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A pH-driven ring translocation switch against cancer cells

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A molecular switch built with cucurbit[7]uril and a 3-station viologen-phenylene-imidazole compound exhibited pH actuated ring translocation with high fatigue resistance (up to 10^2 cycles). The switch movement was harnessed toward selectively masking the toxicity of the viologen fragment at neutral pH near normal cells, while exposing it at acid pH near cancer cells.

Despite enormous progress accomplished these last decades in the fight against cancer, one of the still dominant options relies on chemotherapy where most of the anticancer agents are non-specific toxic compounds.[1] In this context, innovative strategies aimed at decreasing the side effects of chemotherapeutic agents are highly sought after. Recently, supramolecular chemistry has come up with new opportunities by applying some of its concepts in supramolecular chemotherapy.[2,3] More specifically, macrocycles such as cucurbit[*n*]urils (CB[*n*]),[4-7] especially CB[7], have been reported to decrease various side-effects, while maintaining the biological activity of included drugs.[8-11] On the other hand, methyl-viologen and some of its derivatives are non-specific, toxic compounds since they induce the generation of reactive oxygen species responsible for cell death,[12,13] but their encapsulation by CB[*n*] resulted in a significant decrease of their toxicity.[14-16] Inspired by the success of CB[*n*] associated with cis- or oxaliplatin,[17-21] camptothecin[22] or doxorubicin,[23-25] we reasoned that CB[7] could be used in combination with a properly derivatized methyl-viologen to enable ring translocation that may reversibly expose the toxic viologen fragment around cancer cells, while masking it around normal cells. In this work, we examined the possibility of selective cytotoxicity of a CB[7], ring translocation switch against cancer cells due to their slightly acidic cellular environment[26-28] where the viologen unit would be exposed as a result of CB[7] translocation (Figure 1, right). At near neutral pH that is typical of non-cancerous cells (pH ≈ 7.4), CB[7] is designed to stay on station *V* (viologen, Figure 1, left), thus protecting non-cancerous cells against the viologen fragment.

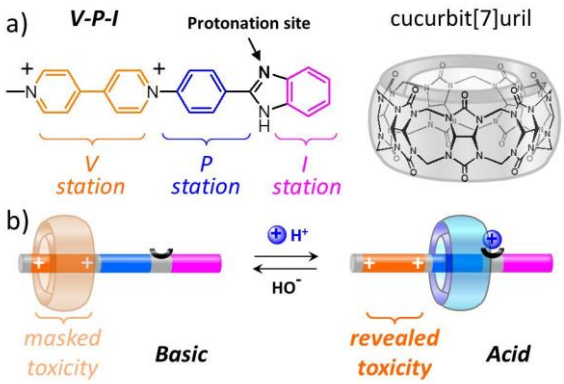

Figure 1. (a) Molecular structures of ***V-P-I*** and CB[7]. (b) Concept of switch triggered toxicity induction.

Such a system should display (i) high binding stations where CB[7] may translocate from one place to another with pH reversibly, (ii) tolerance to large concentrations of salts, ubiquitous in biological media, and (iii) high fatigue resistance to warrant multiple viologen exposure (cancer cells) and masking (healthy cells) during switch circulation. Our design is based on a recently discovered ring translocation switch where CB[7] can reversibly be translated over two stations of a rigid thread in water by silver cations.[29] Because our previous switch[29] possesses a pH responsive imidazole group and a viologen fragment, we decided to investigate the pH responsiveness of this molecular switch, composed of a Viologen-Phenylene-Imidazole (***V-P-I***) rigid axle and CB[7] (Figure 1) in water and test stations binding modulated toxicity (Figure 1b), against macrophages and cancer cells.

