Competitive binding of aristolochic acid between various cyclodextrins and serum albumin as a model for acute poisoning detoxification

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ABSTRACT
We showed that aristolochic acid toxin (ARI) and its fluorescent analogue form inclusion complexes with various cyclodextrins (CDs), including the clinically used γ-CD: Sugammadex. The binding affinity of ARI towards CDs varied on a CD size and rim modifications, whereby the stability of ARI/Sugammadex complex was 2-fold greater than ARI binding to serum albumin (BSA), blood protein well-known as a transporter of small molecules and similar to the ARI complex with ds-DNA. Molecular modelling supported the formation of ARI/Sugammadex complex with carboxyl group of ARI exposed to water and ARI-condensed aromatic moiety deeply immersed into CD-cavity. These results are a proof-of-concept that CDs could be used as a first aid upon acute intoxication with ARI by binding larger part of the toxin in competition with serum albumin or ds-DNA and significantly reducing ARI absorption in the organism, similarly as Sugammadex extracts drugs from blood to stop their bioactivity.

1 Introduction
The family of aristolochic acid plant toxins (Figure 1c), aristolochic acid I shown, ARI) are planar extended aromatic molecules, well-known for their carcinogenic, mutagenic and nephrotoxic activity and most commonly linked to the Europe Balkan region (particularly Danube – Black Sea basin), causing severe endemic nephropathy [1]. Moreover, ARI-containing herbal remedies were used in obstetrics, the therapy of arthritis, gout, rheumatism, festering wounds and slimming regimen [2,3], and the anti-inflammatory properties were also studied until observation that ARI is a strong carcinogen and cause severe nephropathy [4], which led to FDA advice to immediately discontinue use of any botanical products containing ARI and publication of a list of botanical products containing ARI [2,5].
The ARI intake in humans commonly happens by food or water (depending on soil pH and hydrophobic/hydrophilic partition) [6]. Low aqueous solubility of ARI leads commonly to consequent hydrophobic-driven binding to serum albumin in the blood [7], thus being distributed over the organism, strongly supported by ARI hydrophobicity and consequent slow clearance kinetics. ARI itself is of low toxicity, but after bioactivation by enzymes [8] causes covalent damage of ds-DNA as the dominant mode of toxicity [9,10]. The studies [2–5] in southeastern Europe, as well as in USA and China, suggested chronic poisoning by ARI as a powerful nephrotoxic and carcinogenic substance with an extremely short latency period, not only in animals but also in humans [2]. The most important issue is that to the best of our knowledge, there is no medical protocol to address acutely poisoned people.

In a search of medicinal protocols for the fast capturing of small-molecule drugs/toxins in blood, we found several intriguing approaches based on the formation of supramolecular complex between small molecule (drug/toxin) and large synthetic host. Application of macrocyclic compounds in biomedicine is generally well-known and intensively studied [11]. There were several reports about application of macrocycles as supramolecular antidotes, relying on the ability of calixarenes, cyclodextrins, cucurbiturils or pillararenes to form inclusion complexes with small molecule ligands, the most relevant nicely summarised in the comprehensive review [12].

Particularly, cyclodextrins have attracted our attention since one derivative is currently clinically used: Rocuronium and Vecuronium drugs are very efficiently harvested by cyclodextrin analogue Sugammadex ((SMX), Figure 1(b)) [13]. Further, the crystal structure

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**Figure 1.** (a) Structure similarity of studied aristolochic acid (ARI, red lines) and neuromuscular blocking agent (Rocuronium, black lines), (b) Rocuronium in a complex with γ-cyclodextrin Sugammadex (SMX) [14]; (c) ARI I and ARIfluoro [17] structures. (d) Schematic presentation of α-, β-, γ- and SMX cyclodextrin size.
of Rocuronium/Sugammadex inclusion complex showed excellent fit of small molecule inside extended cyclodextrin structure [14], and the overlay of Rocuronium with ARI (Figure 1(a-c)) revealed their structural similarity. Namely, although ARI is a condensed aromatic molecule, while Rocuronium is a more flexible polycyclic-aliphatic molecule, they both occupy similar van der Waals space and have similar hydrophobicity – both properties essential for the formation of cyclodextrin inclusion complex.

