Journal of Enzyme Inhibition and Medicinal Chemistry, August 2003 VOL. 18 (4), pp. 333–338

Taylor & Francis healthsciences

Carbonic Anhydrase Inhibitors. Inhibition of Cytosolic Isozymes I and II and Transmembrane, Cancer-associated Isozyme IX with Lipophilic Sulfonamides

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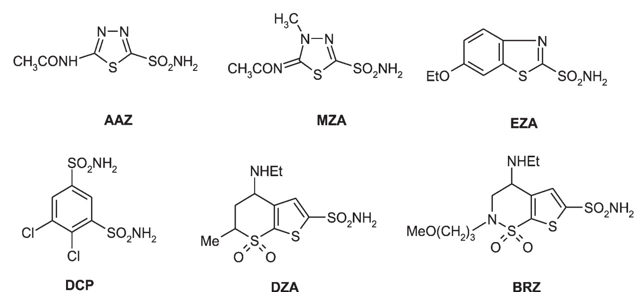
(Received 2 April 2003; In final form 22 April 2003)

A series of new compounds was obtained by reaction of aromatic/heterocyclic sulfonamides incorporating amino groups with *N,N*-diphenylcarbamoyl chloride and diphenylacetyl chloride. These sulfonamides were assayed for the inhibition of three carbonic anhydrase (CA, EC 4.2.1.1) isozymes: the cytosolic CA I and CA II, and the transmembrane, cancer-associated isozyme CA IX. Good inhibitors against all these isoforms were detected, and the inhibition profile of the newly investigated isozyme IX was observed to be different from that of the cytosolic isozymes, I and II. This may lead to the development of novel anticancer therapies based on the selective inhibition of CA IX.

Keywords: Carbonic anhydrase; Isozyme I, II, IX; Lipophilic; Sulfonamide; Anticancer drug

INTRODUCTION

Among the zinc enzymes extensively studied in the last decade, the carbonic anhydrases (CAs, EC 4.2.1.1) occupy a special place for several reasons: (i) these enzymes are ubiquitous in all kingdoms, starting with *Archaea*, *Bacteria*, algae and green plants, and ending with superior animals, including vertebrates [1–4]; (ii) their physiological function is essential for these organisms, as CAs catalyze a very simple physiological reaction, the interconversion between carbon dioxide and bicarbonate.[1–4] This reaction is critical for respiration and transport of CO_2 between metabolizing tissues and excretion sites, secretion of electrolytes in a variety of tissues and organs, pH regulation and homeostasis, CO_2 fixation (for algae and green plants), several metabolic biosynthetic pathways (in vertebrates), etc.[1–4]; (iii) inhibition (but also activation) of these enzymes may be exploited clinically in the treatment or prevention of a variety of disorders.[1–3] In consequence, CA inhibitors (CAIs) and to a lesser extent up to now, CA activators possess a variety of applications in therapy.[1,2] Four such pharmacological agents, acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, and dichlorophenamide **DCP**, have been used for more than 40 years as systemic CAIs, whereas an additional two drugs dorzolamide **DZA** (clinically launched in 1995) and the structurally-related brinzolamide **BRZ** (used since 1999) are topically acting antiglaucoma CAIs.[1,2]



CAs are encoded by three distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, *Bacteria*, algae and cytoplasm of green

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1475636031000138778

plants), the β-CAs (predominantly in *Bacteria*, algae and chloroplasts of both mono- as well as dicotyledons) and the γ-CAs (mainly in *Archaea* and some *Bacteria*), respectively.[1,2,5] In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) were described, with very different subcellular localization and tissue distribution.[1,2,5] Basically, there are several cytosolic forms (CA I-III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), one mitochondrial form (CA V) as well as a secreted CA isozyme, CA VI.[1,2,5] Not much is known about the cellular localization of other isozymes.

Some of the isozymes mentioned above, such as CA IX and CA XII, are predominantly found in cancer cells.[6] The first tumor-associated CA isozyme discovered was CA IX, a transmembrane protein with a suggested function in maintaining the acid-base balance and intercellular communication. It consists of an N-terminal proteoglycan-like domain that is unique among the CAs, a highly active CA catalytic domain, a single transmembrane region and a short intracytoplasmic tail.[7] CA IX is particularly interesting for its ectopic expression in a multitude of carcinomas derived from cervix uteri, kidney, lung, esophagus, breast, colon etc., contrasting with its restricted expression in normal tissues, namely in the epithelia of the gastrointestinal tract.[7–14]

