Novel inhibitor by modifying oseltamivir, based on neuraminidase structure, for treating drug-resistant H5N1 virus using molecular docking, NMR and DSC methods.

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INTRODUCTION

The danger of the highly pathogenic H5N1 influenza virus is not only its high fatality, but also its resistance to commercially available drugs.

The influenza virus has two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA)

NA activity is necessary in the elution of newly formed viruses from infected cells

NA plays essential role in influenza virus replication.

It has highly conserved active sites.

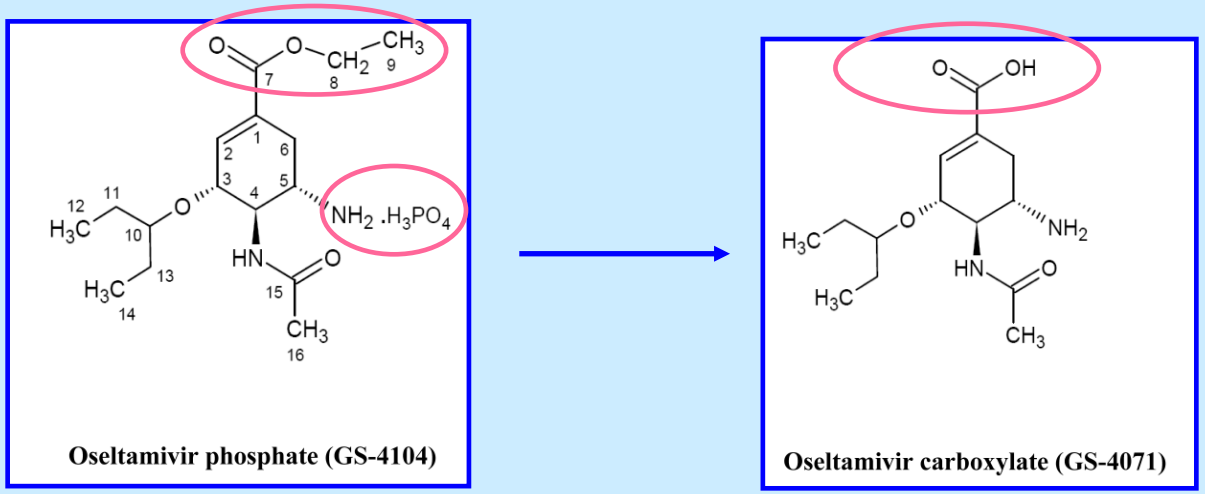
Inhibiting NA can delay the release of progeny virus from the surface of infected cells, thereby suppressing the viral population.

Therefore, NA has become the main target for drug design against influenza viruses

Oseltamivir & Zanamivir (Most commonly used NA inhibitors currently in the market)

Oseltamivir, first orally active neuraminidase inhibitor commercially developed by Gilead Sciences and is currently marketed by Roche under the trade name Tamiflu, used in the treatment and prophylaxis of both influenza A and influenza B.

It is a prodrug (usually administered as phosphate), it is hydrolysed hepatically to the active metabolite, the free carboxylate of oseltamivir (GS4071) which has poor oral bioavailability compared to the prodrug being more hydrophobic



The antiviral activity ensues from the interaction of the parent drug (carboxyllate) with the NA receptor and this aspect can be understood by carrying out its docking with the receptor.

Zanamivir, due to its high polarity, is administered by inhalation

Mutations in the active site of NA are responsible for the resistance to oseltamivir. However, such a resistance has not been observed for zanamivir.

Molecular Docking

docking is a method which predicts the preferred orientation of one molecule to a second when **bound** to each other to form a stable **complex**.

This Knowledge can be used to predict the strength of association or **binding affinity** between two molecules .

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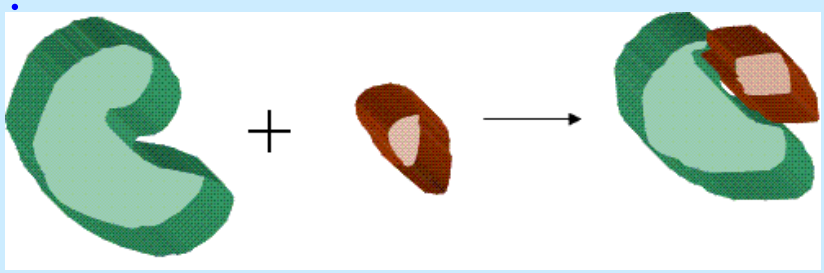
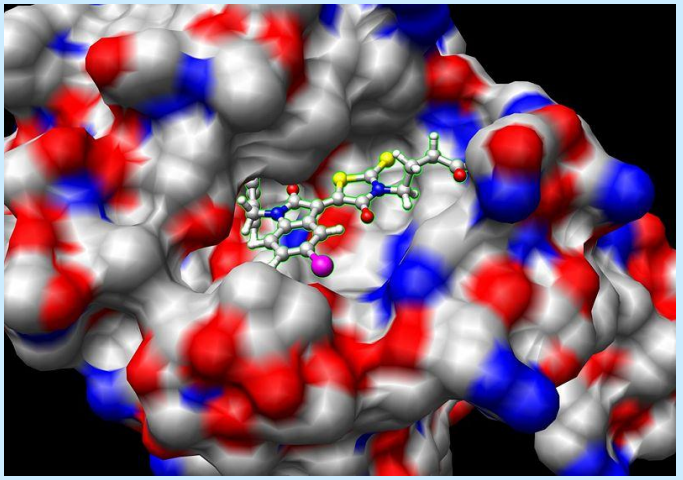


docking of a ligand to a protein receptor



Small molecule docked to a protein

Small molecule docked to a protein

we have used the approach of molecular docking of oseltamivir to the enzyme H5N1-NA to explore the active site and to provide clues to favorable binding interactions that can be used to modify the existing drugs.

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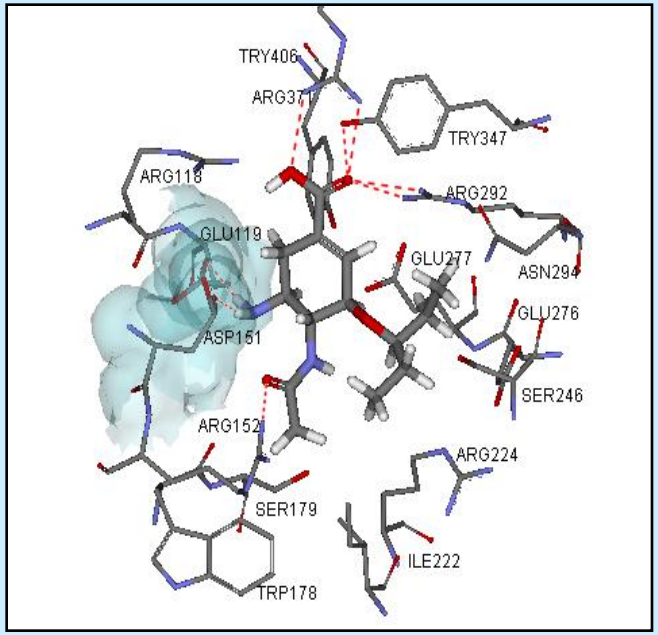
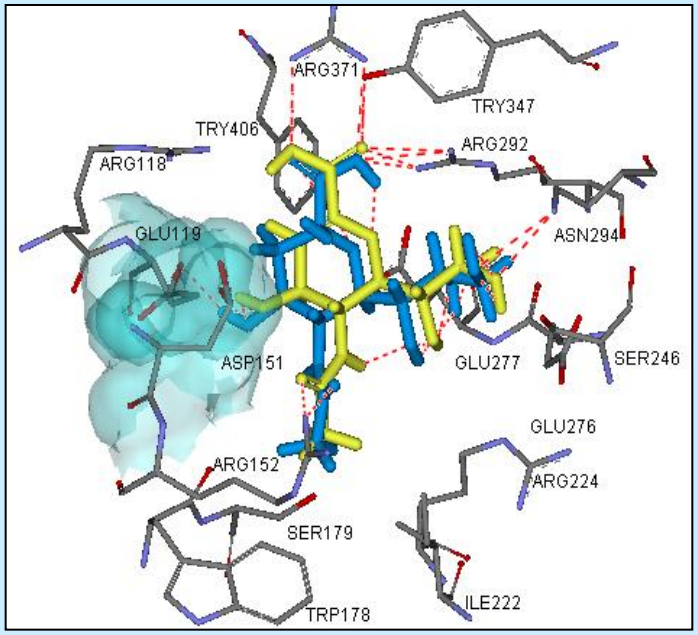
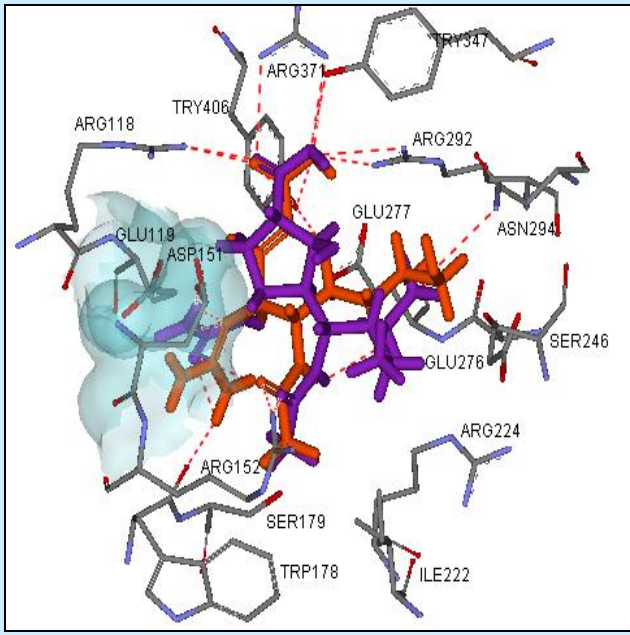
Molecular Docking….Results



