The Abdus Salam
International Centre for Theoretical Physics

United Nations
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2039-7

Conference on 2nd Drug Development for the Third World: From Computational Molecular Biology to Experimental Approaches

1 - 5 June 2009

Multivalent Drugs and Diarrheal Diseases

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Diarrheal diseases & The Power of Multivalency in Drug Design

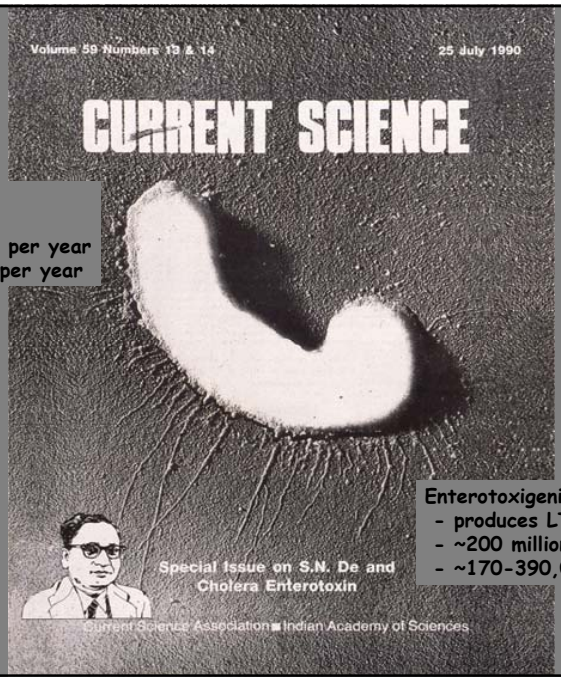
Wim Hol
University of Washington, Seattle
-
Drug Development for the Third World
International Center of Theoretical Physics (ICTP)
Trieste, Italy
June 2009

Multivalent Inhibitors of Cholera Toxin (CT)

produced by *Vibrio cholerae.*

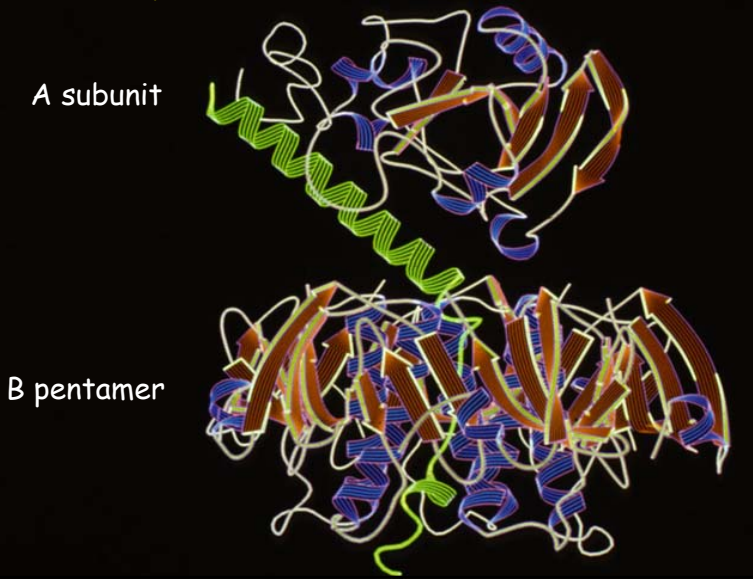
CT is a close relative of
Heat-Labile Enterotoxin (LT)
produced by enterotoxigenic *E. coli,*
the cause of much of children's and travellller's diarrhea

Vibrio cholerae :
- produces CT
- ~3-5 million cases per year
- ~120,000 victims per year

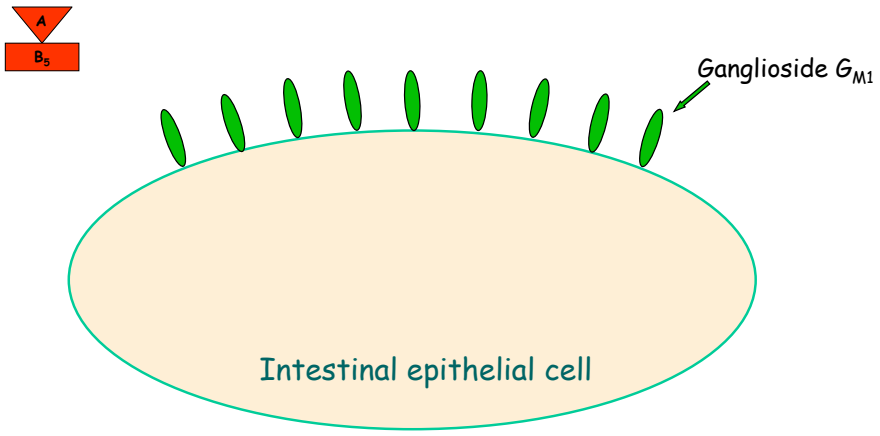


Enterotoxigenic *E. coli* :
- produces LT and ST
- ~200 million cases per year
- ~170-390,000 victims per year

Heat-labile Enterotoxin (LT), a very close relative of Cholera toxin (CT)