It has recently been demonstrated that such tumor-associated CAs (mainly CA IX) may be of considerable value as markers of tumor progression. This is mostly due to their induction by hypoxia, a clinically important factor of tumor biology that significantly affects treatment outcome and disease progression.[9] Strong association between CA IX expression and intratumoral hypoxia (either measured by microelectrodes, or detected by incorporation of a hypoxic marker pimonidazole, or by evaluation of extent of necrosis) has been demonstrated in the cervical, breast, head and neck, bladder and non-small cell lung carcinomas (NSCLC).[10–13] Moreover, in NSCLC and breast carcinomas, correlation between CA IX and a constellation of proteins involved in angiogenesis, apoptosis inhibition and cell–cell adhesion disruption has been observed, possibly contributing to the strong relationship of this enzyme to a poor clinical outcome.[13] Hypoxia is linked with acidification of the extracellular milieu that facilitates tumor invasion and CA IX is believed to play a role in this process via its catalytic activity.[14] Thus, inhibition of this enzyme may constitute a novel approach to the treatment of cancers in which CA IX is expressed.

Acetazolamide, one of the best-studied, classical CAI used clinically, was on the other hand shown to function as a modulator in anticancer therapies, in combination with different cytotoxic agents (such as alkylating agents, nucleoside analogs, platinum derivatives, etc.) and to reduce the invasive capacity of several renal carcinoma cell lines (Caki-1, Caki-2, ACHN and A-498).[15,16] Such valuable studies constitute a proof-of-concept demonstration that CAIs may be used in the management of tumors that overexpress one or more CA isozymes. It should also be mentioned that our group reported the design and *in vitro* antitumor activity of several classes of sulfonamide CA inhibitors, shown to act as nanomolar inhibitors against the classical isozymes known to possess critical physiological roles, such as CA I, CA II and CA IV.[17–20] No data regarding CA IX inhibition with different types of sulfonamides are available up to the present, although inhibition of this isozyme may be clinically exploited for designing novel anticancer therapies. However, a key issue for a possible cancer therapy approach by means of CA inhibition would be the selectivity of such compounds for different CA isozymes, in order to avoid or limit the side effects associated with this kind of therapy. In fact, this is also a major aim for improving the present treatment of CA-related diseases based on systemic or topical delivery of carbonic anhydrase inhibitors.[1,2]

On these premises, we report here one of the first CA IX inhibition studies with a series of aromatic and heterocyclic sulfonamides incorporating lipophilic tails, as well as their inhibitory activity against the classical isozymes CA I and II.

MATERIALS AND METHODS

Chemistry

Melting points were determined with a heating plate microscope and are not corrected. NMR spectra were recorded using a Varian Gemini 300BB apparatus, operating at 300 MHz for ^{1}H-NMR and at 75 MHz for ^{13}C-NMR. Chemical shifts are expressed as δ values (ppm) relative to Me_4Si as internal standard for proton spectra and to the solvent resonance for carbon spectra. Elemental analysis were performed with a Carlo Erba Instruments CHNS Elemental Analyzer, Model 1106, the results being found $\pm$ 0.4% within the theoretical values for the proposed structures. All reactions were monitored by thin-layer chromatography (TLC), using 0.25 mm-thick precoated silica gel plates (E. Merck) eluted with MeOH:$CHCl_3$ 3:7 v/v. Preparative column chromatography was performed on silica gel 60 (0.063–0.200 mm) (Merck) eluted with MeOH:$CHCl_3$ 1:9 v\v.

Diphenylacetyl chloride, and diphenylcarbamyl chloride, were from Aldrich. Orthanilamide (**A**), sulfanilamide (**C**), homosulfanilamide (hydrochloride) (**D**) and 4-(2-aminoethyl)-benzenesulfonamide

(**E**) were from Sigma-Aldrich (Milan, Italy). 5-Amino-1,3,4-thiadiazole-2-sulfonamide (**I**) was prepared from acetazolamide (Sigma-Aldrich) by deprotection with concentrated hydrochloric acid, followed by neutralization of the hydrochloride salt. 5-Imino-4-methyl-Δ^2-1,3,4-thiadiazoline-2-sulfonamide hydrochloride (**J**) was similarly prepared from methazolamide (Sigma-Aldrich) as described in Ref. 21. 3-Aminobenzenesulfonamide (**B**), 4-sulfanilyl-sulfanilamide (**F**), 4-(sulfanilylamino-methyl)benzenesulfonamide (**G**), 4-[(2-sulfanilyl) aminoethyl]benzenesulfonamide (**H**) and 4-amino-benzolamide (5-(4-aminophenylsulfonylamido)-1,3,4-thiadiazole-2-sulfonamide) (**K**) were prepared as described in Refs. 22–25 Acetonitrile, pyridine, triethylamine and other solvents used were from Sigma-Aldrich or Fluka (Milan, Italy) and were kept on molecular sieves in order to maintain them in anhydrous conditions.

