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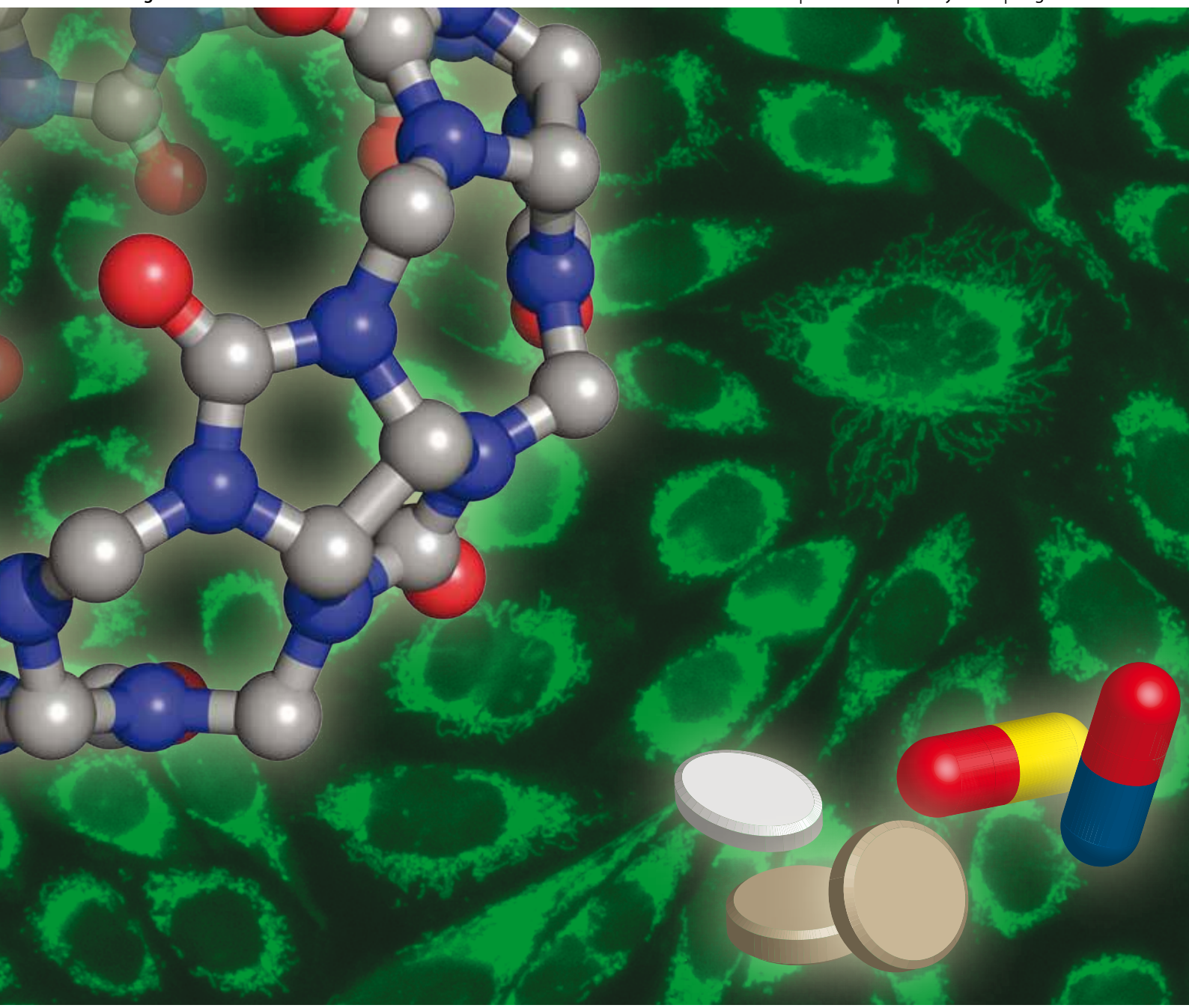

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Toxicity of cucurbit[7]uril and cucurbit[8]uril: an exploratory *in vitro* and *in vivo* study