Oseltamivir



sialic acid (endogenous ligand-blue) and DANA (yellow)



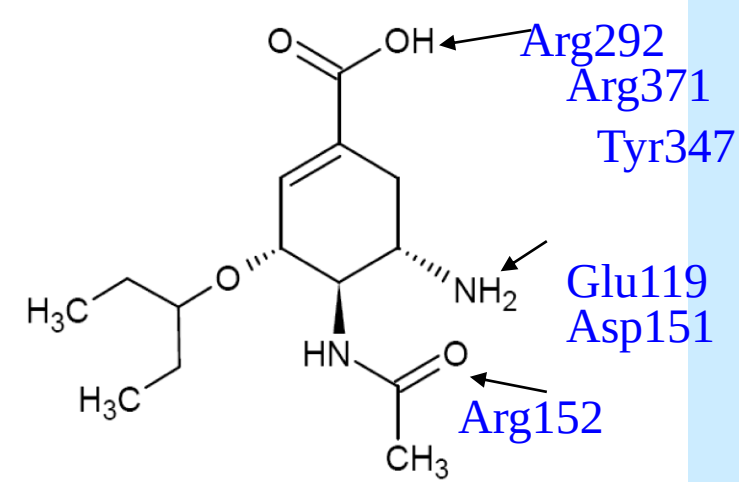
zanamivir (GG-167) (orange) and peramivir (RWJ-270201) (violet)

Docked pose of oseltamivir and the other ligands in H5N1-NA active site. H-bonds between the drug and the enzyme are indicated in red and solid surface indicates site 2.

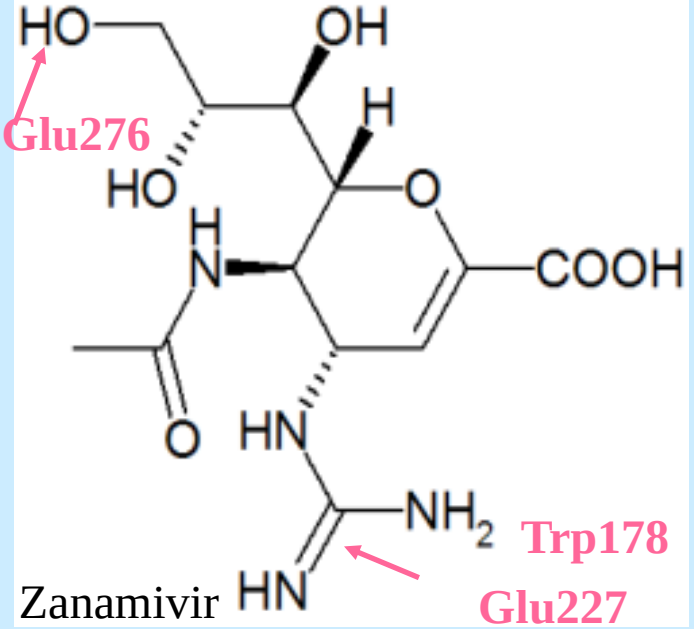
Results of docking studies with H5N1-NA

Amino acid residues of H5N1-NA	Ligands: Oseltamivir GS-4071	Zanamivir GG-167	Peramivir RWJ-270201	DANA	Sialic Acid
ARG118		HO-C=O....HN	HO-C=O....HN		HO-C=O....HN
GLU119	NH2....O=C				C-OH....O=C
ASP151	NH2....O=C	NH2....O=C	NH2....O=C		
ARG152	HN-C=O....HN	HN-C=O....HN	HN-C=O....HN	HN-C=O....HN	HN-C=O....HN
TRP178		NH2....O=C			
GLU227		NH2....O=C	NH2....O=C		
GLU276		C-OH....O=C			
		C-OH....O=C			
ARG292	HO-C=O....HN	HO-C=O....HN	HO-C=O....HN	HO-C=O....HN	HO-C=O....HN
	HO-C=O....HN			HO-C=O....HN	HO-C=O....HN
TYR347	HO-C=O....HO	O=C-OH ....HO	HO....HO	HO-C=O....HO	
ARG371	O=C-OH....HN	O=C-OH....HN	O=C-OH....HN	O=C-OH....HN	O=C-OH....HN
	HO-C=O....HN	HO-C=O....HN	HO-C=O....HN	HO-C=O....HN	HO-C=O....HN
H bonds	8	11	8	6	7

INTERACTIONS….



Oseltamivir carboxylate (GS-4071)



Zanamivir

number of hydrogen bonds in the case of oseltamivir is smaller compared to the two contemporary drugs zanamivir and peramivir.

The additional interactions in case of Zanamivir are the binding of the guanidine amino group and glycerol hydroxyl group.

These additional interactions in case of zanamivir provided by the guanidine and glycerol moieties, probably contribute to its lower resistance to NA seen among the currently available drugs.

INTERACTIONS….

- **Docking of sialic acid, the endogenous substrate shows a binding pattern very similar to inhibitor oseltamivir except that it does not interact with Asp151.**

- **DANA, on the other hand, shows less hydrogen bonding interaction compared to other inhibitors. This is also supported by the lack of binding at Asp151 in our docking studies thereby the observed less activity of DANA as compared to other NA inhibitors.**

- **The results correlate with the fact that lack of positive charge on sialic acid and DANA (presence of hydroxyl group instead of amine/guanidine) does not facilitate the interaction with Asp151 in these two cases.**

Conclusions from Docking Studies….

- To accommodate the hydrophobic side chain of oseltamivir in the active site, the NA molecule undergoes rearrangement to create an additional binding pocket.

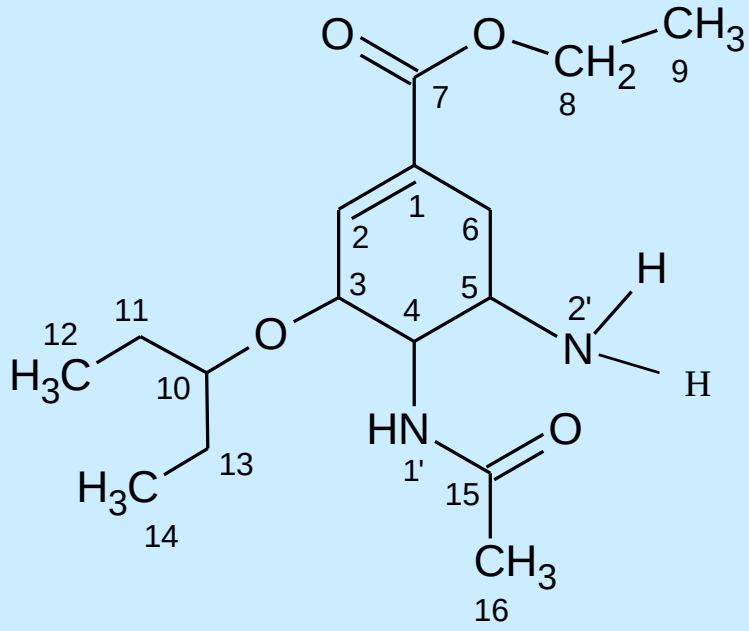
- This involves rotation of Glu276 and binding with Arg224 to form such pocket.

- This process is inhibited by the mutations R292K, N294S, and H274Y preventing the Glu276-Arg224 ionic interaction as a result the formation of additional binding pocket is hindered resulting resistance to oseltamivir.

- The mutations nonetheless allow the binding of the natural sialic acid substrate, so that the mutated virus can survive and propagate.

