Supplementary information

as part of the manuscript

Design of gadoteridol-loaded cationic liposomal adjuvant CAF01 for *in vivo* lung MRI of the multistage tuberculosis vaccine candidate H56/CAF01

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Table S1. Quality target product profile (QTPP) of gadoteridol-loaded CAF01 liposomes.

Response	**Target**	**Reason**
z-average (nm)	≤ 200	Important for adjuvant activity
Polydispersity index (PDI)	≤ 0.3	Quality marker
Zeta potential (mV)	≥ 10	Important for adjuvant activity and antigen adsorption
Encapsulation efficiency (%)	Maximize	Increase contrast activity and reduce dose
Loading (%)	Maximize	Improve cost-effectiveness
Phase transition temperature (T_m, ˚C)	42 - 43	Stability marker
Enthalpy change (ΔH, kJ/mol)	36 - 45	Stability marker
Peak width at half height ($\Delta T_{1/2}$, ˚C)	Assess	Stability marker

Table S2. Experimental design showing the individual experiments.

Experiment number	Gadoteridol concentration (mM)	Lipid concentration (mg/ml)	pH
1	10	1.0	7.00
2	90	1.0	7.00
3	10	6.0	7.00
4	90	6.0	7.00
5	10	1.0	8.50
6	90	1.0	8.50
7	10	6.0	8.50
8	90	6.0	8.50
9	10	3.5	7.75
10	90	3.5	7.75
11	50	1.0	7.75
12	50	6.0	7.75
13	50	3.5	7.00
14	50	3.5	8.50
15	50	3.5	7.75
16	50	3.5	7.75
17	50	3.5	7.75

Table S3. Raw data from the design of experiments (DoE), including the *z*-average, PDI, encapsulation efficiency loading and enthalpy.

Experiment Number	*z*-average (nm)	PDI	*z*-average (nm)	PDI	Encapsulation efficiency (%)	Loading (%)	Enthalpy (kJ/mol/°C)
	Before dialysis		After dialysis				
1	146.4	0.250	147.6	0.253	72.58	78.74	36.45
2	138.4	0.259	140.6	0.264	42.19	92.66	18.56
3	155.1	0.257	146.8	0.256	83.01	47.12	39.40
4	150.8	0.289	147.5	0.265	61.64	78.65	35.56
5	139.1	0.338	136.4	0.318	10.58	42.44	21.73
6	132.9	0.263	132.8	0.259	2.50	46.09	18.42
7	146.8	0.242	139.3	0.283	27.70	21.03	33.96
8	147.7	0.247	139.3	0.253	12.12	35.28	24.41
9	133.0	0.237	131.3	0.241	70.23	62.11	36.80
10	165.2	0.288	153.1	0.268	58.21	83.85	26.48
11	143.4	0.278	141.5	0.270	17.15	79.84	20.91
12	146.5	0.258	147.0	0.262	57.40	68.36	30.99
13	149.0	0.266	142.5	0.262	66.56	82.04	36.24
14	137.6	0.254	135.7	0.257	3.96	21.83	26.30
15	144.1	0.252	141.9	0.250	54.14	79.56	34.12
16	140.3	0.298	142.8	0.274	62.59	81.52	35.93
17	139.8	0.257	140.0	0.257	63.36	80.78	31.02

Table S4. Physicochemical properties of gadoteridol-loaded DPPC and DPPC/TDB liposomes in 10 mM Tris buffer (pH 7.4) containing 50 mM gadoteridol (n = 1).

Formulation	***z*-average (nm)**	**PDI**	**Encapsulation efficiency (%)**	**Δ*H* (kJ/mol)**	**T_m (˚C)**	**$\Delta T_{1/2}$ (˚C)**
DPPC	147	0.182	3.83	32.95	40.99	1.28
DPPC/TDB	111	0.048	3.82	31.54	40.30	1.20

Table S5. Comparison of pK_a values for gadoteridol determined *in silico* and reported in the literature.

Group	Model Marvin**	Model Marvin**	Literature [1]	Literature [2]
-COOH	1.46	1.76	2.84	-
-COOH	3.84	3.89	3.26	3.80
-COOH	4.44	4.42	4.30	4.99
-NH-	-	-	-	-
-NH-	-	-	-	-
-NH-	6.61	6.01	9.43	9.33
-NH-	8.30	7.53	11.96	11.17
-OH	15.29	14.13	-	-

References

[1] C.K. Kumar, A. Chang, L.C. Francesconi, D.D. Dischino, M.F. Malley, J.Z. Gougoutas, M.F. Tweedle, Synthesis, Stability, and Structure of Gadolinium(III) and Yttrium(III) Macrocyclic Poly(amino carboxylates), Inorg. Chem. 33(16) (1994) 3567–75.
[2] A. Bianchi, L. Calabi, C. Giorgi, P. Losi, P. Mariani, P. Paoli, P. Rossi, B. Valtancoli, M. Virtuani, Thermodynamic and structural properties of Gd3+ complexes with functionalized macrocyclic ligands based upon 1,4,7,10-tetraazacyclododecane, Journal of the Chemical Society, Dalton Transactions (5) (2000) 697-705.