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Cucurbit[*n*]urils (CB[*n*]) are potential stabilizing, solubilizing, activating, and delivering agents for drugs. The toxicity of the macrocyclic host molecules cucurbit[7]uril (CB[7]), the most water-soluble homologue, as well as cucurbit[8]uril (CB[8]) has been evaluated. *In vitro* studies on cell cultures revealed an IC_{50} value of 0.53 ± 0.02 mM for CB[7], corresponding to around 620 mg of CB[7] per kg of cell material. Live-cell imaging studies performed on cells treated with subtoxic amounts of CB[7] showed no detrimental effects on the cellular integrity as assessed by mitochondrial activity. For CB[8], no significant cytotoxicity was observed within its solubility range. The bioadaptability of the compounds was further examined through *in vivo* studies on mice, where intravenous administration of CB[7] showed a maximum tolerated dosage of 250 mg kg^{-1}, while oral administration of a CB[7]/CB[8] mixture showed a tolerance of up to 600 mg kg^{-1}. The combined results indicate a sufficiently low toxicity to encourage further exploration of CB[*n*] as additives for medicinal and pharmaceutical use.

1. Introduction

Macrocyclic host molecules have the potential to encapsulate biologically relevant guests and act as drug carriers, drug solubilizers, drug stabilizers, and drug bioavailability enhancers. This strategy has been extensively explored both for naturally occurring hosts such as cyclodextrins,[1,2] as well as for synthetic molecular receptors such as calixarenes and crown ethers.[3,4] A very promising emerging class of synthetic molecular hosts are cucurbit[*n*]urils (CB[*n*]). CB[*n*] are macrocyclic containers formed by acid-catalyzed condensation of *n* glycoluril units with formaldehyde.[5] Although first made in 1905,[5] the interest towards the CB[*n*] family has only grown after the full characterization of the parent compound cucurbit[6]uril (CB[6]) in 1981.[6] Subsequently, larger and smaller homologues have been characterized, such that the CB[*n*] family nowadays comprises CB[5], CB[6], CB[7], CB[8], and CB[10].[7-10] All CB[*n*] homologues have highly symmetrical structures with a hydrophobic cavity, accessible on both sides through two identical carbonyl-rimmed portals (Fig. 1). They function as molecular containers forming strong noncovalent 1 : 1 as well as 2 : 1 host–guest inclusion complexes with neutral and positively charged organic molecules.[10,11]

Several biologically and pharmaceutically relevant applications of cucurbiturils, particularly of CB[7], have been recently reported.[12–49] With respect to interactions with biomolecules, cucurbiturils have been shown to form host–guest inclusion complexes with selected naturally occurring amino acid residues,[12] which has been used to recognize and self-sort sequences of short peptides.[13-17] Cucurbituril–dye reporter pairs have been successfully employed for assaying the activity of enzymes[18–20] and for amino acid sensing.[21] Cucurbiturils have also shown the potential to bind with either peptide substrates[22] or inhibitors[23] and in this way affect the activity of enzymes. Cucurbiturils have also been employed to immobilize proteins on surfaces by noncovalent interactions.[24] Furthermore, cucurbiturils have shown the capability to refold synthetic oligomers under chemical stimuli response.[25] The fluorescence enhancement and photostabilization of different dye molecules upon cucurbituril encapsulation provides additional potential for biological analysis. These features are crucial for imaging microscopy applications where improved biological fluorescence markers are in high demand. This last strategy has been applied to several commonly used dyes such as Rhodamine 6G,[26–28] as well as Brilliant Green and Neutral Red in combination with proteins.[29,30]

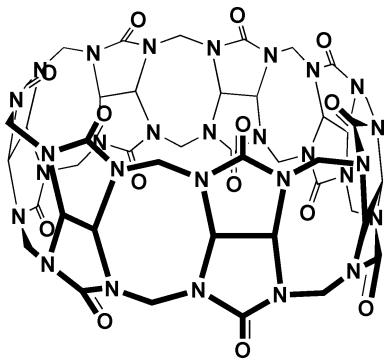

Fig. 1 Structure of cucurbit[7]uril (CB[7]).