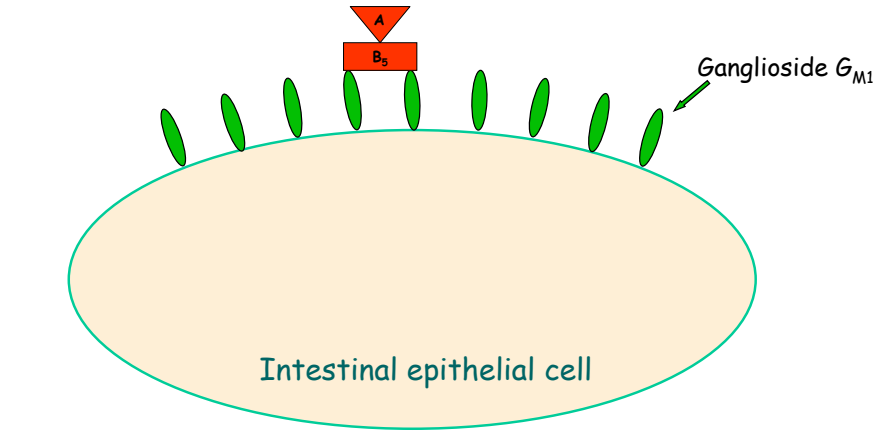


CT and LT *vs.* human cell



CT : Cholera LT : Traveller's & Children's diarrhea

CT and LT *vs.* human cell

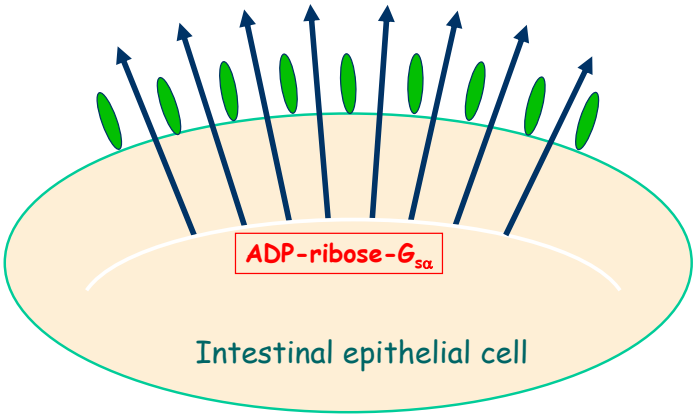


CT : Cholera LT : Traveller's & Children's diarrhea

CT and LT *vs.* human cell

Eventually: DEHYDRATION

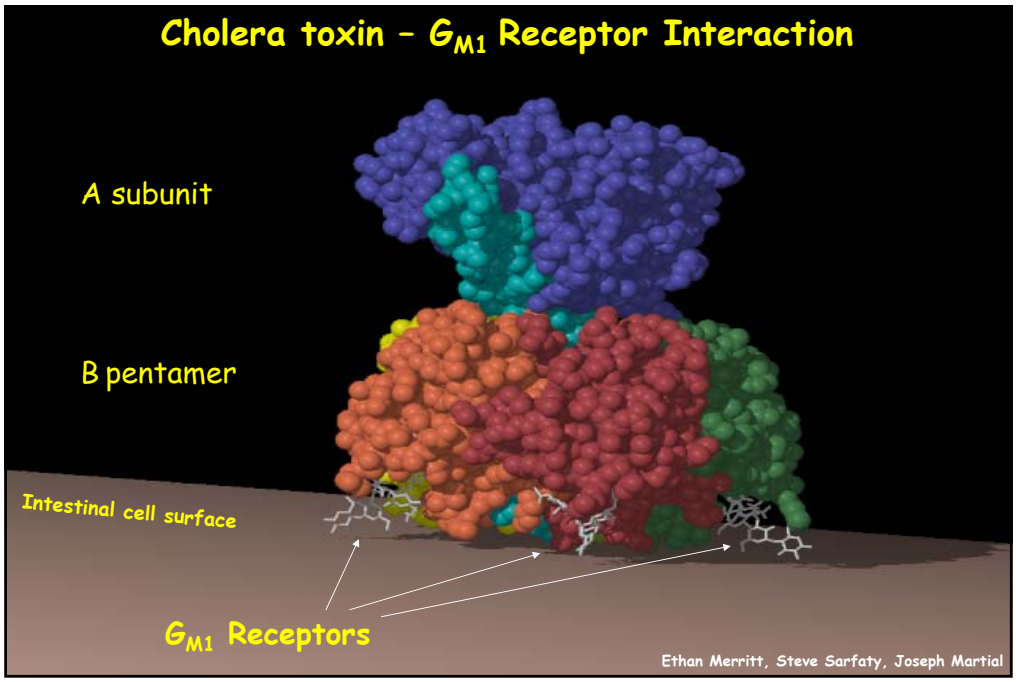
Due to dramatic efflux of salt and water