Furthermore, there are several reports in which interaction of α- or β-cyclodextrins (CDs) with ARI was used, mostly in a process of development of novel chromatographic detection methods [15,16]; however, none of the works in detail characterise the stability of ARI/CD complex or study the impact of CD α-, β-, γ- cyclodextrin size (Figure 1(d)) on ARI complexation. To the best of our knowledge, there are no reports on Sugammadex–ARI interactions.

Therefore, we decided to study the interactions of ARI and its fluorescent analogue ARIflu [17] (Figure 1(c)) with commonly studied series of α-, β-, γ- cyclodextrins (CDs), as well as with extended γ-cyclodextrin Sugammadex (SMX). For comparison, under the same experimental conditions, the interactions of both ARI analogues with serum albumin (BSA) will be characterised to determine the ability of the studied CDs to compete with BSA in binding ARI, and also ARI non-covalent interactions with ds-DNA will be assessed to estimate the eventual accumulation of ARI close to DNA before biotransformation [8–10] to the toxic ARI metabolites.

2. Materials and methods

2.1. Chemicals

Aristolochic acid I (ARI, M.W. = 341.27, Purity ≥ 90%) was purchased from Sigma-Aldrich (Taufkirchen, Germany), dimethyl sulphoxide (DMSO), BSA (M.W. = 66.5 kDa, Purity ≥ 96%), as well as calf thymus-DNA (ct-DNA) and buffers were purchased from Sigma-Aldrich (Deisenhofen, Germany). Cyclodextrins (CD) were purchased: α-CD (M.W. = 972.90, Purity ≥ 98%; Sigma-Aldrich, Steinheim, Germany), β-CD (M.W. = 1134.37, Purity ≥ 97%; Thermo Fisher, Karlsruhe, Germany), γ-CD (M.W. = 1297.12, Purity ≥ 98%; Sigma-Aldrich, Steinheim, Germany) and Sugammadex (SMX) (Bridion®, CYC-Br; c = 0.077 M, Merck Sharp & Dohme Limited, Hertfordshire, United Kingdom). Vecuronium (M.W. = 637.73, Purity ≥ 99%) was purchased from Selleck Chemicals (Houston, USA). Deionised water was produced by Millipore Milli-Q Gradient A10 water purification equipment (Merck Millipore, Croatia). All other chemicals were from Kemika (Zagreb, Croatia).

2.2. UV-Vis and fluorescence spectroscopy

Calf thymus (ct)-DNA (Sigma–Aldrich) was dissolved in sodium cacodylate buffer, I = 0.05 M, pH 7.0. The ct-DNA was additionally sonicated and filtered through a 0.45 μm filter to obtain mostly short (approx. 100 base pairs) rod-like B-helical DNA fragments.

Bovine serum albumin (BSA) (Sigma–Aldrich) was dissolved in sodium cacodylate buffer, I = 0.05 M, pH 7.0, and its concentration was determined spectroscopically using a NanoDrop spectrophotometer at 280 nm using its molar extinction coefficient of 43824 M⁻¹ cm⁻¹.

UV-Vis spectra were recorded on a Varian Cary 100 Bio spectrometer, fluorescence spectra were recorded on a Varian Cary Eclipse fluorimeter and CD spectra were recorded on JASCO J815 spectropolarimeter at 25.0°C using appropriate quartz cuvettes (path length: 1 cm). Fluorimetric titrations were performed by adding aliquots of a protein stock solution (to cover c(protein) = 1–20 μM) into a solution of the compound, subsequently collecting emission spectrum, allowing the establishment of thermodynamic equilibrium for 120 s. After the end of the titration, the fluorescence spectra of dye/protein complexes remained constant for more than 1 h, proving that equilibrium was reached and no other processes (e.g. cleavage) are involved. Titration data were plotted in Origin 7.0 software, corrected for dilution (cumulative addition during titration was <5% of starting volume) and fitted to the 1st exponential equation, adequate for 1:1 stoichiometry of compound/protein complex. For titration of ARIflu with ds-DNA, data were fitted by non-linear regression to McGhee, vonHippel formalism of Scatchard equation [18]. In a specific case of several complexes formed (Figure 2(a,b)), titration data were analysed by the Specfit program for multivariate analysis [19,20]. For all cases, good agreement between experimental and fitted data was observed, correlation coefficients being > 0.99 for all calculated Ks values, with error of ±10%.

