Electronic Supplementary Information

Tailoring amphotericin B as an ionic liquid: an upfront strategy to potentiate the biological activity of antifungal drugs

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Synthesis of cholinium amphotericin B [chol][AmB]:

(2-Hydroxyethyl)-trimethylammonium chloride (0.0355g; 0.254 mmol) was dissolved in methanol and passed through an ion-exchange Amberlite IRA-400(OH) (5 eq., flux rate 0.133 $mL.min^{-1}$ = 8 BVh^{-1}). Then, the hydroxide solution formed was slowly added to amphotericin B (0.249 g; 0.269 mmol) dissolved in 1.0 M dried triethylamine methanolic solution. The mixture was stirred at room temperature for 1 h. After solvent evaporation the residue was dried *in vacuum* for 24 h to provide the desired product as an orange solid (0.1335 g; 51.1 %).

$[\alpha]_D^{25}$ = 50.0 ± 5.8 (c = 1 $mg.mL^{-1}$ in MeOH);

^{1}H-NMR (400.13 MHz, $(CD_3)_2SO$) δ = 6.47-5.97 (m, 14H), 5.68 (bs, 1H), 5.46-5.40 (m, 1H), 5.35-5.32 (m, 1H), 5.24-5.20 (m, 1H), 4.98-4.74 (m, 1H), 4.63 (bs, 1H) 4.34 (bs, 1H), 4.37-4.32 (m, 1H), 4.26-4.24 (m, 1H), 4.08-4.04 (m, 1H), 3.84-3.83 (m, 6H), 3.61-3.64 (m, 4H), 3.60-3.38 (m, 10H), 3.10 (s, 9H), 2.33-2.27 (m, 3H), 2.15 (d, 1H, *J* = 5.8 Hz), 1.91-1.70 (m, 1H), 1.65-1.31 (m, 10H), 1.23 (s, 3H), 1.14 (d, 3H, *J* = 5.6 Hz), 1.10 (d, 3H, *J* = 6.1 Hz), 1.03 (d, 3H, *J* = 6.0 Hz), 0.91 (d, 3H, *J* = 7.0 Hz), 0.83 (t, 3H, *J* = 6.7 Hz) ppm;

IR (KBr): υ = 3398, 3018, 2917, 2077, 1638, 1577, 1559, 1506, 1460, 1401, 1387, 1324, 1270, 1183, 1130, 1110, 1073, 1035, 982, 956, 851, 721 cm^{-1}.

MALDI-TOF-MS analysis in the positive ion mode: *m/z* calcd for $[C_5H_{14}NO]^+$: 104.1070, found 104.1080; MALDI-TOF-MS analysis in negative ion mode: *m/z* calcd for $[C_{47}H_{72}NO_{17}]^-$ 922.4806, found $[M-2H]^-$ 920.6717.

Synthesis of cetylpyridinium amphotericin B [C_{16}py][AmB]:

Cetylpyridinium chloride (0.088g; 0.246 mmol) was dissolved in methanol and passed through an ion-exchange Amberlite IRA-400(OH) (5 eq., flux rate 0.133 $mL.min^{-1}$ = 8 BVh^{-1}). Then, the hydroxide solution formed was slowly added to amphotericin B (0.250 g; 0.271 mmol) dissolved in 1.0 M dried triethylamine methanolic solution. The mixture was stirred at room temperature for 1 h. After solvent evaporation the residue was dried in vacuum for 24 h to provide the desired pure product as an orange solid (0.215 g; 71.3 %).

$[\alpha]_D^{25}$ = 49.7 ± 5.8 (c = 0.2 $mg.mL^{-1}$ in MeOH);

^{1}H-NMR (400.13 MHz, $(CD_3)_2SO$) δ = 9.10 (d, 2H, *J* = 5.6 Hz), 8.60 (t, 1H, *J* = 7.6 Hz), 8.16 (t, 2H, *J* = 6.7 Hz), 6.47-5.97 (m, 14H), 5.65 (bs, 1H), 5.51-5.40 (m, 1H), 5.33-5.31 (m, 1H), 5.21-5.20 (m, 1H), 4.79-4.78 (m, 3H), 4.59 (t, 3H, *J* = 7.4 Hz) 4.34 (bs, 1H), 4.24-4.23 (m, 2H), 4.17-4.12 (m, 1H), 4.07-4.05 (m, 1H), 3.74-2.81 (m, 12H), 2.41-2.25 (m, 3H), 2.15 (d, 1H, *J* = 5.7 Hz), 1.91-1.89 (m, 2H), 1.82-1.37 (m, 16H), 1.27-1.23 (m, 28H), 1.14 (d, 3H, *J* = 5.8 Hz), 1.11 (d, 3H, *J* = 6.1 Hz), 1.03 (d, 3H, *J* = 5.9 Hz), 0.90 (d, 3H, *J* = 6.9 Hz), 0.85 (t, 3H, *J* = 6.7 Hz) ppm;

