Correlation between structure, aggregation behaviour and cellular toxicity of anti-HIV catanionic analogues of galactosylceramide

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The self-association process of catanionic analogues of galactosylceramide and in particular the arrangement of their hydrophobic part seems to play a key role in their cellular toxicity

Amongst the variety of glycolipids involved in molecular recognition of pathogenic agents,[1] galactosylceramide was identified as an alternative receptor of HIV-1,[2] having a highly specific affinity for the viral glycoprotein gp120.[3] This discovery opened new perspectives in the field of anti-HIV polytherapies. Recently, we developed and successfully tested *in vitro* galactosylceramide analogues as chimeras for the virus.[4] The acidobasic reactions performed in water between aminolactitol[5] moieties and fatty acids lead to water-soluble ion pair surfactants (Scheme 1), the so-called catanionic surfactants.[6] Preliminary information has been obtained from biological *in vitro* tests (Table 1): *(i)* all the compounds exhibited an antiviral activity (IC_{50}: inhibition concentration for 50% of HIV-infected cells) below their critical aggregation concentrations (CAC), this means in their ion-pair form (compounds 1 and 2), whereas their cellular toxicity (CC_{50}: cytotoxic

concentration for 50% of non-infected cells) was principally associated to their aggregation state; *(ii)* for the two-chain analogues, increasing the lipophilicity† improved the antiviral activity, with a parallel increase of the cytotoxicity; *(iii)* in the particular case of gemini[7] analogues, the toxicity did not follow this rule. The highly hydrophobic gemini 2b, exhibited a high anti-HIV activity with a low toxicity for non-infected cells. Within this family of catanionic analogues, this gemini is the only one to have a CC_{50} above its critical aggregation concentration, indicating that the compound is toxic only in its aggregated state. It also displays a CC_{50} of more than 100 µM for a log P of 8.4, whereas the corresponding two-chain analogue exhibited a higher toxicity (CC_{50} = 2.5 µM) for a lower lipophilicity (log P = 6.5). Parameters other than the lipophilicity seem to contribute to the cytotoxicity of the gemini analogues.

Therefore, it appeared essential to establish a correlation between the structure and the cytotoxicity of anti-HIV catanionic analogues of galactosylceramide so as to optimise these future antivirals. The toxic concentrations of compounds 1 and 2 being in the same range as the corresponding CAC, we first looked at the self-association behaviour of these compounds in water solutions, to obtain information on the arrangement of the ion-pair surfactants inside the aggregates.

Catanionic surfactants,[8] and in particular derivatives 1 and 2,[9] are known to spontaneously self-assemble in aqueous solutions. The macroscopic and microscopic structures of these aggregates were elucidated by freeze-fracture[10]‡ (Fig. 1) at concentrations higher than the corresponding CAC and CC_{50}. Every two-chain analogue 1 displayed unilamellar vesicles as illustrated in Fig. 1A for compound 1b. The bilayer is cross-fractured, which means that the fracture is propagated along the membrane, generating convex and concave surfaces.[11] The elongated shape and the fracture surface relief of many vesicles indicate a vesicular fusion process. However, a different organization was observed on freeze-fracture for both gemini amphiphiles 2a and 2b. As reported earlier using transmission electron microscopy,[9] spontaneous formation of vesicles was also revealed by freeze-fracture electron microscopy (Fig. 1B) for gemini analogue 2a, appearing in the present case as circles. Amphiphile 2b, on the other hand, principally displayed lamellae or multilamellar vesicles (Fig. 1C). Although they showed different types of aggregates, the replica images of both 2a and 2b provide information on the arrangement of the bilayer. The absence of a fracturable midplane in the membrane indicates the particular arrangement of the gemini spacer inside the bilayer. In fact, it is noteworthy that the alkyl spacer of the gemini analogues can be bent or straight.[12] From the images obtained by freeze-fracture for amphiphiles 2a and 2b (Figs. 1B and 1C), it can be seen that both gemini surfactants are arranged with the spacer straight, spanning the membrane to form a monolayer preventing bilayer fracture (Fig. 2C). Nevertheless, a partial mixture of the two arrangements (U-bent and straight spacer) can be considered. In fact, the defaults generated by the limited presence of U-bent spacers inside the transmembrane organization, could explain the curvature of the monolayer to form vesicles in the case of gemini 2a (Fig. 2B).


1a n = 4, m = 4
1b n = 4, m = 8
1c n = 12, m = 4
1d n = 12, m = 8

2a n = 4
2b n = 12
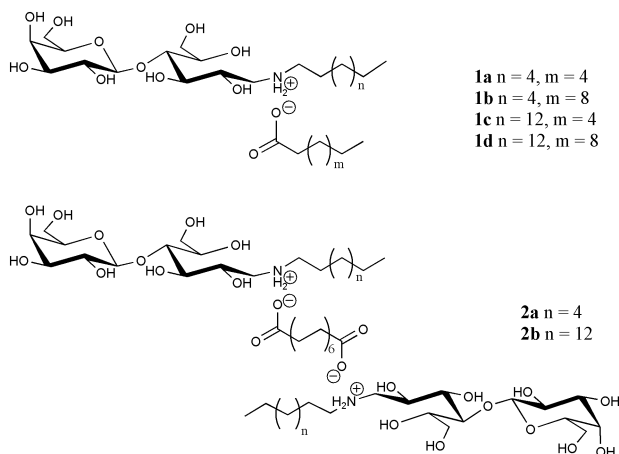
Scheme 1 Structure of two-chain 1 and gemini 2 catanionic analogues of galactosylceramide.