General Procedure for the Preparation of the New Compounds 1A–1K and 2A–2K

Five mmoles of aminosulfonamide **A–K** were suspended/dissolved in 20–30 mL of anhydrous acetonitrile, then 5.5 mmoles triethylamine or pyridine (depending on the reactivity of the sulfonamide) were added under stirring at room temperature. The corresponding diphenylacetyl/*N*,*N*-diphenylcarbamoyl chloride dissolved in 3–5 mL MeCN was then added dropwise to the reaction mixture under stirring at 0°C, during a period of 0.5–1 h. The reaction mixture was stirred a few hours at 0°C and then stirred overnight or until a reasonable conversion was reached (TLC control). The solvent was removed in vacuum and the resultant product (precipitate or oil) was washed with 15–20 mL water, in order to eliminate the ammonium/pyridinium salts. The crude solid was filtered and recrystallized from anhydrous methanol or ethanol, affording the pure desired products. In some cases, the recrystallized product was further purified by column chromatography, using silicagel 60 as stationary phase and MeOH/$CHCl_3$ gradients as eluent. The overall yields were in the range of 75–30%, depending on the reactivity of the aminosulfonamide **A–K**.

All new compounds were characterized by IR and NMR spectra, and by elemental analysis. Examples for several representatives of both classes are shown below.

5-(*N*,*N*-Diphenylcarbamoylamido)-1,3,4-thiadiazole-2-sulfonamide **1I**

White crystals, m.p. 243–4°C, IR (KBr), $cm^{-1:}$ 1162 (SO_2), 1375 (SO_2), 1610 (C=N), 1670 (C=O), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 7.08–7.75 (m, 10H, ArH), 7.81 (s, 1H, CONH), 8.15 (br s, 2H, SO_2NH_2), ^{13}C-NMR (DMSO-d_6), δ, ppm: 118.8, 119.9, 128.1, 130.3, 159.5 (C-2 of thiadiazole), 171.3 (C-5 of thiadiazole), 176.2 (CONH). Found: C, 48.23; H, 3.36; N, 18.54. $C_{15}H_{13}N_5O_3S_2$ requires: C, 47.99; H, 3.49; N, 18.65 %.

5-[4-(Diphenylacetamido)phenylsulfonylamido]-1,3,4-thiadiazole-2-sulfonamide **2K**

White crystals, m.p. 179–81°C. IR (KBr), $cm^{-1:}$ 1166(SO_2), 1370 (SO_2), 1610 (C=N), 1673 (C=O), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 5.15 (s, CH), 7.28 (m, 8H, ArH), 7.64 (m, 4H, ArH), 7.73 (m, 2H, ArH), 8.23 (br s, 2H, SO_2NH_2), 10.61 (s, 2H, CONH + SO_2NH), 8.15 (br s, 2H, SO_2NH_2); ^{13}C-NMR (DMSO-d_6), δ, ppm: 57.70 (CH), 118.9, 127.1, 127.2, 128.7, 128.8 (all CH arom), 157.7 (C-2 of thiadiazole), 170.5 (C-5 of thiadiazole), 176.8 (CONH)., Found: C, 59.19; H, 4.58; N, 11.85. $C_{23}H_{20}N_4O_3S_2$ requires: C, 59.47; H, 4.34; N, 12.06 %.

CA Inhibition

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II as described by Lindskog's group.[26] Cell growth conditions were those described in Ref. 27 and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[28] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1} cm^{-1}$ for CA I and 54 $mM^{-1} cm^{-1}$ for CA II, respectively, based on M_r = 28.85 kDa for CA I and 29.3 kDa for CA II, respectively.[29,30]

The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek *et al.*[7]) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene, Milan, Italy). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenised in a buffered solution (pH 8) of 4 M urea and 2% Triton X-100, as described by Wingo *et al.*[31] The homogenate thus obtained was extensively centrifuged ($11,000 \times g$) in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenisation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap

