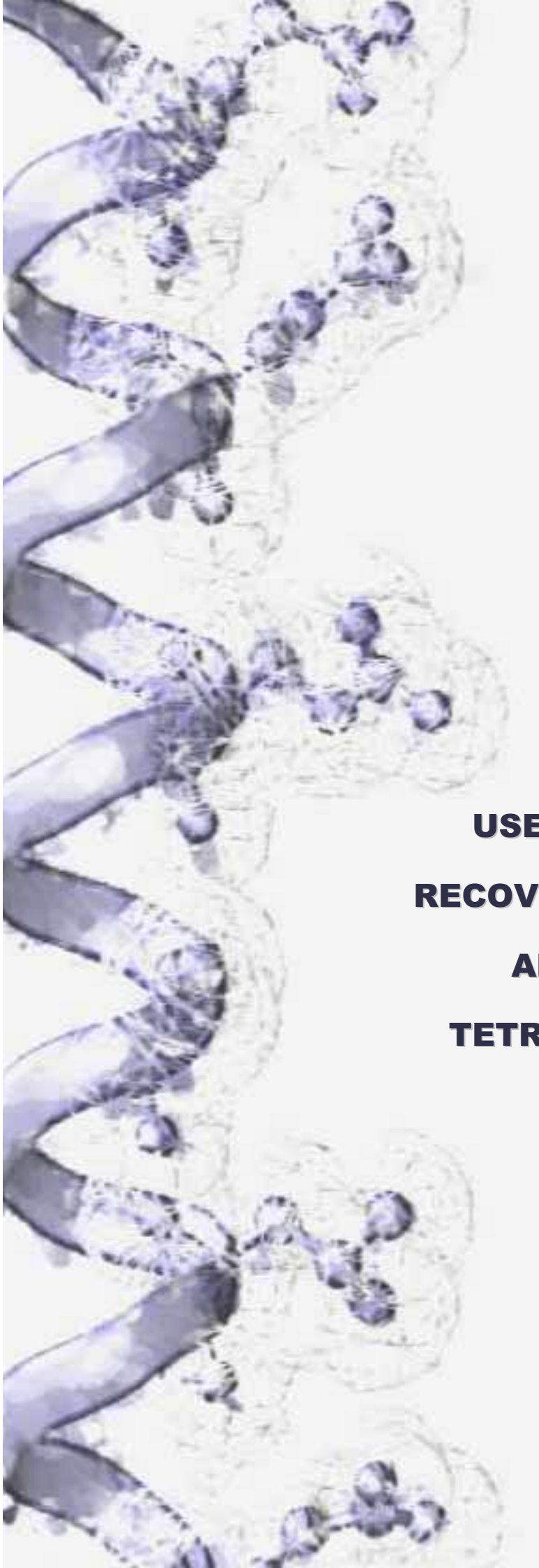

USE OF CALIX[4]ARENES TO RECOVER THE SELF-ASSEMBLY ABILITY OF MUTATED p53 TETRAMERIZATION DOMAINS

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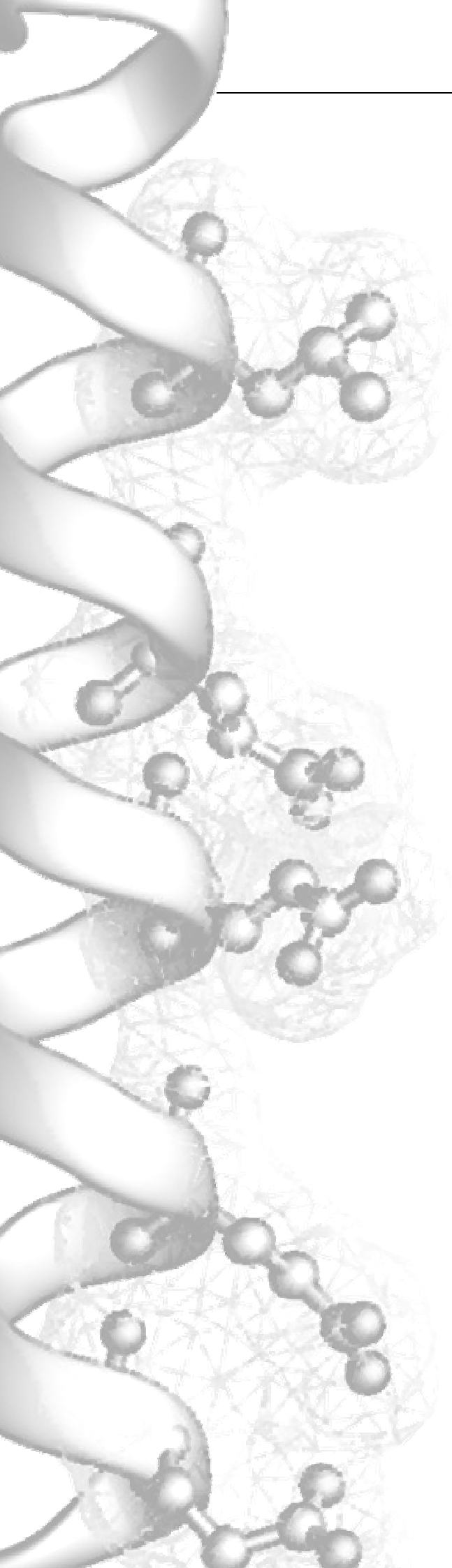

RESULTS

RESULTS 4

Other molecular recognition properties of the calix[4]arenes compounds

Calixarenes compounds are very versatile platforms widely employed in a variety of fields due to their particular chemical and physical properties. This fourth chapter presents some additional –and initially unexpected– properties of our calix[4]arenes.