Table 1

| Product	n	m	IC_{50} (µM)	CC_{50} (µM)	SI (CC_{50}/IC_{50})	CAC (µM)	log P
1a	4	4	> 1000	> 1000	1	50000	1.7
1b	4	8	100	> 100	1	2100	3.3
1c	12	4	16	38	2.3	170	4.9
1d	12	8	0.9	2.5	2.7	10	6.5
2a	4		500	600	1.1	2500	2.1
2b	12		0.5	> 100	> 200	10	8.4
DDI 2′,3′-dideoxy-inosine			5.5	1050	190		
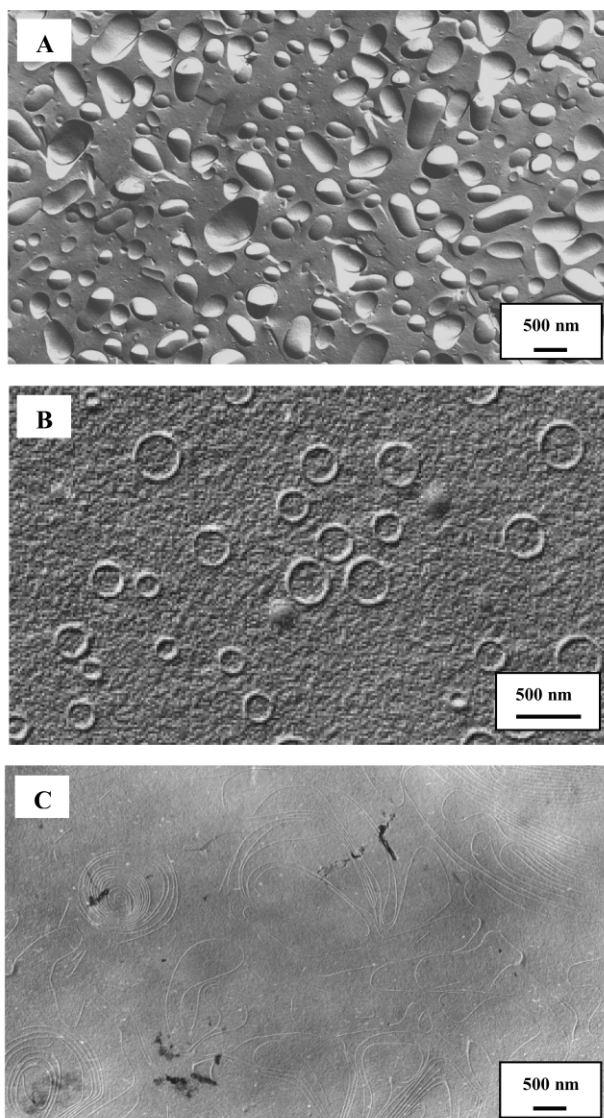
Fig. 1 Freeze-fracture images of catanionic amphiphiles (A) two-chain analogue **1b**, (B) gemini **2a** and (C) gemini **2b**.
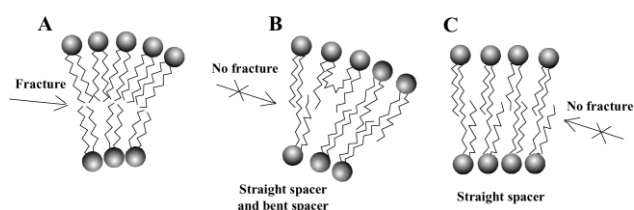
Fig. 2 Supramolecular arrangement of catanionic amphiphiles.

Considering that the cytotoxicity of the pure analogues is related to the modification of the cell membrane,[13] the incorporation of the alkyl chains of the analogue into the lipid bilayer would then influence the toxicity of these compounds. For two-chain analogues, the free alkyl chains can easily penetrate the cell membrane and disturb the cell function (Fig. 3A). In contrast, for gemini analogues, the three alkyl chains lie between the two polar heads, as demonstrated in the freeze-fracture experiments. Biological media being highly hydrophilic, the hydrophobic chains are packed together to minimize their contact with water (Fig. 3C). The hydrophobic part is then masked, decreasing the ability of the alkyl chains to penetrate the cell membrane. In the case of gemini **2a**, the mixture of the two possible arrangements of the spacer can be correlated to its higher toxicity in relation with its CAC. It should be remembered that only gemini **2b** has a CC_{50} higher than its
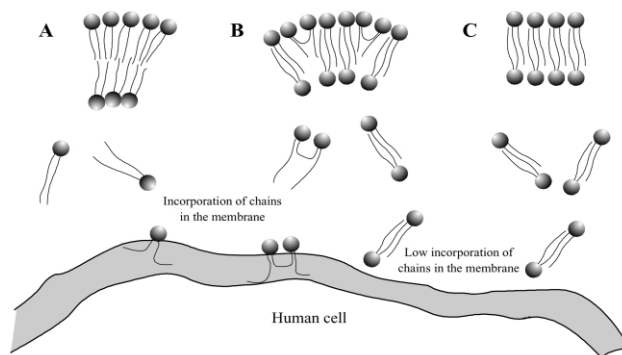
Fig. 3 Galactosylceramide analogues while approaching the cell membrane (A) two-chain **1** and (B) gemini **2a** (C) gemini **2b**.

CAC. Membrane penetration of compound **2a** happens more easily (Fig. 3B) than for gemini analogue **2b** (Fig. 3C). This new information on the arrangement of the gemini analogues is very promising, because gemini **2b** presents a similar *in vitro* antiviral efficiency (selectivity index SI = CC_{50}/IC_{50}, as shown in Table 1) to DDI (2′,3′-dideoxyinosine) which is already used in anti-HIV polytherapies.[14]

X-ray scattering experiments on mixtures of phospholipids and gemini analogues are in progress to confirm the proposed correlation between the structure of the galactosylceramide analogues, their aggregation behaviour and their toxicity related to their cell membrane incorporation.

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