With respect to potential pharmaceutical applications, cucurbiturils have also exerted beneficial effects upon interaction with different classes of drugs. An extensively investigated class of drug

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molecules are the platinum-based anticancer drugs, where cucurbituril encapsulation has been used in an attempt to reduce their toxicity,[31] to act as a drug delivery vehicle that would specifically target cancerous cells, and to improve drug solubility, stability, or specificity.[32–37] No cytotoxic effects of CB[7] have become obvious from those studies. The poorly soluble organic cytotoxic drugs albendazole and camptothecin also showed improved properties *in vitro* when encapsulated in CB[7] and CB[8].[38,39] Another prominent example is CB[7] complexation of several drugs administrated through the gastrointestinal tract, such as lansoprazole and omeprazole; their encapsulation in cucurbituril shifts the pK_a values of these drugs, thereby improving their activation and stabilization.[40] The histamine H_2-receptor antagonist ranitidine shows a pK_a shift through encapsulation and is thereby stabilized.[41] Similarly, the local anaesthetics procaine, tetracaine, procainamide, dibucaine, and prilocaine also increase their pK_a values upon CB[7] encapsulation.[42] With respect to peptide-based drugs, cucurbiturils have also shown preservation effects on peptide substrates by inhibiting their enzymatic hydrolysis.[22] Moreover, cucurbiturils have been proposed as carriers in gene delivery,[43] and in drug delivery by employing multivalent vesicles formed from amphiphilic CB[6] derivatives.[44] Two examples of CB[7] complexation of the vitamins B_2 and B_{12} have also been reported.[45,46] Furthermore, a recent study has indicated that CB[*n*]–dye complexes are able to cross the cell membrane of mouse embryo cells.[47]

Surprisingly, although the number of biologically related cucurbituril applications is dramatically increasing, the intrinsic toxicity of cucurbituril macrocycles, which constitutes a critical parameter for their pharmaceutical use, has not been reported until now. Herein, we present a toxicity profile of cucurbiturils, in particular cucurbit[7]uril (CB[7]), based on an *in vitro* cytotoxicity assay, supported by live-cell imaging microscopy, and combined with an *in vivo* oral and intravenous administration study on mice. The CB[7] homologue has been selected for the cytotoxicity and intravenous studies since it possesses the highest solubility in aqueous solution of the known homologues[48,49] and is, consequently, an obvious first choice for drug-related applications. CB[7] is also sufficiently large to fully or at least partially include numerous common drugs, a precondition which is not fulfilled for the smaller water-soluble CB[5] and poorly water-soluble CB[6] homologues. The larger CB[8] has most potential as an oral drug delivery vehicle, where dissolution of this poorly water-soluble macrocycle would present no principal limitation. In general, the thermal stability of CB[7] and CB[8] in the pH range 1.3–9.0, that of the gastrointestinal tract, makes them ideal candidates for oral applications.[10,50]

2. Results and discussion

We have designed our experimental study such that we initially tested the toxicity of CB[7] and CB[8] in cell culture in order to determine the subtoxic dosage of the compound. We then worked in the identified subtoxic range to monitor the effect of CB[7] incubation on the integrity and metabolic activity of viable cells by fluorescence microscopy. The scope of the investigation was augmented by *in vivo* studies on mice. Firstly, a maximum tolerated dose (*MTD*) was determined by performing CB[7] intravenous injection, and subsequently the effect of oral administration of a 1 : 1 mixture of CB[7] and CB[8] was investigated.

2.1 *In vitro* cytotoxicity and effect of metabolic activity

The cucurbituril-induced cytotoxicity was measured in an MTT assay on Chinese Hamster Ovary (CHO-K1) cells. The MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay presents a quantitative colorimetric method for studying cytotoxic agents, where the amount of MTT reduced by cells to its blue formazan derivative is quantified spectroscopically at 570 nm and is equivalent to the number of viable cells.[51,52] In order to determine the subtoxic levels of CB[7], we screened a broad range of CB[7] concentrations (0.1–4.5 mM) by dissolving the additive in the cell culture medium and incubating CHO-K1 cells for a period of 48 h. Concentrations above 2 mM were found to inhibit cell proliferation, while concentrations below 1 mM were tolerable (data not shown). To determine the exact concentration of CB[7] exhibiting cytotoxic effects, we investigated the subtoxic range of up to 1 mM for the same time interval of 2 days (Fig. 2A), relative to a control sample lacking CB[7], and obtained an IC_{50} value of 0.53 ± 0.02 mM. The IC_{50} value, which represents the concentration of CB[7] at which the cell viability is 50% of the viability at control conditions, corresponds to approximately 620 mg of CB[7] per kg of CHO-K1 cells, a number that can be more directly compared to the oral and intravenous studies (see below). Similar studies were performed over 48 h for CB[8] as additive, but due to the lower solubility of this macrocycle the accessible concentration range was limited (Fig. 3). At the highest

