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Enhancement of transcriptional activity of mutant p53 tumor suppressor protein through stabilization of tetramer formation by calix[6]arene derivatives

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ABSTRACT

Li–Fraumeni syndrome, a hereditary disorder characterized by familial clusters of early-onset multiple tumors, is caused by mutation of the *TP53* gene, which encodes the p53 tumor suppressor protein. Mutation of Arg337 to histidine in the tetramerization domain of p53 is most frequently observed in Li–Fraumeni syndrome. This mutation is reported to destabilize the tetrameric structure of p53. We designed and synthesized calix[6]arene derivatives, which have six imidazole or pyrazole groups at the upper rim. In this study, we report, for the first time, the enhancement of the in vivo transcriptional activity of the most common Li–Fraumeni p53 mutant by imidazole-calix[6]arene through stabilization of the oligomer formation.

Li–Fraumeni syndrome (LFS) is a disorder characterized by familial clustering of early-onset tumors, including sarcomas, breast cancer, brain tumors, and adrenocortical carcinomas (ADCs).[1] The *TP53* and *CHEK2* genes are associated with Li–Fraumeni syndrome. More than half of all families with LFS have inherited mutations in the *TP53* gene, which encodes the p53 tumor suppressor protein. Arg337His (CGC to CAG), located within the tetramerization domain (TD) of p53, is the most frequent germ-line mutation of the *TP53* gene. This mutation was first reported in Brazilian children with ADC.[2] Arg337His *TP53* carriers have a high prevalence of ADC, breast cancers, and sarcomas. Structural study has revealed that the Arg337His mutant exhibits pH-dependent instability of the mutant p53 tetramer in the physiological range.[3] This sensitivity correlates with the protonation state of the mutated His337 residue. In functional studies, p53 proteins with the Arg337His mutation showed defects in oligomerization and decreased transcriptional activity.[4,5]

The tumor suppressor protein p53 is a 393 amino acid phosphoprotein that controls apoptosis and cell-cycle arrest in response to DNA damage by inducing or repressing the transcription of several genes. Tetramer formation of p53 is essential to its tumor-suppressor activity. The TD consists of a β strand (Glu326 to Arg333), a tight turn (Gly334), and an α helix (Arg335 to Gly356).[6] Two monomers form a primary dimer by means of an antiparallel β sheet and α helices; two primary dimers associate into a tetramer with an unusual four-helix-bundle structure.[7] The guanidinium group of Arg337 stabilizes the tetrameric structure through a salt bridge (plus hydrogen bond) with the carboxyl group of Asp352 and the methylene group forms hydrophobic interactions. Recently, Gordo et al. reported that guanidinium-calix[4]arene, which has four guanidinium groups, could stabilize the tetrameric structure of the p53 Arg337His mutant in water with an adjusted pH to 7.0.[8] HSQC-NMR analysis has revealed that this compound stabilizes the mutant tetrameric structure through interactions with a hydrophobic pocket and glutamic acid residues (Glu336 and Glu339) on the tetrameric surface of p53. In this study, we report, for the first time, stabilization of the Arg337His mutant tetrameric structure under physiological conditions and rescue of the transcriptional activity of the p53 Arg337His mutant by calix[6]arene derivatives.

On the tetrameric surface of p53, six glutamic acid residues (Glu346, Glu339, and Glu346) from two different monomers are located as shown in Figure 1. In the tetrameric structure, distances between each glutamic acid residue are: Glu336–Glu336, 9.7 Å; Glu339–Glu339, 16.4 Å; Glu343–Glu343, 13.7 Å. We designed and synthesized two calix[6]arene derivatives that possess six

Abbreviations: ADC, adrenocortical carcinoma; CD, circular dichroism; EGFP, enhanced GFP; HSQC, Hetero-nuclear single Quantum Coherence; LFS, Li–Fraumeni syndrome; PDB, Protein Data Bank; RE, responsive element; TD, tetramerization domain.

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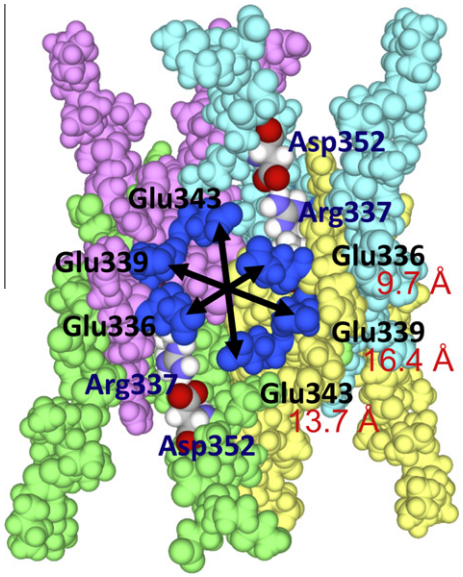


Figure 1. Structure of the p53TD. Space-filling model of the p53TD (PDB code 3sak) prepared with MolFeat version 4.0 (FiatLux Corp.). Glutamic acid residues that interact with the calixarene derivatives (Glu336, Glu339, and Glu343) from two different monomers are shown in blue.