^{13}C-NMR (150 MHz, $(CD_3)_2SO$) δ = 170.45, 145.37, 144.64, 133.66, 133.63, 133.61, 133.47, 133.17, 132.70, 132.66, 132.25, 132.20, 131.99, 131.90, 131.84, 131.80, 127.99, 99.28, 96.65, 79.10, 78.88, 78.66, 73.50, 72.87, 69.18, 68.75, 67.70, 66.15, 60.68, 44.65, 44.29, 44.27, 41.96, 35.08, 31.20, 30.61, 28.95, 28.91, 28.81, 28.68, 28.61, 28.28, 25.31, 22.00, 18.41, 18.00, 16.91, 13.87, 11.99 ppm;

IR (KBr): υ = 3435, 3010, 2920, 2852, 1638, 1579, 1563, 1488, 1456, 1401, 1383, 1340, 1324, 1272, 1181, 1130, 1108, 1069, 1037, 1009, 982, 908, 887, 853, 772, 719, 683 cm^{-1}.

MALDI-TOF-MS analysis in the positive ion mode: *m/z* calcd for $[C_{21}H_{38}N]^+$: 304.2999 found 304.3117; MALDI-TOF-MS analysis in negative ion mode: *m/z* calcd for $[C_{47}H_{72}NO_{17}]^-$ 922.4806, found $[M-2H]^-$ 920.5183.

Synthesis of trihexyltetradecylphosphonium amphotericin B [$P_{6\,6\,6\,14}$][AmB]:

Trihexyl(tetradecyl)phosphonium (0.127 g; 0.246 mmol) was dissolved in methanol and passed through an ion-exchange Amberlite IRA-400(OH) (5 eq., flux rate 0.133 $mL.min^{-1}$ = 8 BVh^{-1}). Then, the hydroxide solution formed was slowly added to amphotericin B (0.251 g; 0.270 mmol) dissolved in 1.0 M dried triethylamine methanolic solution. The mixture was stirred at room temperature for 1 h. After solvent evaporation the residue was dried *in vacuum* for 24 h to provide the desired product as orange solid. (0.1953 g; 75.0 %).

$[\alpha]_D^{25}$ = 95.0 ± 3.8 (c = 1 $mg.mL^{-1}$ in MeOH);

^{1}H-NMR (400.13 MHz, $(CD_3)_2SO$) δ = , 6.35-6.02 (m, 16H), 5.71 (bs, 1H), 5.53-5.21(m, 6H), 4.79-3.89 (m, 24H), 2.12 (t, 12H, *J* = 14.3 Hz), 1.47- 137 (m, 24H), 1.29-1.24 (m, 47H), 1.13 (dd, 2H, *J* = 16.5, J = 5.9 Hz), 1.06-0.99(m, 3H), 0.87 (t, 12H, *J* = 7.3 Hz) ppm;

IR (KBr): υ = 3435, 2921, 2848, 1656, 1648, 1579, 1561, 1490, 1480, 1456, 1385, 1322, 1262, 1179, 1130, 1106, 1069, 1039, 1009, 901, 853, 776, 719, 685 cm^{-1}

MALDI-TOF-MS analysis in the positive ion mode: *m/z* calcd for $[C_{32}H_{68}P]^+$: 483.51, found 483.4919; MALDI-TOF-MS analysis in negative ion mode: *m/z* calcd for $[C_{47}H_{72}NO_{17}]^-$ 922.4806, found $[M-2H]^-$ 920.6147.

Table S1. Fungal strains used in this study.

Strains	Genotype	Source	Catalog Number
Aspergillus fumigatus Af293	wild-type	FGSC[a]	A1100
Aspergillus terreus NIH2624	wild-type	FGSC[a]	A1156

[a]Fungal Genetics Stock Center.