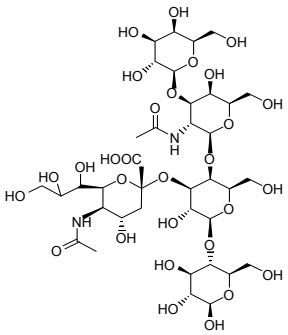
CT : Cholera

LT : Traveller's & Children's diarrhea

Cholera toxin – G_{M1} Receptor Interaction

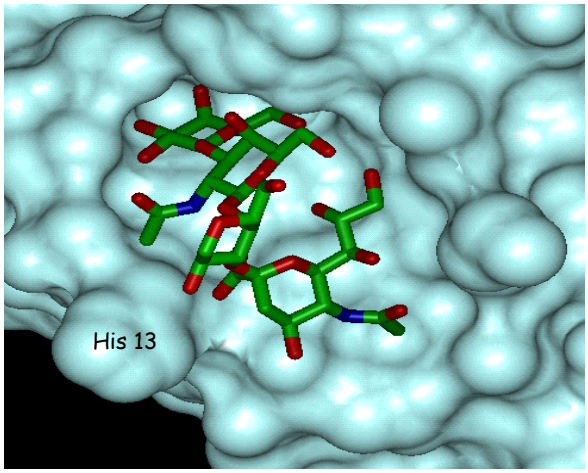


G_{M1} Pentasaccharide bound by CT



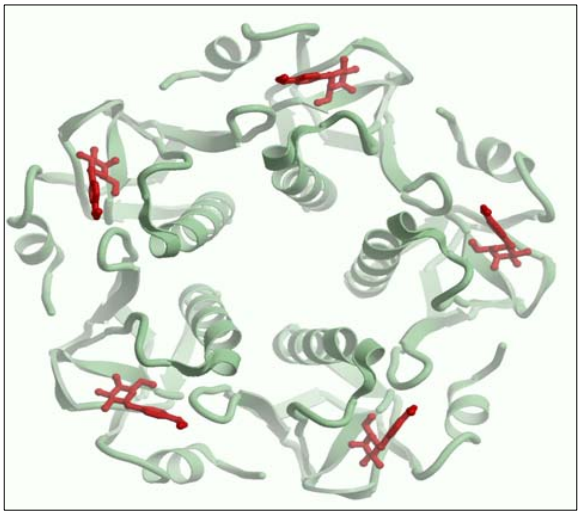
IC_{50} = 14 nM

Extensive hydrophobic and H-bonding interactions

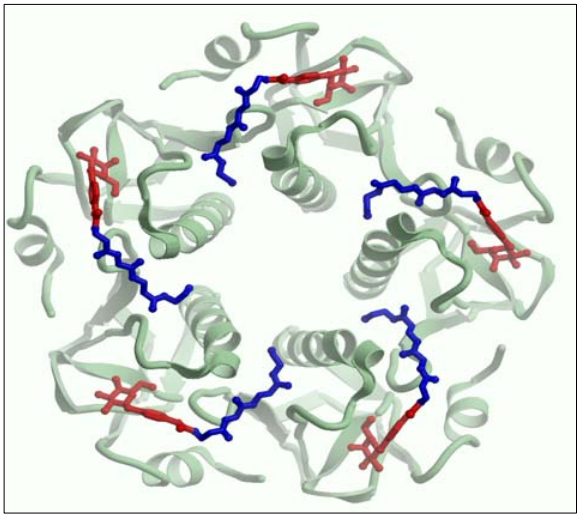


The enemy

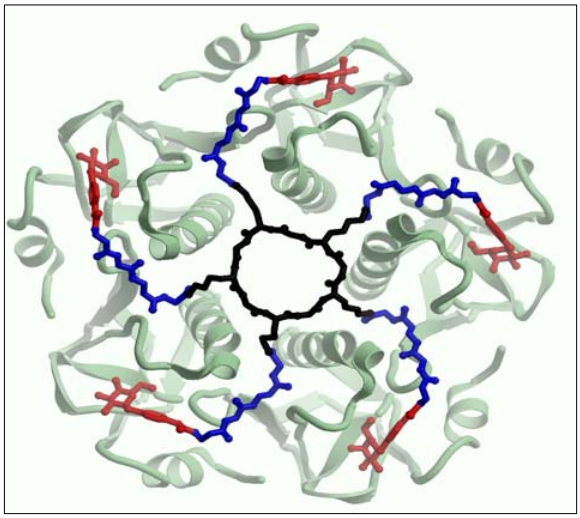
Five receptor binding sites



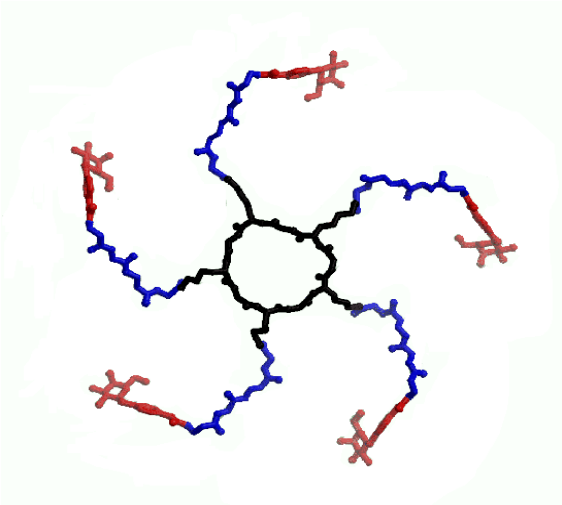
Making ligands longer



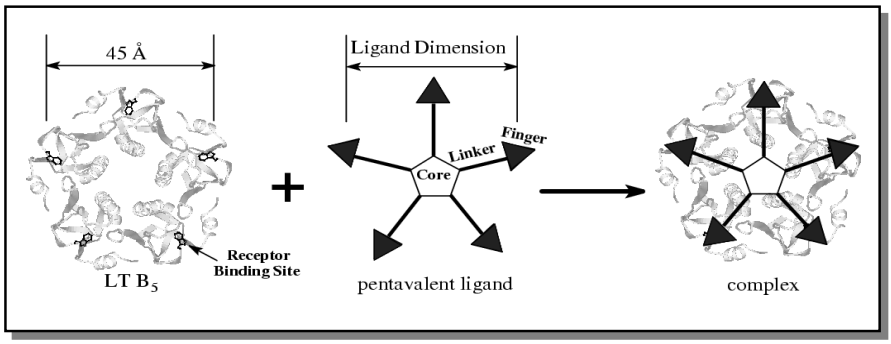
Ligand-Protein Complex



Pentavalent Ligand

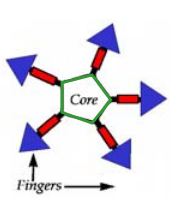
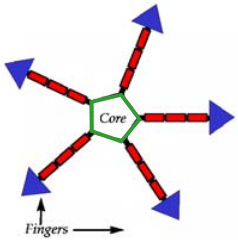
