:2), C:M(1:0), C:M(2:1) and C:M(1:2) liposomes and LDH assay was performed after 4 h. The % of membrane integrity was expressed as the percentage of the intracellular LDH activity relative to the total (extracellular + intracellular) LDH activity. Results are expressed as mean ± S.E.M. of three independent experiments.

The disruptive effects of DODAX:MO liposomes on plasma cell membranes were cell line-dependent, but once again the same trend was observed: DODAC-based formulations strongly affected the plasma membrane integrity of 293T, MDA-MB-435, MDA-MB-468, K562, THP1 and BJ5-ta, for the same molar fraction. B:M(1:0) liposomes induced less disruptive effects when compared to the other nanoformulations.

ROS accumulation after incubation with DODAX:MO liposomes

The accumulation of reactive species of oxygen (ROS) was determined by the DCFH-DA assay as described in Materials and Methods. Cells were seeded into 24-multiwell plates (TPP, Switzerland) at a cell density of 100×10^3 cells per well for MDA-MB-468, and at a density of 200×10^3 cells per well for K562 cells.

Results shown on Figure 3 show that incubation of BJ5-ta cells with 80 µg mL^{-1} liposomes gave a peak of ROS accumulation after 4 h incubation, which decreased after 8 h of exposure. This was the result of the higher cytotoxicity associated with DODAC-based nanoformulations (Fig. 1, S1, S2, and S3), that compromised cellular integrity, and therefore decreased ROS detected. Therefore, for the DCF assays with MDA-MB-435 and K562 cell lines, 4 h incubation with DODAX:MO liposomes was chosen.

Figure S4 shows that ROS accumulation was cell-line dependent, and MDA-MB-435 and K562 seemed to be less sensitive than BJ5-ta (Fig. 3) to DODAX:MO liposomes.

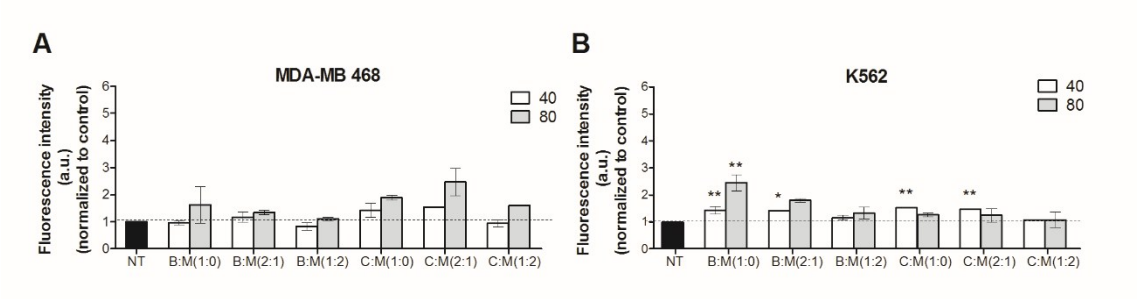

Figure S4 - Reactive oxygen species (ROS) accumulation induced by DODAX:MO liposomes. MDA-MB-468 (**A**) and K562 (**B**) cells were incubated with 40 and 80 µg/ml DODAX:MO liposomes for 4 h, and DCFH-DA assay was performed to determine ROS accumulation. Results are expressed mean ± S.E.M. of three independent experiments. NT - non-treated cells.