Journal of Pharmaceutical Research International

34(43A): 59-66, 2022; Article no.JPRI.88632
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Inhibitory Effect on Hexokinase II by Benzimidazoles and the Insight into Interactions

Hillary J. Navarro [a], Jhawn G. Saul [a], Andrew E. Huckleby [a] and Sung-Kun Kim [a*]

[a] Department of Natural Sciences, Northeastern State University, Broken Arrow, OK 74014, USA.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2022/v34i43A36311

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/88632

Original Research Article

Received 17 April 2022
Accepted 28 June 2022
Published 01 July 2022

ABSTRACT

Background: Benzimidazoles are aromatic, heterocyclic organic molecules which exhibit pharmacological potential as anti-inflammatory, antiulcer, anti-hypertensive, anticancer agent, and anthelmintic treatments. The benzimidazoles could inhibit human hexokinase II, which is a critical factor in the glycolysis pathway in humans.
Methods: Autodock analyses were performed for the initial docking between human hexokinase II and benzimidazoles. To determine the IC_{50} values by benzimidazole compounds, hexokinase enzyme assay was executed using continuous colorimetric detection. In addition, the insight into binding interactions was revealed primarily by Gromacs molecular dynamics simulations.
Results: We tested the most common benzimidazoles such as Fenbendazole, Albendazole, and Mebendazole on the target enzyme hexokinase II. The Autodock vina showed that the binding affinity values were between -7.9 and -6.1 kcal/mol for all three benzimidazoles. The IC_{50} values were 0.29, 2.5, and 10 μM for Fenbendazole, Albendazole, and Mebendazole, respectively.
Conclusions: Taken altogether, Fenbendazole appears to show most effective inhibition on hexokinase II. This research may give better insight in development of target-specific benzimidazole derivatives as potential anticancer therapeutics.

Keywords: Benzimidazole; albendazole; mebendazole; fenbendazole; hexokinase.

**Corresponding author: E-mail: kim03@nsuok.edu;*

1. INTRODUCTION

Human hexokinase II is the main isoform in many cell structures that increases cancer and can be found in the muscle, heart, and in mitochondrial outer membranes [1]. A compound called lonidamine was synthesized by researchers to present itself as a hexokinase inhibitor, antitumor agent, mitochondrial pyruvate carrier, and plasma membrane monocarboxylate transporter inhibitor [2]. Consequently, lonidamine disrupts energy metabolism of cancer cells, specifically inhibiting aerobic glycolytic activity affecting the mitochondrial complex II, therefore, lonidamine may impair energy metabolism that relies on benzimidazoles and impairs enzymatic activity of hexokinase II, leading to apoptotic signaling activation [2]. Additionally, benzimidazoles are a large family of medications used to treat parasitic infections caused by nematodes in veterinary medicine. Benzimidazoles are bicyclic aromatic compounds with an imidazole ring fused with benzene. Commonly used benzimidazoles such as Fenbendazole, Albendazole, and Mebendazole have shown the ability to disrupt cellular growth in human cancer cells by depolymerizing microtubules and interfering in critical transitions of the cell cycle, ultimately causing cell death [3]. This results in multiple downstream effects including reduced glucose uptake from the glucose transporter isoform 4 (GLUT4) and reduced expression of glycolytic enzymes like hexokinase II. A nitrogen heterocycle benzimidazole was first synthesized by Hoebrecker [4], and after 80 years the therapeutic activity as an anthelmintic drug was recognized. Today, benzimidazoles are highly esteemed anthelmintics that serve as effective drugs for intestinal and tissue-dwelling parasites.