Circular dichroism (CD) spectra were recorded with a scanning speed of 200 nm/min (an average of three accumulations). A buffer background was subtracted from each spectrum. CD experiments were performed by adding portions of the compound stock solution into the solution of the polynucleotide (c = 2 × 10⁻³ M).

Thermal melting curves for ds-DNA and its complexes with studied compounds were determined as previously described [21] by monitoring the absorption change at 260 nm as a function of temperature. The absorbance of
Figure 2. Changes of ARI UV spectra ($c = 2 \times 10^{-5}$ M, MQ water) upon titration with a) SMX; b) dependence of ARI changes at 391 nm upon titration with SMX; note the breakpoint at exactly 1:1 stoichiometry. Specfit multivariate non-linear regression analysis giving best fit for ARI/SMX = 2:1 and 1:1 stoichiometry; red lines represent calculated fit and $\beta_{21}$ and $\beta_{11}$ represent estimated binding constants; c) titration with $\gamma$-CD; d) titration with BSA. Insets to c) and d): red line represents non-linear fitting to 1:1 complex model with resulting binding constants ($K_s$). Correlation coefficients for all fitted data (red lines) $R > 0.999$.

the ligands was subtracted from every curve, and the absorbance scale was normalised. $T_m$ values are the midpoints of the transition curves determined from the maximum of the first derivative and checked graphically by the tangent method. The $\Delta T_m$ values were calculated by subtracting the $T_m$ of the free nucleic acid from the $T_m$ of the complex. Every $\Delta T_m$ value here reported was the average of at least two measurements. The error in $\Delta T_m$ is ±0.5°C.

3. Results

3.1. Characterisation of aristolochic acid derivatives in biorelevant medium

ARI was purchased from Sigma Aldrich, and ARIfluox was prepared by the procedure described previously [17]. Due to low solubility in water, stock solutions of ARI derivatives were prepared in DMSO (5 mM), stored in refrigerator at +4 to +8°C and diluted in a buffer before the experiment. The absorbance of ARI and ARIfluox in water was proportional to concentration up to $c = 2.0 \times 10^{-5}$ M (Supp. Info. S1), whereas at higher concentrations, appearance of colloidal species was observed, causing a systematic increase of UV/Vis baseline at range >400 nm, where ARIs do not absorb. Eventually, with time, precipitation was observed. To avoid colloidisation/precipitation problems, we used in all experiments freshly prepared ARI solutions of $<c = 5.0 \times 10^{-6}$ M. A temperature increase of ARIfluox solution caused changes in UV spectra (Supp. Info. S2), namely loss of increased baseline >400 nm, attributed to dissolving off the traces of aggregate. Upon cooling back to room temperature, the aggregate was not reconstituted. Fluorescence and UV spectra of ARIfluox (Supp. Info. S3) agreed well with literature data [17], spectroscopic response in our conditions proportional to concentration up to $c = 5.0 \times 10^{-6}$ M.
3.2. Interactions of ARI with various cyclodextrins and bovine serum albumin (BSA) and calf thymus DNA

Studied cyclodextrins (CD) were dissolved in water as stock solution $c = 0.001$ M, all stored in refrigerator at +4 to +8°C and diluted in water or the buffer before use. Stock solutions of bovine serum albumin (BSA) and ct-DNA in buffer were prepared and stored by standard procedures.

The addition of γ-CD, SMX or BSA caused small but measurable changes in the UV/Vis spectrum of ARI aqueous solution (Figure 2), while titration with α- or β-CD yielded much weaker, almost negligible changes (Supp. Info S4). Only SMX showed the formation of two types of complexes (Figure 2(b)), likely due to extension arms in respect to γ-CD. Analysis of complete titration data by Specfit program for multivariate analysis [19,20] gave the best fit for two complexes formed; at excess of ARI over SMX dominant was ARI: SMX = 2:1 complex, while after the clear breakpoint, further excess of SMX over ARI dominantly yielded 1:1 stoichiometry complex. Small changes in UV spectrum allowed only estimation of binding constants ($\beta_{21}$ and $\beta_{11}$); however, at excess of SMX over ARI (biologically relevant conditions, since in clinical use SMX is added at high concentration) 1:1 stoichiometry is dominant and characterised by binding constant ($\beta_{11}$) in the $10^5$ M$^{-1}$ range.