Table S6. Physicochemical properties of CAF01 liposomes loaded with 5, 10, 25, or 50 mM gadoteridol (3 mg/mL lipid concentration and Tris buffer pH 7.4), which were used for *in vivo* MR imaging.

Formulation	***z*-average (nm) before dialysis**	**PDI before dialysis**	***z*-average (nm) before dialysis**	**PDI before dialysis**	**Encapsulation efficiency (%)**
CAF01	136.6	0.278	-	-	-
CAF01 + 5 mM gadoteridol	139.1	0.241	136.7	0.246	48.31
CAF01 + 10 mM gadoteridol	134.3	0.245	130.7	0.241	69.05
CAF01 + 25 mM gadoteridol	133.8	0.257	133	0.256	65.97
CAF01 + 50 mM gadoteridol	143.4	0.323	146.8	0.287	66.15

Table S7: Normalized T_1 map values and % T_1 change in mice lungs administered with either non-loaded CAF01 or CAF01 loaded with 5, 10, 25, or 50 mM gadoteridol formulations.

Animal	CAF01			CAF01-5 mM gadoteridol			CAF01-10 mM gadoteridol			CAF01-25 mM gadoteridol			CAF01-50 mM gadoteridol		
	Normalized T_1 (untreated lungs)	**Normalized T_1 after administration**	**% T_1 change**	**Normalized T_1 (CAF01-treated lungs)**	**Normalized T_1 after administration**	**% T_1 change**	**Normalized T_1 (CAF01-treated lungs)**	**Normalized T_1 after administration**	**% T_1 change**	**Normalized T_1 (CAF01-treated lungs)**	**Normalized T_1 after administration**	**% T_1 change**	**Normalized T_1 (CAF01-treated lungs)**	**Normalized T_1 after administration**	**% T_1 change**
1	0.47113	0.40419	14.20	0.58295	0.43609	25.19	0.58295	0.39157	32.82	0.58295	0.37064	36.42	0.58295	0.42285	27.46
2	0.45155	0.37922	16.10	0.57328	0.35004	38.93	0.57328	0.31802	44.52	0.57328	0.45047	21.42	0.57328	0.42456	25.94
3	0.44758	0.38761	13.39	0.55797	0.41588	25.46	0.55797	0.26894	51.79	0.55797	0.37739	32.36	0.55797	0.41522	25.58

Table S8: Normalized T_1 map values and % T_1 change in mice lungs administered with either non-loaded CAF01 or CAF01 loaded with 10 mM gadoteridol formulations.

Animal	CAF01			CAF01-10 mM gadoteridol		
	Normalized T_1 (untreated lungs)	**Normalized T_1 after administration**	**% T_1 change**	**Normalized T_1 (untreated lungs)**	**Normalized T_1 after administration**	**% T_1 change**
1	0.48264	0.41044	14.96	0.48264	0.33250	31.10
2	0.54270	0.42013	22.58	0.54270	0.35499	34.58
3	0.46587	0.40343	13.40	0.46587	0.32836	29.51
4	0.39782	0.42712	-7.36	0.39782	0.33295	16.30
5	0.37913	0.43738	-15.36	0.37913	0.31660	16.49
6	0.45369	0.34759	23.38	0.45369	0.31127	31.39

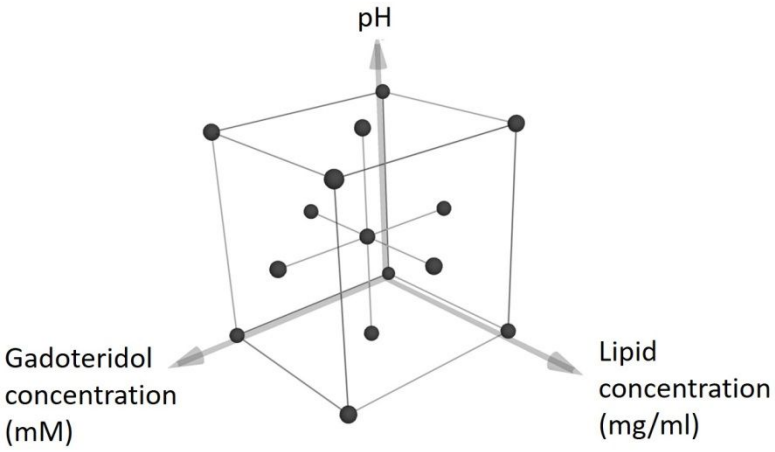

Figure S1. Design region of the central composite face (CCF) centered model. The individual points refer to the 17 experimental runs presented in Supplementary Table S2.

