SUPPORTING INFORMATION

Cell membrane composition drives selectivity and toxicity of designed cyclic helix-loop-helix peptides with cell penetrating and tumor suppressor properties

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Table S1: Properties of cHLH-p53-R and analogues.

Peptides	Theoretical mass (Da)	Observed mass (Da)[a]	ε_{280} ($M^{-1}.cm^{-1}$)[b]	Retention time (min)[c]	Charge[d]
cHLH-p53-R	4330.5	4331.6	5500	21.6	+6
[F30A]cHLH-p53-R	4254.5	4255.2	5500	21.8	+6
[G17K]cHLH-p53-R	4401.6	4402.8	5500	21.3	+7
cHLH-pDI1-R	4524.4	4526.8	6990	23.0	+4
cHLH-pDI2-R	4440.5	4441.6	6990	21.8	+6
cHLH-KD3-R	4399.3	4399.2	6990	21.8	+4
[G17K]cHLH-p53-R p1[e]	4919.5	4918.8		22.5	+6
[G17K]cHLH-p53-R p2[e]	4919.5	4918.2		24.2	+6
[G17K]cHLH-p53-R p3[e]	4919.5	4918.8		25.0	+6

[a]Observed masses were calculated from the M^{+4} obtained by m/z values from ESI-MS, [b]Coefficient of extinction at 280 nm (**ε_{280}**) was estimated from the contribution of the Trp (5500 $M^{-1}cm^{-1}$) and Tyr (1490 $M^{-1}cm^{-1}$) as previously reported*1*, [c]Retention time as observed on HPLC-MS with a 2%/min gradient (0-60%) of solvent B in solvent A after a 5 minutes bypass, [d]Overall charge of the peptide at pH 7.4, [e][G17K]cHLH-p53-R labelled with one Alexa fluor® 488 at different lysine positions.