Also, small changes in ARI UV/Vis spectra caused by γ-CD or BSA (Figure 2(c,d)) allowed collection of less than 10 data points, and analysis of titrations by non-linear regression to 1:1 stoichiometry complex could only give an estimate of binding constant, nevertheless, suggesting somewhat stronger binding of ARI to γ-CD ($K_s = 1 \times 10^5$ M$^{-1}$) in comparison to BSA ($K_s = 3 \times 10^4$ M$^{-1}$).

Next, we modified ARI according to the literature procedures [17] to fluorescent analogue ARIflu, intending to directly monitor changes in ARI chromophore upon binding, with the aim to collect more titration points due to the higher fluorescence sensitivity (Figure 3(c,d)). Also, in parallel, we performed fluorimetric titrations monitoring changes in emission of BSA upon addition of non-emissive ARI (Figure 3(a,b)) – latter experiment agreeing excellently with previously reported [7]. The excellent agreement of $K_s$ values obtained by monitoring two different fluorophores (Figure 3, BSA and ARIflu) strongly supported binding of ARIflu to the same binding site on BSA as binding of a parent, non-emissive ARI. Also, excellent agreement of titration data to the 1:1 stoichiometry model supported one dominant binding site for ARI on BSA [22].

The titration of ARIflu with SMX (Figure 4) also resulted in emission increase (Figure 3), whereby non-linear regression fitting of titration data to 1:1 stoichiometry complex gave binding constant ($K_s = 1 \times 10^6$ M$^{-1}$); value approximately two-fold higher in comparison to ARIflu affinity to BSA ($K_s = 6 \times 10^5$ M$^{-1}$). It should be stressed that binding constants estimated by UV/Vis titrations (Figure 2) also showed approximately double affinity of ARI towards γ-CD or SMX in comparison to BSA.

To check the binding affinity of ARI to SMX by the method independent of spectrophotometric titrations, we opted for isothermal titration calorimetry (ITC), established as a standard method for characterisation of SMX/drug complexes [13]. The first attempt to

Figure 3. a) Changes in fluorescence spectra of BSA ($c = 2.0 \times 10^{-6}$ M, $\lambda_{exc} = 295$ nm) upon titration with ARI (non-fluorescent); dependence of BSA emission at $\lambda_{max} = 350$ nm on $c$[ARI], at pH 7.05, sodium carboxylate buffer, $I = 0.05$ M. b) Changes in fluorescence spectra of ARIflu ($c = 2.0 \times 10^{-6}$ M, $\lambda_{exc} = 375$ nm) upon titration with BSA; dependence of ARIflu emission at $\lambda_{max} = 401$ nm on $c$ (BSA), at pH 7.05, sodium carboxylate buffer, $I = 0.05$ M. Correlation coefficients for all fitted data (red lines) R > 0.995.
follow the literature procedure commonly used for SMX-studies [13] by adding ARI to SMX was hampered by the low solubility of ARI in water. The reverse protocol attempt by adding SMX to ARI was hampered by the precipitation during titration, observed in the control experiment monitored by UV/Vis spectroscopy. Subsequently, we opted for a competition experiment, first performing a common ITC experiment by adding drug (Vecuronium) to SMX (Supp. Info., Figure S9a) and then performing second ITC titration in which SMX/ARI complex (1:1 stoichiometry, positioned in the cell, similar concentration conditions as in fluorimetric titration) was titrated with Vecuronium (Supp. Info., Figure S9b). Both ITC micrographs (Supp. Info., Figure S9a and S9b) were similar, thus hampering precise quantitative analysis. However, there is a difference in total enthalpy change (ΔH), whereby the presence of ARI lowers ΔH by about 15% (ΔΔH =−1000 cal/mol), which could be attributed to the competitive binding of ARI and Vecuronium to the SMX and agrees with the order of magnitude lower affinity ARI towards SMX in respect to Vecuronium (Ks = 1.8 × 10^−2 M^−1 [13]). Thus, the ITC experiment at least qualitatively supported the binding of ARI to SMX in a similar manner as Vecuronium.