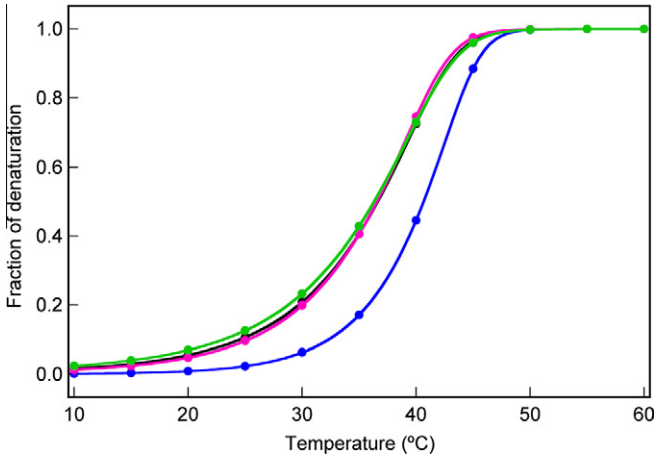
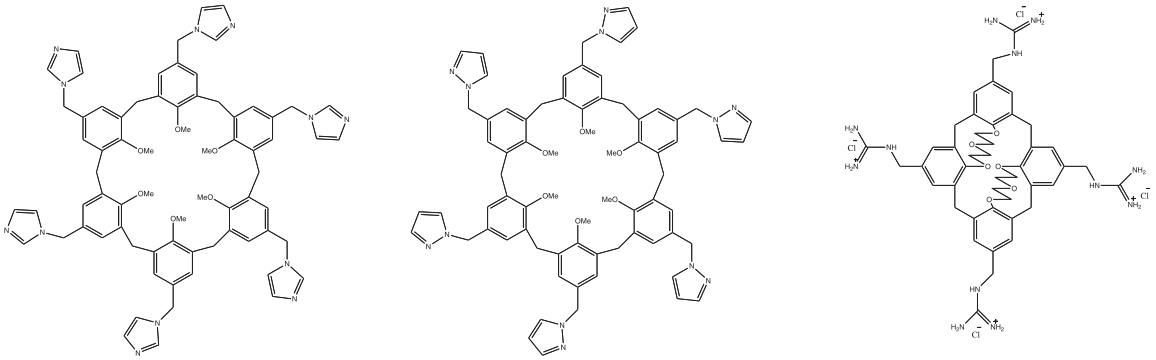


Figure 3. Thermal denaturation curves of the p53TD-Arg337His peptide in the presence of calixarene derivatives. The unfolding process of the p53TD peptide was fitted to a two-state transition model in which the native tetramer directly converts to an unfolded monomer. Black, no derivative present; blue, 40 μM imidazole-calixarene **1**; green, 10 μM pyrazole-calixarene **2**; magenta, 40 μM guanidinium-calixarene **3**. Because pyrazole-calixarene **2** precipitated in phosphate buffer at the high concentrations used, the CD experiment was performed at 10 μM for pyrazole-calixarene **2**.

imidazole (imidazole-calixarene **1**)[9] or six pyrazole (pyrazole-calixarene **2**)[10] groups at the upper rim to interact with the glutamic acid residues (Fig. 2). The diameter of the calix[6]arenes, including the functional groups, is about 10–20 Å. The imidazole and pyrazole groups from the calix[6]arenes can interact with the glutamic acid residues and stabilize the mutant tetrameric structure. The guanidinium-calix[4]arene **3** was also synthesized, as described by Gordo et al.[8]