V-P-I was prepared according to a previously reported procedure.[29] Protonation of ***V-P-I***$^{2+}$ or ***V-P-I***$^{2+}$•CB[7], affording

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V-P-I^{3+} or ***V-P-I***$^{3+}$•CB[7] respectively, increases the HOMO-LUMO gaps and shifts the absorption to the UV region, in line with yellow-coloured solutions turning colourless at lower pH (UV-vis spectra in Figure S1). The pK_a values for ***V-P-I*** alone were found to be 4.0 and 10.4 (Figure S2),[29] and are assigned to benzimidazole protonation (at acid pH, ***V-P-I***$^{3+}$ is formed) and deprotonation (at basic pH, a precipitate formed, maybe due to a ***V-P-I***$^{1+}$ species).[30] In the presence of CB[7], the pK_a values shifted to 6.8 and 11.5 (Figure S2) in line with previous reports.[31,32] To investigate the ring translocation, we focused on the pH range 3 to 9.5 corresponding to the formation of ***V-P-I***$^{3+}$ and ***V-P-I***$^{2+}$, respectively.

Downfield shifts of ≈ 0.21 ppm for proton signals 8,9 on ^{1}H NMR spectra for solutions of ***V-P-I***$^{2+}$ gradually acidified below pH 6.8 (Figure 2 top and bottom spectra and Figure S3) toward ***V-P-I***$^{3+}$ likely reflect imidazole protonation.

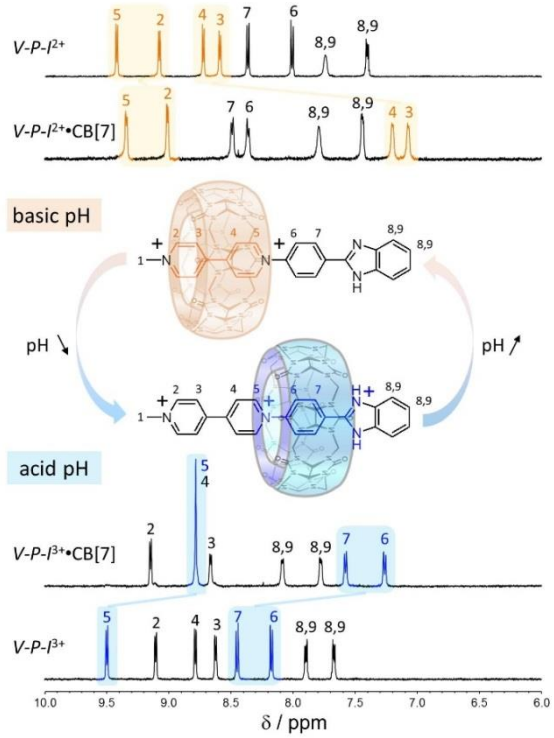

Figure 2. Excerpt of the aromatic region of the 500 MHz ^{1}H NMR spectra of ***V-P-I*** (1 mM) and ***V-P-I***•CB[7] (1 mM each) at basic (top) and acid (bottom) pH and proposed switching process of the CB[7] position along ***V-P-I*** actuated by pH.

Next, 1 equiv CB[7] induced dramatic changes either below or above pH 7. At basic pH, signals assigned to protons 3 and 4 shifted upfield by ≈ 1.5 ppm (Figure 2) in line with viologen complexation (*V* station).[29,33,34] In marked contrast, lowering the pH below 7 resulted in signals assigned to protons 5, 6 and 7 shifted upfield by ≈ 0.70, 0.90 and 0.85 ppm respectively while signals of protons 2, 3 and 4 are almost unaffected (Figure 2). This shows that CB[7] now sits just next to the viologen station, on the phenyl ring (*P* station) connecting the viologen and the imidazolium function (Figures S4 and S5). Actually, 1 equiv of trifluoroacetic acid (TFA) was sufficient to induce full ring translocation (Figure S6). This translocation is similar to what was obtained with 8-10 equiv of $AgNO_3$,[29] while only 1 equiv of TFA triggered the ring translocation. When the pH was raised to the basic region, the spectrum of ***V-P-I***$^{2+}$•CB[7] showing viologen complexation was again obtained. The system could sustain several cycles of pH adjustment between acid and basic conditions, respectively showing the signatures of the bound *P* or bound *V* stations.