The low solubility of ARI analogues in water hampered NMR studies and preparation of a single crystal for the x-ray crystallography is unpredictable and long-lasting, thus to evaluate the structural details of the ARI/SMX complex, we opted for a molecular modelling approach, quite often used for the analysis of CD-inclusion complexes [23]. We initially relied on the Rocuronium/SMX structure (Figure 1(b)) obtained by X-ray diffraction (CCDC ref: 172247 [14]). The crystal structure of ARI (CCDC ref: 673035) [14] was used for ligand structure. First, molecular docking and molecular mechanics calculations were performed using the Tripos force field as implemented in the SYBYL-X software suite, using the FlexiDock module [24]. The Rocuronium in the SMX cavity was replaced by ARI in two different orientations: the polar carboxyl group is located inside the β-CD cavity and the opposite. The geometry optimisation (with a distance-dependent dielectric constant for water, ε = 78) yielded a family of conformers of the lowest minimum energy with the carboxyl group of ARI facing the alkyl chains on the primary hydroxyl group of glucopyranose of SMX, while the aromatic part of ARI was in the hydrophobic interior of SMX and formed several lipophilic interactions (Figure 5). Opposite ARI orientation (carboxyl group inserted into SMX cavity) was about 4 kcal/mol higher energy.

Further, molecular dynamics simulations on the structure optimised by molecular docking (Figure 5(a)) were performed in the Amber2020 program package [25]. SMX and ARI were described using GAFF2 force field parameters, recommended for organic ligands [26]. The system was simulated in TIP3P water cubical box (dimension 41.8 Å) [27], with periodic boundary conditions, consisting of a few thousand water molecules treated explicitly. The simulations were performed in 4 parts: minimisation, heating, equilibration and final dynamics. Geometry optimisation was done with the steepest descent and conjugated gradient method. The system was heated from 100 to 300 K using Langevin thermostat at constant volume (NVT ensemble) [28], followed by equilibration step at constant temperature and pressure: T = 300 K, p = 1 atm (NpT ensemble, using Berendsen barostat [29]). The molecular dynamics simulation was performed for 5 ns with a timestep of 0.1 fs. The cut-off radius for nonbonded van der Waals and short-range Coulomb interactions was 9 Å. Long-range Coulomb interactions were treated by the Ewald method, as implemented in the particle mesh Ewald (PME) procedure [30]. The figure of the optimised molecular structure (Figure 5(b)) was created using Pymol software [31].

Analysis of molecular modelling results showed that ARI can neatly insert into SMX cavity, occupying the space similarly to Rocuronium (Figure 1(b)) and with hydrophilic carboxyl-group oriented towards bulk water. Dominant binding interactions seem to
be a combination hydrophobic effect of the large condensed aromatic moiety of ARI, combined with VdW interactions within the SMX cavity.

Since according to the literature, covalent conjugation of ARI with ds-DNA is one of the reasons for ARI toxicity, we also tested non-covalent interactions of ARI with ct-DNA to see whether non-covalent binding could precede chemical conjugation. The addition of ct-DNA resulted in negligible changes in ARI UV/Vis spectrum (Supp. Info. Figure S6). However, fluorimetric titration with ct-DNA caused a considerable increase (about 50%) of ARIflu emission (Figure 6(a)). Analysis of titration data using non-linear fitting to Scatchard eq [18] yielded binding constant of $K_s = 6 \times 10^6$ M$^{-1}$ with Scatchard ratio $n$[bound ARIflu]/[DNA] = 0.3 (Figure 6(b)).

In thermal denaturation experiments (Supp. Info. Figure S7), addition of ARI or ARIflu did not stabilise ct-DNA against thermal denaturation, thus excluding intercalative binding mode [21,32]. Circular dichroism spectroscopy was applied as a very informative method about structural changes in ds-DNA secondary structure caused by binding of achiral small molecules (e.g. ARIflu), which do not possess intrinsic CD spectrum [33]. Indeed, the addition of ARIflu caused a minor but significant increase of intensity of

Figure 5. a) Molecular docking optimised energy structure of the ARI (VdW balls)/SMX (sticks) complex; b) molecular dynamics optimised structure in water, after 5 ns at T = 300 K, p = 1 atm. Water molecules are omitted for clarity.