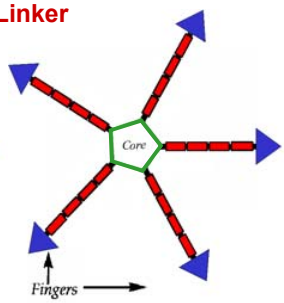


THE PENTAVALENT CONCEPT



"Proper Pre-organization"

Gains in surface-receptor binding inhibition

One-Unit Linker	Two-Unit Linker	Three -Unit Linker	Four-Unit Linker
			
??? x Single Finger	???? x Single Finger	????? x Single Finger	?????? x Single Finger

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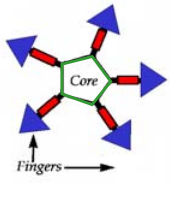
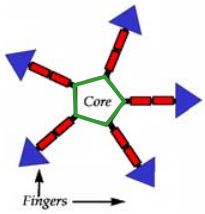
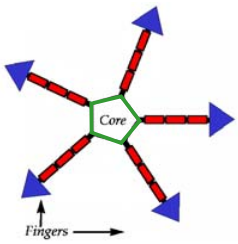
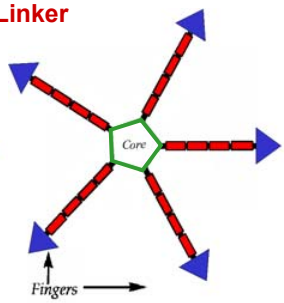
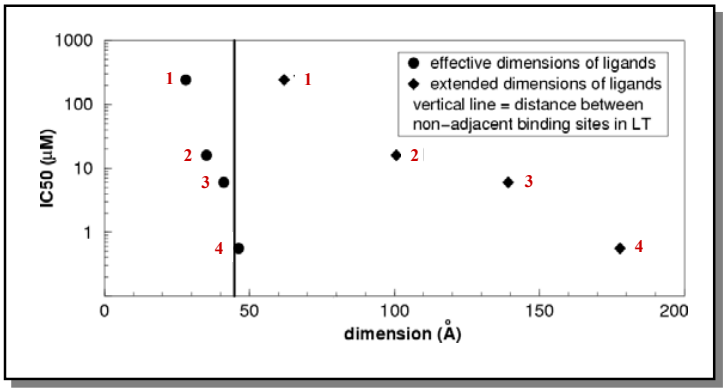
Gains in surface-receptor binding inhibition

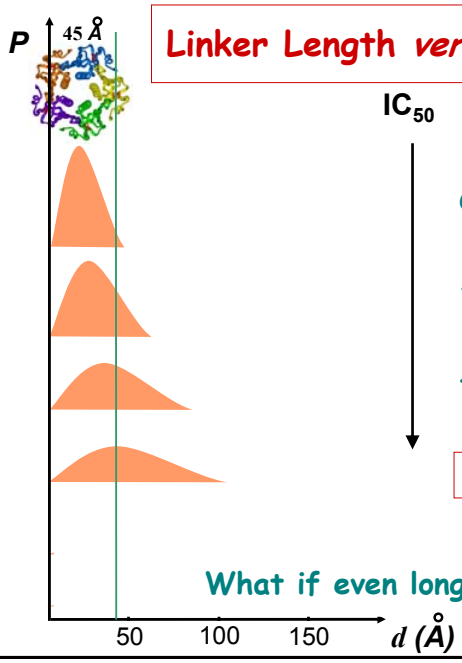
One-Unit Linker	Two-Unit Linker	Three -Unit Linker	Four-Unit Linker
			