A mutant p53TD-Arg337His peptide, corresponding to p53TD residues 319–358, was chemically synthesized as described previously.[11] Circular dichroism (CD) spectra were recorded in 50 mM sodium phosphate buffer containing 100 mM NaCl, pH 7.5.[12] The thermal denaturation curves of mutant p53TD-Arg337His peptide (10 μM monomer) in the presence of calixarenes were calculated from changes in CD ellipticity at 222 nm, which corresponds to an α helix (Fig. 3A and Table 1). The results showed that imidazole-calixarene **1** significantly stabilized tetramer formation by the p53TD-Arg337His peptide, with the T_m value shifted from 36.6 to 38.9 °C and 40.7 °C at 10 and 40 μM, respectively. The $\Delta\Delta G_u$ value calculated at 37 °C was $\Delta\Delta G_u^{37°C} = -2.06$ kcal/mol at 40 μM. For pyrazole-calixarene **2** (10 μM), the stability of the p53TD-Arg337His peptide was not significantly altered. This difference in stabilization could be explained by the pK_a values of the imidazole and pyrazole groups. The pK_a of a 1-methylimidazole is 7.06,[13] so half of the imidazole groups of the calix[6]arene derivative could be protonated under the conditions used in the CD experiment. By contrast, the pyrazole group would be mainly unprotonated, because the pK_a of a 1-methylpyrazole is 2.04.[14] It is reported that guanidinium-calix[4]arene **3** stabilized the p53TD-Arg337His peptide tetrameric structure in the absence of salt.[8] However, compound **3** showed no stabilization in the presence of 100 mM NaCl, under a more physiological condition. For the wild-type p53TD peptide, all calixarene derivatives did not enhance the stability of the tetrameric structure (Fig. S1 and Table S1 in Supplementary data). The data suggested that imidazole-calixarene **1** stabilized the tetrameric structure of the mutant peptide under physiological conditions, possibly through interactions with the glutamic acid residues.

The imidazole-calixarene **1** stabilized the mutant tetrameric structure of the Arg337His peptide in in vitro assays. We analyzed the effects of imidazole-calixarene **1** on the transcriptional activity of the p53 Arg337His protein in cells. The transcriptional activity of



imidazole-calixarene **1** pyrazole-calixarene **2** guanidinium-calixarene **3**

Figure 2. Structures of the calixarene derivatives.

Table 1
Thermodynamic parameters for the p53 Arg337His peptide in the presence of calixarenes

	Concentration (μM)	T_m (°C)	ΔT_m (°C)	$\Delta H_u^{T_m}$ (kcal/mol)	$\Delta\Delta G_u^{37°C}$ (kcal/mol)
No calixarene		36.6 ± 0.1	—	116.9 ± 2.4	—
Imidazole-calixarene **1**	10	38.9 ± 0.2	2.3	133.1 ± 5.9	−0.93
	40	40.7 ± 0.2	4.1	167.6 ± 5.4	−2.06
Pyrazole-calixarene **2**	10	36.6 ± 0.2	0.0	124.2 ± 3.4	0.04
	40	n.d.	n.d.	n.d.	n.d.
Guanidinium-calixarene **3**	10	36.9 ± 0.2	0.3	152.3 ± 11.5	−0.07
	40	36.3 ± 0.2	−0.3	108.2 ± 4.3	0.10

Concentration, concentration of calixarene derivatives; T_m, transition temperature; $\Delta H_u^{T_m}$, variation in the enthalpy of unfolding at T_m; $\Delta\Delta G_u^{37°C}$, the difference in ΔG between Arg337His in the absence of calixarene derivatives and Arg337His in the presence of calixarene derivatives at 37 °C; n.d., not determined due to poor solubility of compound **2**. The standard errors of fittings are indicated.