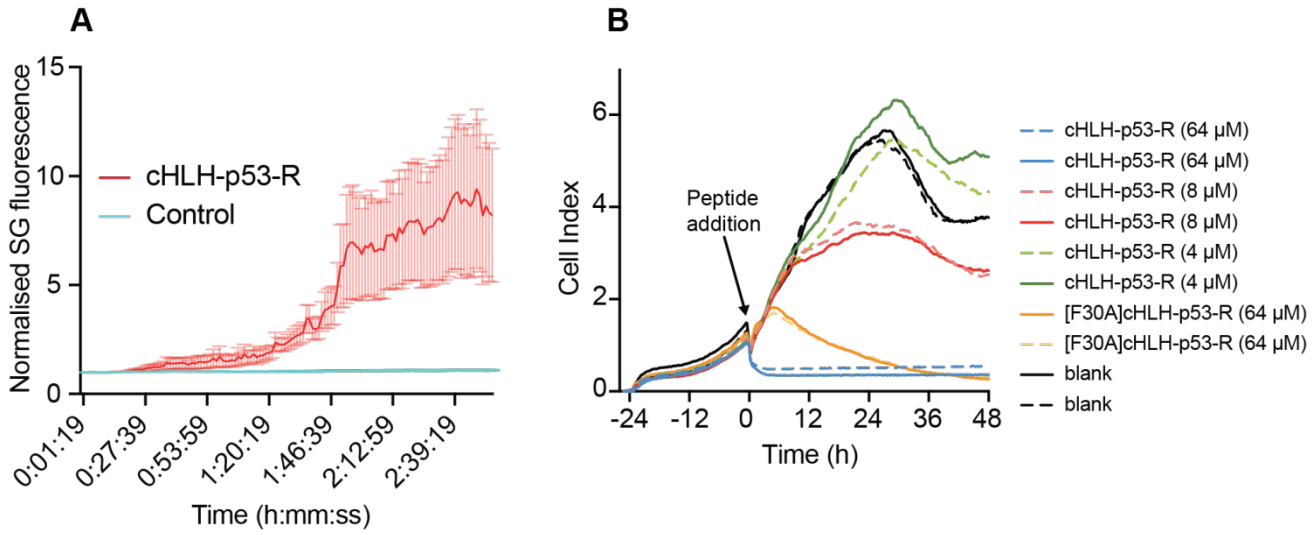

Figure S1: Cell membrane permeabilization and cell proliferation followed in real time. (A) Normalised fluorescence recorded using time lapse microscopy of MCF-7 incubated with SYTOX® Green in the absence or presence of 32 µM cHLH-p53-R. SYTOX® green is a non-permeable dye that becomes fluorescent upon binding to nucleic acids. Live cells were imaged every 79 seconds for 2 hours, and 40 minutes. Fluorescently-labelled cells were tracked and measured over time, using a custom macro, where end-point nuclei intensity levels, along with multiple user-generated selections were used to generate ROIs which were measured individually at each time-point for quantification. Increase in the fluorescence emission intensity indicates cell membrane permeabilization, and agrees with the observation of membrane damage following 2 h incubation of peptides using the LDH assay. Micrographs suggest that SYTOX® green starts binding to the nuclei after 20 minutes of treatment with peptide. At around ~ 1 h 45 min a fast increase in fluorescence emission intensity occurs; observation of the cells on the bright field shows that the increase in the fluorescence signal occurs when the overall cell structure is compromised. Such events facilitates the entry of SYTOX® Green into cells resulting in increased overall fluorescence emission signal. Disruption of the integrity of intracellular components corroborates with the observations made on videos S1 and S2. (B) Impedance of MCF-7 cells followed in real time using an xCELLigence assay. Cell viability and proliferation correlates with cell index monitored over time. 10,000 cells were seeded in each well of the plate; cells were treated 24 h after seeding with 4, 8, 64 µM cHLH-p53-R, or with 64 µM [F30A]cHLH-p53-R, and the cell index monitored for an extra 48 h and compared with cells without treatment (blank). Samples were run in duplicate. 4 µM of cHLH-p53-R did not interfere with cell proliferation, 8 µM cHLH-p53-R slowed the cell proliferation after 12 h of treatment, and 64 µM cHLH-p53-R killed the cell immediately after addition. When treated with 64 µM [F30A]cHLH-p53-R cell proliferation/viability decreased 5 h after treatment and eventually lead to a cell index close to zero.

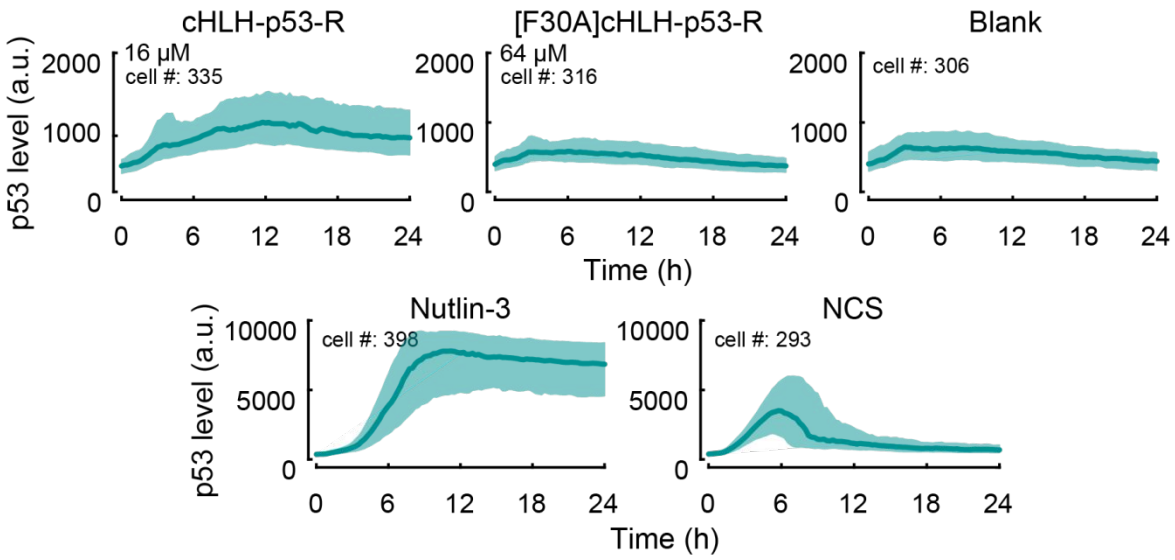

Figure S2: Replicate of the time lapse microscopy obtained with Lung cancer cell A549. cHLH-p53-R and [F30A]cHLH-p53-R are unable to activate the p53 pathway, in contrast to the controls nutlin-3a (20 µM) and neocarninostatin (NCS; 500 ng/mL) (see Figure 5).