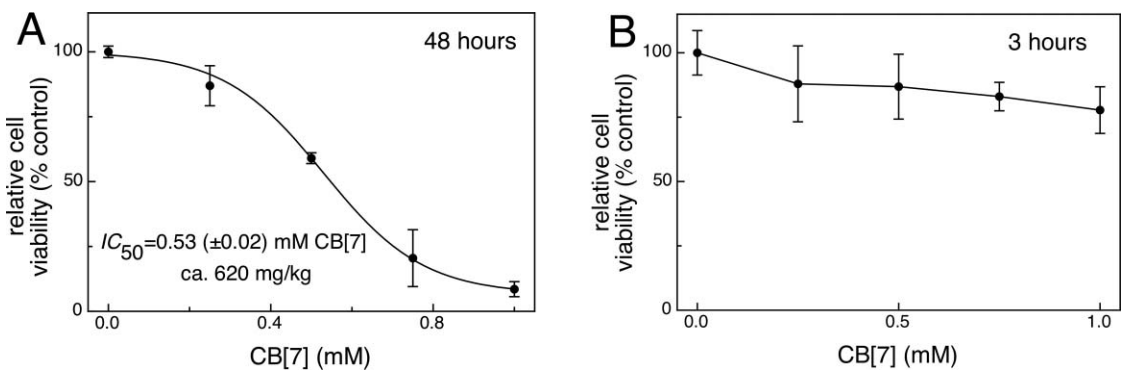

Fig. 2 Relative cell viability of CHO-K1 cells in dependence on CB[7] concentration (0–1 mM) and incubation time determined using the MTT assay by monitoring formazan absorbance at 570 nm. Mean values and standard deviations were obtained from five independent experimental determinations.

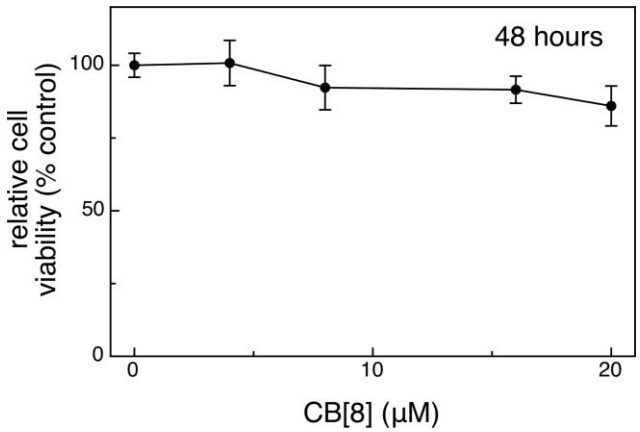

Fig. 3 Relative cell viability of CHO-K1 cells in dependence on CB[8] concentration (0–20 μM) after 48 h incubation time determined using the MTT assay by monitoring formazan absorbance at 570 nm. Mean values and standard deviations were obtained from five independent experimental determinations.

investigated concentration of 20 μM after 48 h (the solubility limit in the employed cell culture medium), the relative cell viability dropped marginally to 86 ± 7%. While this effect is too small to rigorously substantiate an emerging cytotoxic effect, it prevented in any case an accurate determination of the IC_{50} value of CB[8].