240 x Single Finger	3,600 x Single Finger	10,000 x Single Finger	104,000 x Single Finger

Erkang Fan, Zhongsheng Zhang, Jason Pickens, Jiyun Liu, *et al*

IC_{50} *versus* EXTENDED & EFFECTIVE DIMENSIONS OF PENTAVALENT LIGANDS



Erkang Fan and co-workers



Linker Length *versus* affinity

IC_{50}

One-unit linker

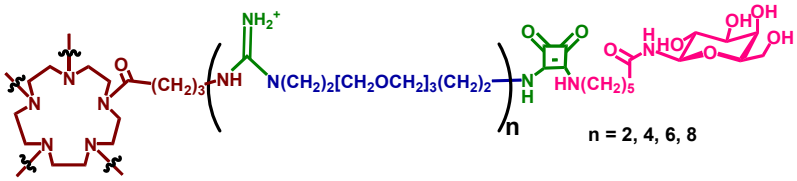
Two-unit linkers

Three-unit linkers

Four-unit linkers

What if even longer linkers?

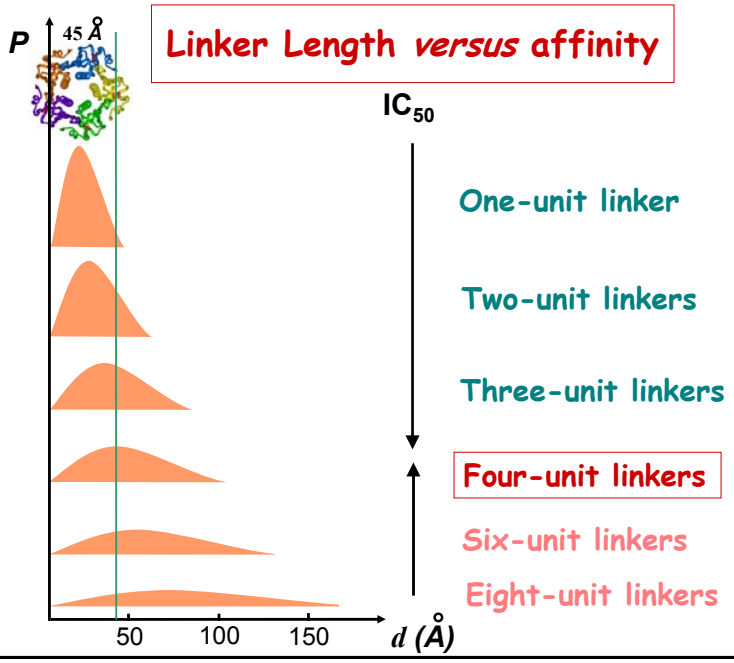
Linker too long : *less* affinity



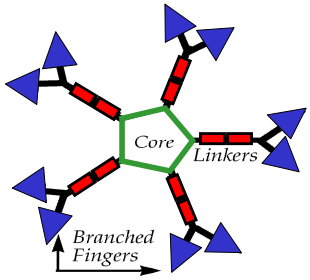
Linker Units	IC_{50} (μM)
n = 2	13.26 ± 0.95
n = 4	1.50 ± 0.10
n = 6	4.63 ± 0.46
n = 8	7.25 ± 0.38

(A single galactose "finger": IC_{50} = ~ 100 mM =~100,000 μM)

Erkang Fan and co-workers

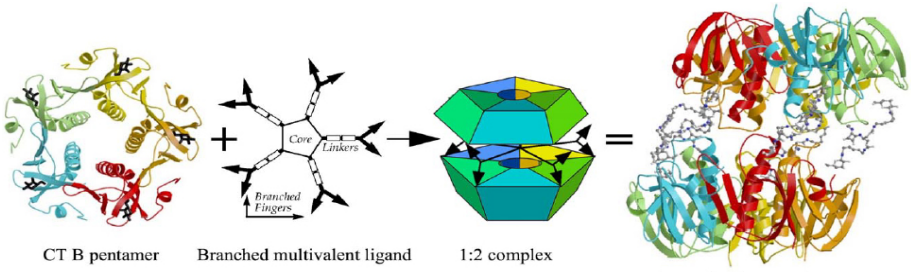


Decavalent Inhibitor



Would "pre-organization" of a decavalent inhibitor,
by binding to one CT B-pentamer,
enhance the affinity for binding to a second CT B-pentamer??