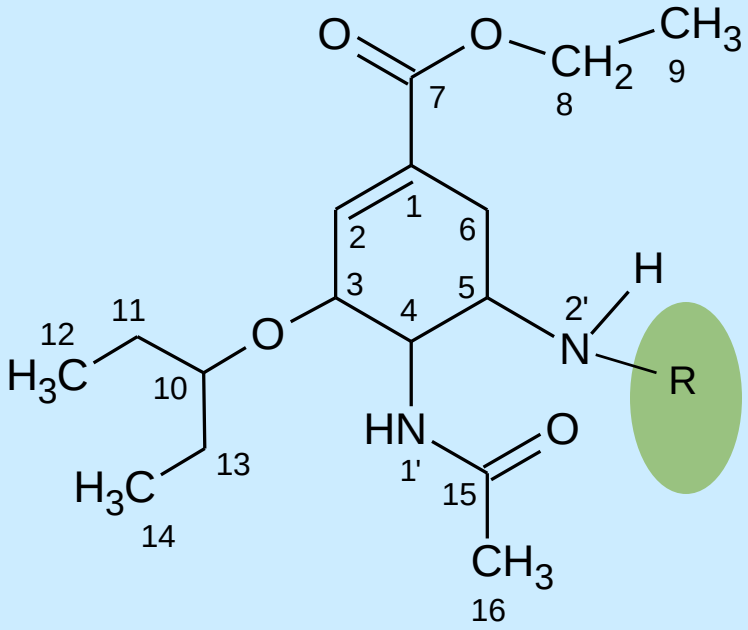
Conclusions from Docking Studies….

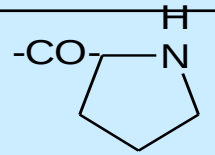
Based on the docking results it turns out that modifying the molecular volume of oseltamivir by extending the length of the C5-amino group, at the same time increasing the lipophilicity may strengthen the binding interactions and overcome the problem of drug resistance



Analogues of oseltamivir (OS)

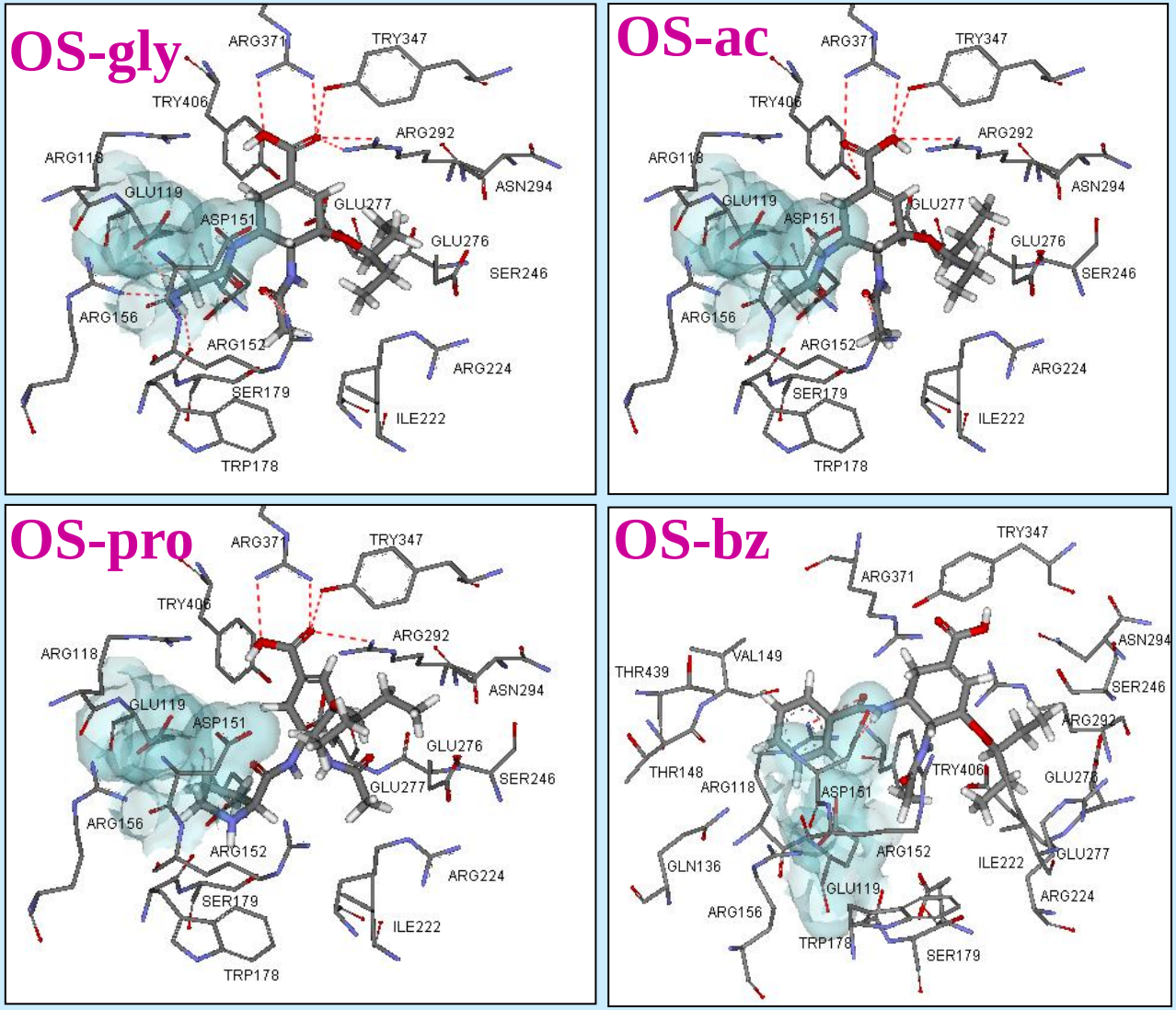
Analogues of oseltamivir (OS) with varying charge and lipophilicity



Compound/ code	Substituent R
OS	H
OS-gly	-COCH$_2$NH$_2$
OS-ac	-COCH$_3$
OS-pro	
OS-bz	-COC$_6$H$_5$

these molecules were docked into the H5N1-NA active site and the binding energies were analyzed and compared

Derivatives of oseltamivir (OS)



Docked poses of oseltamivir analogues in the H5N1-NA active site. H-bonds between the analogues and the enzyme are indicated in red.

Amino acid residues of H5N1-NA	Ligands					
	Oseltamivir (GS-4071)	**Zanamivir (GG-167)**	**OS-gly**	**OS-ac**	**OS-bz**	**OS-pro**
ARG118		**HO-C=O....**HN	**HO-C=O….**NH			
GLU119	**NH$_2$….**O=C		**CH$_2$-NH$_2$….**O=C			
ASP151	**NH$_2$….**O=C	**NH$_2$….**O=C	**O=C-NH….**O=C	**O=C-NH….**O=C	**bz-CONH….**O=C **bz-CONH….**O=C	
ARG152	**HN-C=O….**HN	**HN-C=O….**HN	**HN-C=O….**HN	**O=C-NH….**NH **O=C-NH….**NH		
ARG156			**CH$_2$-NH$_2$….**HN			
TRP178		**NH$_2$….**O=C	**CH$_2$-NH$_2$….**O=C			
GLU227		**NH$_2$….**O=C				
GLU276		**C-OH….**O=C **C-OH….**O=C				
ARG292	**HO-C=O….**HN **HO-C=O….**HN	**HO-C=O….**HN	**HO-C=O….**HN **HO-C=O….**HN	**O=C-OH….**HN		**HO-C=O….**HN
TYR347	**HO-C=O….**HO	**O=C-OH….**HO	**HO-C=O….**HO	**O=C-OH….**HO		**HO-C=O….**HO
ARG371	**O=C-OH….**HN **HO-C=O….**HN	**O=C-OH….**HN **HO-C=O….**HN	**O=C-OH….**HN **HO-C=O….**HN	**O=C-OH….**HN **HO-C=O….**HN		**O=C-OH….**HN **HO-C=O….**HN
TYR406					**O=C-NH….**OH	
H bonds	8	11	11	8	4	4

Analogues of oseltamivir

AS compared to oseltamivir, zanamivir forms additional H-bonds with Trp178 and Glu227 in H5N1-NA, which arise from the guanidino group.

OS-gly forms H-bonds similar to oseltamivir and interacts with Trp178 and Arg 118 (similar to zanamivir). The gly group contributes additional H-bond interactions over the parent drug. These interactions are responsible for the tighter binding with the enzyme, and might contribute to a lower susceptibility to resistance, in parallel with the observation for zanamivir.

Similar interactions are not observed in case of the OS-ac analog; instead the acetyl group interacts with Asp151 and Arg152.