Next, we examined the cytotoxicity effect of CB[7] within 3 h of administration (Fig. 2B). This time scale is particularly relevant for fluorescence microscopic imaging applications of cells and tissues, where standard incubation times range from minutes to hours.[53] Recall, in this context, that CB[7] has been suggested as an additive to increase the brightness and photostability of fluorescent dyes used for fluorescence staining,[26–30] a prospective application which is greatly facilitated by the absence of cytotoxic effects as well as other detrimental effects on cellular integrity. Indeed, at shorter incubation times even a higher CB[7] concentration of 1 mM was found to be tolerable. For comparison, administration of CB[8] caused no reduction in cell viability over the shorter (3 h) incubation time.

We further examined the effect of CB[7] on the metabolic activity of living cells when working near the IC_{50} concentration at short incubation times. For this purpose, the mitochondria of CHO-K1 cells were stained with MitoTracker Red CMXRos, a membrane potential-dependent probe, which can selectively distinguish metabolically active mitochondria.[54,55] The integrity of mitochondria was studied by fluorescence microscopy (Fig. 4). To exclude possible artifacts due to interaction of the probe with CB[7], *e.g.*, due to inclusion complex formation,[26–30] we conducted the experiments by varying the sequence of addition, *i.e.* MitoTracker incubations followed by CB[7] and *vice versa* (Fig. 4B and C, respectively). Mitochondrial activity was found to be retained under all experimental conditions since they preserved the characteristic elongated spaghetti-like structure through the cytosol as seen in the control sample containing no CB[7] (Fig. 4A). Overall inspection of the micrographs showed that the cellular architecture and integrity was preserved in the presence of CB[7], *e.g.*, no signs characteristic for necrosis or apoptosis like rupture or plasma membrane blebbing were observed. To sum up, no cytotoxic effects were observed at a concentration of 0.5 mM CB[7] applied in complete culture medium.

The results of the cytotoxicity study with cultured mammalian cells clearly indicated that CB[7] exhibits almost no cytotoxicity when administered over 2 days at concentrations below 0.5 mM (Fig. 2A), with concentrations of 1 mM being tolerable for shorter time periods in the range of few hours (Fig. 2B). Higher concentrations lead to decreased cell proliferation. The live-cell imaging studies further revealed no signs of cytotoxicity when working near the IC_{50} value (0.53 mM). Most importantly, after treatment with subtoxic CB[7] amounts, the metabolic activity of the cells was preserved, indicated by the intact structure of mitochondria and by their ability to maintain an intact membrane potential across the inner mitochondrial membrane, which is a prerequisite in order to accumulate the MitoTracker probe within the mitochondrial matrix (Fig. 4). CB[8] showed no significant