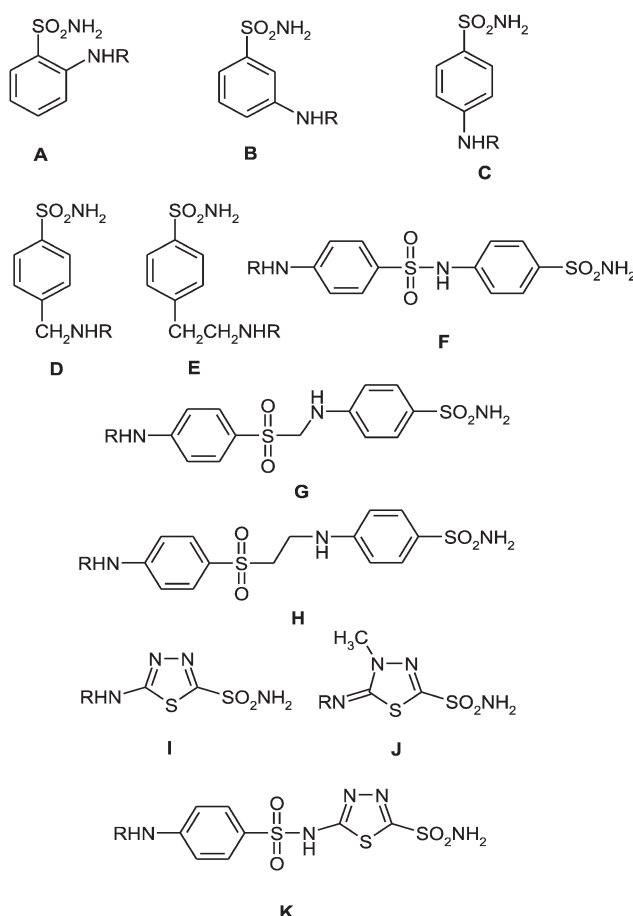
dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM $ZnCl_2$, 2 mM EDTA, 2 mM reduced glutathione and 1 mM oxidized glutathione. Active hCA IX was extensively dialysed into a solution of 10 mM Hepes (pH 7.5), 10 mM Tris HCl, 100 mM Na_2SO_4 and 1 mM $ZnCl_2$. The last step consisted in sulfonamide column affinity purification of the protein,[31,32] which was eluted with 1 mM sodium azide solution. After dialysis in the above mentioned buffer, the amount of protein was determined by spectrophometric measurement and its activity by stopped-flow measurements, with CO_2 as substrate.[32]

Initial rates of 4-nitrophenylacetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC.[33] Solutions of substrate were prepared in anhydrous acetonitrile and the substrate concentrations varied between 2.10^{-2} and 1.10^{-6} M, working at 25°C. A molar absorption coefficient ϵ of 18,400 $M^{-1} cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, under the conditions of the experiments (pH 7.40), as reported in the literature.[33] Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constant K_I was determined as described by Pocker and Stone for isozymes I and II.[33] Enzyme concentrations were 3.6 μM for CA II, and 10.9 μM for CA I.

An SX.18MV-R Applied Photophysics stopped-flow instrument was used for assaying the CA IX CO_2 hydration activity.[32] Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20°C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 1–3 mM (in DMSO-water 1:1, v/v) and dilutions up to 0.1 nM done with the assay buffer mentioned above. The enzyme concentration was 0.1 μM, and inhibition constants were calculated as described in Ref. 32.

RESULTS AND DISCUSSION

In previous studies from these laboratory,[19,20] it has been shown that some aromatic/heterocyclic sulfonamides with powerful CA I, II and IV inhibitory activity also show a certain degree of tumor cell growth inhibition *in vitro* and *in vivo* (usually in low micromolar concentrations). Among such compounds, several derivatives possessed the *N,N*-diphenyl-carbamoyl moiety in their molecule.[19,20] It thus appeared of interest to investigate in detail a series of such compounds incorporating this rather bulky and lipophilic moiety, as well as the isostructural diphenylacetyl one, which has not been investigated previously in connection with CA inhibitors. By applying the tail approach,[1,2] a series of aromatic and heterocyclic sulfonamides of types **A–K** have been derivatized at their amino group by reaction with *N,N*-diphenylcarbamoyl chloride or diphenylacetyl chloride (in the presence of bases), respectively. Compounds **1A–1K** and **2A–2K** have been obtained in this way, by routine chemical procedures (see Materials and Methods).



These new derivatives have been characterized by spectroscopic and elemental analysis data, which confirmed their structures.

The new compounds **1A–1K**, **2A–2K** as well as the standard CA inhibitors used clinically have been assayed for their inhibition against isozymes CA I, II and IX (Table I).