4.1. Calix[4]arenes and DNA molecular recognition

The results presented in the previous chapters proved that the *para*-guanidinomethyl calix4bridge and calix4prop recognized and interacted with p53 tetramerization domain just as they were designed to do.

The results reported by Ungaro and co-workers about the abilities of a family of *para*-guanidinium-calixarenes to bind DNA,[1] prompted us to check if any of our calix[4]arenes molecules could also display such skill. Reproducing the experiments described by those authors, electrophoresis mobility shift assays (EMSA)[2] were performed to evaluate whether or not the calixarenes could recognize and bind to a plasmid (*e.g.* pEGFP). Results for the tetraguanidinomethyl calix4prop and calix4bridge are shown in **Figure 4.1**. Both molecules could interact with plasmidic DNA, although the affinity and the resulting band patterns were not the same.

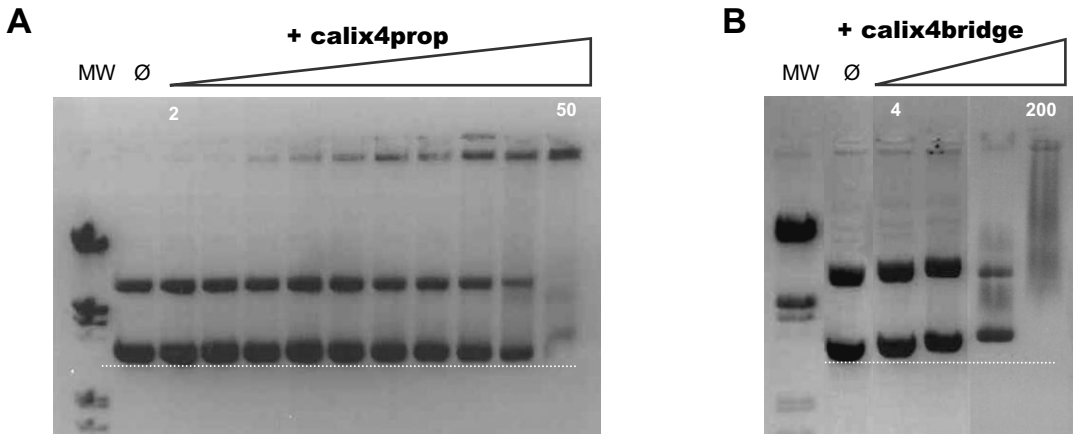

Figure 4.1. Agarose EMSA analysis of 1µg pEGFP plasmid, free (Ø), and incubated for 1h at room temperature with **(A)** calix4prop (at 2, 5, 10, 15, 20, 25, 30, 35, 40, 50µM) and **(B)** calix4bridge (at 4, 40, 80, 200µM), in 20mM Tris-HCl pH 8 buffer (15µL reaction volume). MW: DNA molecular weight ladder.

On the one hand, when calix4bridge (**Figure 4.1B**) interacted with the plasmid, the DNA-complex migrated with lesser mobility. The resulting blurry band suggested a rather weak electrostatic interaction,[3] which could be negatively affected by the ionic strength of the electrophoresis buffer.

On the other hand, calix4prop seemed to bind and compact the plasmidic DNA into complexes too large to migrate into the agarose gel (**Figure 4.1A**).[3] These effects were concentration dependent: started to be detected at 10µM and were completed at *ca.* 50µM. The behavior was the same than the described for the calixarene 4G4Pr-cone from Ungaro *et al.* –which differed from calix4prop because its guanidinium groups were directly attached to the aromatic platform (see section 3.4.1.2). The hypothetical mechanism of DNA-condensation for 4G4Pr-cone proposed by those authors,[4] could be also applied to calix4prop. Their experiments by AFM (Atomic Force

Microscopy) suggested that the exposed calixarene guanidinium groups would first interact with the negatively charged DNA helix. The increased local concentration of calixarenes would then favor the hydrophobic interaction of the lipophilic tails of the DNA-bound calixarenes, thereby joining different portions of the DNA molecule and forming intramolecular condensates (**Figure 4.2A**). Moreover, these authors found that efficiency in DNA condensation depended on the length of the alkyl chains at the lower rim.