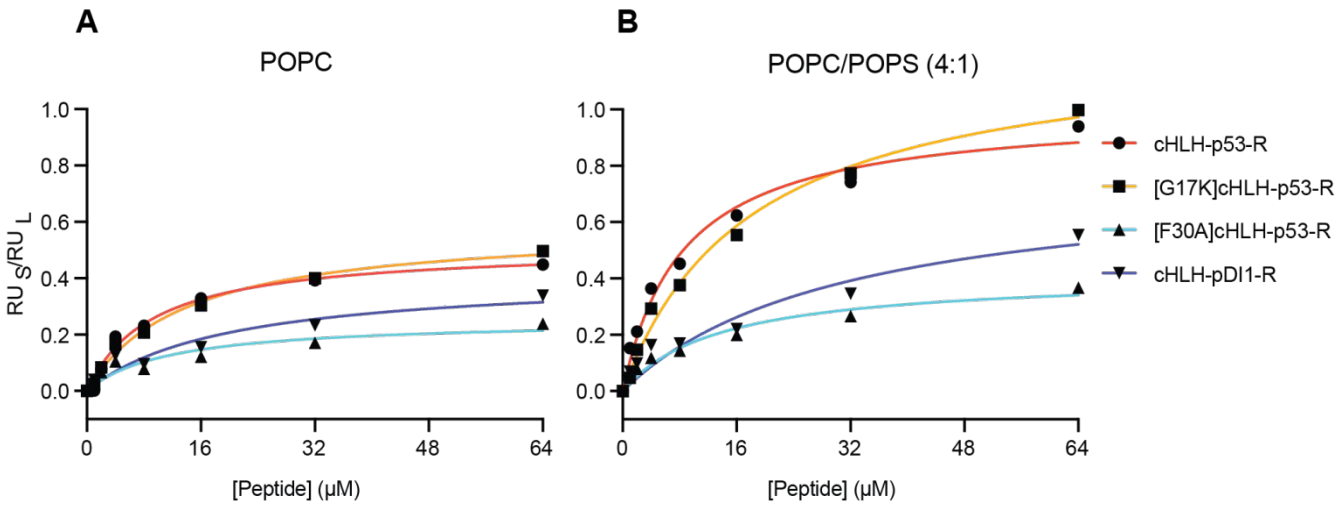

Figure S3: Binding of cHLH-p53-R analogues with lipid bilayers composed of (A) POPC or (B) POPC/POPS (4:1). Ratio of response units (RU) obtained with peptide (RU_S) at the end of the association phase (t = 170s) and of RU obtained for the respective lipid deposition on the L1 chip (RU_L); plotted as a function of peptide concentration. The curves were fitted with a membrane partition equation following a steady-state model previously determined.[2] The partition constants (K_p) were determined by fitting the equation below and added in Figure 6C.

$$\frac{RU_S}{RU_L} = \frac{\gamma_L K_p \frac{M_S}{M_L}[S]_W}{1 + \sigma\gamma_L K_p [S]_W} \quad \text{(eq S1)}$$

RU_s response units of the peptide associated to the lipid membrane; RU_L response units of total lipid deposition; M_L is the molecular mass of the lipids, M_S is the molecular mass of the peptide; γ_L is molar volume of the lipid; σ is the ratio of lipid-to-peptide ($n_L / n_{S,L}$) when the binding reaches saturated; $[S]_W$ is the concentration of peptide in aqueous solution.[2]