Figure 6. a) Changes in fluorescence spectra of ARIflu ($c = 2.0 \times 10^{-6}$ mol dm$^{-3}$, $\lambda_{exc} = 330$ nm; incubation 60 sec.) upon titration with ct-DNA; b) dependence of ARIflu emission at $\lambda_{max} = 401$ nm on c(ct-DNA), fitted by non-linear regression to Scatchard eq [18]. Done at pH 7; sodium cacodylate buffer, $l = 0.05$ M. Correlation coefficient for fitted data (red line) $R > 0.995$. 

$K_s = 6 \times 10^6$ M$^{-1}$

$n$[bound ARIflu]/[DNA]=0.3
the negative maximum of ct-DNA at 245 nm, as well as the bathochromic shift of maximum for +3 nm (Supp. Info. Figure S8). Moreover, nonlinear dependence of CD (245 nm – Figure S8: inset) intensity change supported non-covalent interaction as a cause of spectroscopic change. The CD band at 245 nm is attributed to the DNA backbone, thus observed changes indicate that binding of A Rifluo influenced the secondary structure of DNA double helix. The absence of any measurable changes at ds-DNA positive maximum at 280 nm (attributed to basepairs) suggested that A Rifluo does not form any significant aromatic stacking interactions with nucleobases (again excluding intercalative binding). Further, an achiral small molecule can, upon binding to ds-DNA, acquire an induced (l) CD signal [34], positioned at the absorption bands of the small molecule, in the case of A Rifluo in the range 300–400 nm. However, the absence of any ICD band >300 nm (Supp. Info. Figure S8) suggests that A Rifluo upon binding to ds-DNA is not uniformly oriented in respect to DNA chiral axis but more likely heterogeneously arranged along DNA backbone, thus only causing minor binding of DNA helix backbone.

Thus, here the studied ARI analogues bind non-covalently to ds-DNA by mixed, non-specific binding mode, but with a bio-relevant affinity comparable to the ARI affinity towards BSA. Such non-covalent interaction with ds-DNA might be a precursor of the literature reported biotransformation of ARI [8], which is an essential step for covalent conjugation to ds-DNA responsible for ARI toxic and carcinogenic effects.

4 Conclusions

We showed by UV/Vis, fluorescence and ITC experiments that aristolochic acid I toxin (ARI) forms inclusion complexes with various cyclodextrins (CDs), including the clinically used derivative of γ-CD: Sugammadex (SMX). The binding affinity of ARI-toxin towards CDs varied on a CD size and rim modifications, whereby the stability of ARI-toxin/ Sugammadex complex was 2-fold greater than ARI-toxin binding to serum albumin (BSA), blood protein well-known as a transporter of small molecules, and similar to the affinity of A Rifluo to ds-DNA. Such non-covalent interaction of ARI with ds-DNA might be a precursor of the literature reported biotransformation of ARI [8], which is an essential step for covalent conjugation to ds-DNA responsible for ARI toxic and carcinogenic effects. The difference in binding constants of Rocuronium/SMX complex (Ks = 1.8 x 10\(^7\) M\(^{-1}\) [13]) and A Rifluo/SMX complex (Ks = 5 x 10\(^6\) M\(^{-1}\)) could be attributed to the additional electrostatic interaction between positively charged Rocuronium and negatively charged SMX, which could not be formed by neutral A Rifluo. However, hydrophobic and van der Waals interactions of ARI or Rocuronium with SMX seem to be comparable, as supported by the molecular modelling studies, giving as the lowest-energy structure the ARI/ Sugammadex complex with carboxyl group of ARI exposed to water, and ARI-condensed aromatic moiety deeply immersed into the CD cavity.

These results are a proof-of-concept that, similarly as Sugammadex extracts neuromuscular relaxation agents (e.g. Vecuronium or Rocuronium) from blood to stop their bioactivity, it could be used as first aid upon acute intoxication to encapsulate a larger part of the ARI-toxin in competition with serum albumin or DNA and significantly reduce its absorption and consequent toxic biotransformation in the organism.

Presented results strongly support further detailed study in vivo, since medicinally used CDs (e.g. Sugammadex, SMX) is commonly excreted in the urine, and thus corresponding CD complex bioactive substance (e.g. Rocuronium or ARI) could be monitored in urine and based on that estimated rate of detoxification.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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