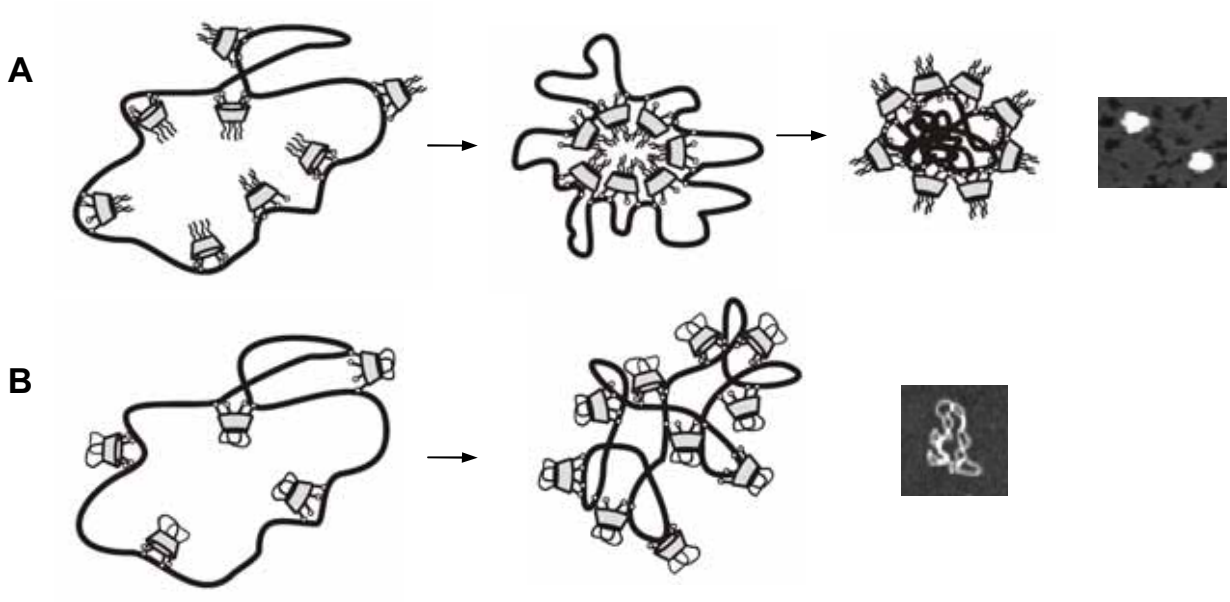

Figure 4.2. (A) Hypothetical mechanism of DNA interaction and condensation of conical *p*-guanidinium-calixarenes functionalized with non-polar alkyl chains in the lower-rim, such as 4G4Pr-cone or calix4prop (adapted from Sansone *et al.*[4]). **(B)** Hypothetic mechanism of the interaction of calix4bridge with a DNA plasmid. The AFM images are shown at the right.

Dr. F. Sansone, from the Ungaro's laboratory, kindly performed the AFM experiments[a] of our calix4prop (**Figure 4.4A**), and the same DNA-condensing phenomenon was also observed, although the larger size of the condensates suggested complexation of several DNA filaments.

The effects of calix4bridge on the plasmid structure were also studied by AFM (**Figure 4.4B** and **C**). As expected from the EMSA results, calix4bridge did not condensate the plasmid efficiently, nor even at high concentrations. The lack of an aliphatic alkyl chain in the lower-rim could be the most likely reason (**Figure 4.2B**).

The role of the lower-rim alkyl chains was further evaluated by studying the amino-version of calix4prop: NH_2-calix4prop. EMSA analysis showed that NH_2-calix4prop could also promote DNA condensation, but it was not so efficiently as for the tetraguanidinium analogs (**Figure 4.3**). The lower affinity displayed by the amino-groups was likely due to both the lack of the four-positive

[a] AFM experiments were initially run here, under the same conditions reported by Sansone *et al.*,[4] but unfortunately, it was impossible to obtain reproducible images.

charges[5] and the lack of effective chelation with the phosphate anions from the nucleic acids.[6] The DNA condensates were also detected by AFM (**Figure 4.4D** and **E**), but they were not as compact as those of calix4prop and, owing to the lower affinity, higher concentrations of ligand were required.

The effects of NH_2-calix4bridge were also evaluated by EMSA, but nothing was detected (**Figure 4.3**).

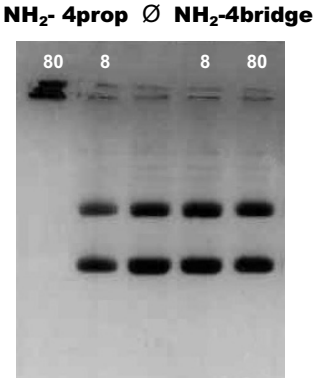

Figure 4.3. Agarose EMSA of pEGFP plasmid incubated with the *p*-aminocalixarenes NH_2-calix4prop (left lines) and NH_2-calix4bridge (right lines) at 8μM and 80μM, for 1h at room temperature in 20mM Tris-HCl at pH 8. The middle line, Ø, corresponds to free pEGFP plasmid.