Among the most common benzimidazoles, Fenbendazole (methyl N-(6-phenylsulfanyl-1H-benzimidazol-2-yl) carbamate) functions as a broad-spectrum anthelmintic that contains anti-proliferative activity. Fenbendazole is typically used in veterinary medicine to target parasitic pinworms with a low degree of toxicity and high degree of safety in animals [5–10]. It works by disrupting microtubules and proteasomes, down regulating the glycolytic enzymes GLUT4 and hexokinase II, thereby reducing glucose uptake, and ultimately starving the cancer cells [11]. Like Fenbendazole, Albendazole is also a common anthelmintic drug, and it is notably low in toxicity and considerably safer for treatment due to its benzimidazole carbamates. Albendazole (5-(propylthio-1H-benzimidazol-2-yl) carbamic acid methyl ester is insoluble in water and most organic solvents, and poorly absorbed in the gastrointestinal tract. It functions by inhibiting polymerization of parasite tubulin into microtubules and specifically targets parasites because it has a higher affinity to the parasite tubulin than host tubulin. This prevents the parasites from maintaining energy production and ultimately leads to death [12]. Albendazole may treat neurocysticercosis, which is an infection by the pork tapeworm in the muscles, brain, and eyes that may lead to seizures, brain swelling, and vision problems [13]. Lastly, Mebendazole (2-benzimidazolecarbamic acid methyl ester), is another class of benzimidazoles that came into use in 1971, and it was the first benzimidazole carbamate to be used on humans, and also functions by reducing glucose uptake. It can also be used as a prescription to treat parasitic helminth infections such as threadworms, tapeworms, roundworms, pinworms, and other nematode infections in humans and livestock [14–16].

While extensive research has been done on benzimidazoles, inhibition studies for human hexokinase II have been limited. Here, the research aim is to examine the anticancer activity of benzimidazoles. More specifically, the inhibition of hexokinase II were explored by the benzimidazoles (Fenbendazole, Albendazole, and Mebendazole) using *In silico* and *In vitro* analyses. We sought to determine potential binding sites of Fenbendazole, Albendazole, and Mebendazole to hexokinase II, and to study the kinetics of hexokinase II inhibition with each benzimidazole compound. Results between the computational and experimental analyses could then be compared. The findings from this study could have future implications in the research of hexokinase II, and possible therapeutic use of these studied inhibitors.

2. MATERIALS AND METHODS

2.1 Materials

The chemicals used here were purchased from Sigma-Aldrich or Fisher Scientific and used as received. Hexokinase Colorimetric Assay Kit was purchased from BioVision Inc (Milpitas, CA, USA).

2.2 Autodock Analysis

Autodock analysis was carried out in a multistep process beginning with selecting a crystal

structure and culminating with successful docking of the receptor and ligand. In order to find a crystal structure to begin with a search for Human hexokinase II was completed through SWISS-MODEL. The crystal structure selected as the best fit for this study was PDB ID: 1IG8 for hexokinase II, which was uploaded to the molecular editing program Avogadro if any editing is needed. The ligands used in this study were the benzimidazoles - Fenbendazole, Albendazole, and Mebendazole. A 3D structure of benzimidazoles was built using the same molecular editing program, Avogadro, to be optimized structurally. With both the receptor and ligand prepared in the same format, PDBQT, Autodock Vina was then performed.

2.3. Experimental Analysis

Inhibition tests were performed using Hexokinase Colorimetric Assay Kit (BioVision, INC., Milpitas, CA, USA) using Mebendazole, Albendazole, and Fenbendazole as potential hexokinase inhibitors. The concentrations of the inhibitors varied from 0.5 µM to 0.5 mM along with 1 µL of human hexokinase II. The assay was monitored by the increased absorbance of 450 nm wavelength.

2.4. Molecular Dynamics

In proceeding with molecular dynamics (MD), the conformation with the best binding affinity from the Autodock Vina results was selected. For this simulation the AMBER 99SB force field was used along with the TIP3P water model. In the MD program GROMACS, typically the first stage of each dynamics simulation is solvating the system. For this MD simulation, it was important to balance the charge of the system. To do so, counter ions such as Na^+ or Cl^- can be added depending on whether the net charge of the system is positive or negative. To balance the net negative charge of the Human hexokinase II and Fenbendazole complex, 16 Na^+ ions were added to the system.

Next, an energy minimization was carried out for 50000 steps using the steepest descent algorithm. The first phase of equilibration was carried out in a 100 ps run using the NVT ensemble with the leap-frog integrator. This run stabilized the temperature around 300 K. The second phase of equilibration was carried over a 100 ps run using the NPT ensemble with the leap-frog integrator as well. This run stabilized the pressure of the system around 1 bar. Position restraints were used for both the receptor and the ligand for each run. The final production MD simulation was run for 10 ns, during which the position restraints were removed. To obtain a final analysis of binding affinity, the Prodigy webserver was used [17].