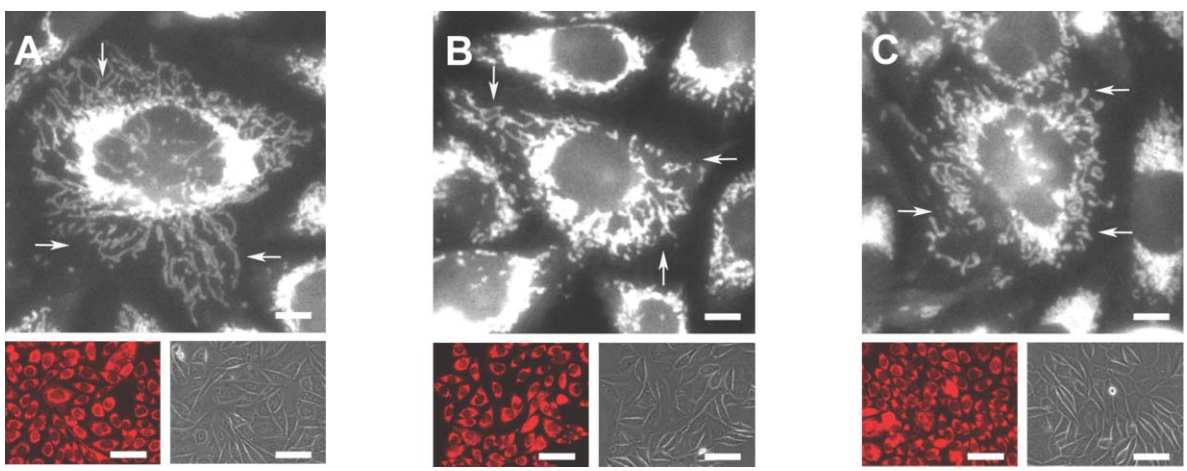

Fig. 4 Micrographs depicting staining of the mitochondria with MitoTracker Red CMXRos in metabolically active CHO-K1 cells: (**A**) MitoTracker applied without CB[7] as a control; (**B**) incubation with MitoTracker (45 min), then 0.5 mM CB[7] (30 min); (**C**) incubation with 0.5 mM CB[7] (30 min), then MitoTracker (45 min). Every micrograph includes a black-and-white magnified image of the fluorescence signal of individual cells (upper panels, 10 μm scale bar), as well as fluorescence and phase contrast images as overviews (lower panels, 50 μm scale bar). White arrows indicate the elongated spaghetti-like structures of the mitochondria.

cytotoxicity within its solubility range (up to 20 μM) after 2 days.

2.2 *In vivo* toxicity by intravenous and oral administration

The effect of intravenous administration was only investigated for CB[7], because of the limited solubility and the absence of an observable cytotoxic effect of the larger homologue CB[8]. Specifically, the effect of a single intravenous dose of CB[7] on mouse body weight was investigated at doses of up to 300 mg kg^{-1} (Fig. 5). No toxicity (assessed and defined here as 10% weight loss) up to levels of 200 mg kg^{-1} was observed. At higher doses and *fast* injection, however, the mice went into a shock-like state immediately after dosing. The maximum tolerated dose of CB[7] was consequently established at the 250 mg kg^{-1} dose level when the compound was administered as a *slow* intravenous push.

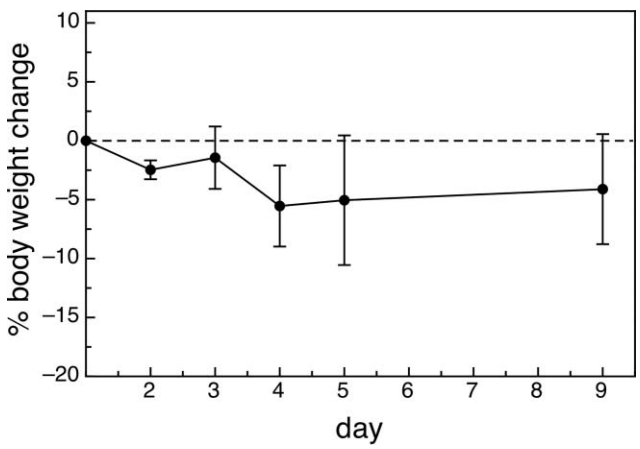

Fig. 5 Effects of CB[7] on mouse body weight over days at the *MTD* of 250 mg kg^{-1} administered by slow intravenous push.

The effects observed for an intravenous single dosing *in vivo* demonstrate that CB[7] has a very low acute toxicity of 250 mg kg^{-1}. Significantly, all of the mice injected with slow infusion into the vein began recovery after 5–8 days. It was beyond the scope of this study to determine the fate of CB[7], but the recovery of the mice may indicate a clearing of CB[7] from the body by either excretion or metabolism. As a drug delivery vehicle or as a biological tool this is certainly a desirable feature.

Potential pharmaceutical applications of CB[7] (as well as CB[8]) would need to compete with those already established with β-cyclodextrin (β-CD), which has a similarly large cavity and could include similarly large drugs. Intuitively, one would expect a higher toxicity for the former synthetic host when compared to the latter naturally occurring and α-D-glucose based one. Indeed, the synthetic CB[7] macrocycle shows a slightly lower intravenous tolerance than β-CD, for which an LD_{50} value of *ca.* 790 mg kg^{-1} has been reported.[56] However, the difference is surprisingly small, especially if one takes into account that the *MTD* of CB[7] (250 mg kg^{-1}) is not equivalent to an LD_{50} value but corresponds to a sub-lethal effect on long-term growth and recovery, which lies generally well below the LD_{50}. Moreover, any applications relying on the supramolecular encapsulation of drugs would depend on the association constants of the host, which are generally much higher for CB[7] (10^4–10^7 M^{-1})[10] than for β-CD (10^1–10^4 M^{-1}).[57] A smaller amount of CB[7] would consequently be required to efficiently complex a given amount of drug. For example, the albendazole drug requires >6 times higher concentration of β-CD than CB[7] to achieve the same solubility.[38,58,59] Moreover, the two macrocycles differ substantially with respect to their selectivity and binding preferences. Accordingly, they could be used in a complementary manner to bind, for example, cationic (for CB[7]) *versus* hydrophobic residues (for β-CD) in peptide-based drugs.[22] Finally, as discussed in the introduction, CB[7] possesses a high solubility in water, which is further increased in the presence of metal ions, as they would be ubiquitous in physiological media.[60] This salt-dependent solubility offers further advantages of the macrocycle as a drug delivery agent, on one hand to improve solubility profiles of encapsulated drugs,[38–40] on the other hand to reduce the risk of any undesirable precipitation (salting out) *in vivo*.