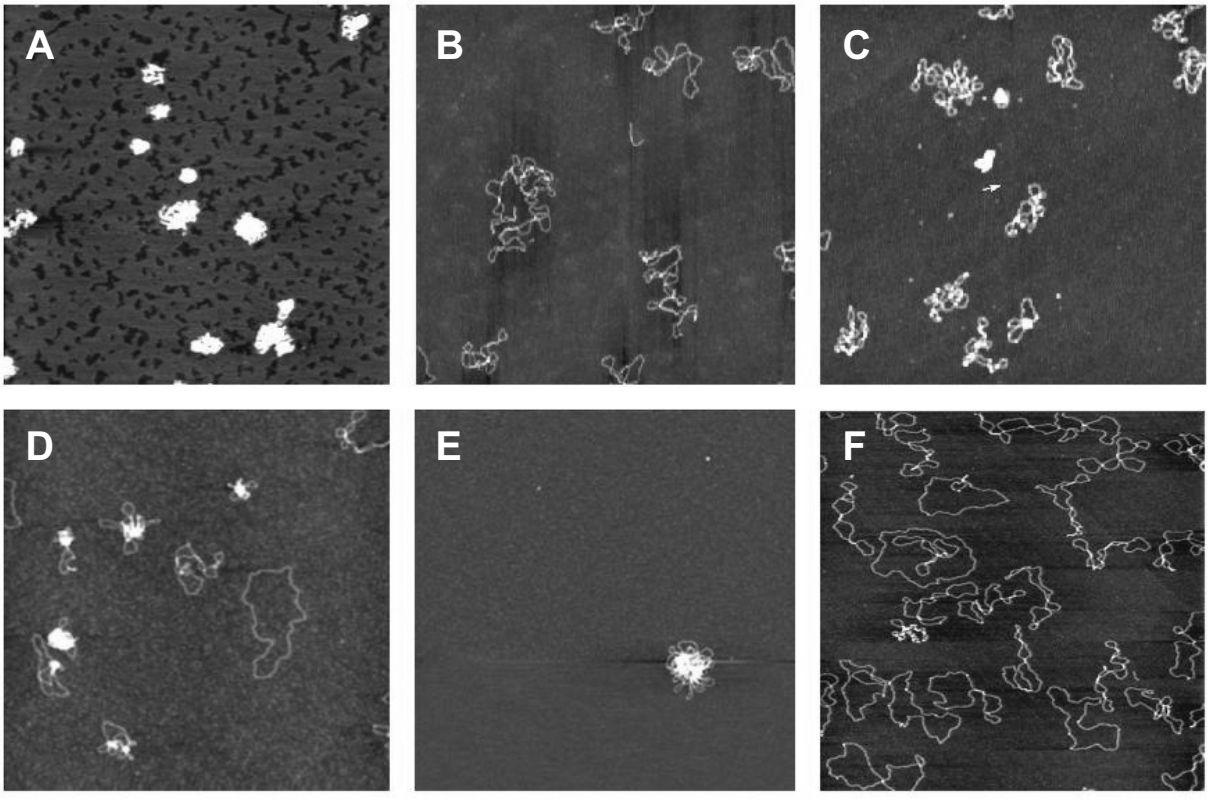

Figure 4.4. AFM images showing the effects of the calix[4]arenes on the plasmidic DNA. Pictures correspond to samples of 1nM pEGFP-Cl incubated with **(A)** calix4prop at 0.5μM; **(B)** calix4bridge at 1μM and **(C)** at 2μM; **(D)** NH_2-calix4prop at 1μM and **(E)** at 2μM. **(F)** Free plasmid; it appears mainly extended over the mica surface with only some supercoils. Deposition buffer: 4mM Hepes pH 7, 10mM NaCl, 2mM $MgCl_2$. *All AFM experiments were kindly done by Dr. Sansone at the Universitá degli Study di Parma.*

4.2. Calix[4]arenes as transfectants for DNA delivery

The DNA-condensation ability of calix4prop further encouraged us to study another more interesting property such is cellular transfection. By the time those experiments were done, nothing had been published on the matter; a few months later, Ungaro's group published a complete work on DNA-complexation and transfection properties of *p*-guanidinium-calixarenes[4] that helped in understanding our results.

Prior to evaluate transfection, calix4prop toxicity was assessed by the MTT assay[7] on HeLa cells (human cervical adrenocarcinoma epithelial cells). Calix4prop was found to be highly toxic (**Figure 4.6**). In poor culture media (*e.g.* OPTI-MEM), in which cells are more sensitive to environment changes, the calixarene was even more lethal –especially at long incubation times. In fact, under toxic concentrations of calix4prop, cells were literally destroyed; this behavior resembled to that of a detergent.[8]

It is worth noting that calix4prop was toxic at much lower concentrations than its analog 4G4Pr-cone. Under the same conditions (but different cell line), the lethal dose for 50% mortality (LD_{50}) of calix4prop was *ca.* 6μM, while for 4G4Pr-cone was *ca.* 25μM, nearly four times higher.[1]

In contrast, calix4bridge did not show any significant lethality at concentrations as high as 64μM. This suggested that the toxicity depended on the lower rim functionalization. Nevertheless, the charged state of calix4prop also was determinant. Lalor *et al.* have recently described the low toxicity of the amino-derivative of 4G4Pr-cone, for which LD_{50} is *ca.* 80mM (although in CHO cells and under slightly different conditions).[12] Taken all together, it seems that, besides the lower rim functionalization, the number of positives charges in the upper rim is also a crucial factor for the lethality of these ligands.[9,10] Perhaps the greater amphipathicity of calix4prop (*i.e.* four positive charges in one side and four non-polar alkyl chains in the other) could enable the molecule to be inserted into the cellular membrane, thereby disrupting and even destroying it.[11]

The toxic concentrations determined by the MTT assay could not be accurately extrapolated into the internalization assays. Firstly, the confluence level employed for the cell viability assays was larger than that used in the internalization assays (7,000cells/cm^2 vs. 2,270cells/cm^2), which means that, for a given concentration, the calixarene might become more toxic because of the higher ratio ligand-to-cells. And secondly –and counteracting the previous effect– the calixarene was not free but forming a complex with the DNA, which might diminish the toxicity. The balance between these two phenomena would certainly affect the toxic concentration of the calixarene. Nevertheless, the toxicologic information found by MTT helped in setting the experimental conditions of the internalization assays.