Table S2. List of *Aspergillus fumigatus* primers used in *q*RT-PCR analyses.

Gene (Code)	Forward Sequence 5´ – 3´	Reverse Sequence 5´ – 3´
Afu4g03630 (*erg6*)	TGGACGAGTACTTCAAGCATTGGG	TAAAGATCCGTGGCCAGGTTGT
Afu7g03740 (*erg11B*)	GGTGCATTGGCGAGCAATTT	AGCGGTTTGGAGAACAGAGAAG
Afu3g10660 (*erg13*)	ACTGCGTGACTTGGATTACGA	TGGCCGTGTACATGTTACCA
Afu3g03500 (*mdr3*)	AAAGTACGGCACCAGTAGAGCA	GTAGTGGACGCAGGGAAGGAGATA
Afu3g07640 (*pma1*)	CGTGAGCTGGTCACTGGTGATATT	TTGAGGGTGTCATCGTTGGCA
Afu1g14550 (*sod3*)	GGTGGATATGTGGGAGCATGCTT	TGTCACCCGCTATGTACCGATTCT
Afu3g07930	ATCTCAAGCACACCAGTACGAC	GGGGACCATGATTTTGACGAAC
Afu1g17250 (*rodB*)	AAGTTCCTCGCTGTTGTCTCTC	GCATTGCTTGTTGAGGAGGT
Afu6g12340	CTGTCCAGCAATCCTGAATCCA	GGTTCCATCCACGGCTCTTTAT
Afu5g01970 (*gpdA*)	CTCTCCAACGCCTCTTGCA	CTTGTTGGAGGGAGCATCGA

Table S3. Energies of the different complexes generated for the molecular dynamics simulations of this study. The aqueous solutions containing the ergosterol or cholesterol and amphotericin B (AmB) or cetylpyridiniym amphotericin B ([C_{16}py][AmB]) were modelled using 1 sterol molecule and 2 AmB or [C_{16}py][AmB] molecules mixed with 4000 water molecules. Three different complexes (at least) were produced for each mixture. All simulations were performed as described in materials and methods. The most stable complex for each combination was chosen (bold). Low density initial configurations were randomly built using the packmol package, placing 10 complexes mixed with 10000 water molecules.

System	Energy kJ/mol	Diff kJ/mol
AmB + ergosterol – complex 1	-133619	3
AmB + ergosterol – complex 2	-133604	18
AmB + ergosterol – complex 3	**-133622**	**0**
AmB + ergosterol – complex 4	-133612	10
[C_{16}py][AmB] + ergosterol – complex 1	-133646	43
[C_{16}py][AmB] + ergosterol – complex 2	-133683	6
[C_{16}py][AmB] + ergosterol – complex 3	**-133689**	**0**
AmB + cholesterol – complex 1	**-133601**	**0**
AmB + cholesterol – complex 2	-133592	9
AmB + cholesterol – complex 3	-133593	8
[C_{16}py][AmB] + cholesterol – complex 1	**-133665**	**0**
[C_{16}py][AmB] + cholesterol – complex 2	-133650	15
[C_{16}py][AmB] + cholesterol – complex 3	-133653	12

Table S4. Gene expression analysis (*q*RT-PCR) of amphotericin B-responsive genes in *Aspergillus fumigatus* after 4 or 24-hour exposure to amphotericin B (AmB), cholinium amphotericin B ([chol][Amb]), cetylpyridinium amphotericin B ([C_{16}py][AmB]) or trihexyltetradecylphosphonium amphotericin B ([$P_{6\,6\,6\,14}$][AmB]). Glyceraldehyde 3-phosphate dehydrogenase gene (*gpdA*) was used as internal control. Values represent the fold-change relative to the negative control followed by their standard deviation. Three biological replicates were performed. The asterisks mark significant differences in expression when compared to AmB for each exposure time (* = p<0.05; ** = p<0.01, *** = p <0.001).