The data in Table I afforded the following SAR conclusions to be drawn for this family of CA inhibitors: (i) derivatizations of sulfonamides **A–K** by means of the lipophilic *N*,*N*-diphenylcarbamoyl and diphenylacetyl tails leads to effective CA inhibitors, as all obtained new compounds **1A–1K**, **2A–2K** were better inhibitors against all three investigated CAs, than the corresponding parent sulfonamide from which they were obtained; (ii) the diphenylacetamido derivatives **2A–2K** were generally slightly better inhibitors than the corresponding diphenylureas **1A–1K**; (iii) as usual,[1,2] heterocyclic sulfonamides (derivatives of sulfonamides **I–K**) were much better CA I and CA II inhibitors as compared to the aromatic derivatives, whereas for this last type of compounds, inhibitors with longer molecules (for example derivatives of sulfonamides **F–H**) were generally more effective than those possessing "short" molecules (derivatives of sulfonamides **A–C**), a behavior explained in two recent QSAR studies of this group.[23,24] However, in the case of isozyme CA IX, this behavior is not so accentuated. In fact, rather small differences were observed in the inhibition of CA IX with some heterocyclic and aromatic new sulfonamides described here; compare for example the aromatic compound **1D** and the heterocyclic one **1K**, which practically possess the same inhibition constant for CA IX, although their inhibition of the other two isozymes (CA I and II) investigated here is very different. Thus, our data (as well as a recent publication from our group on CA IX inhibition with simple aromatic/heterocyclic sulfonamides)[34] clearly show that CA IX has an inhibition profile rather different from that of CA I and II. As seen from the data of Table I, this behavior is also present for the clinically used CA inhibitors: dichlorophenamide **DCP**—an aromatic sulfonamide—is equipotent to dorzolamide **DZA**—a heterocyclic sulfonamide, as CA IX inhibitor; (iv) many of the new derivatives described here act as much better CA IX inhibitors compared to the clinically used compounds **AAZ–BRZ**. For example **2J** (K_I of 11 nM against CA IX) is at present the most effective CA IX inhibitor detected.[25,34] These data may be important for the development of CA IX inhibitor-based anticancer therapies.

TABLE I CA I, II, and IX inhibition data with sulfonamides **1,2(A–K)** and clinically used inhibitors

	K_I* (nM)		
Inhibitor	hCA I[a]	hCA II[a]	hCA IX[b]
1A	330 (45400)	64 (295)	25 (33)
1B	270 (25000)	60 (240)	nt (238)
1C	315 (28000)	73 (300)	168 (294)
1D	265 (25000)	38 (170)	18 (103)
1E	250 (21000)	36 (160)	20 (33)
1F	74 (164)	21 (46)	nt (34)
1G	63 (109)	15 (33)	28 (31)
1H	49 (95)	13 (30)	nt (24)
1I	7 (8600)	8 (60)	15 (41)
1J	9 (9300)	10 (19)	13 (30)
1K	3 (6)	0.8 (2)	16 (38)
2A	335 (45400)	61 (295)	nt (33)
2B	264 (25000)	56 (240)	103 (238)
2C	300 (28000)	69 (300)	85 (294)
2D	260 (25000)	33 (170)	46 (103)
2E	254(21000)	32 (160)	21 (33)
2F	66 (164)	19 (46)	nt (34)
2G	62 (109)	14 (33)	19 (31)
2H	40 (95)	10 (30)	nt (24)
2I	5 (8600)	5 (60)	15 (41)
2J	7 (9300)	8 (19)	11 (30)
2K	1 (6)	0.4 (2)	21 (38)
AAZ	250	12	25
MZA	50	14	27
EZA	25	8	34
DCP	1200	38	50
DZA	50000	9	52
BRZ	-	3	37

*Data in parenthesis represent inhibition constants for the parent sulfonamides **A–K**, from Refs. 20,34. nt = not tested. a Human cloned isozymes, esterase assay method.[26] b Human cloned isozyme (catalytic domain), CO_2 hydrase assay method.[24,25]

In conclusion, we report here two series of lipophilic sulfonamide CA inhibitors incorporating *N*,*N*-diphenylcarbamoyl and diphenylacetyl moieties in their molecule. Many of these derivatives showed very good CA I, II and IX inhibitory properties *in vitro*. The inhibition profile of the tumor-associated isozyme CA IX was on the other hand quite different from those of the cytosolic isozymes I and II, making our findings interesting for the potential development of novel anticancer therapies based on sulfonamide CA inhibitors.

Acknowledgement

This research was financed by CSGI (Florence, Italy).

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