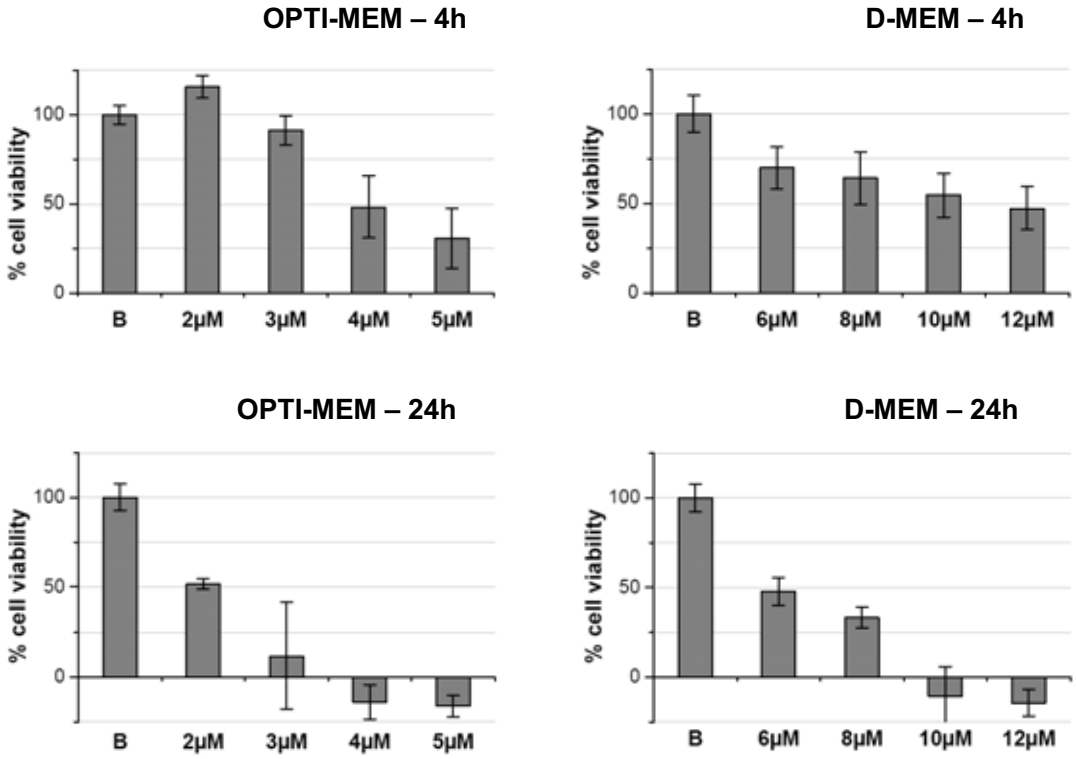

Figure 4.5. Calix4prop toxicity determined by the MTT assay on HeLa cells (3,150 cells in 100μL media). Both OPTI-MEM and D-MEM media were evaluated, at short (4h) and long (24h) incubation times. Percentages in the y-axis correspond to cell viability respect to the blank cells control "B".

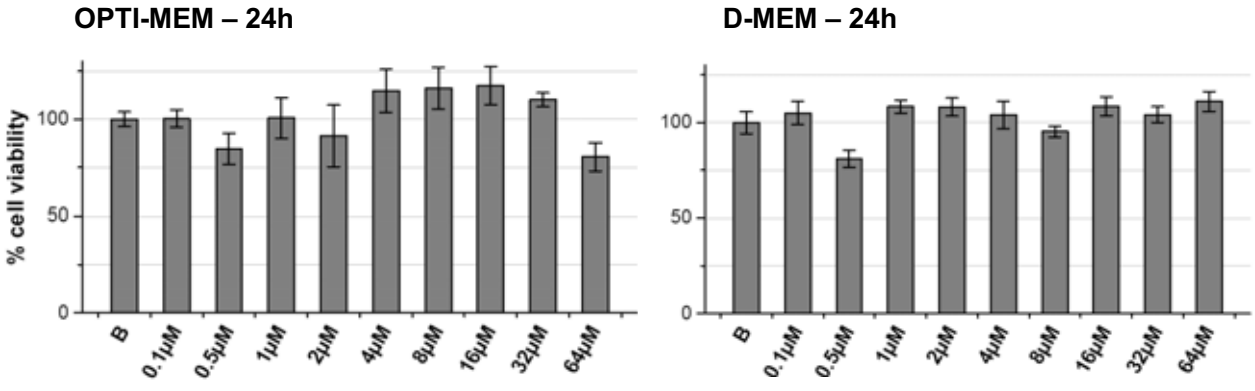

Figure 4.6. Viability of HeLa cells incubated for 24h with calix4bridge in both OPTI-MEM and D-MEM media (obtained by the MTT assay). No significant difference is detected between control cells, "B", and those treated with calix4bridge.