3. RESULTS AND DISCUSSION

3.1. Computational Analysis by Autodock

After obtaining structure files of the benzimidazole ligands and hexokinase II, we carried out Autodock vina and selected the best docking results as shown in Fig. 1. The *in silico* studies showed that a nitrogen in the imidazole group of the Fenbendazole molecule formed a hydrogen bond with the N-Terminus of Ala161. This conformation had the best binding affinity at -7.0 kcal/mol among the 9 docking positions (Fig. 1A). Albendazole binding to hexokinase II was also determined, and as shown in Fig. 1B, the results showed that Albendazole formed hydrogen bonds with Arg470. The range in binding affinity was between -5.4 and -6.1 kcal/mol, and the best affinity energy was -6.1 kcal/mol. Lastly, the docking was performed for Mebendazole, and three hydrogen bonds were found in the complex with the residues Ser70, Gln466, and Arg470 (Fig. 1C). The binding affinity ranged -6.9 to -7.9 kcal/mol based on 9 docking results. The best one was -7.9 kcal/mol. The complete binding affinity results are shown in Table S1.

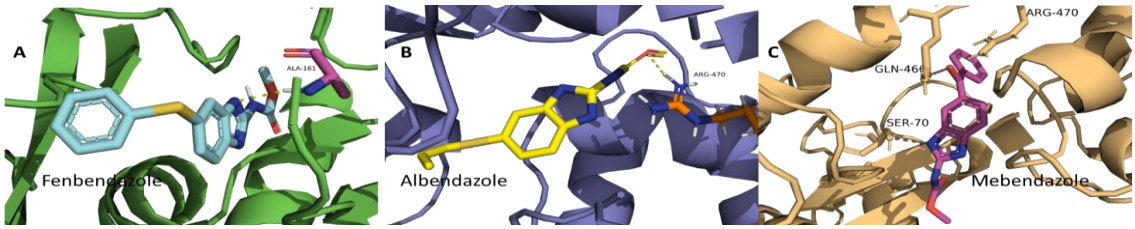

Fig. 1. Molecular docking between hexokinase II and the benzimidazoles (Fenbendazole, Albendazole, and Mebendazole). (A) A snapshot of binding mode of hexokinase II and Fenbendazole. (B) A snapshot of binding mode of hexokinase II and Albendazole. (C) A snapshot of binding mode of hexokinase II and Mebendazole

3.2 Inhibition Tests

To examine the possibility of inhibition of hexokinase II activity by the three benzimidazoles, IC_{50} values were tested. Concentrations of all three benzimidazoles were used from a range of 0.5 µM to 0.5 mM to determine IC_{50} values. The data were plotted in a semilogarthmic graph to see the clear midpoint values, and the data in the presence of the benzimidazoles were fit to a concentration-response plot with the equation $v_i/v_o = 1/ (1 + ([I]/IC_{50})^h)$, where I is inhibitor and h is the Hill coefficient (the Hill coefficients used were between 0.5 and 1). As shown in Fig. 2, the IC_{50} values for Fenbendazole, Albendazole, and Mebendazole were 0.25 ± 0.1 µM, 2.5 ± 0.8 µM, and 10.0 ± 1.2 µM, respectively. Experiments were performed in triplicate.

Based on the inhibition tests, it appeared that Fenbendazole has the lowest IC_{50} values among the benzimidazoles tested. The IC_{50} value was particularly less than 10 µM, which most pharmaceutical companies are looking for. Thus, Fenbendazole may be an effective inhibitor for hexokinase II; however, further investigations are warranted.

3.3 Molecular Dynamics

A 10 nanosecond (ns) molecular dynamics simulation was completed and analyzed to further understand the molecular interactions between the receptor and ligand. The initial binding occurred between the amine group of Fenbendazole and the Ala145 residue. Almost simultaneously the carbonyl oxygen of Fenbendazole had hydrogen interactions with the side chain of Asn194. Interactions between an imidazole nitrogen of Fenbendazole and the C-terminus of Ser142 could also be observed. However, most of the significant hydrogen bonding throughout the simulation was between the imidazole and Glu253, along with the carbonyl oxygen and a hydrogen of the Asn194 side chain.