The bulky benzyl and proline moieties of OS-bz and OS-pro show close van der Waals contacts with Val149, Thr148, Thr439, Gln136 and Glu227, Trp178, Arg156, Asp151 respectively. Strong steric interactions of these bulky groups with the receptor surface changes the overall 3D orientation of these molecules within the receptor cavity.

Thus, the addition of bulky groups at the amino moiety in oseltamivir lowers the affinity for the NA and this may lead to a decrease in the biological activity.

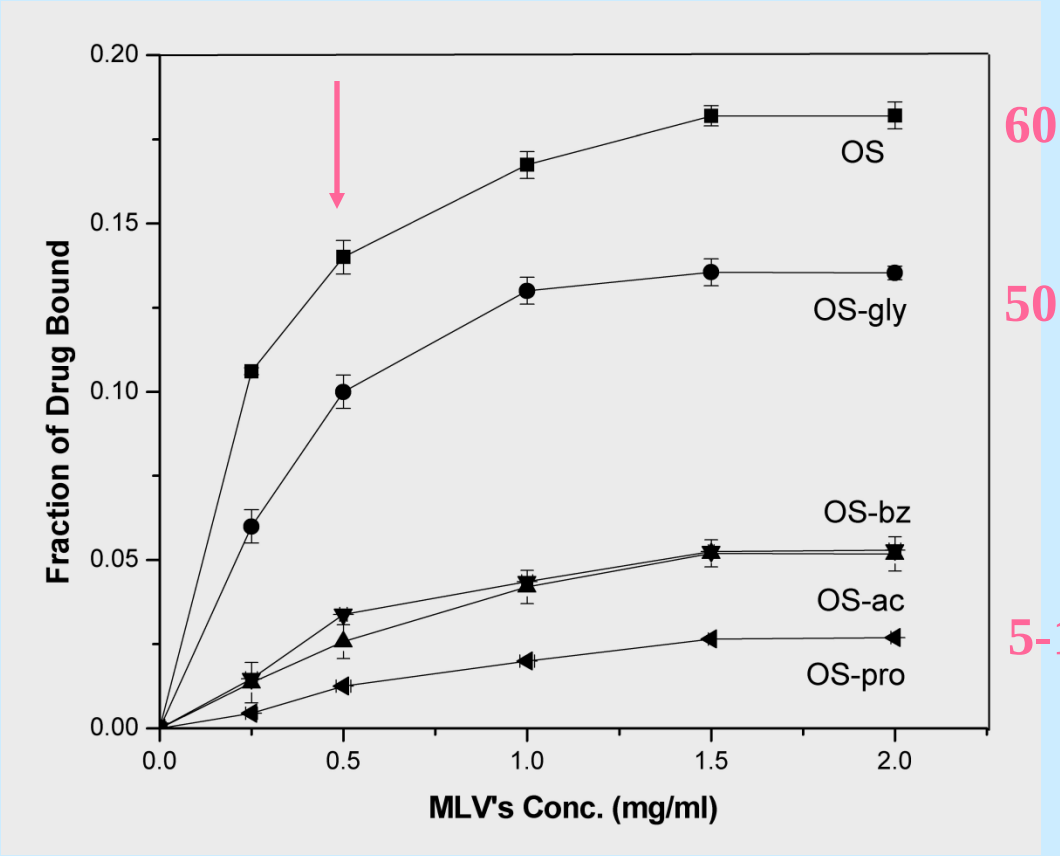
Interaction with Model Membrane

Interaction with Model Membrane ??

NA inhibitors are reported to inhibit the receptor binding of HA and thus its ability to promote attachment and fusion of cell membrane to the virus. By this process it is likely to interfere with cell-cell fusion thus interfering with the virus entry step.
Therefore a detailed investigation on interaction of these ligands with model membranes is warranted.

Moreover, the prodrug which is phosphate salt is orally administered. The interaction of the prodrug with model membranes will provide understanding of the absorption and the transport of the drug through the gastrointestinal tract.

Binding with MLVs



60%

50%

5-10%

apparent binding constants

OS - 8594 M^{-1}

OS-gly - 4725 M^{-1}

OS-ac - 623 M^{-1}

OS-bz - 717 M^{-1}

OS-pro - 187 M^{-1}

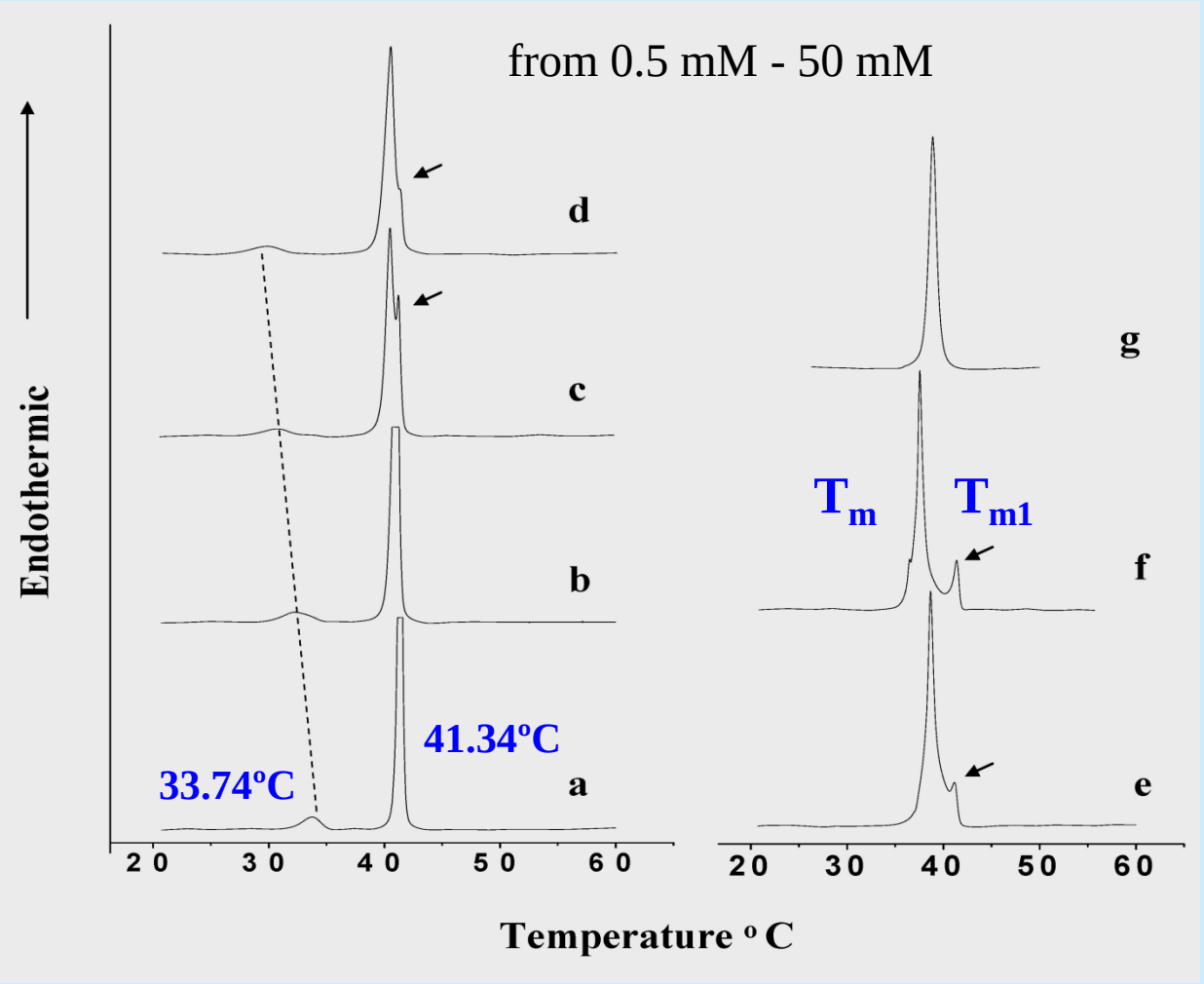
binding affinity increases with increasing hydrophilicity. This may be due to a preferential binding to the head group (polar, hydrophilic portion) of the lipid vesicles.

For OS and OS-gly nearly 60% and 50% respectively of the total drug is bound to the liposomes even at the relatively low lipid concentration of 0.5 mM.

Thermotropic behaviour

Issues concerning the nature of this binding with regard to the thermotropic behaviour and dynamics of the lipid, and also the various groups involved in the intermolecular interactions are further addressed .