The transfection assays were only performed with calix4prop, since this was the only one that could condense efficiently the DNA. The plasmid pEGFP was used as the transfection reporter,[13] and the mammalian HeLa line as the host. If pEGFP were internalized into cells, transient expression of the encoded EGFP (*i.e.* Enhanced Green Fluorescent Protein) would be direct evidence that the calixarene was able to transport plasmidic DNA into cells.[b]

Previous works in the group on cell penetrating peptides[14-17] established the optimal conditions for internalization assays in HeLa cells. Although the calixarene transfection experiment was conceptually different, those conditions were an excellent starting point. Flow cytometry was the technique used for determining cell fluorescence –hence, transfection– and cell death –hence, toxicity– in those experiments.

Initial trials under non-toxic calix4prop concentrations (<4μM) did not showed any clear fluorescence signal, although the flow cytometry profile of treated cells was not the same than that of the blank control.[c] Several experimental parameters were modified in order to enhance the transfection –if any–, starting by the increase of calix4prop concentration (>10μM). To counteract the resulting high mortality, other factors were tuned as follows:

- DNA amount: more plasmid was added to form the calix4prop-DNA complex (2μg instead of 1μg). Therefore, although the calixarene was at a higher concentration, it would not be free. In addition, a larger plasmid-to-cells ratio would also increase the transfection chance.
- Time of transfection: toxicity could also be reduced by shortening the incubation period, although too short times would negatively affect the transfection. The final period was set to 4 hours.
- Transfection media: frequently, OPTI-MEM is used for transfection assays because its minimal serum content causes cells to be more sensitive and prone to be transfected –although this is not a “real” medium. Cell sensitivity in those poor conditions also results in higher toxicity (**Figure 4.6**); hence, standard D-MEM was used.
- Besides OPTI-MEM, antibiotics are also frequently avoided in transfection experiments. Neither calix4prop nor pEGFP were sterile, and therefore, antibiotics were used in order to minimize another source of toxicity.
- Since DNA condensates (*i.e.* plasmidic particles) are likely internalized by endocytosis,[18] some chloroquine (10μM) was added to the medium during the transfection period in order to help in breaking those hypothetic endosomes.[19] In fact, a fluorescence “improvement” was detected in the presence of chloroquine.
- Finally, after the transfection treatment, cells were incubated for 48h (instead of only 24h) before measuring the fluorescence. Long incubation times would enable cells to better get over from the damages of the calixarene treatment; additionally, more EGFP would be expressed and thereby the fluorescence signal would increase.

[b] Transient expression of plasmid encoded products usually occurs over a period of several hours after transfection and continues for 72 to 96 hours after introduction of plasmid DNA into mammalian cells; it can be monitored by the observation of fluorescence emission

[c] Experimental data from flow cytometry can not be shown because the original files were regrettably lost.

After all those sequential modifications, it was possible to detect by flow cytometry some weak but clear fluorescence in treated cells. However, samples showing the best –or the less worse– transfection levels (*i.e.* <30% of total cells being fluorescent), were also those with the highest mortality (*i.e.* >70% death cells); this was likely the reason why fluorescence results could not be accurately reproduced.

In conclusion, calix4prop could deliver plasmidic DNA into cells but under too toxic conditions.

Once DNA-delivery was detected by flow cytometry, transfected cells were studied by confocal laser scanning microscopy (CLSM). The toxic concentrations used only left a few number of cells alive; within those, most displayed EGFP fluorescence, although it was insignificant if compared with FuGENE® (Roche), which is a non-liposomal multicomponent reagent used as positive transfection control (**Figure 4.7**).