	4 hours			
	AmB	**[chol][AmB]**	**[C_{16}py][AmB]**	**[$P_{6\,6\,6\,14}$][AmB]**
erg6	-2.81 ± 0.03	**-1.79 ± 0.08***	**-1.59 ± 0.09***	**1.74 ± 0.19*****
erg11B	-7.52 ± 0.01	**-8.99 ± 0.01***	**-3.98 ± 0.04***	**-2.88 ± 0.07***
erg13	-3.16 ± 0.04	**-1.89 ± 0.03****	**-1.71 ± 0.08***	**1.17 ± 0.08*****
mdr3	-3.19 ± 0.07	-2.06 ± 0.15	-2.56 ± 0.15	**1.40 ± 0.15*****
pma1	-3.05 ± 0.08	-3.03 ± 0.08	-2.12 ± 0.12	**1.14 ± 0.08*****
sod3	-15.14 ± 0.02	-19.80 ± 0.02	-14.91 ± 0.02	**-4.68 ± 0.02****
GST	-3.30 ± 0.05	-2.99 ± 0.08	-2.57 ± 0.08	**-1.09 ± 0.09*****
rodB	-7.91 ± 0.02	**-3.74 ± 0.06***	-4.98 ± 0.05	**-1.36 ± 0.13****
GTPase	-4.39 ± 0.04	-2.62 ± 0.07	-2.68 ± 0.11	**0.98 ± 0.18****

	24 hours			
	AmB	**[chol][AmB]**	**[C_{16}py][AmB]**	**[$P_{6\,6\,6\,14}$][AmB]**
erg6	1.29 ± 0.19	1.20 ± 0.06	1.36 ± 0.22	**2.63 ± 0.38****
erg11B	-1.63 ± 0.05	**-1.13 ± 0.08***	-1.79 ± 0.05	-1.22 ± 0.12
erg13	1.02 ± 0.14	-1.10 ± 0.04	-1.12 ± 0.19	**2.23 ± 0.58***
mdr3	5.49 ± 1.77	3.76 ± 0.95	4.84 ± 1.29	**25.75 ± 7.84***
pma1	-1.01 ± 0.15	1.12 ± 0.09	1.08 ± 0.09	**1.87 ± 0.15****
sod3	-2.34 ± 0.06	-1.91 ± 0.01	**-1.74 ± 0.01***	**-1.07 ± 0.03****
GST	1.15 ± 0.16	-1.09 ± 0.07	1.07 ± 0.19	**2.34 ± 0.69***
rodB	-1.26 ± 0.14	-1.25 ± 0.20	-2.00 ± 0.10	**-3.39 ± 0.10****
GTPase	1.70 ± 0.19	1.61 ± 0.10	1.45 ± 0.07	**3.84 ± 1.08***

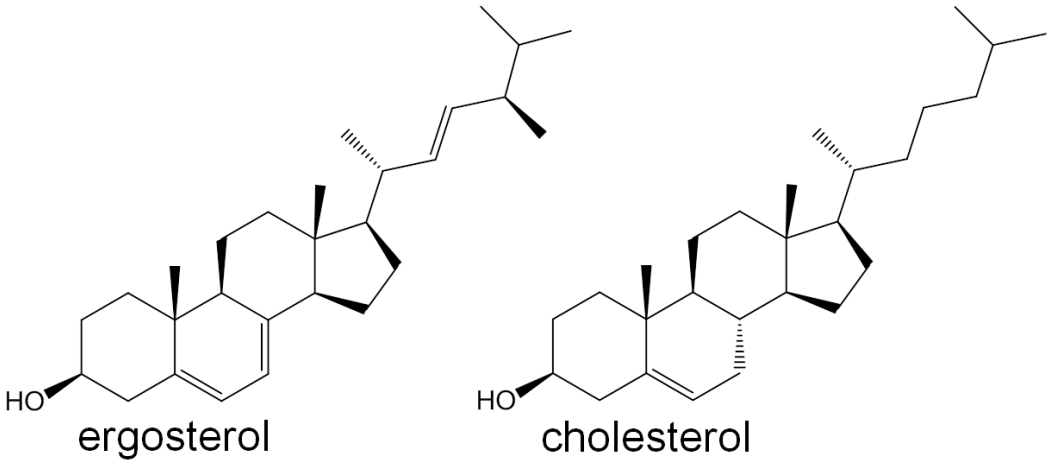

Figure S1. The structure of ergosterol (main fungal sterol) and cholesterol (main animal sterol).