Fig. 3 shows some snapshots of the molecular dynamics simulation between hexokinase II and Fenbendazole. In reviewing the dynamics, the hydrogen interactions were observed at different stages throughout the simulation (Table 1). The amino acids, Ala145, Asn194, Ser142, and Glu253 appear to form frequent hydrogen bonds with the carbonyl oxygen, the nitrogen of imidazole, and the amine group hydrogen of Fenbendazole. These observations suggest that the movement of the amide functional group and imidazole seem to be critical in the interaction of the ligand with the target protein hexokinase II.

In order to examine conformational variations of the binding within a hydrated environment, molecular dynamics simulations were performed, and the root-mean-square deviation (RMSD) values of the atomic positions were analyzed. Fig. 4A shows the RMSD for the ligand as a function of the 10 ns simulation time. The obtained RMSD values varied around 0.5 Å and stabilized after 0.5 ns, indicating that Fenbendazole maintained a stable complex with the enzyme. As shown in Fig. 4B, the RMSD of the peptide backbone of the enzyme varied around 0.2 Å after 0.2 ns and then leveled off. This indicates that the system was well equilibrated throughout the simulation. To better understand the overall change in binding energy of the complex, the online PRODIGY program was used to calculate the affinity at 9.55 ns, revealing the binding affinity value to be -6.9 kcal/mol, showing minimal change in comparison to the value obtained by Autodock vina (i.e., -7.0 kcal/mol). This observation demonstrated that during the molecular dynamics simulation, the binding affinity fluctuation would not be significant.

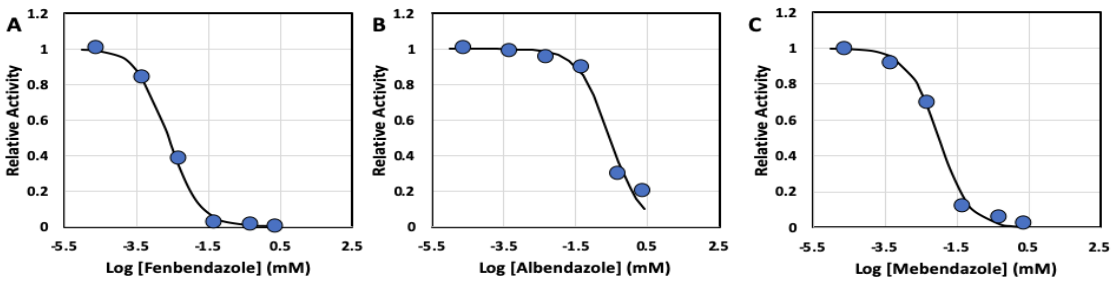

Fig. 2. Concentration-response plots for hexokinase II inhibition with compounds, Fenbendazole, Albendazole, and Mebendazole