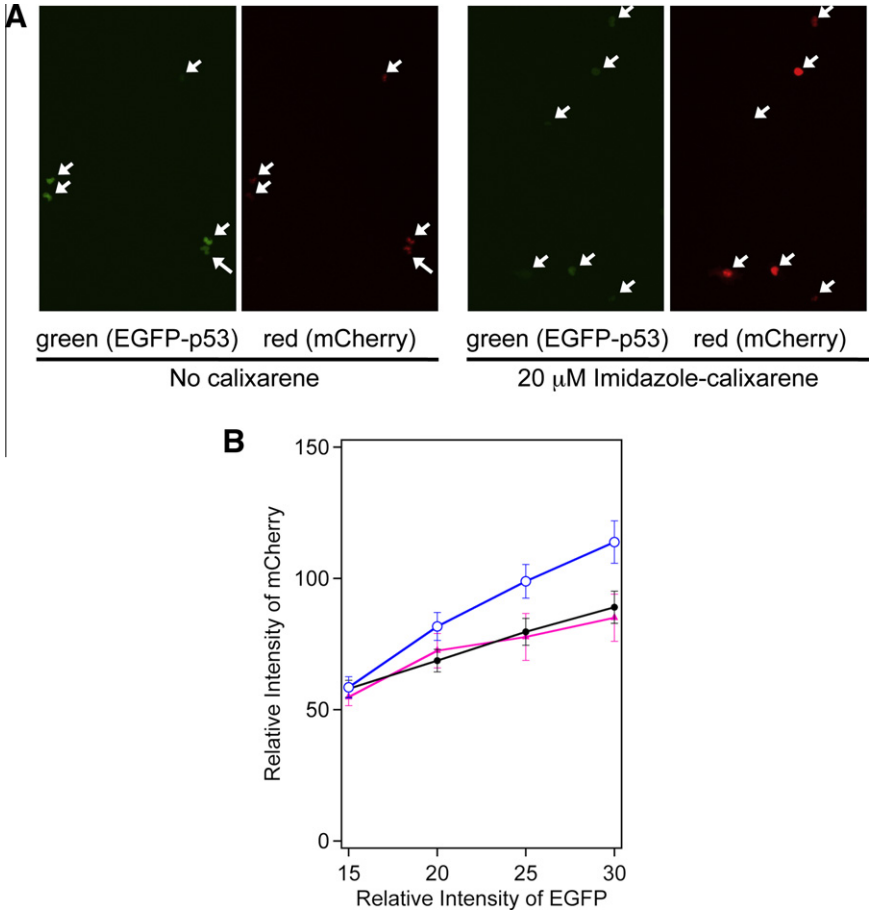


Figure 4. Effect of imidazole-calixarene **1** on the transcriptional activity of the p53 Arg337His mutant. (A) H1299 cells were transfected with two plasmids (pEGFP-p53-Arg337His and p53RE-mCherry) using Lipofectamine 2000. Derivative **1** was added to H1299 cells (final 20 μM) 1 h after transfection. After 11 h incubation, cells were fixed with 3.5% formaldehyde, and green and red fluorescence signals (arrows) were quantified in each cell. (B) Relative transcriptional activity of EGFP-p53-Arg337His with imidazole-calixarene **1** (20 μM) or guanidinium-calixarene **3** (20 μM). The mCherry (red) signals in each cell, which expressed EGFP-p53 at same level as endogenous p53 in A549 were quantified.[4] The averages of the mCherry signals in each EGFP signal are shown. Black, no derivative present; blue, 20 μM imidazole-calixarene **1**; magenta, 20 μM guanidinium-calixarene **3**. The standard error is indicated.

the p53 Arg337His mutant in the presence of calixarene was measured by a modified method as previously described.[4,15] H1299 cells were transfected with the EGFP-p53-Arg337His expression vector and the reporter plasmid p53RE-mCherry(NLS). In this study, we used p53RE-mCherry(NLS) plasmid as the reporter vector, instead of p53RE-DsRed, to enhance the red fluorescence signal. The amount of p53 expressed in each cell and its transcriptional activity were estimated from the green and red fluorescence signals, respectively. Cells expressing EGFP-p53-Arg337His mutant in the absence of calixarene derivatives showed weak mCherry signals (Fig. 4A, left panels). The mCherry signal in each cell increased in the presence of imidazole-calixarene **1** (Fig. 4A, right panels). We quantified the red signal in each cell, which expressed EGFP-p53 at the same level as endogenous p53 in A549, estimated by immunostain with anti-p53 monoclonal antibody.[4] Many human normal cells and cancer cell lines with the wild-type p53 are reported to express p53 protein mostly in the same range.[16] Figure 4B shows that the transcriptional activity of EGFP-p53-Arg337His increased to ~130% in the presence of imidazole-calixarene **1** compared with the absence of the

compound. On the other hand, guanidinium-calixarene **3**, which showed no stabilization of the mutant tetrameric structure, did not enhance the transcriptional activity of EGFP-p53-Arg337His in cells (Fig. 4B). For the wild-type p53 or an inactive mutant p53 with the mutation of Arg273His in DNA binding domain, imidazole-calixarene **1** did not show any enhancement of the transcriptional activities (Fig. S2 in Supplementary data). These results suggested that imidazole-calixarene **1** could restore the transcriptional activity of p53 Arg337His through stabilization of its tetrameric structure.

In summary, the calix[6]arene derivative with six imidazole groups could stabilize the p53 tetrameric structure under physiological conditions. Guanidinium-calix[4]arene **3**, which has four guanidinium groups, does not show stabilization of the mutant p53 under the same conditions. These data indicate one advantage of using calix[6]arene as a template in restoring mutant p53 function. Furthermore, we have demonstrated that imidazole-calixarene **1** could enhance the transcriptional activity of p53 Arg337His in cells. The future optimization of this calix[6]arene derivative might lead to the development of a candidate for cancer therapy.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.053.

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