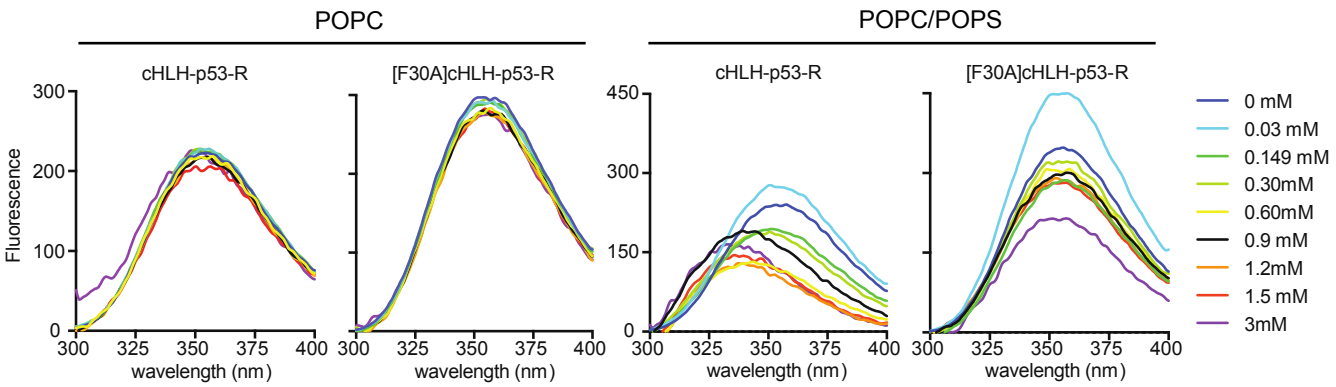

Figure S4: Fluorescence emission spectra of cHLH-p53-R and [F30A]cHLH-p53-R (12.5 μM) upon addition of increasing concentration of vesicles composed of POPC or POPC/POPS (4:1). Spectra were corrected for the dilution and light scattering from addition of vesicle suspensions. The fluorescence quantum yield decreases as the concentration of POPC/POPS increases suggesting quenching of Trp when bound to the lipid bilayer.

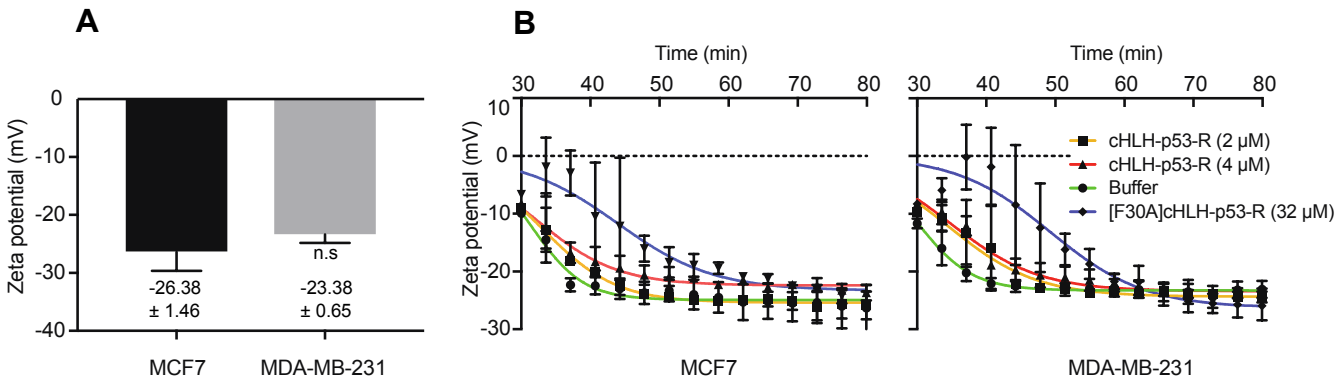

Figure S5: Cell membrane surface charge calculated using zeta potential. (A) Mean zeta potential of untreated MCF-7 and MDA-MB-231 cells. The surface of MCF-7 is more negative than that of MDA-MB-231 cells. (B) Zeta potential was measured every 3.5 min for 50 minutes. The cell surface charge did not vary upon treatment with peptide after 80 minute incubation (in MCF-7, the charge is -23.65 ± 0.95 mV with 4 µM cHLH-p53-R and -24.00 ± 0.5 mV with 32 µM [F30A]cHLH-p53-R. In MDA-MB-231, the charge is -24.15 ± 0.85 mV with 4 µM cHLH-p53-R and -26.23 ± 2.97 mV with 32 µM [F30A]cHLH-p53-R). Variation of the overall charge obtained with cells treated with 32 µM [F30A]cHLH-p53-R suggest an initial charge neutralization, but a decrease to the initial surface charge at 80 min, -26 mV, identical to non-treated cells. A neutralization of the charge immediately after addition of peptide, followed by recovering of the negative charge characteristic of untreated cells agrees with internalization of the peptide inside cells over time.