DFT calculations were performed gradually shifting CB[7] along ***V-P-I***$^{2+}$ and ***V-P-I***$^{3+}$ from the *V* to the *I* station (see ESI for details). The viologen station is more favoured at basic pH (***V-P-I***$^{2+}$, Figure S7), than the phenyl or benzimidazole ones which agrees well with NMR results. For ***V-P-I***$^{3+}$ instead, CB[7] prefers the phenylene station which is again consistent with NMR results. A number of charge-assisted C-H•••O and N-H•••O interactions are present in the minimized structures.

Intrigued by the occurrence of a 1:2 complex with 2 CB[7] when $AgNO_3$ was used,[29] we recorded ^{1}H NMR spectra with increasing concentrations of CB[7] at basic and acid pH. The 1:1 complex only forms in basic conditions (Figure S8) with CB[7] on station *V* while broad signals appear above 1 equiv CB[7] at acid pH before new resonances are observed around 3.5-4 equiv CB[7] pointing to a weaker binding of a 2nd CB[7] (Figure S8). CB[7]$_1$ is on station *P* in the 1:1 complex (1 equiv CB[7]) while at ≈ 4 equiv CB[7], upfield shifts of protons 3,4 (≈ 1.55 ppm) and 8,9 (≈ 0.75 ppm) indicate CB[7]$_1$ location on station *V* and CB[7]$_2$ location on station *I* (Figures S9 and S10). Mass spectrometry confirms the possibility to form 1:1 and 1:2 complexes (Figure S11). Job plots determined by UV-vis spectroscopy at basic pH (***V-P-I***$^{2+}$) showed a typical curve with an inflexion point at 0.5 in line with a 1:1 stoichiometry (Figure S12). Results are similar at acidic pH (***V-P-I***$^{3+}$) except that the inflexion is less clearly marked at 0.5 and showed a slight deviation closer to 0.66 which could be an indication for the presence of a 1:2 complex (Figure S13). However, the Job plot technique can be inaccurate for systems involving multiple equilibria.[35] The binding constant of ***V-P-I***$^{2+}$ toward CB[7] was determined before ($K_{a\,VPI2+•CB[7]}$ = 7.25 (± 1.65) × 10^5 M^{-1}).[29] Isothermal titration calorimetry was used to determine the binding constant of ***V-P-I***$^{3+}$ toward CB[7] but afforded inconsistent results presumably due to strong affinities perhaps close to the limit of the technique or to the presence of TFA used to acidify the solutions. We thus relied on NMR experiments using a competitor[36,37] that could be used to determine the affinity of ***V-P-I***$^{3+}$ toward CB[7] for the 1st and the 2nd binding. After having tried several compounds, 1-Butyl-3-methylimidazolium chloride (BMIC, Table S1 and Figure S14) was chosen and, in the presence of 1 equiv of ***V-P-I*** and 1 or 2 equiv of CB[7] affording binding constants $K_{a(VPI3+•CB[7])1}$ = 1.04 (± 0.07) × 10^7 M^{-1} (Table S2 and Figure S15) and $K_{a(VPI3+•CB[7])2}$ = 2.3 (± 0.7) × 10^3 M^{-1} (Table S3 and Figure S16) in D_2O.