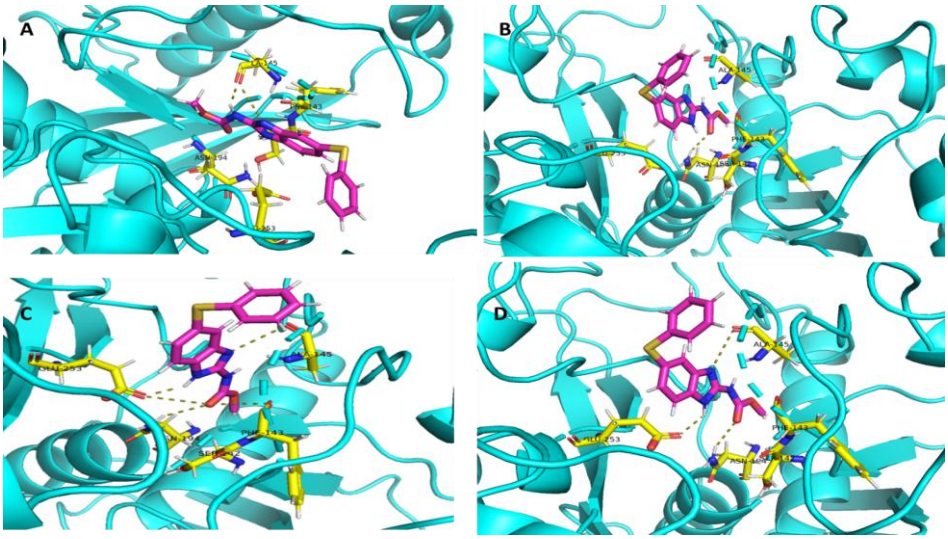

Fig. 3. Snapshots of the molecular dynamics simulation for Fenbendazole at 0.01 ns (A), 1.90 ns (B), 4.00 ns (C), and 9.55 ns (D)

Table 1. A snapshot of hydrogen interactions during the molecular dynamics simulation

Ligand	Residue	Time
Amine group H	Ala145 C-terminus	0.01 ns
Carbonyl O	Asn194 H amide group	0.02 ns
NH of Imidazole Group	Ser142 O of C-terminus	0.07 ns
NH of Imidazole Group	Glu253 COO^- of R' group	1.90 ns
Carbonyl O	Asn194 H amide group	4.00 ns
Carbonyl O	Asn194 H amide group	6.40 ns
NH of Imidazole Group	Glu253 COO- of R' group	6.40 ns
Carbonyl O	Asn194 H amide group	9.55 ns
NH of Imidazole Group	Glu253 COO- of R' group	9.55 ns

After docking, the selected complex was also analyzed by the aid of the Protein-Ligand Interaction Profiler (PLIP) web server of Technical University, Dresden [17]. Two main types of interactions are established upon docking: hydrogen bonding and hydrophobic interactions. Table 2 lists the interactions pattern analyzed by PLIP software. The PLIP analysis shows three hydrogen bonds through Ala145, Asn194, and Glu253 and three hydrophobic interactions through Pro144, Glu156, and Glu253. The average hydrogen bond length is 2.27, and the average hydrophobic interaction length is 3.72.

We have demonstrated the enzyme inhibition by benzimidazoles and provided the insight into the binding interaction between the enzyme and Fenbendazole, which showed the best inhibitory effect. In previous literature, a couple of potential inhibitors for hexokinase II were developed. For example, the glucose analogue 2-dexoy-D-glucose was shown to affect the metabolism of the entire glycolysis [18]; however, signs of toxicity to the brain were detected on the high doses [19]. As other examples, the microRNA MicroRNA-199a-5p, 3-bromopyruvate, and metformin showed some inhibition for hexokinase II, but all had problems for druggability [20–22]. Recently a compound, (E)-4-Nitro-*N*′-(2,3,4-trihydroxybenzylidene) benzohydrazide (Benitrobenrazide, BNBZ), was designed and synthesized as a hexokinase II inhibitor [23]; IC_{50} value was determined to be 2.20 ± 0.12 μM [24]. The IC_{50} value of BNBZ is thought to be promising, but the IC_{50} value of Fenbendazole appears to be ten times better than the case of BNBZ, suggesting that Fenbendazole seems much promising for further development.