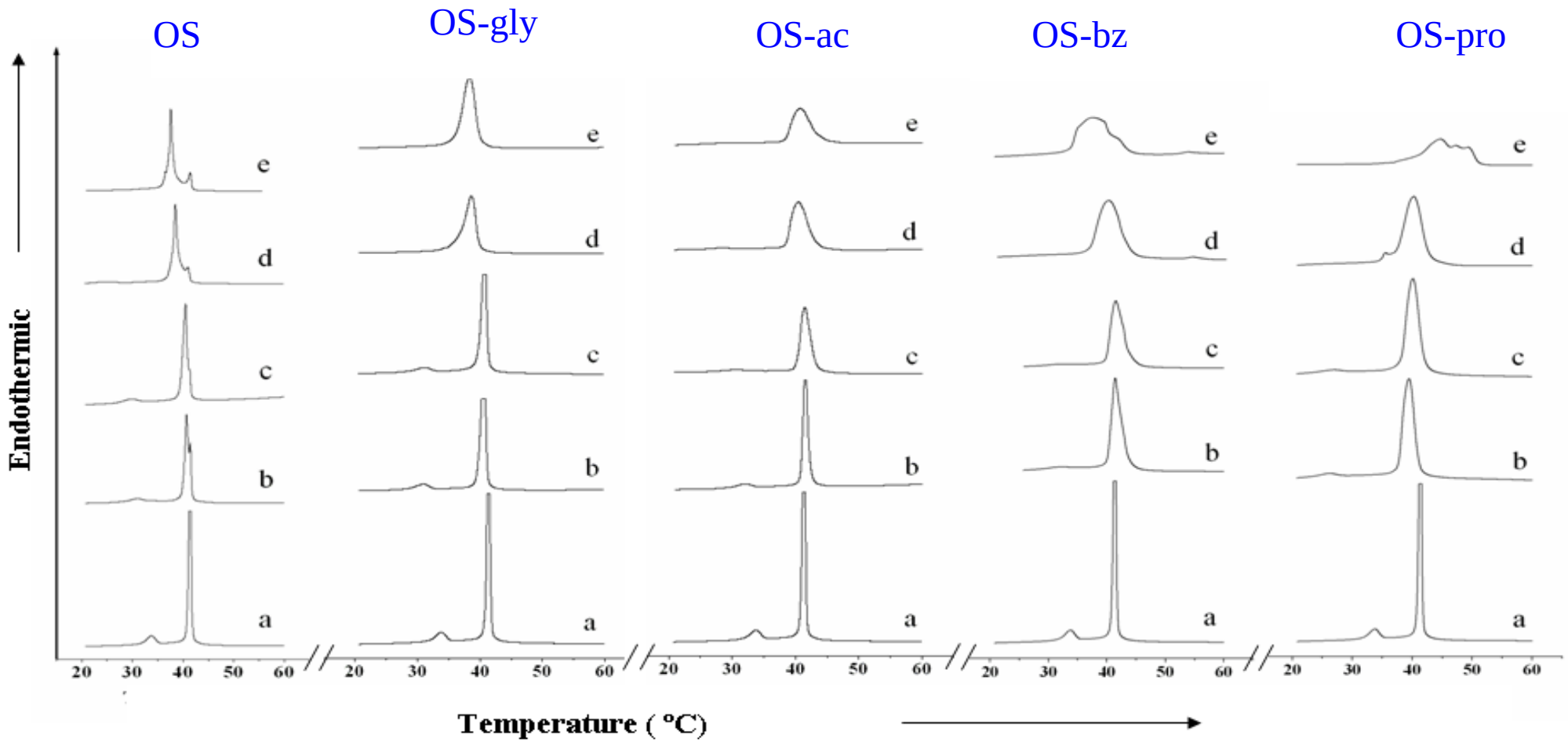
Thermotropic aspect of drug-lipid interaction using DSC



Changes in the melting point (Tm) shape of the DSC trace.

The T_m and T_{m1} transitions, may be due to the coexistence of drug-rich and drug-poor DPPC domains (phases) within the bilayer. The presence of coexisting phases indicates phase segregation, which may arise from osmotic stress on the DPPC bilayer, due to the strong interaction of the phosphate group in OS with the polar head group of the lipid.

Thermotropic aspect of drug-lipid interaction using DSC

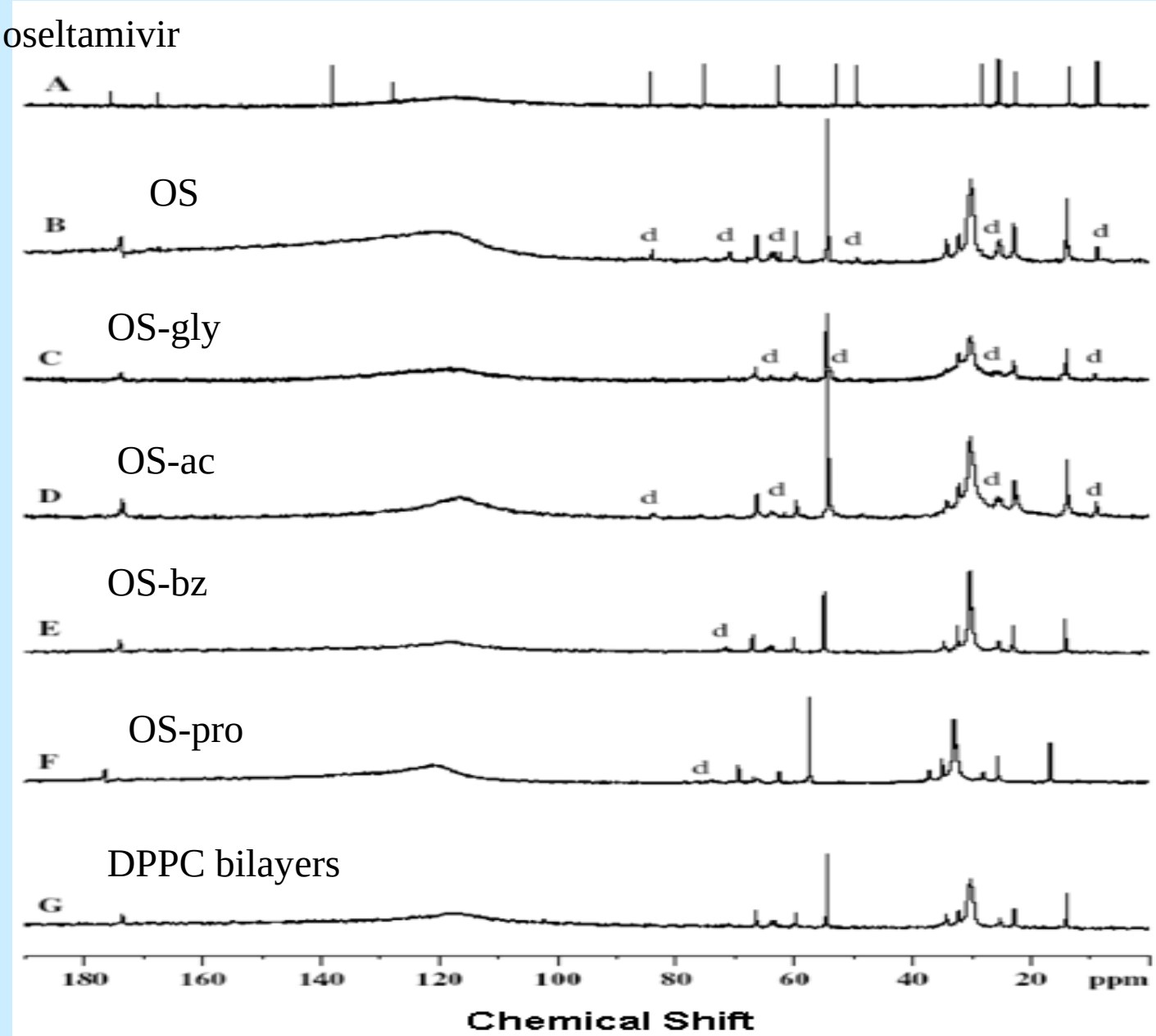


behavior of OS-gly parallels that of GS-4104 (OS).

OS-ac causes little change to both transition temperatures

benzoylic or prolyl moiety lead to remarkable changes in the isotherms.

Nature of the intermolecular interaction



resonances remain sharp indicating **fast exchange** between bound and free form.