Cyclic voltammograms (CV) of solutions containing ***V-P-I***$^{2+}$, ***V-P-I***$^{3+}$, ***V-P-I***$^{2+}$•CB[7] and ***V-P-I***$^{3+}$•CB[7] showed two successive and reversible redox waves ($E^1_{1/2}$ and $E^2_{1/2}$, Figure S17), respectively assignable to (i) the one electron reduction of the viologen fragment in the corresponding radical cation and (ii) a neutral species. The CB[7] complexation only weakly affected

the redox potentials of ***V-P-I***, irrelevant of the protonation state and in line with previous reports about CB[7] complexation of methyl-viologen.[34,38]

The evaluation of fatigue resistance in terms of cycling has become a crucial criterion for applied systems and efforts are made toward expanding the number of cycles a switch can sustain by measuring its fatigue resistance.[39] Fostered by the reversibility of the switching position of CB[7] observed over several cycles, we tried to estimate the limit of this pH switch in terms of cyclability. In this system, a good fatigue resistance is necessary to increase the likelihood of toxicity turnover, and target a safe circulation around normal cells (shielded *V* station) while improving toxicity at the cancer site. We first reduced the amount of necessary stimuli to actuate ring translocation by switching between pH 4.5 and 9.5, and we tested the reversibility of the switch over 10 cycles and then extended the work to 100 cycles (Figure 3).

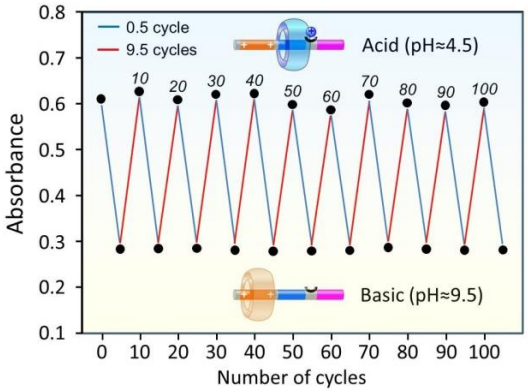

Figure 3. Fatigue resistance of the switch process of ***V-P-I*** (0.04 mM) with CB[7] (1.1 equiv) as monitored by UV-vis spectroscopy at λ = 335 nm in water over 100 cycles.

The pH changes were processed 200 times and successive changes in absorbance at λ = 335 nm have been monitored each 10 cycles (acid and basic pH, Figure S18). After 100 cycles of pH changes, the UV spectra are still those of the complexed species which agrees with the weak salt accumulation amounting to ≈ 10 mM NaCl (for [***V-P-I***] = 4 × 10^{-5} M) and its moderate competitive effect. So this system could be processed for at least 100 times without signs of decay or fatigue. Controls of the switching process in conditions corresponding to 100 cycles of actuation ([NaCl] = 10 mM) by NMR still showed ring translocation with ***V-P-I*** complexed on station *V* or station *P* (Figure S19). The pH-driven translocation NMR tests were then extended to NaCl concentrations of 110 and 150 mM (physiologically relevant, i. e. in blood) and showed almost quantitative complexation of ***V-P-I*** in acid and basic conditions (Figure S19). These results indicate that 1000 pH cycles can in principle be reached, and that the relatively high concentration of Na^+ in cells should not hamper quantitative ring translocation in biological conditions.

Cytotoxicity assays were then conducted in vitro on RAW 264.7 (murine macrophage cells) and BEL 7402 (human liver cancer cells) cell lines, as representative normal and cancerous cell lines respectively. As shown in Figure 4a, MTT assays of cells treated with ***V-P-I***•CB[7] (IC_{50} = 70.23 μM) showed significantly lower cytotoxicity on RAW 264.7 cells after 24 h of incubation, in comparison with that of ***V-P-I*** only (IC_{50} = 21.16 μM).