For the oral administration studies the body weight change with time was monitored for mice, which had been fed a single dose of 600 mg kg^{-1} of a 1 : 1 CB[7]/CB[8] mixture. Both CB[7] and CB[8] are promising additives for the oral administration of drugs and were consequently studied in parallel, also with the intention to limit the amounts of test animals. Fortunately, no significant toxicity, as assessed by lack of weight loss, was observed. Thus, the *MTD* for the combined cucurbituril mixture by the oral route is in excess of 600 mg kg^{-1} or, excluding the very unlikely possibility of an interaction between these two hosts, in excess of 300 mg kg^{-1} CB[7] as well as CB[8]. The complete lack of any toxic effects observed for the oral single dosing demonstrated that the toxicity *via* this route is even lower than that for intravenous administration. Presumably, this points to a low absorption of cucurbituril across the gastrointestinal tract into the blood stream. The alternative explanation, a destruction of the macrocycle in the gastrointestinal tract, appears highly unlikely in view of the high chemical stability of cucurbiturils.[10] The intrinsic stability of CB[*n*] macrocycles provides another important feature for their use as drug delivery agents since it could also influence and improve the gastrointestinal stability of the complexed drugs.

3. Conclusions

Our combined results demonstrate a very low toxicity of the macrocyclic compounds CB[7] and CB[8] through *in vitro* studies in CHO-K1 cells and *in vivo* intravenous injection, as well as oral administration studies on mice. The IC_{50} and *MTD* values are mutually consistent and suggest that CB[7] is essentially nontoxic when applied in concentrations below 250 mg kg^{-1} tissue or body weight, or below concentrations of 0.5 mM, which would be far above the concentrations required for drug delivery applications. The potential benefits that can be imparted upon a pharmacological agent by cucurbit[*n*]uril such as improved solubility, stability, and specificity are consequently reinforced by our present finding of a low cytotoxicity.

4. Experimental

In vitro studies with CB[7]

Acetone, ethanol, absolute ethanol and paraformaldehyde were purchased from AppliChem GmbH (Darmstadt, Germany). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and

10 times concentrated phosphate buffered saline (PBS) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Dimethyl sulfoxide (DMSO), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT Formazan) and 1% penicillin–streptomycin solutions were all purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Ham's F12 medium was purchased from CC Pro (Neustadt/Weinstrasse, Germany). MitoTracker Red CMXRos (M-7512) and 10% fetal calf serum (FCS) were purchased from Gibco™ Invitrogen GmbH (Karlsruhe, Germany). CB[7] was synthesized according to previously reported literature procedures.[7,8,61] Flasks, cell culture flasks, and 6- or 24-well plates were purchased from Nunc (*via* OmniLab-Laborzentrum GmbH & Co. KG, Bremen, Germany).

All cell culture manipulations were performed in a sterile environment using a Hera safe cell culture bench (KS12, Kendro Laboratory Products GmbH, Langenselbold, Germany). The cells were kept and manipulated under standard conditions of 37 °C in 5% CO_2 atmosphere using a CB 210 CO_2-incubator (Binder GmbH, Tuttlingen, Germany). Chinese Hamster Ovary Cells (CHO-K1) were grown in Ham's F12 medium, supplemented with 10% FCS and a 1% penicillin–streptomycin solution. The Ham's F12 medium and Ham's F12 medium without phenolred were purchased from CC Pro (Neustadt/Weinstrasse, Germany).