Decavalent Inhibitor



CT B pentamer | Branched multivalent ligand | 1:2 complex | 1:2 complex in crystal structure

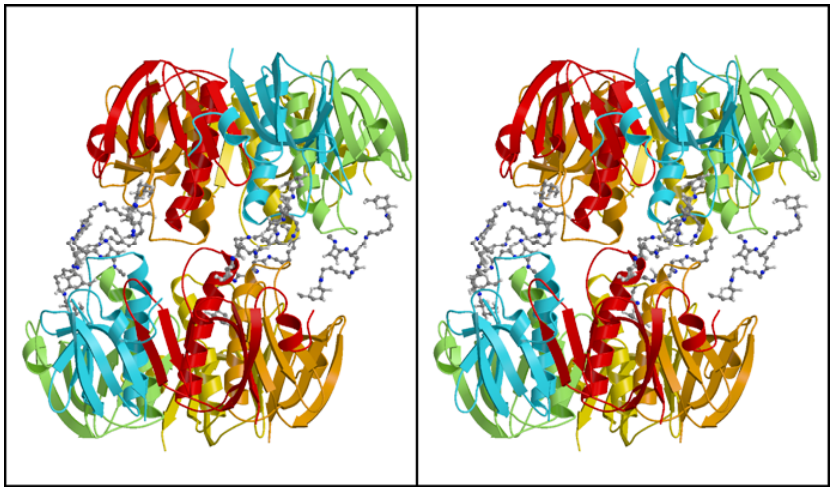
Tenfold higher affinity than Pentameric ligand with same linker length

One million fold higher affinity than a single "Galactose Finger"

Zhongsheng Zhang
Erkang Fan

Misol Ahn
Ethan Merritt

Decavalent Inhibitor



Two CT-B Pentamers Linked by a Decavalent Ligand of 10.6 kDa

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CT and LT Multivalent Inhibitors

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Seattle

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Seattle

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University of Liège
Belgium

Joseph Martial

References

Fan, E., Zhang, Z., Minke, W. E., Hou, Z., Verlinde, C. L. M. J. & Hol, W. G. J. (2000). High affinity pentavalent ligands of *Escherichia coli* heat-labile enterotoxin by modular structure-based design. *J. Am. Chem. Soc.* **122**, 2663-2664. [Highlighted in *Chemical & Engineering News* (March 27, 2000) vol. 78, p. 22]

Merritt, E. A., Zhang, Z., Pickens, J. C., Ahn, M., Fan, E. & Hol, W. G. J. (2002). Characterization and crystal structure of a high-affinity pentavalent receptor-binding inhibitor for Cholera toxin and E. coli heat-labile enterotoxin. J. Am. Chem. Soc. 124, 8818-8824. [Editor's Choice in Chemistry, Science 297, 3003 (2002)].

Zhang, Z., Merritt, E. A., Ahn, M., Roach, C., Hou, Z., Verlinde, C. L. M. J., Hol, W. G. J. & Fan, E. (2002). Solution and Crystallographic Studies of Branched Multivalent Ligands that Inhibit the Receptor-Binding Process of Cholera Toxin. *J. Am. Chem. Soc.* **124**, 12991-12998.

Zhang, Z., Pickens, J. C., Hol, W. G. J. & Fan, E. (2004) Solution and solid-phase syntheses of guanidine-bridged, water-soluble linkers for multivalent ligand design. *Org. Lett.* **6**, 1377-1380.

Fan, E., O'Neal, C. J., Mitchell, D. D., Robien, M. A., Zhang, Z., Pickens, J. C., Tan, X.-J., Korotkov, K., Roach, C., Krumm, B. E., Verlinde, C. L. M. J., Merritt, E. A. & Hol, W. G. J. (2004). Structural Biology and Structure-based Inhibitor Design of Cholera Toxin and Heat-labile Enterotoxin. *Int. J. Med. Microbiol.* **294**, 217-223.

Zhang, Z., Liu, J., Verlinde, C. L., Hol, W. G. & Fan, E. (2004). Large Cyclic Peptides as Cores of Multivalent Ligands: Application to Inhibitors of Receptor Binding by Cholera Toxin. *J. Org. Chem.* **69**, 7737-7740.

The Art and Science of Linking Ligands

The Science of Linking Ligands

In quite a number of cases two different Ligands to the same Target have been connected which resulted in a significant gain in free energy of binding. However, in other cases the free energy gain appeared to be negligible and in some cases the linked ligands have no affinity at all for the Target. How to connect Ligands to obtain higher affinity linked-ligands is an important art in Ligand Design.

In this case we have to consider several entities:

- the Ligand L1
- the binding site S1 for Ligand L1
- the Ligand L2
- the binding site S2 for Ligand L2
- the connector C, linking L1 and L2
- a possible binding site SC for the connector C

It is useful to start with a few simple equations where we will ignore water for simplicity reasons only:

L1	+ T	<>	T:L1	ΔG(L1)	(1)
L2	+ T	<>	T:L2	ΔG(L2)	(2)
L1-C-L2	+ T	<>	T:L1-C-L2	ΔG(L1-C-L2)	(3)

Now it is very common, but very wrong, to state that:

$$\Delta G(L1\text{-}C\text{-}L2) = \Delta G(L1) + \Delta G(L2) \qquad (4)$$

We will discuss below why this is wrong - but the fact is that many authors are happy about applying equation (4) since by sheer luck they obtain a correct answer for the wrong reasons! There is a fundamental point here which will be discussed in the next paragraphs.

The Science of Linking Ligands (ctd.)

First, lets list a number of options for the characteristics of the connector C. This linker C can :

- be completely rigid, or very flexible
- have very favorable, unfavorable, or no interactions with the Target
- allow the ligands L1 and L2 to bind in exactly the same mode to Target when connected as when unconnected, *or* does force L1, or L2, or both L1 and L2, to bind in a different mode when connected compared to unconnected.
- the connector does or does not cause a conformational change in the Target, affecting S1, or S2, or both.