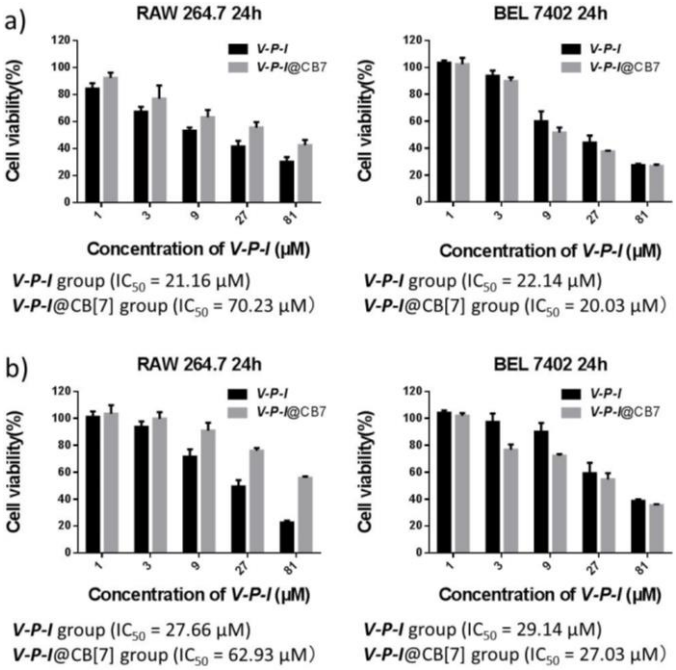

Figure 4. Cell viability (%) of RAW 264.7 cell line (left) and BEL 7402 cell line (right) treated with different concentrations of ***V-P-I*** (1-81 μM) and (a) 1 equiv of CB[7] or (b) 0.5 mM CB[7].

Conversely, there was no significant difference between ***V-P-I*** and ***V-P-I***•CB[7] treated groups on BEL 7402 cell line after 24 h of incubation with IC_{50} of ***V-P-I*** and ***V-P-I***•CB[7] calculated as 22.14 and 20.03 μM respectively (Figure 4a). In the presence of excess amount of CB[7], the reduced toxicity of ***V-P-I*** on RAW 264.7 cells was more pronounced (Figure 4b), with again, highly preserved cytotoxicity against cancer cells. These results suggest that the CB[7] complexation could improve the specific toxicity of ***V-P-I*** against cancer cells by lowering non-specific toxicity against non-cancerous cells. Moreover, controls using increasing host guest concentrations at 1:1 ratio or fixing ***V-P-I*** concentration to 9 μM and increasing that of CB[7] (Figure S20), afforded similar results. Considering the differences in microenvironmental pH between normal cells (≈ 7.4) and cancer cells (≈ 6.8), we inferred that in normal cells, CB[7] could stay on the viologen fragment thus reducing ***V-P-I*** toxicity, similar to what observed by Zhang,[16] whereas in cancer cells, a significant part of CB[7] could have shuttled on station *P* (since actual pH ≈ pK_a), thus contributing to the observed toxicity. At present, the mechanistic origin of the CB[7] protection for normal cells is unclear. Since CB[7] hardly changed the redox potentials of ***V-P-I*** (Figure S17), be it on station *V* or on station *P*, CB[7] would unlikely hinder electron transfer. However, in vivo, methyl-viologen is involved in redox cycling by means of several enzymes including diaphorases.[12] Reaching the various enzyme active sites for the *V* station is

believed to be impeded by CB[7] complexation around and in normal cells,[13] but significantly less around and in cancer cells where CB[7] should be on station *P*. Although the exact mechanism for the observed modulated toxicity is not entirely clear, these preliminary results are still encouraging and support further work toward improved designs targeting increased IC_{50} differences between normal and cancerous cells and thus reach more secured usages.

In summary, we have described a pH triggered ***V-P-I•***CB[7] molecular switch that can easily sustain 100 forward and backward ring translocations. Since cucurbituril complexes are often characterized by very high binding constants, reaching 10^3 or 10^4 cycles appears possible. The good fatigue resistance for ring translocation in ***V-P-I•***CB[7] was exploited to selectively increase (ring on station *P*) or decrease (ring on station *V*) the toxicity of the viologen station, likely due to the pH responsive ring translocation. The exposed strategy could afford new avenues for molecular switches with selective toxicity against cancer cells and foster further investigations of molecular machines into biomedical sciences.

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Conflicts of interest

There are no conflicts to declare.

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