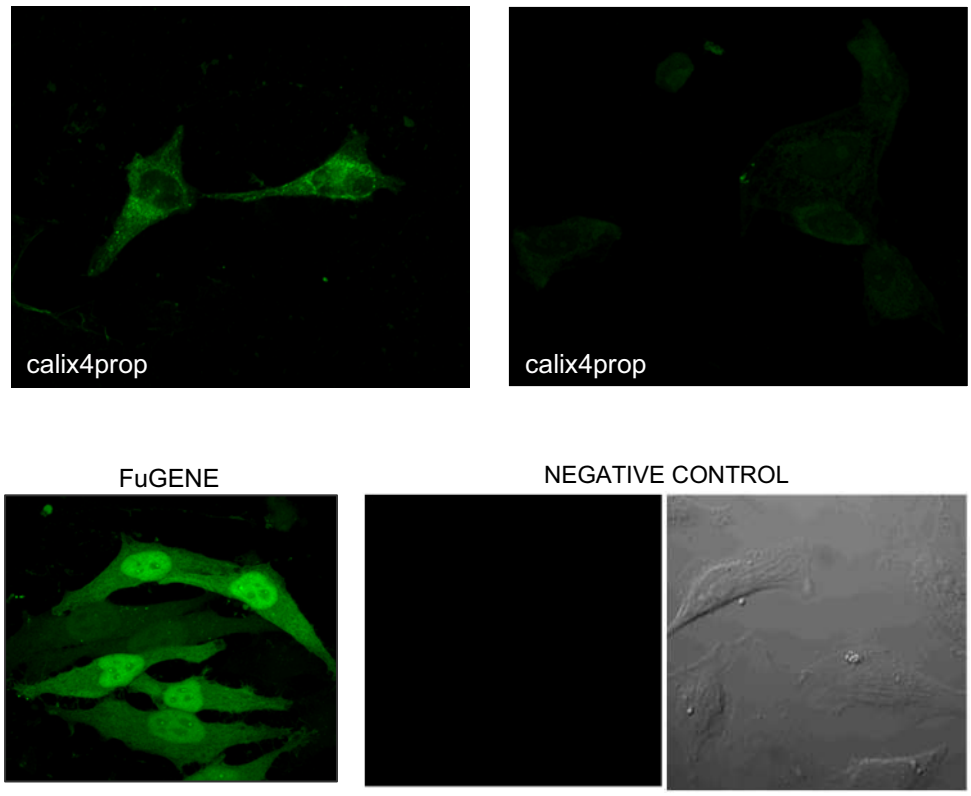

Figure 4.7. Transient pEGFP transfection with calix4prop visualized by CLSM (top pictures). HeLa cells were transfected with 2μg of plasmid at 10μM calix4prop during 4h in D-MEM, and further incubated for 48h. FuGENE (bottom, right picture) was used as positive tranfection control. Negative control, *i.e.* cells incubated with only pEGFP (bottom, left pictures) did no display any significant fluorescence (contrast phase picture shows the non-fluorescence cells).

The transfection results for calix4prop agreed with those published by Sansone *et al.*, although their less toxic 4G4Pr-cone could not transfect by itself. Those authors used DOPE (dioleoyl-L-α-

phosphatidylethanolamine), a neutral phospholipid transfection adjuvant,[20] to enhance the "transfectant" capacity of 4G4Pr-cone (at 15μM of calixarene and 7.5-30μM of DOPE). Regarding the transfection conditions, it was nicely assessed that our movements in tuning several parameters went in the right direction and they could have been pushed even further. Despite using a different cell line (RD-4), they worked with 3μg of plasmid, in serum-free media; incubate 5 hours with the calixarenes and read the fluorescence after 72 hours.

4.3. Discussion

The results evidence that our calix4arenes compounds can interact with DNA and form macromolecular complexes with plasmids. The way they interact depends on the lower rim functionalization. As expected for an electrostatic interaction, the affinity for the DNA is better in the tetraguanidinium species than in the tetraamino ones.[21]

Calix4prop is able to condense plasmidic DNA into particles of proper size for being transfected into mammalian cells –likely via endocytosis; unfortunately, the high concentration of calixarene required for plasmid delivery is also too toxic.

The lower rim functionalization also seems to play a crucial role in the toxicity of the calix[4]arenes. While calix4bridge does not present significant toxicity at concentrations as high as 64μM, for calix4prop toxicity is detected at concentrations as low as 2μM. The appearance of the dead cells in the presence of calix4prop suggests a detergent-like behavior; perhaps, the amphipathicity of the ligand makes it able to disrupt the phospholipid bilayer.

Comparing the results of calix4prop with those published by Ungaro *et al.* for 4G4Pr-cone,[1,4] it seems that, besides the key role of the propyl chains in the lower rim for condensing DNA, the higher basicity of the guanidinium groups for calix4prop (*i.e.* the pK_a for phenyl-guanidina is 10.9, while for benzylguanidina is ~13) confers additional properties to the molecule that lead to cell transfection and higher toxicity.

Transfection experiments with calix4bridge have not been performed; it was initially consider that, if calix4prop led to such little transfection, calix4bridge would be even worse. However, it would be interesting to test it. Despite not condensing the plasmid into particles, calix4bridge also interacts with DNA and it might be able to transport it into the cell. In addition, its low toxicity would allow working at higher concentration.

Likewise, testing the toxicity and transfectant properties of the amino-ligand NH_2-calix4porp would also prove very informative. On the one hand, NH_2-calix4prop can also condense plasmidic DNA into particles likely suitable for cellular transfection. On the other hand, the amino-ligand can also be a less toxic species. For instance, 4G4Pr-cone toxicity (LD_{50} ~25μM)[1] decreases significantly when its guanidium groups are substituted by amino ones (LD_{50} ~80μM).[22] For those ligand the

strong change is likely to be related with the important drop in basicity (*i.e.* the pK_a for phenylguanidina is 10.9, while for the conjugated acid of aniline is 4.9). For calix4prop the change in basicity between the guanidium and the amino groups is not so drastic (*i.e.* the pK_a for benzylguanidina is ~13, while for the conjugated acid of benzylamine is 9.3); hence, that study would further provide insights on how the protonation state of the upper rim influences the toxicity.

It is worth mentioning that, in the field of the delivery of plasmidic DNA, very little examples of small single molecules with such ability have been reported to date. The most usual non-viral vehicles for gene delivery are large positively charged lipids, polymers, peptides or peptoides, that form large complexes with DNA and promote the cellular uptake via endocytosis.[23-25] Novel non-toxic small synthetic molecules able to deliver plasmids into cells are thus very attractive. Despite the fact that calix4prop could not be used on that purpose because its high toxicity, modification of the guanidinium-calixarene structure might lead to more promising transfectants with better ADME properties.