Broadening arises due to an **exchange at intermediate time scale** between the bound and the free form

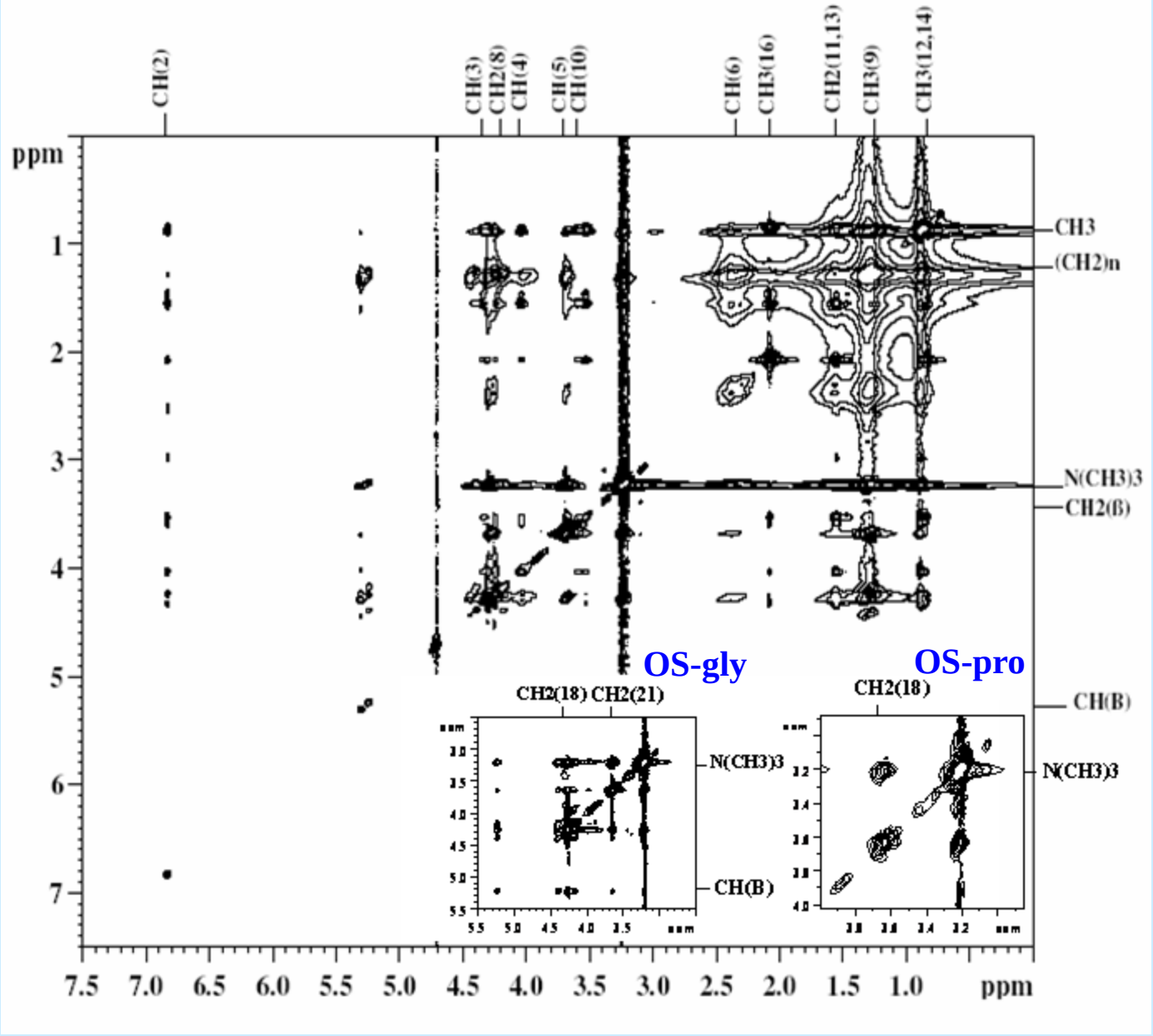
The estimate of T2 from the line widths which is about 100 ms indicates that the molecules lose their mobility and become strongly bound to lipid bilayers resulting in a loss of motional freedom. This also indicates an increase in the motional ordering of the lipid acyl chain and the head group.

binding of these molecules to DPPC largely depends on the nature of the analogue.

OS-gly and OS-ac show an average binding characteristic like OS where the molecules are in a free motion/fast exchange, with most of the signals continuing to remain sharp.

in case of OS-bz and OS-pro, the molecules are strongly bound to the lipid bilayer and loose their motional freedom

Nature of the intermolecular interaction



In magnetic resonance spectroscopy, the transfer of spin polarization from one spin population to another via cross-relaxation is generally called the **Overhauser Effect (NOE)**

the **NOE** between nuclear spins is used to establish the correlations. Hence the cross-peaks in the resulting two-dimensional spectrum connect resonances from spins that are spatially close

Intermolecular NOEs

Intermolecular NOE's seen between the lipid and OS, OS-gly, OS-ac, OS-bz and OS-pro

DPPC	Oseltamivir (GS-4104)	OS-gly	OS-ac	OS-bz	OS-pro
CH_3	CH_2(11,13), CH_3(12,14)	CH_2(11,13), CH_3(12,14)	CH_2(11,13), CH_3(12,14)	CH_2(11,13), CH_3(12,14)	CH_2(11,13), CH_3(12,14)
$(CH_2)_n$	CH_2(11,13), CH_3(12,14),CH(6)	CH_2(11,13), CH_3(12,14),CH(6)	CH_2(11,13), CH_3 (12,14),CH(6)	CH_2(11,13), CH_3(12,14),CH(6)	CH_2(11,13), CH_3 (12,14),CH(6)
	CH(10)	-	CH_3(18)	CH(19-23)	-
CH(B)	CH_2(8)	CH_2(8), CH_2(18)	-	-	CH_2(8),CH(3), CH_2(18)
$CH_2(\beta)$	CH(2)	CH_2(18)	-	-	-
$N(CH_3)_3$	CH_2(8)	CH_2(8), CH_2(18)	-	-	CH_2 (8),CH_2(18), CH(10),CH_2(21)

OS-gly and OS-pro a number of additional intermolecular NOEs are seen compared to OS; these additional interactions are with the head group of the lipid

In complete contrast, for the OS-bz and OS-ac derivatives, the interactions with the lipid head group are absent.

Lipid polymorphism

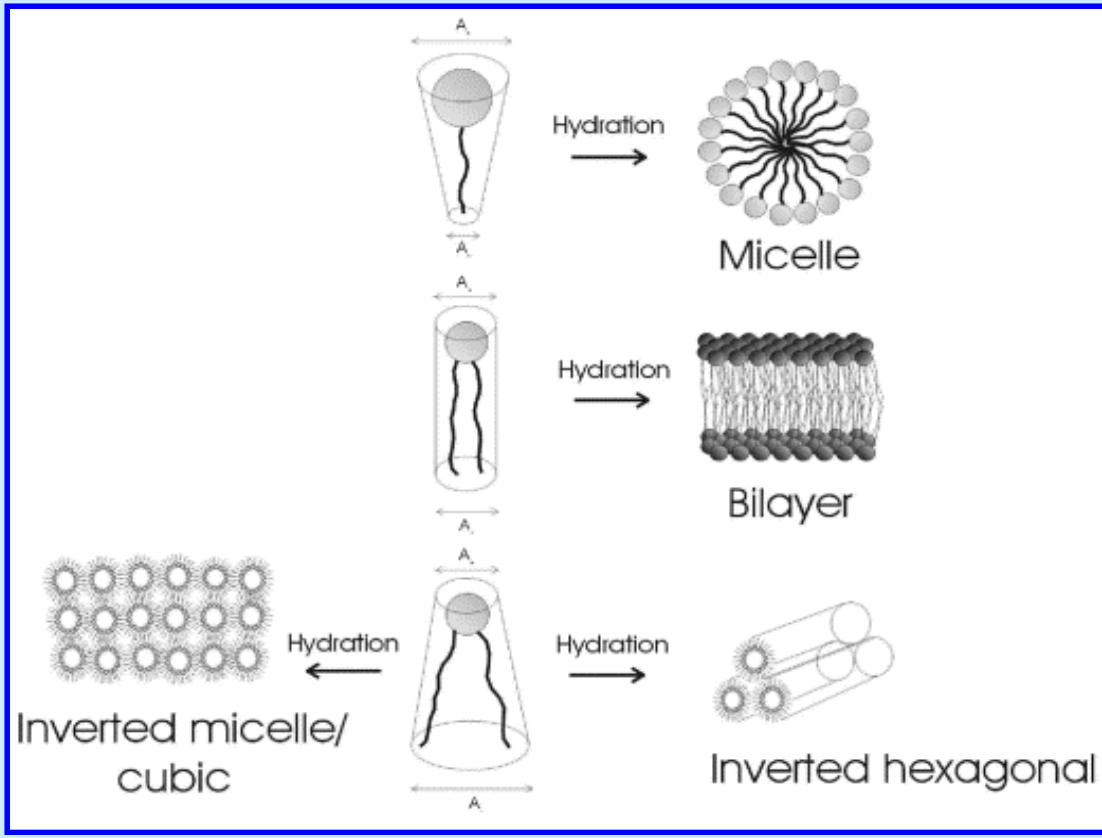
Lipids can assemble into a variety of structures