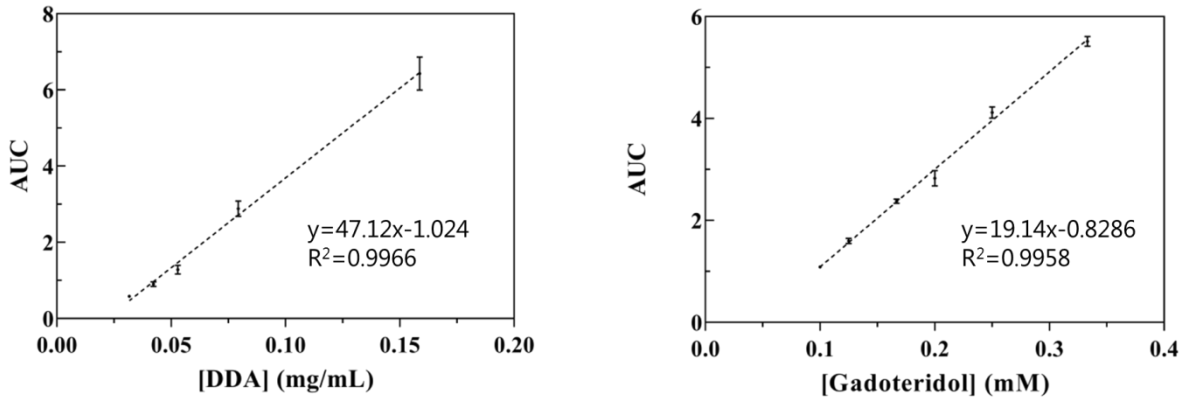

Figure S2. Calibration curve for DDA (left) and gadoteridol (right) by high performance liquid chromatography (HPLC).

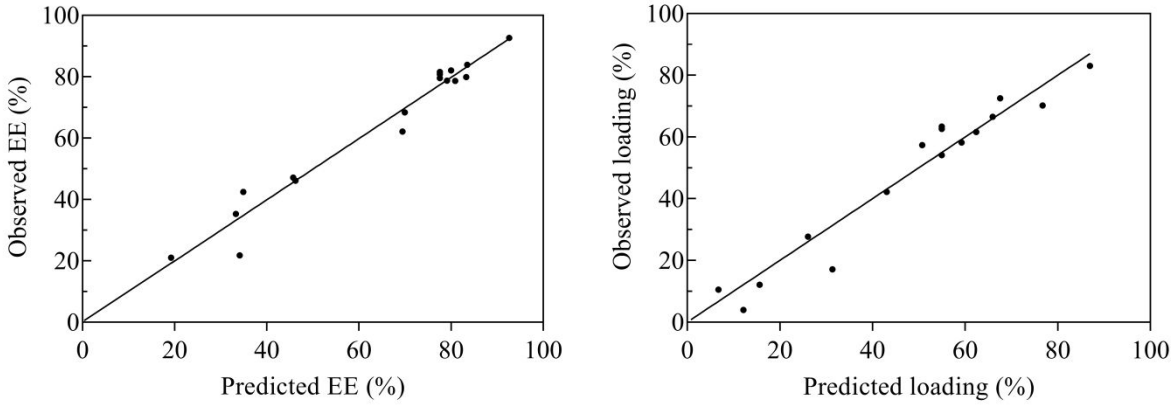

Figure S3. Correlation between the measured and the predicted encapsulation efficiency (left) and loading (right) after establishment of the model. The full lines represent linear fits.

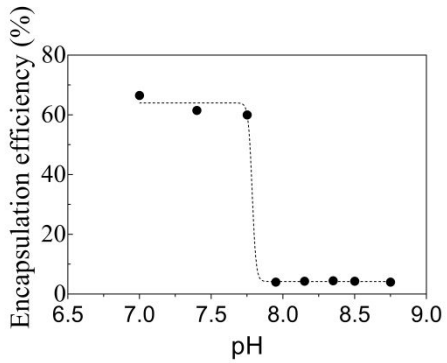

Figure S4: Effect of pH on the encapsulation efficiency of gadoteridol loaded in CAF01 liposomes at a lipid concentration of 3 mg/ml and a gadoteridol concentration of 50 mM. The dotted line represents a sigmoidal fit.