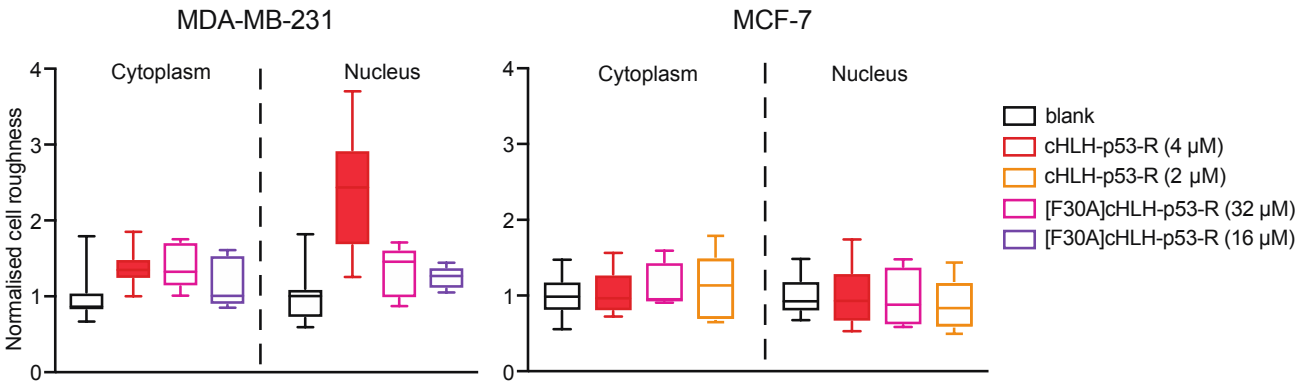

Figure S6: Cell membrane roughness (R_{ms}: Root-mean-square roughness) of breast cancer cells MDA-MB-231 and MCF-7 determined before and after treatment of peptides for 24 h, as followed by atomic force microscopy. R_{ms} values were evaluated from the average of five regions (2.5 × 2.5 µm) of the nucleus or of the cytosol of each cell using AFM height image analysed on the Gwyddion software version 2.24. In MDA-MB-231, the average roughness was determined with 15 untreated cells (blank), ten cells treated with cHLH-p53-R (4 µM) and five cells treated with both concentrations of [F30A]cHLH-p53-R. In MCF-7, 16 untreated cells were used for the blank, 15 cells treated with cHLH-p53-R (4 µM), ten cells treated with cHLH-p53-R (2 µM) and four cells treated with [F30A]cHLH-p53-R (32 µM).

Associated content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

File 1: Supplementary figures and tables (pdf)

File 2: Video S1: Permeabilization of MCF-7 cells when treated with 32 µM cHLH-p53-R peptide monitored by binding of SYTOX® Green with nucleic acids using time lapse microscopy. Cells with green fluorescence have been permeabilized. Scale bar is 50 µm.

File 3: Video S2: Spinning disc confocal microscopy of MCF-7 cells incubated with 4 µM of Alexa Fluor® 488 labelled cHLH-p53-R. 4D rendering was obtained from the Arivis Vision4D software pseudocoloured with a red-yellow gradient LUT ("Muscle and Bones") and cropping the full field of view to a single cell. Scale bar is 10 µm.

File 4: Video S3: Spinning disc confocal microscopy of MCF-7 cells incubated with 4 µM of Alexa Fluor® 488 labelled cHLH-p53-R. Images were acquired every 30 seconds for 120 min. Scale bar is 10 µm.

File 5: Video S4: Side-by-side observation on bright-field microscopy of MCF-7 cells treated or non-treated with 32 µM cHLH-p53-R. The treated cells are strongly affected by the peptide. Scale bar is 50 µm.

File 6: Video S5: Fluorescence microscopy of mVenus-p53 MCF-7 reporter cells with 20 µM cHLH-p53-R. Images were recorded with EX = 500/24 nm and EM = 542/27 nm every 15 min for 24 h, peptide was added after 1 h. Scale bar is 50 µm.

File 7: Video S6: Bright-field microscopy of mVenus-p53 MCF-7 reporter cells treated with 20 µM cHLH-p53-R. Images were recorded every 15 min for 24h; peptide was added after 1 h.

References

[1] Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to Measure and Predict the Molar Absorption-Coefficient of a Protein, *Protein Science* **4**, 2411-2423.
[2] Figueira, T. N., Freire, J. M., Cunha-Santos, C., Heras, M., Goncalves, J., Moscona, A., Porotto, M., Salome Veiga, A., and Castanho, M. A. (2017) Quantitative analysis of molecular partition towards lipid membranes using surface plasmon resonance, *Sci Rep* **7**, 45647.