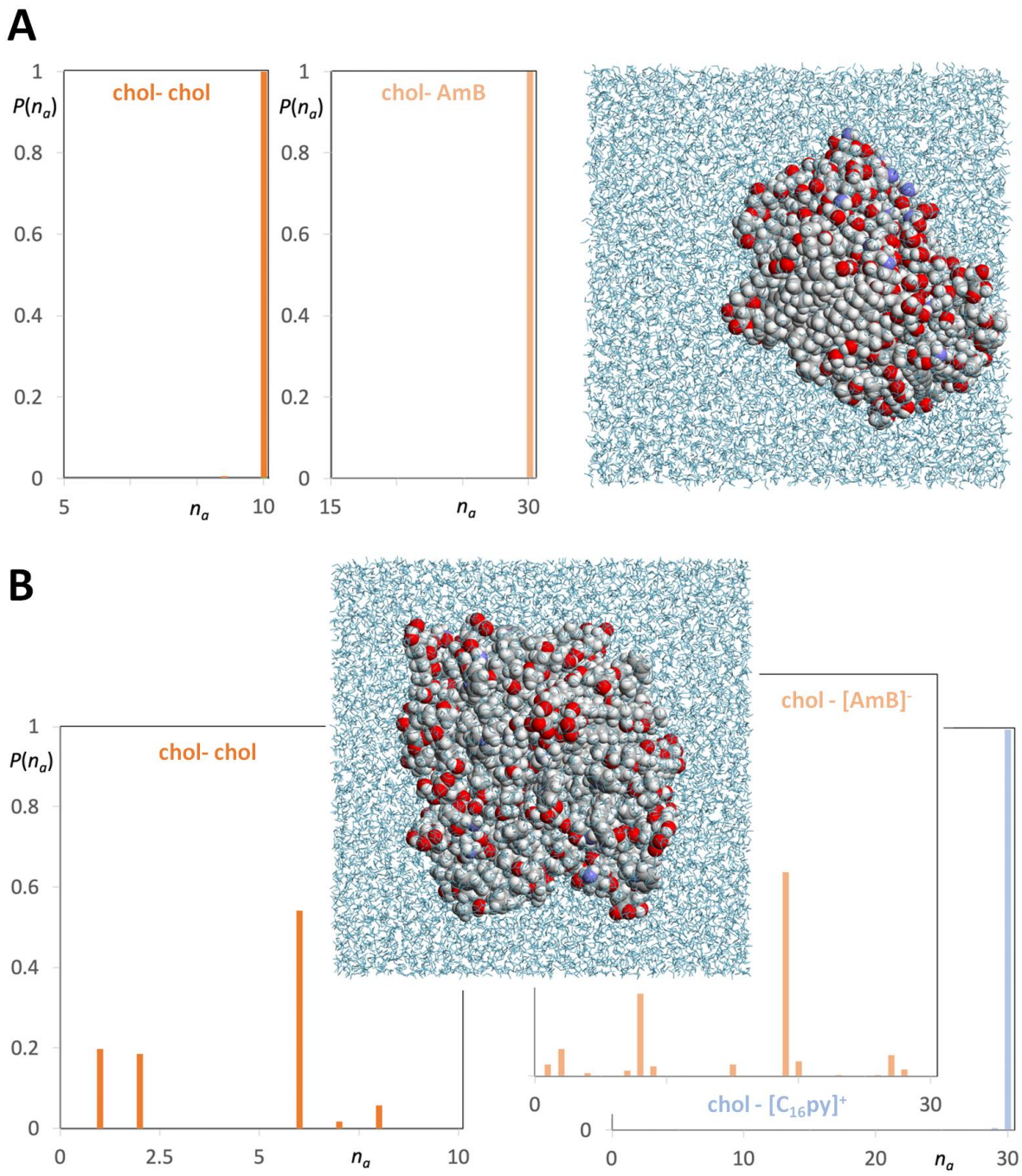

Figure S2. Molecular dynamics simulation snapshots and discrete probability distribution functions of aggregate sizes ($P(n_a)$) for different aggregate types of cholesterol (chol), amphotericin B (AmB) and cetylpyridinium amphotericin B ([C_{16}py][AmB]). **(A)** AmB–cholesterol simulations (dark orange graph: cholesterol clusters; light orange graph: cholesterol–AmB aggregates). **(B)** [C_{16}py][AmB]–cholesterol simulations (dark orange graph: cholesterol clusters; light orange graph: cholesterol–$[AmB]^-$ anion aggregates; blue graph: cholesterol–$[C_{16}py]^+$ cation aggregates).