Cytotoxicity assay (MTT test)

Cells were seeded in 24-well plates at a density of 500 cells per microlitre and incubated for periods of 48 h with a range of CB[7] (0.1–4.5 mM) and CB[8] (4–20 μM) concentrations in complete culture medium. For the 3 h CB[7]-incubation period cells were grown for 45 h with addition of CB[7] containing media in the last 3 h. The MTT cytotoxicity assay was performed by adding 100 μL ml^{-1} of an MTT solution (5 mg ml^{-1}) to each well and incubating for 2.5 h. The MTT-containing media were discarded and 1 ml of DMSO was added to each well. The absorbance of the formazan dye was measured at 570 nm with a Spectronic Genesys 10 spectrophotometer (Fisher Scientific GmbH, Schwerte, Germany). The IC_{50} value was calculated from the obtained dose-response curve by sigmoidal fitting.

Live-cell imaging

The cells were plated on glass coverslips in 6-well plates at a density of 1000 cells per microlitre, incubated for 12 h until they reached 70% confluency, and washed with 1 ml HEPES-buffered culture medium (20 mM HEPES) for 3 min. The cells were stained with 500 nM of MitoTracker Red CMXRos solution for 45 min, washed with HEPES-buffered culture medium, and subsequently incubated with 0.5 mM CB[7] for 30 min. For the experiment with reverse addition, the cells were first incubated with 0.5 mM CB[7] for 30 min, washed with HEPES-buffered culture medium, and then stained with 500 nM of MitoTracker Red CMXRos solution for 45 min. For direct observation of the living cells under the fluorescence microscope, cells were washed in pre-warmed PBS and immersed in HEPES-buffered culture media without phenolred. Phase-contrast and fluorescent images with resolutions of 1024 × 1024 pixels were taken using an Axiovert 25 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany), equipped with an HBO lamp with an excitation wavelength of 543 nm. The scans were obtained with a 40-fold magnification and an exposure time of 1.22 s. Color-coding and image analysis was performed using the Axio Vision LE Rel. Image Browser version 4.5 (Carl Zeiss Jena GmbH, Jena, Germany).

In vivo studies

These studies were conducted in accordance with animal welfare guidelines.[62] All samples of CB[7] and CB[8] were prepared according to previously published procedures and rigorously purified.[7,8,61] Saline solutions (0.9% NaCl) for intravenous use were phosphate-buffered. For both the oral and the intravenous study the mice were female Balb/c mice (age: 8–11 weeks). The dose that caused not more than 10% weight loss, which is recovered within 1 week of ending the treatment, was defined as the maximum tolerated dose (*MTD*).

Intravenous administration of CB[7]

CB[7] was dissolved in buffered saline. Intravenous injections of CB[7] solutions (0.1 ml per 10 g body weight) were given on day 1 as a single dose to each mouse. Separate mice were injected following the results of the previous smaller dose, in dose sizes of 1, 10, 100, 150, 200, 250 and 300 mg kg^{-1}. Mouse weights were recorded daily for at least 1 week. At the determination of the *MTD* at 250 mg kg^{-1}, toxic shock was observed in response to the rate of injection at the time of dosing. The toxic shock-like response was immediate and passed quickly. Toxic shock was minimized or eliminated by slower injection rates and the experiment was repeated for confirmation. A group of 4 mice was injected with single doses of 250 mg kg^{-1} (slow injection) and their weights monitored on a daily basis for 9 days. These results confirmed that the *MTD* was 250 mg kg^{-1}.

Oral administration of CB[7]/CB[8]

The doses of CB[7]/CB[8] were prepared as a slurry in saline solution. The CB[7]/CB[8] mixture (0.1 ml/10 g body weight) was given on day 1 by oral gavage to a single mouse. Separate mice were dosed orally following the results of the previous smaller dose, in dose sizes of 1, 10, 100, 200, 300, 450 and 600 mg kg^{-1}. Mouse weights were recorded daily for at least 1 week. The experiment was terminated at the largest dose of 600 mg kg^{-1} without reaching *MTD*.

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