Lipid organization changes from bilayer to hexagonal (Lipid Polymorphism)

type of lipids,
experimental condition
(pH, temperature, hydration, ions, salt etc)
lead to polymorphic phase behaviour

lipids with small headgroup areas adopt "inverted" lipid phases such as the inverted hexagonal (H_{II}) phase or cubic phases and are said to exhibit negative membrane curvature

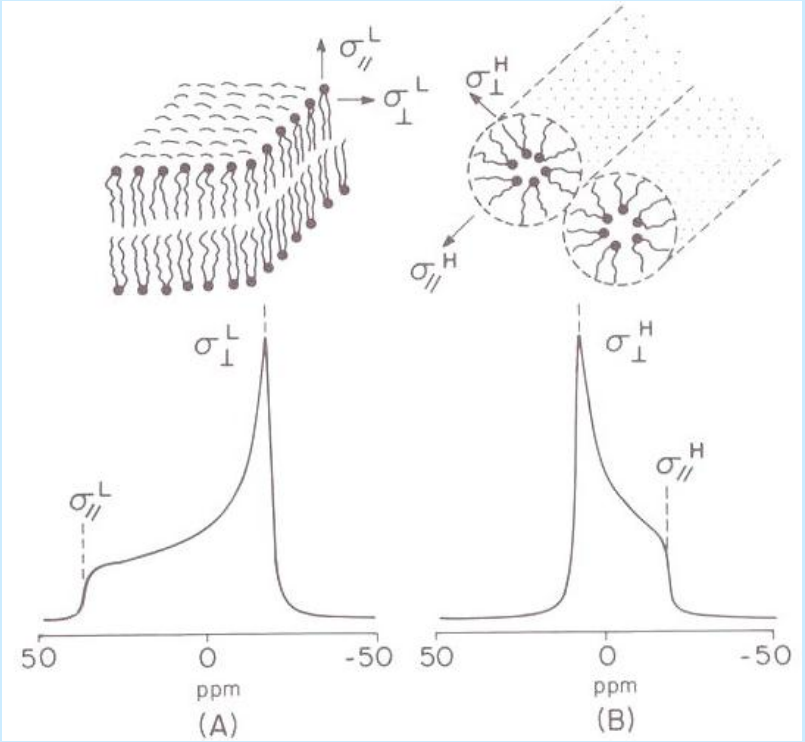
biological involvement of lipid polymorphism is in membrane fusion, exocytosis, transport etc.



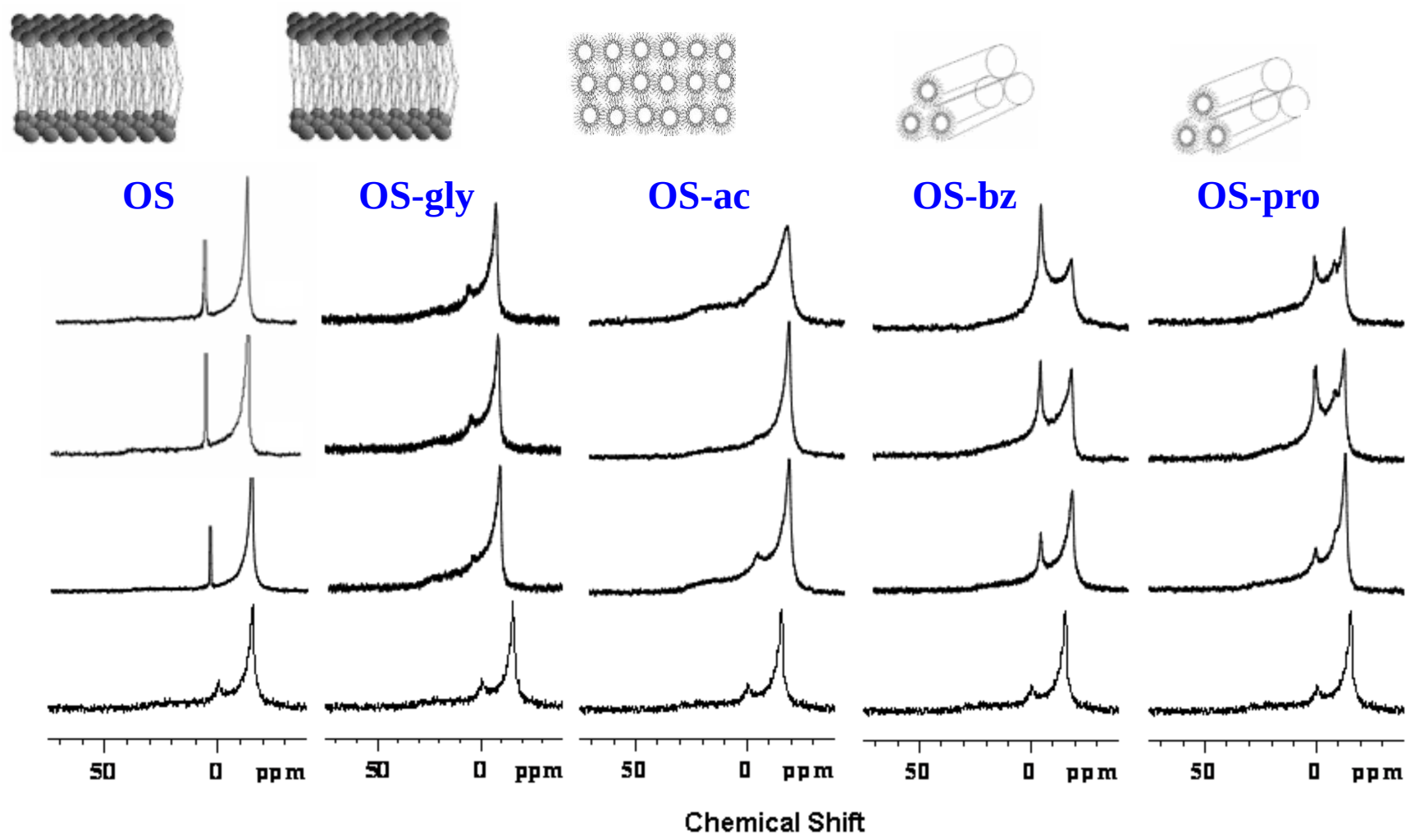
^{31}P NMR (Lipid polymorphism)

•^{31}P NMR spectroscopy is sensitive to local motions and the orientation of the phosphate group in the membrane, making it well suited for monitoring structural changes and detecting polymorphism .

•The ^{31}P NMR resonance line shape is determined by the chemical shift anisotropy (CSA) of the phosphate group coupled with the molecular motions near the head groups.



^{31}P NMR (Lipid polymorphism) in presence of drug molecules



Characteristic feature of the bilayer remains unchanged. At high conc, width of the ^{31}P resonances is narrower and there is a small change in the CSA parameter as seen by the sharpening of the parallel component at -20 ppm. This is due to the increased motional freedom/ decreased order of the phospholipid head group. Overall, this seems to indicate that OS stabilizes the lipid bilayer phase.

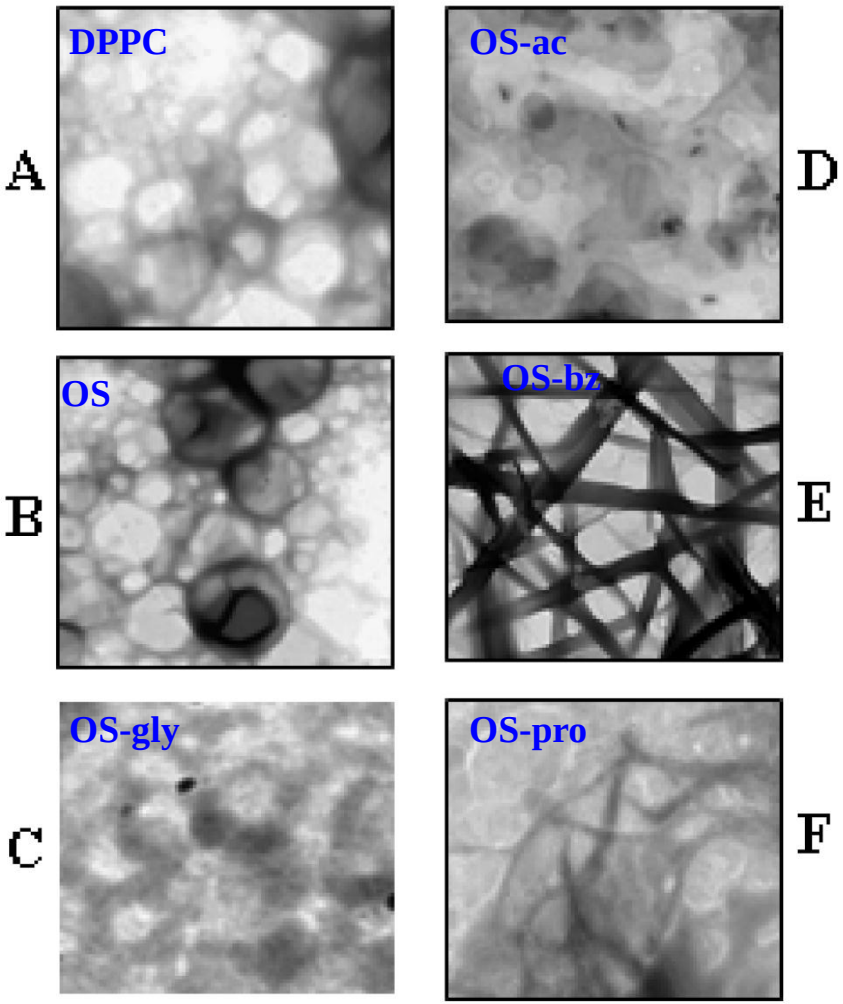
In case of OS-gly, line shape is not affected and remains very similar to the pure DPPC bilayer..