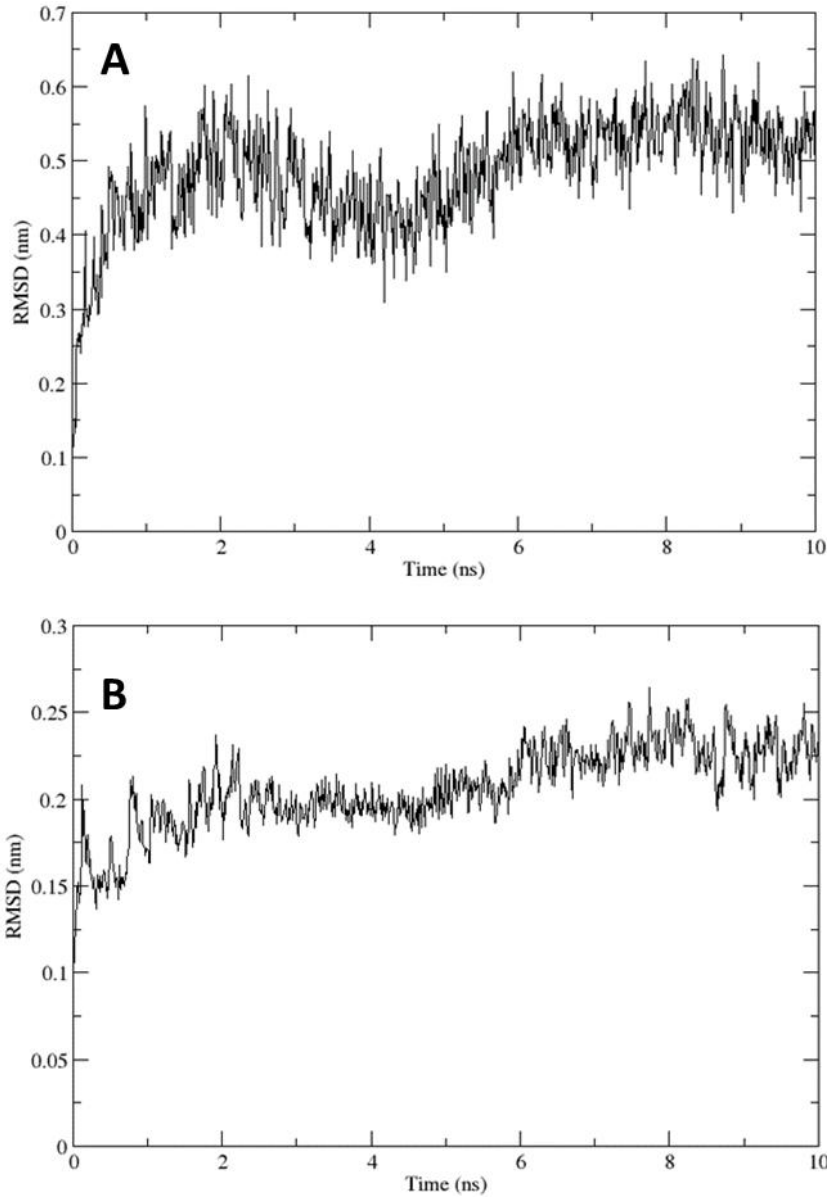

Fig. 4. The RMSD derived from molecular dynamics simulations. (A) the RMSD for the ligand Fenbendazole to human hexokinase II. (B) The RMSD of the peptide-backbone of human hexokinase II with the ligand

Table 2. The interactions formed between hexokinase II and Fenbendazole after the molecular dynamics simulation at 9.55 ns

Hydrogen bonding		Hydrophobic interaction	
Residue	**Distance (Å)**	**Residue**	**Distance (Å)**
Ala145	2.34	Pro144	3.57
Asn194	2.39	Glu156	3.95
Glu253	2.10	Glu253	3.64

4. CONCLUSIONS

While the effects of modern medicine have advanced over the past years, there is still yet to be a cure for cancer. There still needs to be more clinical trials and research done for benzimidazoles to be used as a treatment for human cancer. For this to be accepted in cancer

therapy, more research must be done to better understand the efficacy, side effects, and nature of the drug itself. The long-term effects of benzimidazole anthelmintics are still unknown.

The Autodock vina results showed that the binding affinity values were between -7.9 and -6.1 kcal/mol for all three benzimidazoles. The IC_{50} values were 0.29, 2.5, and 10 μM for Fenbendazole, Albendazole, and Mebendazole, respectively. These observations showed the potential for Fenbendazole to be an effective inhibitor hexokinase II.

This study has provided evidence in the advancement of using benzimidazoles in potential cancer therapy. This research and experimentation bring insight into the use of benzimidazoles to inhibit hexokinase II and opportunities for future study of these compounds in the context of their inhibitory potential. By providing new evidence and supporting previous research data, the market could open to benzimidazoles as a novel treatment in human cancer therapy with great results.

SUPPLEMENTARY MATERIALS

Supplementary materials available in this link: https://journaljpri.com/index.php/JPRI/libraryFiles/downloadPublic/38

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENT

This research was in part supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under award number P20GM103447 and by the Faculty Research Committee from Northeastern State University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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