Regardless of the origin of calix4prop and calix4bridge –initially designed for p53 molecular recognition– the versatility of the guanidinium group confers other molecular recognition properties to these molecules as important as DNA binding and transfection, which emphasizes once again the central role of the guanidinium group in Nature. Moreover, the strength of hydrophobic interactions –as those displayed by the propyl chains of calix4prop– can also be essential in molecular recognition processes.

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Future directions

Throughout the previous chapters several questions have arisen which remain to be answered. These could be addressed with some new experiments that are described below:

> Regarding the study of the mutated tetramerization domains, one of the most intriguing things to be understood is the unfolding/misfolding mechanisms of G334V. Some kind of β-aggregation has been detected for this protein, although not as previously described in the literature. It would be convenient to first determine the conditions required for the β-amyloidogenesis of the recombinant fragment, and then understand what promotes the structural transformation, as well as to characterize the final structure itself.

> The detection of the non-covalent tetrameric protein by ESI-MS opens a new door to the study of how the forces in the gas phase can be correlated with those in the solution phase. Experiments have shown that mutant proteins in solution present a lesser shifted tetramerization equilibrium and lower thermal stability than the wild-type domain. Now the question is: how much of the behavior in the solution phase can be transferred to the gas phase? Namely, can the intensity of the detected tetramer be correlated with the shift in the equilibrium? Can the stability of the tetramer in the gas phase be correlated with that in solution? To what extent does the absence of surrounding water molecules affect the stability of the protein? Again, the system of p53TD and its mutants constitutes an excellent model.

> The interaction of p53 with calix4bridge has been sufficiently characterized as to understand the recognition mechanisms. Further structural characterization would probably corroborate what has already been hypothesized.

Determination of the bound-ligand conformation –for instance, by tr-nOe– would be highly valuable.

Likewise, crystallization of the complex –with the wild-type protein as well as with the mutants– and elucidation of the structure by X-ray diffraction would be invaluable. However, this will not be an easy task and, eventually, the crystal structure might not accurately reproduce the complex in solution.

ESI-MS experiments could also help in understanding the forces behind the interaction. Evaluation of NH_2-calix4bridge would likely help in determining the balance between electrostatic and hydrophobic interactions.

> More questions remain to be answered for the interaction with calix4prop, the most essential of which is establishing what really happens to the structure of the protein upon binding: is the hypothesized mechanism correct?

NMR characterization of the structure of the complex would be fairly difficult. Crystallization of the complex would be better, although, as mentioned before, this is a long term goal, and the crystal complex might not reproduce the solution structure.

Further exploration of the lower affinity ligand NH_2-calix4prop –for instance, by tr-nOe experiments– might help in gaining insights into the conformation of the calixarene propyl chains in the complex.

Evaluation of other calixarene ligands, in which the upper or the lower rim, or even the number of aromatic units are modified, would also help in validating the hypotheses.

For example, the presumably “insertion of the propyl chains” into the hydrophobic core of the protein could be assessed by using longer chains (*e.g.* butyl, penthyl), bulkier groups (*e.g.* isopropyl) or more polar pendants (*e.g.* hydroxypropyl) in the lower rim.

From the point of view of the “positive charge distance”, a longer branch (*e.g.* two or three carbons) in the upper rim could indicate if –and to what extent– the affinity depends on the optimal guanidinium-carboxylate chelation, or if affinity is just a matter of basicity.

For larger calixarenes, entropic penalties would increase. The calixarene can also be made smaller, by shortening or even removing the chains in its lower rim. In this case, the interaction with the protein might be reduced or inhibited due to the large entropic penalties (*i.e.* the calixarene has free rim rotation) and/or to the smaller surface of interaction (*i.e.* lesser energetic terms to counteract the entropic penalty).

> The particular case of G334V and calix4prop, and the enhanced β-amyloidogenesis, is replete with unknowns. Evaluation of NH_2-calix4prop or 4G4Pr-cone at higher temperatures might help in assessing the role of the guanidinium groups in this structural transformation.

> Of course, having proven that the two calix[4]arenes discussed here can recover the tetramerization stability of the mutated proteins –and hence, the active conformation–, the most immediate suggestion is to test these compounds in biological assays. Unfortunately, there are many drawbacks that complicate said experiments.

First, and most important, the guanidinium calixarenes are insoluble in buffered media.

Second, the affinity of these ligands for p53TD (in the micromolar range) may not be high enough for an efficient interaction. Moreover, the multivalency of these ligands, could lead to numerous nonspecific interactions with other biomolecules (*e.g.* DNA, RNA and lipids). This premise is clearly reflected in the high toxicity of calix4prop.

Finally, experiments have been done only with the tetramerization domain of the protein. Although this domain is structurally independent of the rest of the protein (as recently shown by Fersht and collaborators), the calixarenes might not interact with the entire protein or might interfere in other functions of the tetramerization domain. In fact, biophysical experiments using entire p53 were originally envisioned, but were not pursued due to the insolubility of the ligand in buffered media.

> Finally, now that the concept of a synthetic ligand able to stabilize the tetrameric state of mutated p53TD is proved, similar approaches can be undertaken using ligands with better ADME properties. Moreover, the strategy can be extended to other many systems in which stabilization –or even recovery– of a protein assembly is sought.