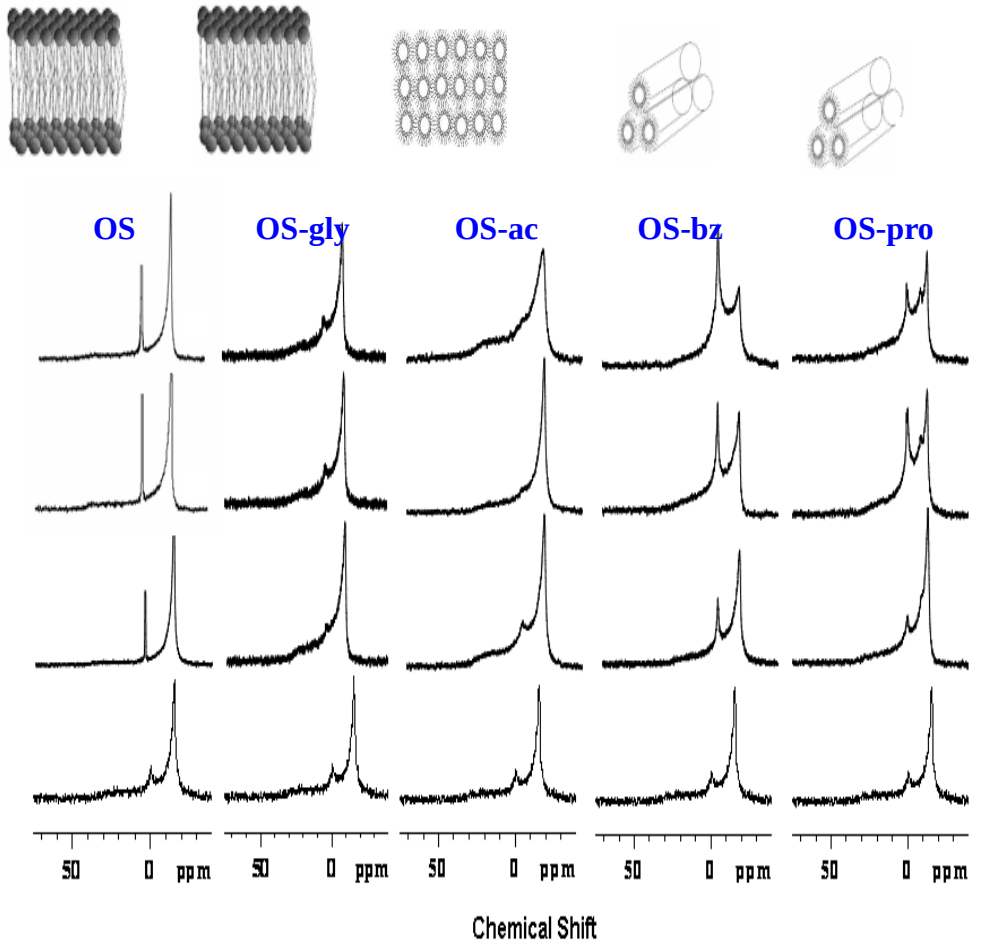
The peak at 0 ppm broadens completely and the main peak at -20 ppm also broadens to a very large extent at 1:5 OS-ac:lipid ratio. This increase in CSA indicates the formation of even larger vesicles. However, the bilayer features remain intact to a large extent keeping the vesicles highly rigid. This suggests that OS-ac causes very little perturbation of the bilayer.

with increasing conc the peak at 0 ppm becomes sharper and increases in intensity. The main peak at -20 ppm decreases in intensity with ensuing broadening. This is characteristic of the transformation of the bilayer phase to the hexagonal phase.

OS-pro-, the peak at 0 ppm increases in intensity and the peak at -20 ppm splits into sharp multiple peaks. The multilamellar bilayer phase is thus completely disrupted with the breaking of the multilamellar vesicles into nearly hexagonal rods. These results thus indicate that the two derivatives OS-bz and OS-pro lead to a complete transformation of the original multilamellar bilayer phase with decreased motional freedom/enhanced acyl chain ordering.

Phase behavior and the aggregate structure of the liposomes with TEM





bilayer characteristics are largely retained, no polymorphism is observed, fusion bodies are absent.

OS and OS-gly bind to the cell membrane and stabilize the bilayer character preventing the cell-cell fusion which is an important phenomenon during the viral entry in the host cell.

Conclusion

❖docking studies indicate that increasing the molecular volume of OS changes the intermolecular interaction to a large extent. The strength of this interaction depends on the size/type of the substituent.

❖small group such as glycyl with its amino group charged at physiological pH enhances the interaction with the receptor site and will possibly increase its inhibitor activity. On the other hand bulky groups are not suitable substituents, as these groups bump into the receptor surface, shifting the locus of the binding, and will probably lead to a lower activity.

❖Thus for improvement in antiviral activity, the introduction of functional moieties at the amino group, with proper shape; size, electronic charge and lipophilicity are critical.

❖these molecules interact with the head group in a manner that stabilizes the membrane architecture to a large extent. Secondly, a decrease in the main transition temperature indicates that they impart fluidity to the bilayer by positioning themselves within the hydrophobic core.

❖bilayer structure is retained in these three cases except for a slight perturbation by OS-ac.
Os-bz and OS-pro show anomalous behavior, perturbing the bilayer structure completely by disturbing the strong hydrophobic interactions between the lipid molecules..

Conclusion

❖OS & OS-gly prevent membrane fusion and are seen to have a stabilizing effect on the membrane.

❖OS-ac causes marginal disruption of the lipid bilayers.

❖OS-pro and OS-bz are identical in their action, disrupting the bilayer structure.

❖On the basis of these results, we conclude that of the four derivatives, OS-gly is the most promising candidate for further *in vivo/in vitro* pharmacological evaluation.

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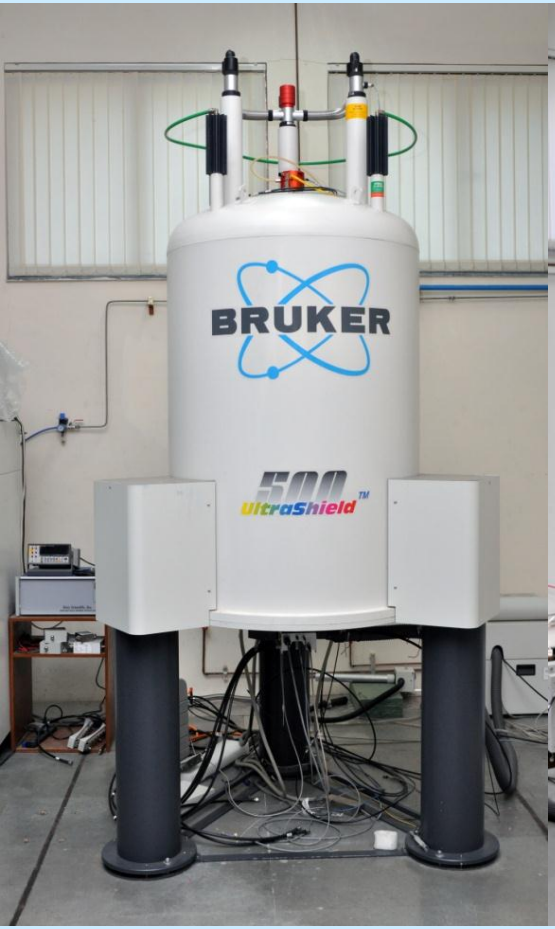
NMR SPECTROMETERS AT NATIONAL NMR FAC ILITY, TIFR, MUMBAI



BRUKER AVANCE I
500MHz

VARIAN INOVA
600MHz

BRUKER AVANCE III
700MHz

BRUKER AVANCE II
800MHz

THANKYOU….