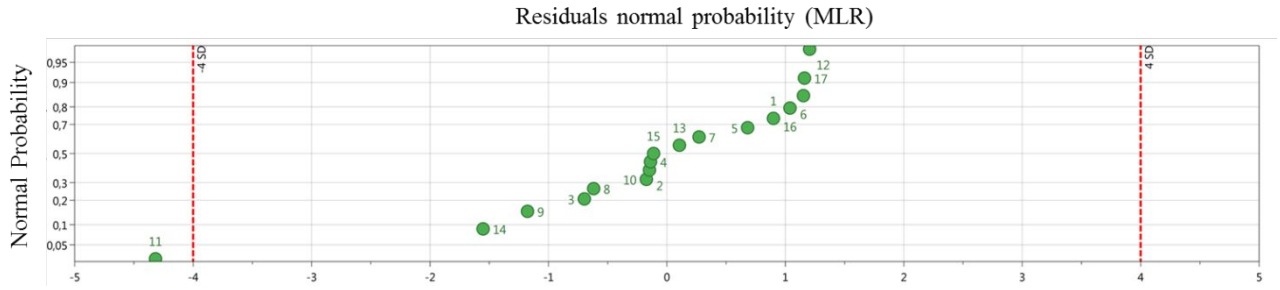

Figure S5. Normal probability plot of residuals for multiple linear regression (MLR) analysis. Formulation 11 represents an outlier.

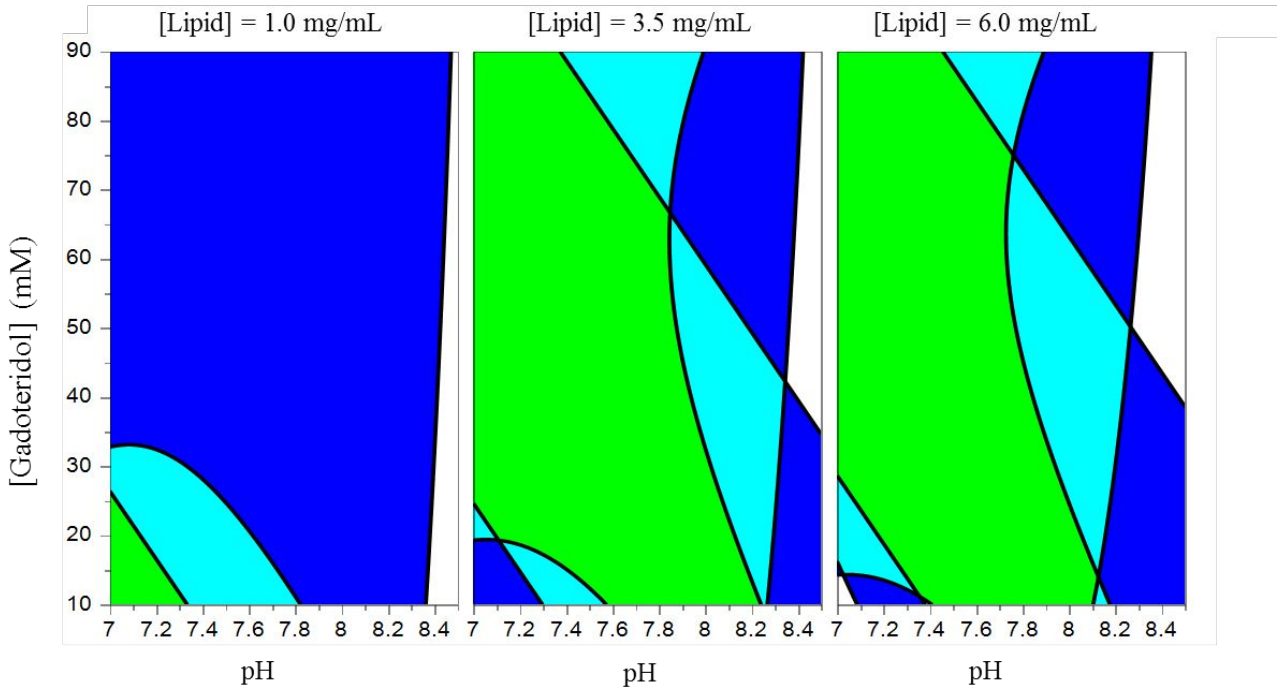

Figure S6: 4D modelling of the optimal operating space (OOS). Restrictions were set for the encapsulation efficiency above 50%, a loading higher than 50% and a Δ*H* of 30 - 40 kJ. Color code: white (no restriction met), dark blue (one restriction met), light blue (two restrictions met) and green (all restrictions met). The largest OOS with respect to pH and gadoteridol concentration appears as a green area in the center figure.