Let us start with a **"perfect non-interacting" Connector**, i.e. a "completely rigid, non-interacting, not binding-site changing Connector which allows L1 and L2 to bind to the Target in exactly the same mode as when L1 and L2 bind individually". The Target is also perfect that is, it undergoes no conformational changes upon binding L1, L2 or L1-C-L2.

If this is the case, then we can rewrite the ΔG-values of equations (1), (2) and (3) above by considering an "intrinsic affinity" of L1 and L2 for S1 and S2, respectively, and the loss of overall rotational and translational entropy of L1, L2 and L1-C-L2.
If we now rewrite the right hand sides of eqs 1,2,3 as:

ΔG(L1)	= ΔG(L1)intr -	TΔS(r+t)	(5)
ΔG(L2)	= ΔG(L2)intr -	TΔS(r+t)	(6)
ΔG(L1-C-L2)	= ΔG(L1-C-L2)intr -	TΔS(r+t)	(7),

and with a perfectly rigid non-interacting Connector:

$$\Delta G(L1\text{-}C\text{-}L2)intr = \Delta G(L1)intr + \Delta G(L2)intr \qquad (8)$$

A key point is that, in spite of significant uncertainty about the numerical values of ΔS(r+t), statistical mechanics considerations indicates that these values are quite independent of molecular weight, and we assume these to be the same for L1, L2 and L1-C-L2.

The Science of Linking Ligands (ctd.)

Since the Connector is perfectly rigid, no freezing of bonds of the Connector, i.e. no loss of conformational entropy of the Connector has to be taken into account.

By adding (5) and (6) we obtain:

$\Delta G(L1) + \Delta G(L2) = \Delta G(L1)intr + \Delta G(L2)intr - 2*T\Delta S(r+t)$ (9)

or:

$\Delta G(L1)intr + \Delta G(L2)intr = \Delta G(L1) + \Delta G(L2) + 2*T\Delta S(r+t)$ (10)

By substituting (8) into (7):

$\Delta G(L1\text{-}C\text{-}L2) = \Delta G(L1)intr + \Delta G(L2)intr - T\Delta S(r+t)$ (11)

By substituting (10) into (11) we obtain:

$\Delta G(L1\text{-}C\text{-}L2) = \Delta G(L1) + \Delta G(L2) + T\Delta S(r+t)$ (12)

Since $T\Delta S(r+t)$ has a significant negative value we have the remarkable and very desirable result that in this specific case:

$\Delta G(L1\text{-}C\text{-}L2)$ is much MORE favorable than $\Delta G(L1) + \Delta G(L2)$!!!!

The estimates of the Rigid Body Entropy in the literature span a wide range of values. This is evident from the Table below which is kindly provided by Dr. Christophe Verlinde.

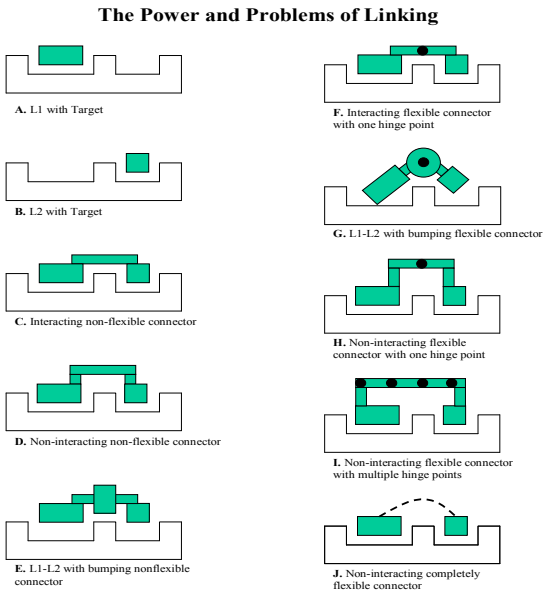
Thermodynamics of Affinity Gain through Multi-valence

Key Papers:

Reference	Notes	$T\Delta S_{trans\text{-}rot}$ at 298 K (kcal/mol)
Jencks W.P. (1981). On the attribution and additivity of binding energies. Proc. Natl. Acad. Sci. USA 78, 4046-4050.	Refers to 1971 paper!	12
Nakamura, Abeles (1985). J. Am. Chem. Soc. 24, 1364-1376.	Experimental fragmentation of HMG-CA inhibitor.	6.0
Finkelstein A.V., Janin J. (1989). The price of lost freedom: entropy of bimolecular complex formation. Prot. Eng. 3, 1-3.	Theoretical.	14 - 16
Tamura A., Privalov P.L. (1997). The entropy cost of protein association. J. Mol. Biol. 273, 1048-1060.	Calorimetric study: non-bonded versus S-S cross-linked dimers of subtilisin.	1.2
Amzel L.M. (1997). Loss of translational entropy in binding, folding and catalysis.	Theoretical. Rotational entropy is neglected (assumed to be small).	5.4 - 8.5
Sharp K.A. (1997). Energetics of cyclic dipeptide crystal packing and solvation. Biophys. J. 72, 913-927.	Calculations. Includes also vibrational entropy.	4.2
Shuker S.B., Hajduk P.J., Meadows R.P., Fesik S.W. (1996). Discovering high-affinity ligands for proteins: SAR by NMR. Science 274, 1531-1534.	Experimentally derived from comparing Kds.	
Carlow D., Wolfenden R. (1998). Substrate connectivity effects in the transition state for cytidine deaminase. Biochem. 37, 11873-11878.	Experimental fragmentation of substrate and inhibitors.	5.1 - 10.6
Williams D.H., Westwell M.S. (1998). Aspects of weak interactions. Chem. Soc. Rev. 27, 57-63.	Measurements in apolar solvents. No conclusion for water as solvent.	12 - 14
Hermans J., Wang L. (1997). Inclusion of loss of translational and rotational freedom in theoretical estimates of free energies of binding. Application to a complex of benzene and mutant T4 lysozyme. J. Am. Chem. Soc. 119, 2707-2714.	MD simulation of binding of benzene to T4 lysozyme mutant.	7

Courtesy of Dr. Christophe Verlinde

The art of Linking Ligands (ctd.)



The Science of Linking Ligands (ctd.)

In view of the many possible ways the Linker (also called the "Connector"), can (i) affect the mutual orientation of the two Ligand fragments, (ii) interact with the Target, (iii) can be flexible or not, (See Fig above) it is not too surprising that different experiments have resulted in very different estimates of the rigid body entropy.

There are, unfortunately, very few - if any - cases known where we have all the high-resolution structures and affinities of T, T:L1, T:L2 and T:L1-C-L2 to discuss this topic further. But there are some very nice examples in the literature of significant gains in affinity upon linking ligands.

A recent paper on this topic *(Murray and Verdonck, J. Comp Aided Mol Design 16, 741-753 (2002))* summarizes available data quite nicely – and also provides the statistical mechanical equations (pp 745 etc) which result in an equation relating the overall rigid-body entropy (i.e. the sum of translational and rotational entropy) S_{rigid} to the molecular weight m of the Ligand:

$$S_{rigid} = A + B.\ R \ln m$$

Where A and B are molecular weight-independent constants, and R is the gas constant. S_{rigid} is logarithmically dependent on the molecular weight of the Ligand. These authors suggest that there is a considerable dependence of S_{rigid} on molecular weight. Specifically the difference in the ΔG of binding of a ligand, at room temperature, for Ligands with molecular weights of 20 and 800 is about 46 kJ/mol. With B about 5, and R about 8.3 J per mol per K, and T = 298 K, we obtain a free enthalpy difference :

$$\Delta\Delta G = T\Delta\Delta S = 5*8.3*298*\ln 3 = 13.6\ \text{kJ/mol} = 3.2\ \text{kcal/mol}$$

for any pair of molecules differing by a factor of 3 in molecular weight. These authors conclude therefore that:
"it would appear that it is a poor approximation to assume that the loss of rigid-body entropy upon [Ligand] binding to a protein is a constant."

In the following section this possibly significant molecular weight-dependence of rigid-body entropy will be ignored to keep the discussion simple.

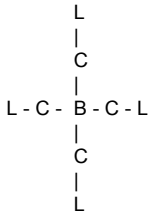
The Real Great Fun of Multivalent Ligands.

In principle one can link together all sorts of weak binding ligands, making strings like L1-C1-L2-C2-L3, and on and on. This does, however, require each time the design or discovery of a new additional ligand, and that can be real tough going.

Multi-subunit proteins, composed of identical monomers, provide a real interesting case where one only has to discover a decent binding Ligand once, which then can be incorporated multiple times into a multivalent ligand. So for an N-meric Target macromolecule, almost always a protein, one can make different sorts of multi-valent Ligands.

One class is like strings on a bead, which for a tetrameric case results in something like : L-C-L-C-L-C-L .

Another class of multi-valent Ligands is spider-like with a central base B and identical connectors C extending from that base and ending in the originally monovalent Ligands L. For a tetravalent case this leads to:



The Real Great Fun of Multivalent Ligands (ctd.)

In each of these cases the gain in free energy of binding is related to the free energy of the monovalent ligand L by a variation of equation (12):

$$\Delta G(N\text{-valent}) = N^{*}\Delta G(L) + (N-1)^{*}T\Delta S(r+t) \qquad (13)$$

Even for relatively small TΔS(r+t)values, which are always negative, this can quickly lead to tremendous effects. However remember, the equations above are only true **for the perfect, rigid, non-interacting Connectors while ignoring any molecular-weight dependency of the overall translational and rotational entropy TΔS(r+t) .**

Note: It is an interesting exercise to see what **a perfectly rigid Connector interacting favorably with the Target** might be able to achieve in terms of binding affinity!

Note: And even more surprising is what a **perfectly flexible, non-interacting Connector** might achieve in increasing affinity in Multi-valent Ligands compared to its constitutive Monovalent Ligands. As we will see in a second.

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Linking and Multivalency

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